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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 September 7; 24(33): 3677-3812

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 33 September 7, 2018

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 33 September 7, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Somchai Amornyotin, MD, Associate Professor, Staff Physician, Department of Anesthesiology and Siriraj Gastrointestinal Endoscopy Center, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
September 7, 2018

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3677-3680

DOI: 10.3748/wjg.v24.i33.3677

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

EDITORIAL

Time to think: Selecting patients who may benefit from synchronous resection of primary pancreatic cancer and liver metastases

Si Shi, Xian-Jun Yu

Si Shi, Xian-Jun Yu, Department of Pancreatic Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, China

Si Shi, Xian-Jun Yu, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China

Si Shi, Xian-Jun Yu, Pancreatic Cancer Institute, Fudan University, Shanghai 200032, China

ORCID number: Si Shi (0000-0002-6652-0629); Xian-Jun Yu (0000-0002-6697-7143).

Author contributions: Shi S and Yu XJ conceived the study and drafted the manuscript; both authors approved the final version of the article.

Supported by the National Science Foundation for Distinguished Young Scholars of China, No. 81625016; and the Shanghai Sailing Program, No. 17YF1402500.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Xian-Jun Yu, MD, PhD, Professor, Surgeon, Surgical Oncologist, Department of Pancreatic Surgery, Fudan University Shanghai Cancer Center, No. 270 Dong'an Road, Xuhui District, Shanghai 200032, China. yuxianjun@fudanpci.org
Telephone: +86-21-64175590
Fax: +86-21-64031446

Received: June 16, 2018
Peer-review started: June 17, 2018
First decision: July 6, 2018
Revised: July 11, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: September 7, 2018

Abstract

Pancreatic cancer remains a lethal disease and is associated with poor prognosis, particularly for patients with distant metastasis at diagnosis. Recently, Oweira reported a retrospective study that included 13233 metastatic pancreatic cancer patients from the Surveillance, Epidemiology and End Results database. They demonstrated that pancreatic cancer patients with isolated liver metastases had worse outcomes than patients with isolated lung metastases or distant nodal metastases. At present, the standard treatment for metastatic pancreatic cancer is chemotherapy. However, improvement in the safety of pancreatic surgery has led to the consideration of more aggressive surgical approaches. Schneitler reported two cases of hepatic metastatic pancreatic cancer in which negative margin (R0) resection and long survival were achieved after effective preoperative chemotherapy. In general, these two studies indicate that although pancreatic cancer patients with liver metastasis have a poor prognosis, surgical approaches may prolong survival for a few of these patients. A strategy to select hepatic metastatic pancreatic cancer patients who may benefit from surgical intervention is urgently needed.

Key words: Liver metastasis; Chemotherapy; Pancreatic cancer; Surgery

Core tip: Pancreatic cancer patients with liver metastasis have worse prognoses than pancreatic cancer patients with metastasis at other sites. Improvement in the safety of pancreatic surgery has led to the consideration of more aggressive approaches. There is increasing agreement that synchronous resection of pancreatic cancer and liver metastases may selectively benefit some patients. A prospective multicenter, randomized, controlled phase three trial has been launched by the Chinese Study Group for Pancreatic Cancer with a goal of establishing such a selection strategy.

Shi S, Yu XJ. Time to think: Selecting patients who may benefit from synchronous resection of primary pancreatic cancer and liver metastases. *World J Gastroenterol* 2018; 24(33): 3677-3680 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3677.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3677

INTRODUCTION

Pancreatic cancer remains a challenging disease to treat and is associated with a poor prognosis[1,2]. Surgery remains the only curative treatment and provides an opportunity for long-term survival. Unfortunately, however, approximately 50% of pancreatic cancer patients are diagnosed with distant metastases; these patients are then deemed incurable and are generally not considered to be suitable for radical surgeries with curative intent[3]. At present, chemotherapy is the standard treatment for patients with metastatic pancreatic cancer. However, improvement in the safety of pancreatic surgery has led to the consideration of more aggressive approaches. Synchronous resection of primary tumors and metastatic sites continues to be attempted[4-6]. Synchronous pancreas and liver resection procedures account for the largest proportion of these attempts. However, the most relevant studies have only indicated the safety of such operations, which have failed to produce survival benefits. Nevertheless, there are many case reports demonstrating that certain pancreatic cancer patients can achieve long-term survival after resection of both the primary tumor and liver metastases[7,8].

STUDY ANALYSIS

In a recent issue of World Journal of Gastroenterology, Oweira reported a retrospective study performed using data from the Surveillance, Epidemiology and End Results database. A total of 13233 patients with stage IV pancreatic cancer and distant metastases at known sites were included for analysis. Metastatic pancreatic cancer patients were classified according to the site of metastases (liver, lungs, bone, brain or distant lymph nodes). Survival analysis indicated that pancreatic cancer patients with isolated liver metastases had worse outcomes than patients with isolated lung metastases or distant nodal metastases. This research demonstrated that we can reasonably provide different treatment strategies for pancreatic cancer patients with metastases at different sites.

Another interesting study by Schneitler *et al*[9] in *World Journal of Gastroenterology* merits attention. Two cases of hepatic metastatic pancreatic cancer were described in which negative margin (R0) resection and long survival were achieved after a preoperative FOLFIRINOX chemotherapy regimen consisting of Fluorouracil (5-FU), folinic acid, irinotecan and oxaliplatin. This study showed that certain pancreatic cancer patients with liver metastasis would benefit from surgical resection after effective chemotherapy.

Taken together, the results of these two studies indicate that although pancreatic cancer patients with liver metastasis generally have poor prognoses, surgical approaches may prolong survival for a few of these patients. There is increasing agreement that synchronous resection of pancreatic cancer and pancreatic liver metastases should be performed in a highly selective manner in some patients[10,11]. Thus, the determination of how to select for and then treat patients who would benefit from such approaches is urgently required.

PERSPECTIVE

In 1995, Hellman and Weichselbaum first proposed the clinically significant condition of oligometastasis, which is a state between local and systemic disease, and advocated for the potential for curative local treatments[12]. Further studies have identified distinct biological differences between limited metastatic lesions and widely disseminated disease for multiple tumor types, including pancreatic cancer[13-15]. Radical surgery to treat both primary and metastatic sites has been accepted and conducted for an increasing number of tumor types[16-18]. Thus, pancreatic cancer patients with few liver metastases may benefit from aggressive surgical approaches. Zanini *et al*[5] indicated that the number of liver metastases had a detrimental effect on survival after surgical resection. However, the number of liver metastases alone is insufficient to identify patients who are likely to be surgically cured and achieve improved overall survival.

Chemosensitivity is another important factor that could influence long-term survival and should therefore also be considered and evaluated. In previous studies on surgical resection of primary pancreatic cancer, preoperative chemotherapy was more common than direct surgery for three reasons[19,20]. First, recurrence and new metastases were observed within a short time after surgery and were the main causes of surgical failure[5]. Preoperative chemotherapy can inhibit tumor activity

and increase both the R0 and negative lymph node (N0) rates[21]. Second, the preoperative chemotherapy period can provide an opportunity to verify biological characteristics of cancers and select patients with less aggressive tumors[22,23]. Last, tumor burden may be reduced after preoperative chemotherapy, resulting in decreased surgical difficulty and increased safety.

Another important issue is the time at which to conduct surgical intervention. Although Response Evaluation Criteria In Solid Tumors (RECIST) are commonly employed to evaluate the efficacy of chemotherapy, these criteria are not appropriate for determining the optimal time point for an operation. Carbohydrate antigen 19-9 (CA19-9) is the most commonly used serum tumor marker of pancreatic cancer. It has been reported that CA19-9 response could be used to improve the selection of borderline and locally advanced pancreatic cancer patients who can benefit from resection after primary chemotherapy[24]. This conclusion may also be generalized to pancreatic cancer patients with liver metastases. However, approximately five to ten percent of the population are Lewis-negative individuals; it is known that such individuals exhibit little to no CA19-9 secretion (34, 35). Carbohydrate antigen 125 and carcinoembryonic antigen are alternative markers because they are the most common serum tumor markers for pancreatic cancer other than CA19-9. In addition, the capacity for resection of both the primary tumor and liver metastases should be carefully evaluated before surgical intervention.

Based on the above ideas, the Chinese Study Group for Pancreatic Cancer (CSPAC) has launched a prospective multicenter, randomized, controlled phase three trial (NCT03398291) named CSPAC-1. Their goal is to establish a treatment strategy to select patients who can benefit from simultaneous resection of primary pancreatic cancer and liver metastatic sites. The results of this trial are planned to be released in 2025; we are looking forward to their release because they may alter current the treatment modes for pancreatic cancer.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

2 **Chen W**, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]

3 **Mayo SC**, Nathan H, Cameron JL, Olino K, Edil BH, Herman JM, Hirose K, Schulick RD, Choti MA, Wolfgang CL, Pawlik TM. Conditional survival in patients with pancreatic ductal adenocarcinoma resected with curative intent. *Cancer* 2012; **118**: 2674-2681 [PMID: 21935914 DOI: 10.1002/cncr.26553]

4 **Shrikhande SV**, Kleeff J, Reiser C, Weitz J, Hinz U, Esposito I, Schmidt J, Friess H, Büchler MW. Pancreatic resection for M1 pancreatic ductal adenocarcinoma. *Ann Surg Oncol* 2007; **14**: 118-127 [PMID: 17066229 DOI: 10.1245/s10434-006-9131-8]

5 **Zanini N**, Lombardi R, Masetti M, Giordano M, Landolfo G, Jovine E. Surgery for isolated liver metastases from pancreatic cancer. *Updates Surg* 2015; **67**: 19-25 [PMID: 25702263 DOI: 10.1007/s13304-015-0283-6]

6 **Hackert T**, Niesen W, Hinz U, Tjaden C, Strobel O, Ulrich A, Michalski CW, Büchler MW. Radical surgery of oligometastatic pancreatic cancer. *Eur J Surg Oncol* 2017; **43**: 358-363 [PMID: 27856064 DOI: 10.1016/j.ejso.2016.10.023]

7 **Buc E**, Orry D, Antomarchi O, Gagnière J, Da Ines D, Pezet D. Resection of pancreatic ductal adenocarcinoma with synchronous distant metastasis: is it worthwhile? *World J Surg Oncol* 2014; **12**: 347 [PMID: 25407113 DOI: 10.1186/1477-7819-12-347]

8 **Neofytou K**, Giakoustidis A, Smyth EC, Cunningham D, Mudan S. A case of metastatic pancreatic adenocarcinoma with prolonged survival after combination of neoadjuvant FOLFIRINOX therapy and synchronous distal pancreatectomy and hepatectomy. *J Surg Oncol* 2015; **111**: 768-770 [PMID: 25556724 DOI: 10.1002/jso.23867]

9 **Schneitler S**, Kröpil P, Riemer J, Antoch G, Knoefel WT, Häussinger D, Graf D. Metastasized pancreatic carcinoma with neoadjuvant FOLFIRINOX therapy and R0 resection. *World J Gastroenterol* 2015; **21**: 6384-6390 [PMID: 26034375 DOI: 10.3748/wjg.v21.i20.6384]

10 **Nentwich MF**, Bockhorn M, König A, Izbicki JR, Cataldegirmen G. Surgery for advanced and metastatic pancreatic cancer--current state and trends. *Anticancer Res* 2012; **32**: 1999-2002 [PMID: 22593478]

11 **Michalski CW**, Erkan M, Hüser N, Müller MW, Hartel M, Friess H, Kleeff J. Resection of primary pancreatic cancer and liver metastasis: a systematic review. *Dig Surg* 2008; **25**: 473-480 [PMID: 19212120 DOI: 10.1159/000184739]

12 **Hellman S**, Weichselbaum RR. Oligometastases. *J Clin Oncol* 1995; **13**: 8-10 [PMID: 7799047 DOI: 10.1200/JCO.1995.13.1.8]

13 **Wuttig D**, Baier B, Fuessel S, Meinhardt M, Herr A, Hoefling C, Toma M, Grimm MO, Meye A, Rolle A, Wirth MP. Gene signatures of pulmonary metastases of renal cell carcinoma reflect the disease-free interval and the number of metastases per patient. *Int J Cancer* 2009; **125**: 474-482 [PMID: 19391132 DOI: 10.1002/ijc.24353]

14 **Lussier YA**, Xing HR, Salama JK, Khodarev NN, Huang Y, Zhang Q, Khan SA, Yang X, Hasselle MD, Darga TE, Malik R, Fan H, Perakis S, Filippo M, Corbin K, Lee Y, Posner MC, Chmura SJ, Hellman S, Weichselbaum RR. MicroRNA expression characterizes oligometastasis(es). *PLoS One* 2011; **6**: e28650 [PMID: 22174856 DOI: 10.1371/journal.pone.0028650]

15 **Al-Taee KK**, Ansari S, Hielscher T, Berger MR, Adwan H. Metastasis-related processes show various degrees of activation in different stages of pancreatic cancer rat liver metastasis. *Oncol Res Treat* 2014; **37**: 464-470 [PMID: 25231686 DOI: 10.1159/000365496]

16 **Jin K**, Xu J, Chen J, Chen M, Chen R, Chen Y, Chen Z, Cheng B, Chi Y, Feng ST, Fu D, Hou B, Huang D, Huang H, Huang Q, Li J, Li Y, Liang H, Lin R, Liu A, Liu J, Liu X, Lu M, Luo J, Mai G, Ni Q, Qiu M, Shao C, Shen B, Sheng W, Sun J, Tan C, Tan H, Tang Q, Tang Y, Tian X, Tong D, Wang X, Wang J, Wang J, Wang W, Wang W, Wang Y, Wu Z, Xue L, Yan Q, Yang N, Yang Y, Yang Z, Yin X, Yuan C, Zeng S, Zhang R, Yu X. Surgical management for non-functional pancreatic neuroendocrine neoplasms with synchronous liver metastasis: A consensus from the Chinese Study Group for Neuroendocrine Tumors (CSNET). *Int J Oncol* 2016; **49**: 1991-2000 [PMID: 27826620 DOI: 10.3892/ijo.2016.3711]

17 **Silberhumer GR**, Paty PB, Denton B, Guillem J, Gonen M, Araujo RLC, Nash GM, Temple LK, Allen PJ, DeMatteo RP, Weiser MR, Wong WD, Jarnagin WR, D'Angelica MI, Fong Y. Long-term oncologic outcomes for simultaneous resection of synchronous metastatic liver and primary colorectal cancer. *Surgery* 2016; **160**: 67-73 [PMID: 27079362 DOI: 10.1016/j.surg.2016.02.029]

18 **Margonis GA**, Buettner S, Sasaki K, Kim Y, Ratti F, Russolillo N, Ferrero A, Berger N, Gamblin TC, Poultsides G, Tran T, Postlewait LM, Maithel S, Michaels AD, Bauer TW, Marques H, Barroso E, Aldrighetti L, Pawlik TM. The role of liver-directed surgery in patients with hepatic metastasis from primary breast cancer: a multi-institutional analysis. *HPB* (Oxford) 2016; **18**: 700-705

[PMID: 27485066 DOI: 10.1016/j.hpb.2016.05.014]
19 **Ma T**, Bai X, Li G, Wei S, Liang T. Neoadjuvant modified-FOLFIRINOX followed by surgical resection of both the primary and metastatic tumors of a pancreatic hepatoid carcinoma with synchronous liver metastasis: A case report. *Medicine* (Baltimore) 2017; **96**: e8413 [PMID: 29069039 DOI: 10.1097/MD.0000000000008413]
20 **Shimura M**, Mizuma M, Hayashi H, Mori A, Tachibana T, Hata T, Iseki M, Takadate T, Ariake K, Maeda S, Ohtsuka H, Sakata N, Morikawa T, Nakagawa K, Naitoh T, Kamei T, Motoi F, Unno M. A long-term survival case treated with conversion surgery following chemotherapy after diagnostic metastasectomy for pancreatic cancer with synchronous liver metastasis. *Surg Case Rep* 2017; **3**: 132 [PMID: 29285651 DOI: 10.1186/s40792-017-0409-9]
21 **Russo S**, Ammori J, Eads J, Dorth J. The role of neoadjuvant therapy in pancreatic cancer: a review. *Future Oncol* 2016; **12**: 669-685 [PMID: 26880384 DOI: 10.2217/fon.15.335]
22 **Mokdad AA**, Minter RM, Zhu H, Augustine MM, Porembka MR, Wang SC, Yopp AC, Mansour JC, Choti MA, Polanco PM. Neoadjuvant Therapy Followed by Resection Versus Upfront Resection for Resectable Pancreatic Cancer: A Propensity Score Matched Analysis. *J Clin Oncol* 2017; **35**: 515-522 [PMID: 27621388 DOI: 10.1200/JCO.2016.68.5081]
23 **Lai TY**, Hu YW. Neoadjuvant Therapy in Resectable Pancreatic Cancer: Immortal Time Bias and Its Correction. *J Clin Oncol* 2017; **35**: 1623 [PMID: 28135147 DOI: 10.1200/JCO.2016.71.2273]
24 **Reni M**, Zanon S, Balzano G, Nobile S, Pircher CC, Chiaravalli M, Passoni P, Arcidiacono PG, Nicoletti R, Crippa S, Slim N, Doglioni C, Falconi M, Gianni L. Selecting patients for resection after primary chemotherapy for non-metastatic pancreatic adenocarcinoma. *Ann Oncol* 2017; **28**: 2786-2792 [PMID: 28945895 DOI: 10.1093/annonc/mdx495]

P- Reviewer: He SQ, Polistina FA **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3681-3694

DOI: 10.3748/wjg.v24.i33.3681

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

REVIEW

From bench to bedside: Fecal calprotectin in inflammatory bowel diseases clinical setting

Maria Gloria Mumolo, Lorenzo Bertani, Linda Ceccarelli, Gabriella Laino, Giorgia Di Fluri, Eleonora Albano, Gherardo Tapete, Francesco Costa

Maria Gloria Mumolo, Linda Ceccarelli, Giorgia Di Fluri, Francesco Costa, Department of General Surgery and Gastroenterology, Gastroenterology Unit, Azienda Ospedaliero Universitaria Pisana, Pisa 56124, Italy

Lorenzo Bertani, Gabriella Laino, Eleonora Albano, Gherardo Tapete, Department of New Technologies and Translational Research in Medicine and Surgery, University of Pisa, Pisa 56122, Italy

ORCID number: Maria Gloria Mumolo (0000-0003-2196-5170); Lorenzo Bertani (0000-0001-8653-1790); Linda Ceccarelli (0000-0002-6480-5778); Gabriella Laino (0000-0001-9412-6525); Giorgia Di Fluri (0000-0002-1644-578X); Eleonora Albano (0000-0002-6887-2863); Gherardo Tapete (0000-0001-6849-771X); Francesco Costa (0000-0002-9150-3345).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: The authors have declared that no potential conflict of interest exists.

Manuscript source: Invited manuscript

Correspondence to: Francesco Costa, MD, PhD, Chief Doctor, Gastroenterology Unit, Azienda Ospedaliero Universitaria Pisana, via Paradisa 2, Pisa 56124, Italy. fcosta@med.unipi.it
Telephone: +39-050997392
Fax: +39-050997392

Received: May 4, 2018
Peer-review started: May 5, 2018
First decision: May 17, 2018
Revised: June 5, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: September 7, 2018

Abstract

Fecal calprotectin (FC) has emerged as one of the most useful tools for clinical management of inflammatory bowel diseases (IBD). Many different methods of assessment have been developed and different cut-offs have been suggested for different clinical settings. We carried out a comprehensive literature review of the most relevant FC-related topics: the role of FC in discriminating between IBD and irritable bowel syndrome (IBS) and its use in managing IBD patients In patients with intestinal symptoms, due to the high negative predictive value a normal FC level reliably rules out active IBD. In IBD patients a correlation with both mucosal healing and histology was found, and there is increasing evidence that FC assessment can be helpful in monitoring disease activity and response to therapy as well as in predicting relapse, post-operative recurrence or pouchitis. Recently, its use in the context of a treat-to-target approach led to a better outcome than clinically-based therapy adjustment in patients with early Crohn's disease. In conclusion, FC measurement represents a cheap, safe and reliable test, easy to perform and with a good reproducibility. The main concerns are still related to the choice of the optimal cut-off, both for differentiating IBD from IBS, and for the management of IBD patients.

Key words: Fecal calprotectin; Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Irritable

bowel syndrome

Core tip: This manuscript is a review of current literature on clinical use of fecal calprotectin in distinguishing irritable bowel syndrome from inflammatory bowel diseases and in the long-term management of inflammatory bowel disease patients, which includes monitoring of disease activity, response to therapy, disease relapse and post-operative recurrence. Concerns about the optimal cut-off in different settings have also been discussed.

Mumolo MG, Bertani L, Ceccarelli L, Laino G, Di Fluri G, Albano E, Tapete G, Costa F. From bench to bedside: Fecal calprotectin in inflammatory bowel diseases clinical setting. *World J Gastroenterol* 2018; 24(33): 3681-3694 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3681.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3681

INTRODUCTION

Calprotectin is a 36 kDa calcium and zinc binding protein, which represent about 60% of soluble proteins of the cytoplasm of granulocytes[1]. It is heat and proteolysis resistant heterocomplex of S100A8 and S100A9 consisting of 2 heavy (14 kDa) e 1 light (8 kDa) chains, each binding 2 Ca^{2+}.

Functions of calprotectin include: competitive inhibition of zinc-dependent enzymes, potential biostatic activity against microbes through chelation of zinc ions, apoptosis induction in malignant cells, and regulation of the inflammatory process[2,3].

Fecal calprotectin (FC) is one of the most sensitive non-invasive marker in distinguishing inflammatory bowel diseases (IBD) from functional disorders. Several factors, however, may influence FC levels, such as colonic cleansing[4], age, diet, exercise[5], and the faecal amount of mucus or blood in stools[6].

A further limitation is a low specificity in discriminating ulcerative colitis (UC) from Crohn's disease (CD), active IBD from non-IBD intestinal inflammation (infections, non-steroidal anti-inflammatory drugs-related damage, cancer, diverticulitis). FC is a more sensitive marker than C-reactive protein (CRP) for detection of mild mucosal inflammation, although in severely active cases CRP better reflects systemic inflammation[7,8].

PITFALLS IN FC ASSESSMENT

Stability

Roseth, in 1992, demonstrated the stability of calprotectin in stools for up to 7 d at room temperature[9,10], which offers advantages for its use in clinical practice[11].

In a more recent study, however, calprotectin concentrations in stool samples were unchanged only for 3 d at room temperature, while after 7 d a significant decrease ($P < 0.01$) was found[12].

Variability

Day-to-day variation of FC was demonstrated by Husebay *et al*[13] in patients without colonic inflammation or neoplasm and confirmed by Moum *et al*[14], in patients with mild-to-moderate active CD, where significant differences in 63 pairs of stool samples collected in 2 consecutive days were found. A lower variability was observed in fecal samples collected for 3 d from 93 CD patients in clinical remission[15]. Dobrzanski *et al*[16] confirmed that variability seems to be relevant only in active IBD, particularly in UC where large amounts of mucus and blood are present in stools.

The most reliable results were provided by analyzing 3 in-wk samples from the first bowel movement in the morning[12]. Higher variability was found in patients with the highest levels of FC; further, the test results were influenced by the sample consistency and by the interval between the bowel movements, supposedly related to the accumulation of leukocyte-derived proteins in the gut lumen.

A good correlation was found between the FC concentrations assessed in two randomly different samples collected from the same bowel movement[12]. Calafat *et al*[6] did not find any influence of the timing of stools sampling, or the presence of blood on FC concentrations, in particular in patients with moderate-to-severe active UC, where the decision-making strategies based on single quantitative FC determinations are not advisable.

Methods of assessment

Different methods can be used for the quantitative assessment of FC, most of them based on the enzyme-linked immunosorbent assay (ELISA); chemiluminescence immunoassays (CLIA), fluoro enzyme immunoassays (FEIA) and particle enhanced turbidimetric immunoassays (PETIA) were also introduced.

Oyaert *et al*[17] compared six automated immunoassays: Thermo Fisher EliA Calprotectin assay on the Phadia 250 (Thermo Fisher Scientific, Uppsala, Sweden), Diasorin Calprotectin assay on the Liaison (Diasorin S.P.A., Saluggia, Italy), Inova QUANTA Flash Calprotectin (research use only) on the Inova BIO-FLASH instrument (Inova Diagnostics, San Diego, CA, United States), Bühlmann fCAL Turbo (Bühlmann Laboratories AG, Schönenbuch, Switzerland) on the Roche Cobas c501 (Roche Diagnostics, Mannheim, Germany), Euroimmun Calprotectin assay (Euroimmun; Lübeck, Germany), on an automated ELISA instrument (QUANTA-Lyser 2, Inova) and Orgentec Calprotectin assay on the Alegria (Orgentec Diagnostika, Mainz, Germany). The authors found that all assays had a sensitivity of 100% when the cut-off of the manufacturer was used (*i.e.*, 50 μg/g),

Table 1 Fecal calprotectin cut off values and performance in different populations

Ref.	Patients and disease (*n*)	Cut-off	Sensitivity (%)	Specificity (%)
Lin *et al*[23]	1471 IBD (active *vs* inactive)	50 μg/g	92	60
		100 μg/g	84	66
		150 μg/g	80	82
Limburg *et al*[24]	110 patients with chronic diarrhea (prediction of inflammation)	100 μg/g	83	83
Von Roon *et al*[25]	IBD *vs* no IBD			
	1267	50 μg/g	89	81
	328	100 μg/g	98	91
D'Haens *et al*[27]	126 IBD (large ulcers)	250 μg/g	60.4	79.5
	87 CD (Endoscopic remission) 39 UC (Active mucosal disease)	250 μg/g	94	62.2
		250 μg/g	71	100
Sipponen *et al*[29]	77 CD (active *vs* inactive)	50 μg/g	91	44
		100 μg/g	81	69
		200 μg/g	70	92
Kittanakom *et al*[21]	40 inactive pediatric CD (prediction of relapse)	400 μg/g (PhiCal Calprotectin - EIA) 500 μg/g (Buhlmann POCT)	100	75.9
		800 μg/g (EliA-Calprotectin)	100	75.9
			100	75.9
Vazquez Moron *et al*[31]	71 CD (active *vs* inactive)	170 μg/g	77.6	95.5

IBD: Inflammatory bowel diseases; UC: Ulcerative colitis; CD: Crohn's disease.

while the specificity at the same cut-off value ranged from 58.4% to 78.5%.

Furthermore, while qualitative correlation among the methods from the different manufacturers was found to be good, quantitative agreement was poor, which means that the result of one method cannot be replaced by the result of another. This data are in line with a study from the United Kingdom National External Quality Assessment Service, where up to 3.8-fold differences among methods from different manufacturers were observed[18]. This suggests that the antibodies used in the different assays were directed against different protein complexes. Alternatively, the difference could be explained by the use of different antibodies (monoclonal *vs* polyclonal) of different origins (recombinant *vs* native) with different immunoassay techniques (ELISA *vs* PETIA *vs* CLIA *vs* FEIA).

Further quantitative tests for calprotectin are available including the Quantum Blue® Calprotectin Rapid Test (Bühlmann Laboratories AG, Schönenbuch, Switzerland), which has been shown to be a suitable alternative to ELISA in a clinical setting[19], although an overestimation of FC levels in comparison with Calprest® ELISA test was found[20].

In pediatric IBD patients, an automated ELISA test (Bühlmann PhiCal Calprotectin-EIA) , an EliA (Phadia 250 EliA-Calprotectin), and Bühlmann immunochromatographic Point-of-Care Test (POCT) displayed similar performance in predicting relapse[21].

Cut-off

Although many studies have suggested different cut-off values, which take into account the type of assay used and the population that the tests were applied to (Table 1), a cut/off value of 50 μg/g of FC has been the most commonly adopted both in literature and by commercially available ELISA kits, for adults and children over 4 years to differentiate IBD from other forms of inflammation[22]. Moreover, Lin *et al*[23] suggested 50 μg/g as a screening cut-off value for further endoscopy examination in clinical practice, with specificity of 60% and pooled sensitivity of 92%.

A single cut-off level of 100 μg/g was agreed by an expert panel as appropriate for this purpose based on results from previous studies, which reported increased diagnostic precision for discrimination of colorectal inflammation in patients with CD and UC at this cut-off[24,25]; a higher cut-off level would be desirable to maximize the negative predictive value (NPV) and reduce incorrect diagnoses of IBD.

A negative test result at the lowest cut-off level (30 to 50 μg/g) suggests a diagnosis of a non-inflammatory condition, such as irritable bowel syndrome (IBS). A positive result at the cut-off of 100 μg/g may indicate IBS, with the recommendation to repeat the test in 6 wk to confirm the initial result[26].

As the cut-off value increases, sensitivity becomes lower and specificity higher. A FC value of 250 μg/g was deemed appropriate for monitoring disease activity in IBD. D'Haens *et al*[27] examinated 126 IBD patients (87 CD and 39 UC) and proposed a FC cut-off of 250 μg/g for indicating IBD remission.

The same cut-off was recommended by a recent meta-analysis[28] to contemplate escalating therapy with pooled sensitivity of 80% and specificity of 82%; in UC the test performed better than in CD.

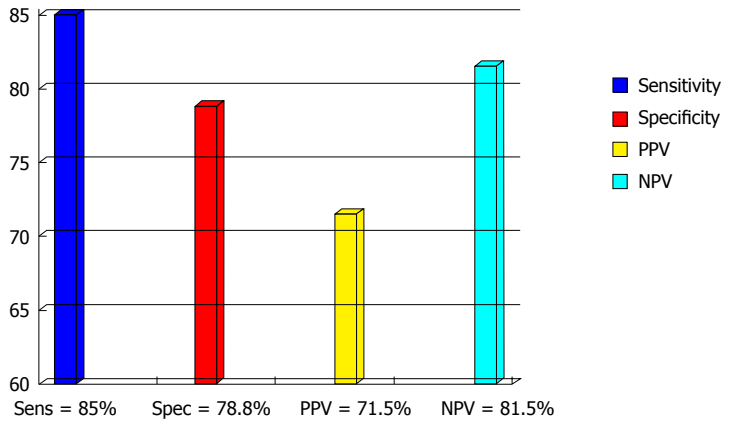


Figure 1 Pooled fecal calprotectin sensitivities, specificities, positive predictive value and negative predictive value of fecal calprotectin in discriminating between intestinal inflammation and functional disorders. PPV: Positive predictive value; NPV: Negative predictive value.

An earlier study by Sipponen *et al*[29] proposed a cut-off value of 200 μg/g for identification of endoscopically inactive CD. In another study of 115 CD patients, FC less than 300 μg/g was associated with a reduced risk of disease relapse[30].

Even a 400 μg/g cut-off was agreed by an expert panel with 1000 μg/g proposed for monitoring response to therapy in patients with severe active IBD[26].

In pediatric patients the optimal FC cut-offs to differentiate active from inactive IBD were 400, 500, and 800 μg/g measured by different methods (PhiCal Calprotectin-EIA, Bühlmann POCT, and EliA-Calprotectin)[21].

In CD, FC concentration of ≥ 170 μg/g predicted endoscopic activity with 77.6% sensitivity, 95.5% specificity and likelihood ratio +17.06, while values ≤ 71 μg/g were predictive of mucosal healing with sensitivity of 95.9%, specificity of 52.3% and likelihood ratio -0.08[31].

Larger prospective studies were suggested to be carried out to validate the cut-off values in clinical practice for UC, using endoscopy as a reference. Once established for UC, cut-off values for CD could then be developed and validated, although harder to establish due to the lack of consistent evidence. In addition, small bowel disease activity is reflected by FC correlates less reliably than in case of colonic involvement[32,33].

Age-related concerns

FC levels have a significant negative correlation with age[28]. Children in the first months of life have high FC concentrations, which could reflect an increased trans-epithelial migration of either granulocytes or newly recruited macrophages as well as inability to regulate the microbial gut flora related to immaturity of the mucosal barrier function[28]. The increase of intestinal permeability during the first weeks of life was suggested in healthy newborns with high FC levels[34]. The type of feeding also influences FC concentrations: breastfed infants have higher FC levels than non-breastfed ones in the first months of life, reflecting the influence of immunomodulatory factors in human milk on the gut mucosa[28].

A statistical difference was found between FC in healthy children aged 1-3 mo and those aged 3-6 mo (375.2 μg/g *vs* 217.9 μg/g, $P < 0.001$), as well as between 1-6 mo and 6-18 mo (median: 282.7 μg/g *vs* 114.9 μg/g; $P < 0.001$)[28]. The results clearly indicate that different cut-offs are necessary for children less than 4 years old. Oord *et al*[35] proposed 538 μg/g for 1-6 mo, 214 μg/g for 6 mo-3 years, and 75 μg/g for 3-4 years.

Finally, in newborns FC concentrations may increase up to 30% when the sample is collected from a diaper, which may be explained by the water absorption into the diaper[35].

FC IN DISCRIMINATING BETWEEN IBD AND IBS

The role of FC as a screening test to differentiate patients with IBD from IBS was firstly proposed by Tibble and co-workers[36,37] who demonstrated the high sensitivity and high NPV of the test. In a population of 602 unselected patients with intestinal symptoms, FC levels < 30 μg/g and Rome I criteria positivity were highly predictive for not having IBD[38]. Since then, a considerable body of literature has been published corroborating these results (Figure 1), yet with wide variation in reported sensitivity and specificity, which may be related to the use of different ELISA kits, different patients populations and different cut off values. Summerton and colleagues[39] reported sensitivity and specificity in line with Tibble, while Carroccio *et al*[40] in a prospective study carried out in adults and children with chronic diarrhea of unknown origin found similar specificity (84%), but lower sensitivity (66%), attributed by the authors to the high number among their referrals of possible celiac patients, where FC levels are usually low. A major problem is represented by the assessment of the optimal threshold, as many authors used only the manifacturer's recommended

cut offs, mostly 50 μg/g. In the first study carried out on southern European patients by our group[41], the ROC curve showed that a value of 60 μg/g offered a diagnostic accuracy of 83% with sensitivity, specificity, positive predictive value (PPV) and NPV of 81%, 88%, 93% and 71% respectively. Li *et al*[42] found median FC concentrations of 466 μg/g in patients with chronic inflammation, 159 μg/g in colorectal cancer and 12.21 μg/g, not statistically different from healthy subjects and IBS patients. In a prospective study[8] where the accuracy of fecal markers, CRP, blood leucocytes, pASCA and pANCA for differentiating IBD from IBS was assessed, fecal tests performed best with overall accuracy of 90% and 89% for fecal lactoferrin (FL) and FC respectively. These results were confirmed by Otten *et al*[43], who found sensitivity and NPV of 100% for FC and 78% and 95% for FL, and by Langhorst *et al*[44] who reported in active UC significantly higher FL and FC levels (152 and 103.5 μg/g respectively) in comparison with IBS (8.3 and 18.6 μg/g)

In the first study which assessed the role of FC in routine general practice in 962 patients with persistent gastrointestinal symptoms[45], at the manufacturer's cut-off of 50 μg/g the NPV was 98% while PPV dropped to a disappointing 28%, showing the impact of evaluating a population with a low prevalence of organic disease on the test performance. Increasing the cut off to 150 μg/g the PPV raised to 71%, saving an acceptable 69% sensitivity. In the systematic review by Waugh *et al*[46] evaluating FC testing for distinguishing between inflammatory and non-inflammatory bowel diseases, 28 studies in both adult and pediatric populations where included; at a cut off level of 50 μg/g, FC showed in adults a pooled sensitivity of 93% (83%-100%), while the specificity ranged between 60% and 100% with a pooled value of 94%. In pediatric patients, at the same cut off sensitivity and specificity ranged from 95% to 100% and from 44% to 93% respectively ; for overlap values between 50 μg/g and 150 μg/g repeated assessments were suggested; point-of-care and ELISA testing proved equally reliable and in a primary care setting FC turned out to reduce the number of referrals and endoscopies.

The potential of FC to discriminate between intestinal inflammation and functional disorders has been highlighted by a large number of further studies. Chang *et al*[47]confirmed significantly higher FC values in IBD than in IBS and healthy controls ($P < 0.0001$). Using the manufacturer cut off value Caviglia *et al*[48] found 100% sensitivity and NPV (with corresponding specificity and PPV 52.4% and 70.6%) for discriminating between patients with and without intestinal inflammation; similar results were reported by Banerjee *et al*[49]. In a systematic online database search, at ≤ 40 μg/g, less than 1% probability of having IBD was reported[50]. Lower values (76% sensitivity and 53% NPP) where reported by Fu *et al*[51] in a comparative study among fecal B cell-activating factor, FC and fecal occult blood test.

Adopting a cut off value > 164 μg/g Kalantari *et al*[52] found sensitivity and specificity of 57% and 75% respectively for discrimination between UC and IBS.

In conclusion, FC is currently the most widely used fecal marker for differentiating between IBD and IBS; due to the high NPV it is highly accurate in ruling out intestinal inflammation both in primary and secondary care. Among IBD patients apparently in remission with IBS-like symptoms, FC tends to be significantly higher than in IBS, suggesting the presence of an undercurrent low-grade inflammation[53,54].

FC IN IBD

Monitoring the disease activity

As IBD are chronic relapsing diseases, regular monitoring is needed for prediction of imminent flares and for tailoring treatment[55]; it includes clinical, biochemical, endoscopic and histologic evaluations.

Many physicians treating IBD still adopt a clinically-based management[56], even if recent data suggest that many IBD patients in clinical remission still have subclinical mucosal inflammation[57]. FC is correlated with clinical activity evaluated either by Sutherland criteria[58] or Partial Mayo Score[59]. In a study by Xiang *et al*[58], FC concentrations were useful to discriminate patients with active UC, inactive UC and control, with a cut-off point of 50 μg/g showing 91.9% sensitivity and 79.4% specificity; in patients with UC, FC had a better correlation with clinical activity than CRP. Moreover, in a prospective study, FC assessment after 3 mo of the initial treatment could predict the clinical course of UC patients after 3 years of follow up[60].

Although colonoscopy is considered the gold standard to assess disease activity, current ECCO guidelines emphasize that routine endoscopy for IBD patients in clinical remission is unnecessary, unless it is likely to change patient management[61]. Therefore, a marker reflecting intestinal inflammation in patients in clinical remission is needed. Many studies showed that FC is the most promising noninvasive marker for assessing mucosal inflammation. In a study by D'Haens *et al*[27] FC had a significant correlation with endoscopic disease scores in both CD and in UC: a cut-off value of 250 μg/g suggested the presence of large ulcers with sensitivity of 60.4%, specificity of 79.5%, PPV 78.4% and NPV 62.0%) in CD, while in UC, a FC > 250 μg/g gave a sensitivity of 71.0% and a specificity of 100.0% (PPV 100.0%, NPV 47.1%) for mucosal disease activity (Mayo > 0). In UC, FC levels reflect the degree of inflammation rather than the disease extent[10].

Interestingly, FC were significantly related to symptom scores in UC ($r = 0.561$, $P < 0.001$), but not in CD. A study by Theede *et al*[62] found a strong correlation both with Mayo Endoscopic Score and Ulcerative Colitis Endoscopic Score. A correlation with Rachmilewitz and modified Baron Score was also

demonstrated. Schoepfer and colleagues[63,64] found that FC was the only marker able to discriminate among mild, moderate and severe disease. A recent Korean study[59] highlighted how not only the ELISA, but also the Quantitative POCT predicted endoscopic inflammation (Mayo endoscopic score ≥ 1) in UC at a cut-off value of 201.3 μg/g and 150.5 μg/g respectively. In CD, a significant correlation with endoscopic activity was found both in colonic[33] and in small bowel CD[36], as well as with capsule endoscopy[65].

In a prospective study on 58 pediatric patients[66] FC showed a high correlation both with endoscopy (r = 0.655) and histology grading (r = 0.699); it proved the most accurate tool (sensitivity 94%, specificity 64%, PPV 81%, NPV 87%) to detect active mucosal inflammation when compared to clinical scores and serum markers. The highest accuracy was found in patients with apparent clinical and laboratory remission (sensitivity 100%, specificity 80%, PPV 67%, NPV 100%).

Guardiola *et al*[67] prospectively evaluated UC patients in clinical and endoscopic remission; those with histologic features of inflammation were reliably identified based on their FC levels at a cut off of 155 μg/g with a sensitivity of 78% and a specificity of 71%. More recently, Zittan *et al*[68] confirmed that FC could predict histological remission, with a cut-off of 100 μg/g. A recent study comparing the predictive value of FC measurement and histological scoring in IBD patients, found that FC performed better, especially in UC[69]. This finding was confirmed in a subsequent study by Theede *et al*[70], who showed that in UC baseline FC more than 321 μg/g predicted relapse both at 6-mo and 12-mo in contrast to histological activity, CRP, or length of remission. A study by Puolanne *et al*[71] confirmed the correlation between FC, clinical activity, and histopatologic findings in 72 patients with colonic IBD.

In an English study[72], calprotectin concentration in the colonic mucosa of UC patients correlated with histological remission; moreover, a median value > 5/HPF were independently associated with worse outcome (corticosteroid use, hospitalisation, or colectomy during a 6-year follow-up). Moreover, in a short report by Roseth *et al*[73], low FC levels were closely associated with mucosal healing.

Predicting disease relapse

A major challenge in managing IBD is a timely detection of patients at risk for impending clinical relapse. Tibble et al. firstly suggested that a high FC concentration could identify those IBD patients in remission who were at risk of early relapse without any difference between UC and CD[74]. Conversely, we showed a 14-fold increase in the relapse risk in patients with UC and a two-fold increase in CD patients in clinical remission with FC concentration higher than 150 μg/g concluding that FC was a stronger predictor of clinical relapse in UC than in CD[32]. In CD, D'Incà *et al*[33] found a significant correlation between a positive FC test and probability of relapse (P < 0.001) only for colonic localization at 130 μg/g cut-off level. On the other hand, a meta-analysis by Mao *et al*[3] was not able to demonstrate that the overall accuracy of FC for predicting relapse was different in UC and CD because of the heterogeneity across studies, due to different criteria to define remission and relapse; however, due to the limited data, the ileal involvement was not assessed.

In asymptomatic patients with IBD, Heida *et al*[75] found that increase of FC levels were correlated with increased (from 53% to 83%) probability of relapse within the next 2 mo to 3 mo, while consecutive normal FC values were associated with 67% to 94% probability of remission in the next 2 mo to 3 mo.

In patients under maintenance therapy with Infliximab (IFX), levels > 160 μg/g were related to probability of relapse higher than 60% over the following 8 wk[76].

In a subanalysis of the STORI trial, serial measurements of FC in CD patients in clinical remission after stopping IFX, showed that in those who relapsed, the FC levels had started to increase 4-6 mo earlier[77]. Despite the test reliability, the ideal FC threshold for monitoring disease relapse is still awaiting to be defined.

Monitoring the therapy effectiveness

In clinical practice, "Treat-To-Target" is currently considered the most important strategy for therapy adjustment.

A study by Wagner *et al*[78] in patients with UC or CD treated with 5-aminosalicilic acid, prednisone or Azathioprine, showed that FC were correlated with clinical scores after 4 wk and 8 wk of treatment in UC and in CD, respectively, and in patients with complete response to therapy there was a significant decline in FC levels (P < 0.01) after 4 wk, which was not observed in partial or non-responders. In children with active disease treated with steroids, FC levels declined in line with clinical improvement but seldom fell within the normal range[79].

In the biologic era, many studies confirmed the role of FC in monitoring the effectiveness of therapy. Molander *et al*[80] demonstrated that a normal FC (< 100 μg/g) after induction therapy with anti-TNFα predicts sustained clinical remission in the majority of patients, both in CD and UC; a cut-off of 139 μg/g for FC had 72% sensitivity and 80% specificity to predict the risk of clinically active disease after 1 year. According to De Vos *et al*[81] two consecutive FC measurements over 300 μg/g are more specific than a single assessment for predicting relapse in UC patients under maintenance treatment with IFX.

Interestingly, even after discontinuation of anti-TNFα therapy, an increase of FC could predict clinical and endoscopic relapse[82]. This data is in accordance with the STORI study[30,77], where FC was comparable to endoscopic assessment in predicting the relapse

risk after stopping TNFα-blocking therapy, starting to increase 4-6 mo before the clinical relapse. A prospective study[83] in IBD patients (20 UC and 52 CD) under treatment with anti-TNFα, showed that the diagnostic accuracy of rapid FC seems to be higher in predicting persistence of endoscopic lesions than clinical remission.

Both in monitoring of therapy and in prediction of relapses FC seems to be more effective in UC than in CD[32]. Nevertheless, in a prospective study of Laharie *et al*[84] in patients responding to IFX induction regimen, FC measurement at w14 could not predict CD clinical relapse at one year. In severe acute colitis, FC evaluation could be helpful in timely prediction of clinical course: Ho et colleagues[85] demonstrated that FC was higher in patients requiring colectomy with a trend toward significance when compared to responders, suggesting that FC in patients with severe acute colitis could be included among the prognostic criteria.

Shifting the therapeutic target from clinical remission to mucosal healing has been supported by population-based cohort studies, post hoc analysis of clinical trials, and meta-analysis, both for CD and UC[86-90]. The STRIDE recommendations[91] defined FC as an adjunctive target in IBD patients, while a Mayo Endoscopic Score ≤ 1 for UC and the resolution of ulcerations in CD are the best target to reach, besides patient reported outcome. The recently published CALM study[92], for the first time used FC as a target despite clinical activity in CD patients, in whom therapy was escalated if FC was ≥ 250 μg/g in a group of patients, while the control group was treated on the basis of clinical activity. The tight control algorithm led to rapid optimization of therapy and, therefore, to a higher proportion of patients achieving mucosal healing [CD Endoscopic Activity Index of Severity (CDEIS) < 4] and no deep ulcers on endoscopy, deep remission [CD Activity Index (CDAI) < 150 and CDEIS < 4 and no deep ulcers, no draining fistula, and no prednisone use for 8 wk or more], biological remission (FC < 250 μg/g, CRP < 5 mg/L, and CDEIS < 4), and steroid-free remission (CDAI < 150 with no prednisone for 8 wk). A limitation is represented by the discretional taper schedule of prednisone at study entry, that, affecting the treatment option at randomization (the use of prednisone defined treatment failure in the tight control group) could have led to an earlier introduction of adalimumab and positively affected the outcomes.

Monitoring the post-operative recurrence

Despite the increasing use of immunosuppressants and biologics, IBD patients frequently need surgery. Approximately 80% of CD patients require intestinal surgery within 20 years after diagnosis and 10%-30% UC patients need colectomy, at 25 years following diagnosis[93]. Surgery is not curative, and is followed by post-operative recurrence (POR, in CD patients) and pouchitis (in UC patients) in a high percentage of cases. The post-operative monitoring, mainly based on endoscopy, is crucial to identify those patients who require early treatment. Non-invasive markers of intestinal inflammation, especially FC, represent an easy, quick and cheap tool for the early diagnosis of post-operative recurrence or pouchitis.

Post-operative recurrence: POR after ileo-colonic resection is a feature of CD. Early studies by the Leuven group reported an endoscopic and histological recurrence rate of 73% within one year from surgery although only 20% of the patients had symptoms[94]. A more recent review, focusing on historical population-based studies, showed that the cumulative risk of POR after 10 years is around 44%-55%[95]. As endoscopic recurrence occurs before the onset of symptoms[94], the early detection of asymptomatic endoscopic lesions may allow a timely treatment in post-operative CD patients. Conventional ileocolonscopy within 6-12 mo is currently recommended to evaluate CD recurrence, graded according to the Rutgeerts' score. The Post-Operative Crohn's Endoscopic Recurrence (POCER) study showed that postoperative endoscopic monitoring, together with treatment escalation for early recurrence, is superior to standard drug therapy alone in preventing disease recurrence, at least in the short term[96]. However, it is not established the timing of endoscopic re-evaluation. Ileocolonoscopy is expensive, time-consuming, often not well accepted by the patient and not devoid of risks. Moreover, endoscopic examination of the neo-terminal ileum is not always technically feasible[97].

Although the role of FC in early detection of POR is still to be established, several studies suggest that FC could avoid unnecessary endoscopies and facilitate earlier diagnosis. FC and FL assay have been suggested as non-invasive, inexpensive and reproducible biomarkers in post-operative CD patients[98].

Orlando *et al*[99] prospectively evaluated 50 CD patients who had undergone surgery; a FC value > 200 μg/g within 3 mo showed 63% sensitivity and 75% specificity in predicting endoscopic recurrence at one year, superior to ultrasound, whose sensitivity and specificity was 26% and 90% respectively.

In asymptomatic CD patients who had undergone ileo-colonic resection with a median follow-up of 40.5 mo, long term high levels of FL and FC were observed, interpreted as sign of ongoing intestinal inflammation, although partially influenced by the systemic post- operative inflammatory status[100].

In a small cohort of 13 post-operative CD patients followed for 1 year, FC and FL were more accurate in predicting clinical disease activity than CRP, platelet count or endoscopic appearance[101]. Accordingly, FC and FL levels positively correlated with both clinical recurrence and severity of endoscopic findings in the neo-terminal ileum who remained in remission during 6-12 mo after

ileocolonic resection[102]. At 170 μg/g cut-off, sensitivity and specificity of FC were higher than FL (83% and 93% *vs* 67% and 71% respectively) in predicting risk of clinical relapse. More recently, the same authors showed that in asymptomatic patients after ileo-colonic resection for CD, sustained low FC levels predict low risk of endoscopic recurrence, avoiding unnecessary endoscopic examinations[103].

These data are in line with Boschetti *et al*[104], who found were significantly higher (473 ± 78 μg/g) FC levels in asymptomatic CD patients with endoscopic recurrence after ileo-colonic resection in the last 18 mo when compared with those in remission (115 ± 18 μg/g, $P < 0.0001$). Sensitivity analysis excluding patients with both ileal and colonic recurrence did not change the results (456 ± 68 μg/g *vs* 115 ± 18 μg/g; $P < 0.0002$). The best cutoff point for FC to distinguish between endoscopic remission and recurrence was 100 μg/g as determined by the ROC curve, and its sensitivity, specificity, PPV and NPV, as well as overall accuracy were 95%, 54%, 69%, 93%, and 77%, respectively. Taking into account the high NPV of FC, a threshold below 100 μg/g could avoid systematic ileocolonoscopies in 30% of patients.

In the retrospective study by Herranz Bachiller *et al*[105] 97 patients with CD and ileocolic resection who had undergone FC measurement and subsequent ileo-colonoscopy were included. FC was related to endoscopic recurrence more than any clinical or serological parameters. Unlike other studies, the optimal cut-off was 60 μg/g.

Lobatón *et al*[106], compared the accuracy of ELISA test with the new quantitative POCT for the prediction of endoscopic activity and POR in CD patients. FC levels correlated more closely with CDEIS than leucocytes, platelets or CRP. The prediction of endoscopic remission (CDEIS < 3), using the quantitative POCT (cut-off 272 μg/g) and the ELISA (cut-off 274 μg/g) presented an area under the curve of 0.933 and 0.935, respectively. Median POCT levels discriminated endoscopic (Rutgeerts) score i0-i1 from i2-i4 (98 μg/g *vs* 234.5 μg/g). These results suggest that FC determined by rapid quantitative test predicts endoscopic remission as well as endoscopic postoperative recurrence in CD patients.

Disappointing results came from a Swedish study[107], that found no significant difference in FC concentrations between patients in endoscopic remission and relapsing patients one year after ileoceacal resection. However, the significant variation over time of FC concentrations highly influenced these results, especially in patients with diarrhea, which implies that a single measurement of FC has limited clinical utility in predicting POR.

The sub-analysis of the POCER study by Wright *et al*[108], demonstrated that FC has good sensitivity and NPV to monitor CD recurrence after intestinal resection. Levels of FC were measured in 319 samples from 135 patients. FC concentration was markedly increased before surgery and decreased substantially after resection of all macroscopically involved segments at 6 mo. Combined 6- and 18-mo FC levels correlated significantly with endoscopic recurrence, whereas CRP and CDAI did not. A cutoff of FC > 100 μg/g detected patients with endoscopic recurrence with 89% sensitivity, 58% specificity and 91% NPV. In this cohort, colonoscopy could be avoided in 47% of cases with endoscopic remission, at the cost of missing 11% of patients with endoscopic recurrence. Also, FC decreased in patients who underwent therapy intensification supporting its role in treatment monitoring. A FC level < 51 μg/g in patients in remission at 6 mo after surgery predicted remission at 18 mo, with 79% NPV; sensitivity, specificity and PPV were less satisfying (50%, 68% and 36%, respectively), suggesting a limited value of FC measurement in long-term prediction of endoscopic recurrence.

Large scale studies should be carried out to clarify controversial points. The optimal cut-off value of FC as a surrogate marker of POR needs to be established and the measurement procedures to be standardized. Nevertheless, our overview suggests the use of FC as promising alternative to ileo-colonoscopy in POR , especially in asymptomatic CD patients after initial negative post-operative endoscopy, and in monitoring response to treatment.

Pouchitis: Ileal pouch anal anastomosis (IPAA) after restorative proctocolectomy is currently the preferred surgical treatment for refractory or complicated UC. *De novo* inflammation of the ileal reservoir, the so-called pouchitis, is reported in about half of the patients. Even though the etiology of pouchitis remains unknown, several influencing factors have been suggested, such as fecal stasis, bacterial overgrowth, dysbiosis, genetic susceptibility and immune alteration. More recently, a CD-like complication of the pouch, has been described which can involve up to 13% of the patients following proctocolectomy with IPAA for UC. This entity is characterized by inflammation in the afferent limb (prepouch ileitis), presence of proximal small bowel strictures, or perianal or internal fistulae unrelated to surgery[109].

In 1994, the pouchitis disease activity index (PDAI), a composite score evaluating symptoms, endoscopic and histologic alteration has been developed to standardize the definition of pouchitis and to assess its severity. Patients with a total PDAI score of ≥ 7 points are classified as having pouchitis. The diagnosis of pouchitis therefore requires endoscopic confirmation with mucosal biopsies. Few studies have evaluated the value of FC measurement in these patients. However, available data show possible benefit with accurate diagnosis and management of pouch disorders as well as cost reduction.

In the small study by Thomas *et al*[110], significantly

increased FC levels were found in all 9 patients with endoscopic and histologic evidence of pouch inflammation compared with those without it. The first-morning FC levels correlated well (r = 0.91, $P \leq 0.0001$) with 24-h stool collection, with endoscopic and histologic scores, and with the percentage of CD15+ mature neutrophils and CD14+ macrophages within the lamina propria.

These findings were confirmed in a larger study carried out in 46 patients with UC and in 8 with familial adenomatous polyposis, who had undergone restorative proctocolectomy[111]. Using a threshold of 92.5 μg/g, FC levels correlated closely with the PDAI with a sensitivity of 90% and a specificity of 76.5%.

In pediatric UC, FC levels after restorative proctocolectomy positively correlated with subsequent pouchitis (r = 0.468, $P < 0.01$), with mean FC values of 71.50 μg/g among patients with no history of pouchitis, 290 ± 131 μg/g among those with a single episode of pouchitis, and highest level 832 ± 422 μg/g among patients with recurrent pouchitis (P = 0.019 between recurrent pouchitis and no pouchitis). A history of recurrent pouchitis was a significant predictor of FC higher than 300 μg/g (OR = 51; 95%CI: 1.2-2200; P = 0.040). Sensitivity, specificity, PPV, and NPV for FC concentration over 300 μg/g in detecting recurrent pouchitis were 57%, 92%, 67%, and 89%, respectively[112].

Yamamoto *et al*[113] prospectively evaluated the serial monitoring of FC and FL for the early detection of pouchitis after restorative proctocolectomy. Stool samples were collected every 2 mo up to 12 mo from 60 patients who had undergone ileostomy closure following total proctocolectomy and IPAA for UC. Endoscopy was performed in all asymptomatic patients at 12 mo and as soon as symptoms suggestive of pouchitis occurred. In the 10 patients (17%) who developed pouchitis FC and FL levels were already increased 2 mo before the diagnosis of pouchitis, while in the others both markers remained constantly at low levels. At cut-off values of 56 μg/g for FC and 50 μg/g for FL, sensitivity and specificity were 100% and 84%, and 90% and 86% respectively. At the time of endoscopy, the median FC and FL levels were significantly higher in patients with pouchitis than those without. Nevertheless, several questions can be raised on how to implement these findings into clinical practice. Current guidelines do not recommend routine pouchoscopy in patients in clinical remission as symptoms seem to reflect underlying inflammation in the pouch[114]. The results by Yamamoto *et al*[113] are in line with these recommendation. None of the 47 asymptomatic patients developed pouchitis during the 12-mo follow-up period, whereas in 10/13 symptomatic patients the inflammation of the pouch was confirmed. Thus, the NPV of 100% of the PDAI score < 7 could be considered as referral criteria for pouchoscopy in symptomatic patients[115].

In conclusion, even in patients with IPAA FC could allow the early detection of subclinical inflammation. Prospective studies need to establish whether this strategy could reduce the rate of chronic pouchitis and subsequent pouch failure.

CONCLUSION AND PERSPECTIVES

We reviewed the role of FC in various settings of IBD clinical management. About 20 years after the study by Roseth[10], FC has been confirmed as one of the most reliable, non-invasive diagnostic tools for management of IBD in clinical practice both in adults and children.

A considerable body of evidence confirms the high sensitivity and NPV of FC in distinguishing IBD from IBS in patients with clinical suspicion of intestinal inflammation. In those with established diagnosis of IBD, a growing number of studies suggest an increasingly recognized role of the test in monitoring disease activity and response to therapy, as well as in predicting disease relapse and POR, including pouchitis. The main concerns are still related to the choice of the optimal cut-off, both for ruling out intestinal inflammation and for the management of IBD patients.

Recently the CALM study[92] included FC measurement among the treatment failure criteria for escalating therapy in patients with early CD, and showed that adjustment of therapy based on the combination of clinical symptoms and biomarkers leads to better outcomes than symptoms-driven decision. These results support the use of FC in the context of the "Treat-To-Target" strategy and may open the way for a higher standard of care in IBD patients, if confirmed by further studies with a longer follow up. Finally, similarly designed studies are awaited in UC, where FC appears to perform best.

REFERENCES

1 **Rodrigo L**. [Fecal calprotectin]. *Rev Esp Enferm Dig* 2007; **99**: 683-688 [PMID: 18290690]

2 **Dale I**, Fagerhol MK, Naesgaard I. Purification and partial characterization of a highly immunogenic human leukocyte protein, the L1 antigen. *Eur J Biochem* 1983; **134**: 1-6 [PMID: 6861753]

3 **Mao R**, Xiao YL, Gao X, Chen BL, He Y, Yang L, Hu PJ, Chen MH. Fecal calprotectin in predicting relapse of inflammatory bowel diseases: a meta-analysis of prospective studies. *Inflamm Bowel Dis* 2012; **18**: 1894-1899 [PMID: 22238138 DOI: 10.1002/ibd.22861]

4 **Kolho KL**, Alfthan H, Hämäläinen E. Effect of bowel cleansing for colonoscopy on fecal calprotectin levels in pediatric patients. *J Pediatr Gastroenterol Nutr* 2012; **55**: 751-753 [PMID: 22614113 DOI: 10.1097/MPG.0b013e31825f4c77]

5 **Poullis A**, Foster R, Shetty A, Fagerhol MK, Mendall MA. Bowel inflammation as measured by fecal calprotectin: a link between lifestyle factors and colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 279-284 [PMID: 14973103]

6 **Calafat M**, Cabré E, Mañosa M, Lobatón T, Marín L, Domènech E. High within-day variability of fecal calprotectin levels in patients with active ulcerative colitis: what is the best timing for stool sampling? *Inflamm Bowel Dis* 2015; **21**: 1072-1076 [PMID: 25793326 DOI: 10.1097/mib.0000000000000349]

7 **Ricanek P**, Brackmann S, Perminow G, Lyckander LG, Sponheim

J, Holme O, Høie O, Rydning A, Vatn MH; IBSEN II Study Group. Evaluation of disease activity in IBD at the time of diagnosis by the use of clinical, biochemical, and fecal markers. *Scand J Gastroenterol* 2011; **46**: 1081-1091 [PMID: 21619483 DOI: 10.3109/00365521.2011.584897]

8 **Schoepfer AM**, Trummler M, Seeholzer P, Seibold-Schmid B, Seibold F. Discriminating IBD from IBS: comparison of the test performance of fecal markers, blood leukocytes, CRP, and IBD antibodies. *Inflamm Bowel Dis* 2008; **14**: 32-39 [PMID: 17924558 DOI: 10.1002/ibd.20275]

9 **Røseth AG**, Fagerhol MK, Aadland E, Schjønsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. *Scand J Gastroenterol* 1992; **27**: 793-798 [PMID: 1411288]

10 **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]

11 **Joishy M**, Davies I, Ahmed M, Wassel J, Davies K, Sayers A, Jenkins H. Fecal calprotectin and lactoferrin as noninvasive markers of pediatric inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2009; **48**: 48-54 [PMID: 19172123 DOI: 10.1097/MPG.0b013e31816533d3]

12 **Lasson A**, Stotzer PO, Öhman L, Isaksson S, Sapnara M, Strid H. The intra-individual variability of faecal calprotectin: a prospective study in patients with active ulcerative colitis. *J Crohns Colitis* 2015; **9**: 26-32 [PMID: 25008478 DOI: 10.1016/j.crohns.2014.06.002]

13 **Husebye E**, Tøn H, Johne B. Biological variability of fecal calprotectin in patients referred for colonoscopy without colonic inflammation or neoplasm. *Am J Gastroenterol* 2001; **96**: 2683-2687 [PMID: 11569695 DOI: 10.1111/j.1572-0241.2001.04126.x]

14 **Moum B**, Jahnsen J, Bernklev T. Fecal calprotectin variability in Crohn's disease. *Inflamm Bowel Dis* 2010; **16**: 1091-1092 [PMID: 19834972 DOI: 10.1002/ibd.21136]

15 **Naismith GD**, Smith LA, Barry SJ, Munro JI, Laird S, Rankin K, Morris AJ, Winter JW, Gaya DR. A prospective single-centre evaluation of the intra-individual variability of faecal calprotectin in quiescent Crohn's disease. *Aliment Pharmacol Ther* 2013; **37**: 613-621 [PMID: 23347334 DOI: 10.1111/apt.12221]

16 **Dobrzanski C**, Pedersen N, Fuglsang H, Munkholm P. Is there a diurnal variation in faecal calprotectin in inflammatory bowel disease patients? *United European Gastroenterol J* 2013; **1**: A135-A587 [PMID: 4070603 DOI: 10.1177/2050640613502900]

17 **Oyaert M**, Boel A, Jacobs J, Van den Bremt S, De Sloovere M, Vanpoucke H, Van Hoovels L. Analytical performance and diagnostic accuracy of six different faecal calprotectin assays in inflammatory bowel disease. *Clin Chem Lab Med* 2017; **55**: 1564-1573 [PMID: 28222018 DOI: 10.1515/cclm-2016-1012]

18 **Whitehead SJ**, French J, Brookes MJ, Ford C, Gama R. Between-assay variability of faecal calprotectin enzyme-linked immunosorbent assay kits. *Ann Clin Biochem* 2013; **50**: 53-61 [PMID: 23129721 DOI: 10.1258/acb.2012.011272]

19 **Coorevits L**, Baert FJ, Vanpoucke HJ. Faecal calprotectin: comparative study of the Quantum Blue rapid test and an established ELISA method. *Clin Chem Lab Med* 2013; **51**: 825-831 [PMID: 23001318 DOI: 10.1515/cclm-2012-0386]

20 **Caviglia GP**, Ribaldone DG, Rosso C, Saracco GM, Astegiano M, Pellicano R. Fecal calprotectin: beyond intestinal organic diseases. *Panminerva Med* 2018; **60**: 29-34 [PMID: 29370679 DOI: 10.23736/S0031-0808.18.03405-5]

21 **Kittanakom S**, Shajib MS, Garvie K, Turner J, Brooks D, Odeh S, Issenman R, Chetty VT, Macri J, Khan WI. Comparison of Fecal Calprotectin Methods for Predicting Relapse of Pediatric Inflammatory Bowel Disease. *Can J Gastroenterol Hepatol* 2017; **2017**: 1450970 [PMID: 28491862 DOI: 10.1155/2017/1450970]

22 **Gisbert JP**, McNicholl AG. Questions and answers on the role of faecal calprotectin as a biological marker in inflammatory bowel disease. *Dig Liver Dis* 2009; **41**: 56-66 [PMID: 18602356 DOI: 10.1016/j.dld.2008.05.008]

23 **Lin JF**, Chen JM, Zuo JH, Yu A, Xiao ZJ, Deng FH, Nie B, Jiang B. Meta-analysis: fecal calprotectin for assessment of inflammatory bowel disease activity. *Inflamm Bowel Dis* 2014; **20**: 1407-1415 [PMID: 24983982 DOI: 10.1097/mib.0000000000000057]

24 **Limburg PJ**, Ahlquist DA, Sandborn WJ, Mahoney DW, Devens ME, Harrington JJ, Zinsmeister AR. Fecal calprotectin levels predict colorectal inflammation among patients with chronic diarrhea referred for colonoscopy. *Am J Gastroenterol* 2000; **95**: 2831-2837 [PMID: 11051356 DOI: 10.1111/j.1572-0241.2000.03194.x]

25 **von Roon AC**, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, Paraskeva P, Tekkis PP. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* 2007; **102**: 803-813 [PMID: 17324124 DOI: 10.1111/j.1572-0241.2007.01126.x]

26 **Rogler G**, Aldeguer X, Kruis W, Lasson A, Mittmann U, Nally K, Peyrin-Biroulet L, Schoepfer A, Vatn M, Vavricka S, Logan R. Concept for a rapid point-of-care calprotectin diagnostic test for diagnosis and disease activity monitoring in patients with inflammatory bowel disease: expert clinical opinion. *J Crohns Colitis* 2013; **7**: 670-677 [PMID: 23517932 DOI: 10.1016/j.crohns.2013.02.014]

27 **D'Haens G**, Ferrante M, Vermeire S, Baert F, Noman M, Moortgat L, Geens P, Iwens D, Aerden I, Van Assche G, Van Olmen G, Rutgeerts P. Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 2218-2224 [PMID: 22344983 DOI: 10.1002/ibd.22917]

28 **Li F**, Ma J, Geng S, Wang J, Liu J, Zhang J, Sheng X. Fecal calprotectin concentrations in healthy children aged 1-18 months. *PLoS One* 2015; **10**: e0119574 [PMID: 25742018 DOI: 10.1371/journal.pone.0119574]

29 **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]

30 **Louis E**, Mary JY, Vernier-Massouille G, Grimaud JC, Bouhnik Y, Laharie D, Dupas JL, Pillant H, Picon L, Veyrac M, Flamant M, Savoye G, Jian R, Devos M, Porcher R, Paintaud G, Piver E, Colombel JF, Lemann M; Groupe D'etudes Thérapeutiques Des Affections Inflammatoires Digestives. Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab therapy is stopped. *Gastroenterology* 2012; **142**: 63-70.e5; quiz e31 [PMID: 21945953 DOI: 10.1053/j.gastro.2011.09.034]

31 **Vázquez Morón JM**, Pallarés Manrique H, Machancoses FH, Ramos Lora M, Ruiz Frutos C. Accurate cut-offs for predicting endoscopic activity and mucosal healing in Crohn's disease with fecal calprotectin. *Rev Esp Enferm Dig* 2017; **109**: 130-136 [PMID: 28071062 DOI: 10.17235/reed.2017.4542/2016]

32 **Costa F**, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005; **54**: 364-368 [PMID: 15710984 DOI: 10.1136/gut.2004.043406]

33 **D'Incà R**, Dal Pont E, Di Leo V, Benazzato L, Martinato M, Lamboglia F, Oliva L, Sturniolo GC. Can calprotectin predict relapse risk in inflammatory bowel disease? *Am J Gastroenterol* 2008; **103**: 2007-2014 [PMID: 18802997]

34 **Campeotto F**, Butel MJ, Kalach N, Derrieux S, Aubert-Jacquin C, Barbot L, Francoual C, Dupont C, Kapel N. High faecal calprotectin concentrations in newborn infants. *Arch Dis Child Fetal Neonatal Ed* 2004; **89**: F353-F355 [PMID: 15210674 DOI: 10.1136/adc.2002.022368]

35 **Oord T**, Hornung N. Fecal calprotectin in healthy children. *Scand J Clin Lab Invest* 2014; **74**: 254-258 [PMID: 24568692 DOI: 10.3109/00365513.2013.879732]

36 **Tibble J**, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S, Foster R, Sherwood R, Fagerhol M, Bjarnason I. A

simple method for assessing intestinal inflammation in Crohn's disease. *Gut* 2000; **47**: 506-513 [PMID: 10986210]
37 **Tibble JA**, Bjarnason I. Non-invasive investigation of inflammatory bowel disease. *World J Gastroenterol* 2001; **7**: 460-465 [PMID: 11819811]
38 **Tibble JA**, Sigthorsson G, Foster R, Forgacs I, Bjarnason I. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease. *Gastroenterology* 2002; **123**: 450-460 [PMID: 12145798]
39 **Summerton CB**, Longlands MG, Wiener K, Shreeve DR. Faecal calprotectin: a marker of inflammation throughout the intestinal tract. *Eur J Gastroenterol Hepatol* 2002; **14**: 841-845 [PMID: 12172403]
40 **Carroccio A**, Iacono G, Cottone M, Di Prima L, Cartabellotta F, Cavataio F, Scalici C, Montalto G, Di Fede G, Rini G, Notarbartolo A, Averna MR. Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from irritable bowel syndrome: a prospective study in adults and children. *Clin Chem* 2003; **49**: 861-867 [PMID: 12765980]
41 **Costa F**, Mumolo MG, Bellini M, Romano MR, Ceccarelli L, Arpe P, Sterpi C, Marchi S, Maltinti G. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. *Dig Liver Dis* 2003; **35**: 642-647 [PMID: 14563186]
42 **Li XG**, Lu YM, Gu F, Yang XL. [Fecal calprotectin in differential diagnosis of irritable bowel syndrome]. *Beijing Da Xue Xue Bao Yi Xue Ban* 2006; **38**: 310-313 [PMID: 16778979]
43 **Otten CM**, Kok L, Witteman BJ, Baumgarten R, Kampman E, Moons KG, de Wit NJ. Diagnostic performance of rapid tests for detection of fecal calprotectin and lactoferrin and their ability to discriminate inflammatory from irritable bowel syndrome. *Clin Chem Lab Med* 2008; **46**: 1275-1280 [PMID: 18597588 DOI: 10.1515/cclm.2008.246]
44 **Langhorst J**, Junge A, Rueffer A, Wehkamp J, Foell D, Michalsen A, Musial F, Dobos GJ. Elevated human beta-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. *Am J Gastroenterol* 2009; **104**: 404-410 [PMID: 19174795 DOI: 10.1038/ajg.2008.86]
45 **Pavlidis P**, Chedgy FJ, Tibble JA. Diagnostic accuracy and clinical application of faecal calprotectin in adult patients presenting with gastrointestinal symptoms in primary care. *Scand J Gastroenterol* 2013; **48**: 1048-1054 [PMID: 23883068 DOI: 10.3109/00365521.2013.816771]
46 **Waugh N**, Cummins E, Royle P, Kandala NB, Shyangdan D, Arasaradnam R, Clar C, Johnston R. Faecal calprotectin testing for differentiating amongst inflammatory and non-inflammatory bowel diseases: systematic review and economic evaluation. *Health Technol Assess* 2013; **17**: xv-xix, 1-211 [PMID: 24286461 DOI: 10.3310/hta17550]
47 **Chang MH**, Chou JW, Chen SM, Tsai MC, Sun YS, Lin CC, Lin CP. Faecal calprotectin as a novel biomarker for differentiating between inflammatory bowel disease and irritable bowel syndrome. *Mol Med Rep* 2014; **10**: 522-526 [PMID: 24788223 DOI: 10.3892/mmr.2014.2180]
48 **Caviglia GP**, Pantaleoni S, Touscoz GA, Adriani A, Rosso C, Smedile A, Pellicano R, Astegiano M, Bresso F. Fecal calprotectin is an effective diagnostic tool that differentiates inflammatory from functional intestinal disorders. *Scand J Gastroenterol* 2014; **49**: 1419-1424 [PMID: 25369978 DOI: 10.3109/00365521.2014.934913]
49 **Banerjee A**, Srinivas M, Eyre R, Ellis R, Waugh N, Bardhan KD, Basumani P. Faecal calprotectin for differentiating between irritable bowel syndrome and inflammatory bowel disease: a useful screen in daily gastroenterology practice. *Frontline Gastroenterol* 2015; **6**: 20-26 [PMID: 28839790 DOI: 10.1136/flgastro-2013-100429]
50 **Menees SB**, Powell C, Kurlander J, Goel A, Chey WD. A meta-analysis of the utility of C-reactive protein, erythrocyte sedimentation rate, fecal calprotectin, and fecal lactoferrin to exclude inflammatory bowel disease in adults with IBS. *Am J Gastroenterol* 2015; **110**: 444-454 [PMID: 25732419 DOI: 10.1038/ajg.2015.6]
51 **Fu Y**, Wang L, Xie C, Zou K, Tu L, Yan W, Hou X. Comparison of non-invasive biomarkers faecal BAFF, calprotectin and FOBT in discriminating IBS from IBD and evaluation of intestinal inflammation. *Sci Rep* 2017; **7**: 2669 [PMID: 28572616 DOI: 10.1038/s41598-017-02835-5]
52 **Kalantari H**, Taheri A, Yaran M. Fecal calprotectin is a useful marker to diagnose ulcerative colitis from irritable bowel syndrome. *Adv Biomed Res* 2015; **4**: 85 [PMID: 26015911 DOI: 10.4103/2277-9175.156647]
53 **Keohane J**, O'Mahony C, O'Mahony L, O'Mahony S, Quigley EM, Shanahan F. Irritable bowel syndrome-type symptoms in patients with inflammatory bowel disease: a real association or reflection of occult inflammation? *Am J Gastroenterol* 2010; **105**: 1788, 1789-1794; quiz 1795 [PMID: 20389294 DOI: 10.1038/ajg.2010.156]
54 **Quigley EM**. Overlapping irritable bowel syndrome and inflammatory bowel disease: less to this than meets the eye? *Therap Adv Gastroenterol* 2016; **9**: 199-212 [PMID: 26929782 DOI: 10.1177/1756283X15621230]
55 **Chen JM**, Liu T, Gao S, Tong XD, Deng FH, Nie B. Efficacy of noninvasive evaluations in monitoring inflammatory bowel disease activity: A prospective study in China. *World J Gastroenterol* 2017; **23**: 8235-8247 [PMID: 29290660 DOI: 10.3748/wjg.v23.i46.8235]
56 **Schoepfer AM**, Vavricka S, Zahnd-Straumann N, Straumann A, Beglinger C. Monitoring inflammatory bowel disease activity: clinical activity is judged to be more relevant than endoscopic severity or biomarkers. *J Crohns Colitis* 2012; **6**: 412-418 [PMID: 22398068 DOI: 10.1016/j.crohns.2011.09.008]
57 **Baars JE**, Nuij VJ, Oldenburg B, Kuipers EJ, van der Woude CJ. Majority of patients with inflammatory bowel disease in clinical remission have mucosal inflammation. *Inflamm Bowel Dis* 2012; **18**: 1634-1640 [PMID: 22069022 DOI: 10.1002/ibd.21925]
58 **Xiang JY**, Ouyang Q, Li GD, Xiao NP. Clinical value of fecal calprotectin in determining disease activity of ulcerative colitis. *World J Gastroenterol* 2008; **14**: 53-57 [PMID: 18176961]
59 **Lee YW**, Lee KM, Lee JM, Chung YY, Kim DB, Kim YJ, Chung WC, Paik CN. The usefulness of fecal calprotectin in assessing inflammatory bowel disease activity. *Korean J Intern Med* 2018; [PMID: 29347813 DOI: 10.3904/kjim.2016.324]
60 **Lasson A**, Simrén M, Stotzer PO, Isaksson S, Ohman L, Strid H. Fecal calprotectin levels predict the clinical course in patients with new onset of ulcerative colitis. *Inflamm Bowel Dis* 2013; **19**: 576-581 [PMID: 23377170 DOI: 10.1097/MIB.0b013e31827e78be]
61 **Elkjaer M**. E-health: Web-guided therapy and disease self-management in ulcerative colitis. Impact on disease outcome, quality of life and compliance. *Dan Med J* 2012; **59**: B4478 [PMID: 22759851]
62 **Theede K**, Holck S, Ibsen P, Ladelund S, Nordgaard-Lassen I, Nielsen AM. Level of Fecal Calprotectin Correlates With Endoscopic and Histologic Inflammation and Identifies Patients With Mucosal Healing in Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2015; **13**: 1929-1936.e1 [PMID: 26051392 DOI: 10.1016/j.cgh.2015.05.038]
63 **Schoepfer AM**, Beglinger C, Straumann A, Safroneeva E, Romero Y, Armstrong D, Schmidt C, Trummler M, Pittet V, Vavricka SR. Fecal calprotectin more accurately reflects endoscopic activity of ulcerative colitis than the Lichtiger Index, C-reactive protein, platelets, hemoglobin, and blood leukocytes. *Inflamm Bowel Dis* 2013; **19**: 332-341 [PMID: 23328771 DOI: 10.1097/MIB.0b013e3182810066]
64 **Schoepfer AM**, Beglinger C, Straumann A, Trummler M, Renzulli P, Seibold F. Ulcerative colitis: correlation of the Rachmilewitz endoscopic activity index with fecal calprotectin, clinical activity, C-reactive protein, and blood leukocytes. *Inflamm Bowel Dis* 2009; **15**: 1851-1858 [PMID: 19462421 DOI: 10.1002/ibd.20986]
65 **Kopylov U**, Yung DE, Engel T, Avni T, Battat R, Ben-Horin S, Plevris JN, Eliakim R, Koulaouzidis A. Fecal calprotectin for the prediction of small-bowel Crohn's disease by capsule endoscopy: a systematic review and meta-analysis. *Eur J Gastroenterol*

Hepatol 2016; **28**: 1137-1144 [PMID: 27415156 DOI: 10.1097/meg.0000000000000692]
66 **Canani RB**, Terrin G, Rapacciuolo L, Miele E, Siani MC, Puzone C, Cosenza L, Staiano A, Troncone R. Faecal calprotectin as reliable non-invasive marker to assess the severity of mucosal inflammation in children with inflammatory bowel disease. *Dig Liver Dis* 2008; **40**: 547-553 [PMID: 18358796 DOI: 10.1016/j.dld.2008.01.017]
67 **Guardiola J**, Lobatón T, Rodríguez-Alonso L, Ruiz-Cerulla A, Arajol C, Loayza C, Sanjuan X, Sánchez E, Rodríguez-Moranta F. Fecal level of calprotectin identifies histologic inflammation in patients with ulcerative colitis in clinical and endoscopic remission. *Clin Gastroenterol Hepatol* 2014; **12**: 1865-1870 [PMID: 24993368 DOI: 10.1016/j.cgh.2014.06.020]
68 **Zittan E**, Kelly OB, Kirsch R, Milgrom R, Burns J, Nguyen GC, Croitoru K, Van Assche G, Silverberg MS, Steinhart AH. Low Fecal Calprotectin Correlates with Histological Remission and Mucosal Healing in Ulcerative Colitis and Colonic Crohn's Disease. *Inflamm Bowel Dis* 2016; **22**: 623-630 [PMID: 26829408 DOI: 10.1097/mib.0000000000000652]
69 **Mooiweer E**, Severs M, Schipper ME, Fidder HH, Siersema PD, Laheij RJ, Oldenburg B. Low fecal calprotectin predicts sustained clinical remission in inflammatory bowel disease patients: a plea for deep remission. *J Crohns Colitis* 2015; **9**: 50-55 [PMID: 25518048 DOI: 10.1093/ecco-jcc/jju003]
70 **Theede K**, Holck S, Ibsen P, Kallemose T, Nordgaard-Lassen I, Nielsen AM. Fecal Calprotectin Predicts Relapse and Histological Mucosal Healing in Ulcerative Colitis. *Inflamm Bowel Dis* 2016; **22**: 1042-1048 [PMID: 26919460 DOI: 10.1097/mib.0000000000000736]
71 **Puolanne AM**, Kolho KL, Alfthan H, Ristimäki A, Mustonen H, Färkkilä M. Rapid Fecal Calprotectin Test and Symptom Index in Monitoring the Disease Activity in Colonic Inflammatory Bowel Disease. *Dig Dis Sci* 2017; **62**: 3123-3130 [PMID: 28948412 DOI: 10.1007/s10620-017-4770-0]
72 **Guirgis M**, Wendt E, Wang LM, Walsh A, Burger D, Bryant RV, Kent A, Adamson R, Brain O, Travis SPL, Keshav S. Beyond Histological Remission: Intramucosal Calprotectin as a Potential Predictor of Outcomes in Ulcerative Colitis. *J Crohns Colitis* 2017; **11**: 460-467 [PMID: 27856523 DOI: 10.1093/ecco-jcc/jjw174]
73 **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 DOI: 10.1080/00365520410007971]
74 **Tibble JA**, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 2000; **119**: 15-22 [PMID: 10889150]
75 **Heida A**, Park KT, van Rheenen PF. Clinical Utility of Fecal Calprotectin Monitoring in Asymptomatic Patients with Inflammatory Bowel Disease: A Systematic Review and Practical Guide. *Inflamm Bowel Dis* 2017; **23**: 894-902 [PMID: 28511198 DOI: 10.1097/MIB.0000000000001082]
76 **Ferreiro-Iglesias R**, Barreiro-de Acosta M, Otero Santiago M, Lorenzo Gonzalez A, Alonso de la Peña C, Benitez Estevez AJ, Dominguez-Muñoz JE. Fecal Calprotectin as Predictor of Relapse in Patients With Inflammatory Bowel Disease Under Maintenance Infliximab Therapy. *J Clin Gastroenterol* 2016; **50**: 147-151 [PMID: 25811118 DOI: 10.1097/mcg.0000000000000312]
77 **de Suray N**, Salleron J, Vernier-Massouille G, Grimaud JC, Bouhnik Y, Laharie D, Dupas JL, Pillant H, Picon L, Veyrac M, Flamant M, Savoye G, Jian R, De Vos M, Piver E, Mary JY, Colombel JF, Louis E. P274 Close monitoring of CRP and fecal calprotectin levels to predict relapse in Crohn's disease patients. A sub-analysis of the STORI study. *ACAAI* 2012; **6**: S118-S119 [DOI: 10.1016/S1873-9946(12)60294-3]
78 **Wagner M**, Peterson CG, Ridefelt P, Sangfelt P, Carlson M. Fecal markers of inflammation used as surrogate markers for treatment outcome in relapsing inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 5584-5589; discussion 5588 [PMID: 18810778]
79 **Kolho KL**, Raivio T, Lindahl H, Savilahti E. Fecal calprotectin remains high during glucocorticoid therapy in children with inflammatory bowel disease. *Scand J Gastroenterol* 2006; **41**: 720-725 [PMID: 16716972 DOI: 10.1080/00365520500419623]
80 **Molander P**, af Björkesten CG, Mustonen H, Haapamäki J, Vauhkonen M, Kolho KL, Färkkilä M, Sipponen T. Fecal calprotectin concentration predicts outcome in inflammatory bowel disease after induction therapy with TNFα blocking agents. *Inflamm Bowel Dis* 2012; **18**: 2011-2017 [PMID: 22223566 DOI: 10.1002/ibd.22863]
81 **De Vos M**, Louis EJ, Jahnsen J, Vandervoort JG, Noman M, Dewit O, D'haens GR, Franchimont D, Baert FJ, Torp RA, Henriksen M, Potvin PM, Van Hootegem PP, Hindryckx PM, Moreels TG, Collard A, Karlsen LN, Kittang E, Lambrecht G, Grimstad T, Koch J, Lygren I, Coche JC, Mana F, Van Gossum A, Belaiche J, Cool MR, Fontaine F, Maisin JM, Muls V, Neuville B, Staessen DA, Van Assche GA, de Lange T, Solberg IC, Vander Cruyssen BJ, Vermeire SA. Consecutive fecal calprotectin measurements to predict relapse in patients with ulcerative colitis receiving infliximab maintenance therapy. *Inflamm Bowel Dis* 2013; **19**: 2111-2117 [PMID: 23883959 DOI: 10.1097/MIB.0b013e31829b2a37]
82 **Molander P**, Färkkilä M, Ristimäki A, Salminen K, Kemppainen H, Blomster T, Koskela R, Jussila A, Rautiainen H, Nissinen M, Haapamäki J, Arkkila P, Nieminen U, Kuisma J, Punkkinen J, Kolho KL, Mustonen H, Sipponen T. Does fecal calprotectin predict short-term relapse after stopping TNFα-blocking agents in inflammatory bowel disease patients in deep remission? *J Crohns Colitis* 2015; **9**: 33-40 [PMID: 25052347 DOI: 10.1016/j.crohns.2014.06.012]
83 **Tursi A**, Elisei W, Picchio M, Giorgetti G, Brandimarte G. Accuracy of Rapid Fecal Calprotectin Test in Monitoring Inflammatory Bowel Diseases Under Treatment with TNFα Antagonists. *Dig Dis Sci* 2015; **60**: 1406-1413 [PMID: 25445163 DOI: 10.1155/2014/342751]
84 **Laharie D**, Mesli S, El Hajbi F, Chabrun E, Chanteloup E, Capdepont M, Razaire S, de Lédinghen V, Zerbib F. Prediction of Crohn's disease relapse with faecal calprotectin in infliximab responders: a prospective study. *Aliment Pharmacol Ther* 2011; **34**: 462-469 [PMID: 21671970 DOI: 10.1111/j.1365-2036.2011.04743.x]
85 **Ho GT**, Lee HM, Brydon G, Ting T, Hare N, Drummond H, Shand AG, Bartolo DC, Wilson RG, Dunlop MG, Arnott ID, Satsangi J. Fecal calprotectin predicts the clinical course of acute severe ulcerative colitis. *Am J Gastroenterol* 2009; **104**: 673-678 [PMID: 19262524 DOI: 10.1038/ajg.2008.119]
86 **Colombel JF**, Rutgeerts PJ, Sandborn WJ, Yang M, Camez A, Pollack PF, Thakkar RB, Robinson AM, Chen N, Mulani PM, Chao J. Adalimumab induces deep remission in patients with Crohn's disease. *Clin Gastroenterol Hepatol* 2014; **12**: 414-422.e5 [PMID: 23856361 DOI: 10.1016/j.cgh.2013.06.019]
87 **Colombel JF**, Narula N, Peyrin-Biroulet L. Management Strategies to Improve Outcomes of Patients With Inflammatory Bowel Diseases. *Gastroenterology* 2017; **152**: 351-361.e5 [PMID: 27720840 DOI: 10.1053/j.gastro.2016.09.046]
88 **Shah SC**, Colombel JF, Sands BE, Narula N. Mucosal Healing Is Associated With Improved Long-term Outcomes of Patients With Ulcerative Colitis: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2016; **14**: 1245-1255.e8 [PMID: 26829025 DOI: 10.1016/j.cgh.2016.01.015]
89 **Shah SC**, Colombel JF, Sands BE, Narula N. Systematic review with meta-analysis: mucosal healing is associated with improved long-term outcomes in Crohn's disease. *Aliment Pharmacol Ther* 2016; **43**: 317-333 [PMID: 26607562 DOI: 10.1111/apt.13475]
90 **Frøslie KF**, Jahnsen J, Moum BA, Vatn MH; IBSEN Group. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 2007; **133**: 412-422 [PMID: 17681162 DOI: 10.1053/j.gastro.2007.05.051]
91 **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W,

Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV Jr, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]

92 **Colombel JF**, Panaccione R, Bossuyt P, Lukas M, Baert F, Vaňásek T, Danalioglu A, Novacek G, Armuzzi A, Hébuterne X, Travis S, Danese S, Reinisch W, Sandborn WJ, Rutgeerts P, Hommes D, Schreiber S, Neimark E, Huang B, Zhou Q, Mendez P, Petersson J, Wallace K, Robinson AM, Thakkar RB, D'Haens G. Effect of tight control management on Crohn's disease (CALM): a multicentre, randomised, controlled phase 3 trial. *Lancet* 2018; **390**: 2779-2789 [PMID: 29096949 DOI: 10.1016/S0140-6736(17)32641-7]

93 **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]

94 **Rutgeerts P**, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963 [PMID: 2394349]

95 **Peyrin-Biroulet L**, Loftus EV Jr, 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]

96 **De Cruz P**, Kamm MA, Hamilton AL, Ritchie KJ, Krejany EO, Gorelik A, Liew D, Prideaux L, Lawrance IC, Andrews JM, Bampton PA, Gibson PR, Sparrow M, Leong RW, Florin TH, Gearry RB, Radford-Smith G, Macrae FA, Debinski H, Selby W, Kronborg I, Johnston MJ, Woods R, Elliott PR, Bell SJ, Brown SJ, Connell WR, Desmond PV. Crohn's disease management after intestinal resection: a randomised trial. *Lancet* 2015; **385**: 1406-1417 [PMID: 25542620 DOI: 10.1016/S0140-6736(14)61908-5]

97 **Garcia-Planella E**, Mañosa M, Cabré E, Marín L, Gordillo J, Zabana Y, Boix J, Sáinz S, Domènech E. Fecal Calprotectin Levels Are Closely Correlated with the Absence of Relevant Mucosal Lesions in Postoperative Crohn's Disease. *Inflamm Bowel Dis* 2016; **22**: 2879-2885 [PMID: 27824646 DOI: 10.1097/MIB.0000000000000960]

98 **Yamamoto T**. The clinical value of faecal calprotectin and lactoferrin measurement in postoperative Crohn's disease. *United European Gastroenterol J* 2015; **3**: 5-10 [PMID: 25653853 DOI: 10.1177/2050640614558106]

99 **Orlando A**, Modesto I, Castiglione F, Scala L, Scimeca D, Rispo A, Teresi S, Mocciaro F, Criscuoli V, Marrone C, Platania P, De Falco T, Maisano S, Nicoli N, Cottone M. The role of calprotectin in predicting endoscopic post-surgical recurrence in asymptomatic Crohn's disease: a comparison with ultrasound. *Eur Rev Med Pharmacol Sci* 2006; **10**: 17-22 [PMID: 16494106]

100 **Scarpa M**, D'Incà R, Basso D, Ruffolo C, Polese L, Bertin E, Luise A, Frego M, Plebani M, Sturniolo GC, D'Amico DF, Angriman I. Fecal lactoferrin and calprotectin after ileocolonic resection for Crohn's disease. *Dis Colon Rectum* 2007; **50**: 861-869 [PMID: 17473939 DOI: 10.1007/s10350-007-0225-6]

101 **Lamb CA**, Mohiuddin MK, Gicquel J, Neely D, Bergin FG, Hanson JM, Mansfield JC. Faecal calprotectin or lactoferrin can identify postoperative recurrence in Crohn's disease. *Br J Surg* 2009; **96**: 663-674 [PMID: 19384912 DOI: 10.1002/bjs.6593]

102 **Yamamoto T**, Shiraki M, Bamba T, Umegae S, Matsumoto K. Faecal calprotectin and lactoferrin as markers for monitoring disease activity and predicting clinical recurrence in patients with Crohn's disease after ileocolonic resection: A prospective pilot study. *United European Gastroenterol J* 2013; **1**: 368-374 [PMID: 24917985 DOI: 10.1177/2050640613501818]

103 **Yamamoto T**, Shimoyama T, Umegae S, Matsumoto K. Serial monitoring of faecal calprotectin for the assessment of endoscopic recurrence in asymptomatic patients after ileocolonic resection for Crohn's disease: a long-term prospective study. *Therap Adv Gastroenterol* 2016; **9**: 664-670 [PMID: 27582878 DOI: 10.1177/1756283X16646562]

104 **Boschetti G**, Laidet M, Moussata D, Stefanescu C, Roblin X, Phelip G, Cotte E, Passot G, Francois Y, Drai J, Del Tedesco E, Bouhnik Y, Flourie B, Nancey S. Levels of Fecal Calprotectin Are Associated With the Severity of Postoperative Endoscopic Recurrence in Asymptomatic Patients With Crohn's Disease. *Am J Gastroenterol* 2015; **110**: 865-872 [PMID: 25781366 DOI: 10.1038/ajg.2015.30]

105 **Herranz Bachiller MT**, Barrio Andres J, Fernandez Salazar L, Ruiz-Zorrilla R, Sancho Del Val L, Atienza Sanchez R. The utility of faecal calprotectin to predict post-operative recurrence in Crohńs disease. *Scand J Gastroenterol* 2016; **51**: 720-726 [PMID: 26758472 DOI: 10.3109/00365521.2015.1130164]

106 **Lobatón T**, López-García A, Rodríguez-Moranta F, Ruiz A, Rodríguez L, Guardiola J. A new rapid test for fecal calprotectin predicts endoscopic remission and postoperative recurrence in Crohn's disease. *J Crohns Colitis* 2013; **7**: e641-e651 [PMID: 23810085 DOI: 10.1016/j.crohns.2013.05.005]

107 **Lasson A**, Strid H, Ohman L, Isaksson S, Olsson M, Rydström B, Ung KA, Stotzer PO. Fecal calprotectin one year after ileocaecal resection for Crohn's disease--a comparison with findings at ileocolonoscopy. *J Crohns Colitis* 2014; **8**: 789-795 [PMID: 24418661 DOI: 10.1016/j.crohns.2013.12.015]

108 **Wright EK**, Kamm MA, De Cruz P, Hamilton AL, Ritchie KJ, Krejany EO, Leach S, Gorelik A, Liew D, Prideaux L, Lawrance IC, Andrews JM, Bampton PA, Jakobovits SL, Florin TH, Gibson PR, Debinski H, Macrae FA, Samuel D, Kronborg I, Radford-Smith G, Selby W, Johnston MJ, Woods R, Elliott PR, Bell SJ, Brown SJ, Connell WR, Day AS, Desmond PV, Gearry RB. Measurement of fecal calprotectin improves monitoring and detection of recurrence of Crohn's disease after surgery. *Gastroenterology* 2015; **148**: 938-947.e1 [PMID: 25620670 DOI: 10.1053/j.gastro.2015.01.026]

109 **Huguet M**, Pereira B, Goutte M, Goutorbe F, Dubois A, Bommelaer G, Buisson A. Systematic Review With Meta-Analysis: Anti-TNF Therapy in Refractory Pouchitis and Crohn's Disease-Like Complications of the Pouch After Ileal Pouch-Anal Anastomosis Following Colectomy for Ulcerative Colitis. *Inflamm Bowel Dis* 2018; **24**: 261-268 [PMID: 29361101 DOI: 10.1093/ibd/izx049]

110 **Thomas P**, Rihani H, Røseth A, Sigthorsson G, Price A, Nicholls RJ, Bjarnason I. Assessment of ileal pouch inflammation by single-stool calprotectin assay. *Dis Colon Rectum* 2000; **43**: 214-220 [PMID: 10696896]

111 **Johnson MW**, Maestranzi S, Duffy AM, Dewar DH, Forbes A, Bjarnason I, Sherwood RA, Ciclitira P, Nicholls JR. Faecal calprotectin: a noninvasive diagnostic tool and marker of severity in pouchitis. *Eur J Gastroenterol Hepatol* 2008; **20**: 174-179 [PMID: 18301296 DOI: 10.1097/MEG.0b013e3282f1c9a7]

112 **Pakarinen MP**, Koivusalo A, Natunen J, Ashorn M, Karikoski R, Aitola P, Rintala RJ, Kolho KL. Fecal calprotectin mirrors inflammation of the distal ileum and bowel function after restorative proctocolectomy for pediatric-onset ulcerative colitis. *Inflamm Bowel Dis* 2010; **16**: 482-486 [PMID: 19685453 DOI: 10.1002/ibd.21069]

113 **Yamamoto T**, Shimoyama T. Can Fecal Biomarkers Detect Ileal Inflammation in Inflammatory Bowel Disease? *Am J Gastroenterol* 2015; **110**: 1370 [PMID: 26348308 DOI: 10.1038/ajg.2015.225]

114 **Van Assche G**, Dignass A, Bokemeyer B, Danese S, Gionchetti P, Moser G, Beaugerie L, Gomollón F, Häuser W, Herrlinger K, Oldenburg B, Panes J, Portela F, Rogler G, Stein J, Tilg H, Travis S, Lindsay JO; European Crohn's and Colitis Organisation. Second European evidence-based consensus on the diagnosis

and management of ulcerative colitis part 3: special situations. *J Crohns Colitis* 2013; **7**: 1-33 [PMID: 23040453 DOI: 10.1016/j.crohns.2012.09.005]

115 **Schoepfer A**, Reinisch W. Serial Fecal Calprotectin and Lactoferrin Measurements for Early Diagnosis of Pouchitis After Proctocolectomy for Ulcerative Colitis: Is Pouchoscopy No Longer Needed? *Am J Gastroenterol* 2015; **110**: 888-890 [PMID: 26052770 DOI: 10.1038/ajg.2015.170]

P- Reviewer: Can G, Esmat SM, Ribaldone DG **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3695-3708

DOI: 10.3748/wjg.v24.i33.3695

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

REVIEW

Updates on the hepatocyte growth factor/c-Met axis in hepatocellular carcinoma and its therapeutic implications

Javier A García-Vilas, Miguel Ángel Medina

Javier A García-Vilas, Department of Pediatrics, University of Alberta, Edmonton T6G 2S2, Canada

Miguel Ángel Medina, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Andalucía Tech, Universidad de Málaga, Málaga 29071, Spain

Miguel Ángel Medina, Unidad 741 de CIBER "de Enfermedades Raras" (CIBERER), Málaga 29071, Spain

Miguel Ángel Medina, Institute of Biomedical Research in Málaga, Málaga 29071, Spain

ORCID number: Javier A García-Vilas (0000-0003-0700-5069); Miguel Ángel Medina (0000-0001-7275-6462).

Author contributions: Both authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the version.

Supported by grants BIO2014-56092-R (MINECO and FEDER), No. P12-CTS-1507 (Andalusian Government and FEDER); and funds from group BIO-267 (Andalusian Government). The "CIBER de Enfermedades Raras" is an initiative from the ISCIII (Spain).

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Miguel Ángel Medina, PhD, Full Professor, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Andalucía Tech, Universidad de Málaga, Boulevar Louis Pasteur 31, Málaga 29010, Spain. medina@uma.es

Telephone: +34-952-137132

Received: May 3, 2018
Peer-review started: May 4, 2018
First decision: May 16, 2018
Revised: June 28, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 7, 2018

Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and is the second leading cause of cancer death. Since the diagnosis of HCC is difficult, in many cases patients with HCC are diagnosed advanced stage of development. Hepatocyte growth factor (HGF)/c-mesenchymal-epithelial transition receptor (c-Met) axis is a key signaling pathway in HCC, either via canonical or non-canonical pathways. Available treatments against HCC based upon HGF/c-Met inhibition can increase patient lifespan, but do not reach the expected therapeutic benefits. In HCC, c-Met monomers can bind other receptor monomers, activating several noncanonical signaling pathways, leading to increased cell proliferation, invasion, motility, and drug resistance. All of these processes are enhanced by the tumor microenvironment, with stromal cells contributing to boost tumor progression through oxidative stress, angiogenesis, lymphangiogenesis, inflammation, and fibrosis. Novel treatments against HCC are being explored to modulate other targets such as microRNAs, methyltransferases, and acetyltransferases, which are all involved in the regulation of gene expression in cancer. This review compiles basic knowledge regarding signaling pathways in HCC, and compounds already used or showing potential to be used in clinical trials.

Key words: Hepatocellular carcinoma; Hepatocyte growth factor/c-MET; Tumor microenvironment; c-Met

canonical and non-canonical pathways

Core tip: Hepatocellular carcinoma (HCC) is a tumor usually arising from previous hepatic diseases as cirrhosis and chronic hepatitis B and C infections. Several studies have shown that a key factor for HCC oncogenesis is chronic inflammation. Inflammation induces changes in the gene expression pattern in surrounding cells. These changes provide an environment with a high level of cytokines, promoting hepatocyte transformation to tumor cells. New therapies against HCC are focused on regulating stromal cells within the tumor microenvironment to avoid HCC progression.

García-Vilas JA, Medina MÁ. Updates on the hepatocyte growth factor/c-Met axis in hepatocellular carcinoma and its therapeutic implications. *World J Gastroenterol* 2018; 24(33): 3695-3708 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3695.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3695

INTRODUCTION

Liver is a vital organ responsible for hundreds of chemical reactions. Among them, it is involved in metabolizing many toxins. These reactions are carried out by the hepatocytes, which represent around 80% of the hepatic tissue cell population. Since the liver is the main detoxifying organ, hepatocytes are exposed to many toxins[1] which may cause insults inducing several anomalies, such as primary liver cancer. There are two types of adult primary liver cancers: cholangiocarcinoma and hepatocellular carcinoma (HCC), the latter being responsible for 85% to 90%[2] of total primary liver cancer instances. HCC is one of the deadliest malignancies worldwide[3]. It can be preceded by chronic inflammation due to cirrhosis, hepatitis B virus or hepatitis C virus infections, all of which increase 20-fold the risk of liver cancer[4].

Hepatocytes have a high regeneration rate, being controlled by multiple growth factors. The first molecule discovered with the ability to stimulate hepatocyte division was hepatocyte growth factor (HGF). HGF is expressed and released by specialized non-parenchymal cells called hepatocyte stellate cells. These cells release HGF into the extracellular space, where it acts in a paracrine manner on its receptor, known as c-Met, which is located on the surface of hepatocytes. HGF was characterized as a potent mitogen due to its ability to induce c-Met dimerization. This activates a canonical signal transduction pathway including effector molecules such as RAS-ERK and PI3K-AKT, which increase DNA synthesis and increase cell cycle progression.

The first therapies developed to treat HCC were focused on inhibiting the HGF/c-Met axis, thus stopping hepatocytes in the G1 phase of the cell cycle[5]. Studies in mice have shown that HGF or c-Met deletion are lethal[6]. Moreover, some tumors overexpress these proteins. Therapies targeting HGF or c-Met have been used for years. However, patients receiving these therapies still presented high rates of mortality, as well resistance to chemotherapy, radiotherapy, immunotherapy or hormonal therapy[7].

Recently, novel studies have elucidated other noncanonical signaling pathways that are modified in HCC. It is also now known that the fate of hepatocytes is determined by the interaction with nearby stromal cells. New therapies are being developed targeting the tumor microenvironment, including endothelial cells, immune cells, fibroblasts and the extracellular matrix.

Nonetheless, HGF/c-Met levels are currently being used to predict tumor aggressiveness and the prognosis of HCC patients.

In this review, we have analyzed HCC literature to generate a comprehensive view about the molecular processes already known and important discoveries that remain to be made.

HGF AND c-MET

The *HGF* gene is located on chromosome 7q21. It contains 20 exons and is expressed by mesenchymal cells. Hepatocyte growth factor (HGF) is a member of the peptidase S1 family of serine proteases, although it lacks peptidase activity. This protein is synthesized as an inactive pro-peptide generating an alpha/beta heterodimer linked by a disulfide bond. Proteolytic conversion of pro-HGF to HGF can be mediated by three enzymes present in the tumor environment: matriptase, hepsin and HGF activator (HGFA). However, there is evidence that urokinase plasminogen activator (uPA), transmembrane protease, serine 13 (TMPRSS13)[8] may also activate it. Although HGF was originally identified as a hepatocyte mitogen, it is now known to be a cytokine with pleiotropic effects. It has roles in enhancing angiogenesis, immune response, cell motility, and cell differentiation.

The gene for c-Met is located in chromosome 7q21-31 and contains 24 exons. Its promoter region, however, is located in chromosome 1. c-Met is expressed in epithelial cells. c-Met is a single pass tyrosine kinase receptor made up of an alpha and a beta subunit linked by disulfide bonds. The beta subunit is a transmembrane monomer that contains 5 catalytic tyrosines in its cytoplasmic tail. Y1003 negatively regulates c-Met by linking it to the ubiquitin ligase casitas beta-lineage lymphoma (c-CBL)[9]. In contrast, Y1234; Y1235; Y1349 and Y1356 positively regulate c-Met. Furthermore, S985 in c-Met can be phosphorylated by protein kinase-C, inducing c-Met degradation (ubiquitination

and endocytosis)[10].

c-Met activation has pleiotropic effects because its cytoplasmic domain can interact with multiple proteins involved in several cellular signaling pathways. Because of this, c-Met is considered an oncogene involved in cell proliferation, invasion, motility, angiogenesis and apoptosis.

SIGNALING PATHWAYS IN HCC

The pathophysiology of hepatocellular carcinoma at a cellular level is complex and it is very possible that there are many unknown c-Met interactions with others signaling pathways. Multiple cell pathways are aberrant in HCC, but this review just focuses on the signaling pathways related to c-Met. The activation of c-Met can take place by the canonical pathway, which involves HGF binding to c-Met resulting in c-Met homodimerization. It can also take place through non-canonical pathways, where c-Met dimerizes with different receptors.

Heterodimers of receptor proteins and c-Met are involved in overstimulation and dysregulation of c-Met signaling pathways. This occurs during hypoxia, which can cause c-Met overexpression, mutations on tyrosine kinase domain or *HGF* gene amplification[11]. However, this latter event rarely occurs in HCC[12].

c-Met canonical downstream signaling pathways

These signaling pathways involve proteins with SH2 domains or phosphotyrosine-binding domains that are able to interact with phosphorylated tyrosine residues[13] that, in turn, interact physically with the cytoplasmic domain of c-Met.

Growth factor receptor-bound protein 2 (Grb-2): Grb-2 interacts with Y1356 of c-Met to transduce HGF signaling to the cytoplasm. Grb-2 is considered a key protein in HGF/c-Met axis because it connects to several signaling transducers, such as Ras, SOS, and Gab1. Grb-2 is involved in cell motility, cycle progression, angiogenesis, amongst other.

GRB2-associated binding protein 1 (Gab1): Activated c-Met is phosphorylated on Y1349 and Y1356 residues which specifically interact and phosphorylate to Gab1. However, Gab1 can also be phosphorylated by Grb-2. Gab1 is involved in many signal transduction pathways by binding to effector proteins that have a role in cell motility and extracellular matrix invasion, such as Shp2, Shc, PLCγ1, p120[14].

Phosphoinositide 3 kinase (PI3K): PI3K is an enzyme able to phosphorylate proteins downstream of c-Met thereby linking oncogenes and many receptors essential for cellular functions. The phospho Y1356 in c-Met can phosphorylate PI3K, inducing cell mobility[9] by activating focal adhesion kinase (FAK). However, PI3K can also be activated by Gab1 where it promotes cell survival[11].

Signal transducer and activator of transcription 3 (STAT3): HGF binds to c-Met inducing the phosphorylation on Y1356. This phosphorylated amino acid interacts and actives STAT3, as was shown by Boccaccio *et al*[15]. When it is activated, it translocates to the nucleus where it binds to DNA and promotes gene expression (related with angiogenesis, and long-term response)[15].

Shc-transforming protein 1 (Shc): SHC is an adaptor protein involved in the mitogenic signal transduction from tyrosine receptors. On the other hand, experiments carried out in fibroblast showed that Shc is highly stimulated by VEGF and that activation correlated with the angiogenic response[16].

Non-canonical c-Met signaling pathways

c-Met activation by non-canonical pathways takes place when this receptor is over-expressed and dimerizes with other receptor subunits, or may bind to ligands other than HGF. Non-canonical pathways are usually associated with c-Met gene amplification, and are common in treatment resistant cancers[17,18], tumor progression, and metastasis, as shown in *in vivo* experiments using mice[19,20]. It has also been reported that c-Met dimerization takes place in the absence of ligand binding[21] when it interacts with the following proteins:

Epithelial growth factor receptor (EGFR): Physical interaction between EGFR and c-Met was found in A431 cells[22]. In HCC, the transactivation between these two receptors takes place, inducing the common downstream signaling effectors PI3K and Ras[23].

Human epidermal growth factor receptor (HER): The dimerization between c-Met and HER increases activation of PI3K/AKT signaling[18], which is associated with resistance to EGFR inhibitors[24] as well as cancer progression.

Integrin $\alpha_6\beta_4$: Trusolino *et al*[25] determined that integrin $\alpha_6\beta_4$ physically interacts with c-Met on the membrane surface of carcinoma cells. This protein is necessary for cancer invasion because the cytosolic domain of β4 induces c-Met activation. In this case, the signaling transduction is performed by Shc and PI3K[14].

β-catenin (β-CAT): Phosphorylated β-CAT may bind to c-Met, activating its downstream signaling. Phosphorylation of Y654 in β-CAT actives FAK, which induces cyclin D1 (CKD1) expression[26]. At the same time, β-catenin is translocated to the nucleus, and promotes c-myc gene expression[27].

Receptor for hyaluronic acid (CD44): The CD44v3

splice variant is the CD44 isoform with high affinity for heparin domains. This v3 may be activated by different growth factors with heparin domains, such as fibroblast growth factor (FGF) and HGF. CD44 may act as a concentrator of HGF to present it to c-Met resulting in downstream signaling transduction[28]. On the other hand, Olaku *et al*[29] reported that CD44v6 splice variant is necessary for c-Met activation. It is thought that three specific amino acid residues (RWH in human) in v6 are necessary for complete c-Met activation.

ICAM-1: This protein can substitute for the role of CD44v6 in c-Met activation, as shown in hepatocytes from Cd44 null mice[29].

Plexin B1: Receptor with high similarity to c-Met, which is also expressed in the same tissues as c-Met. After mutation and expression of exogenous c-Met in cells, Giordano *et al*[30] shown that plexin B1 links to c-Met when it is activated by semaphorin 4D. This interaction was reported in invasive cancer cells growing in response to semaphorin 4D.

Vascular endothelial growth factor A (VEGF-A): HGF can induce VEGF expression[31] by phosphorylation of a key transcription factor called Sp1. This characteristic of HGF increases the expression of Bcl-2[32], which acts as an antiapoptotic protein.

Insulin receptor (INSR) tyrosine kinase: This receptor has an extracellular α-chain and a transmembrane β-chain. This protein has a very similar structure compared to c-Met. Furthermore, it has been reported that insulin and HGF can phosphorylate INSR in its Y1146 and Y1150 or Y1151 residues. In HCC cells, Y1322 is also phosphorylated, thus activating PI3K. HGF-stimulated hepatocytes have shown to form a INSR-c-Met complex. There is evidence that c-Met can also phosphorylate insulin receptor substrates (IRS) on Y895. Likewise, INSR phosphorylates IRS on Y612[33]. In summary, both c-Met and IRS increase downstream signaling through PI3K-AKT, promoting cell growth, cell survival and cell motility.

Fas: This protein is one of the surface death receptors and triggers apoptosis signaling when binds its ligand (FasL). Wang *et al*[34] showed that Fas and c-Met associate with each other using coimmunoprecipitation experiments in Hep G2 cells. These authors proposed that c-Met promotes cell survival by two different pathways. (1) When there are low levels of FasL in the microenvironment, Fas binds to c-Met avoiding to trigger its intracellular signaling pathway. (2) However, in the presence of high levels of HGF, c-Met releases Fas activating death receptor-mediated apoptosis. Nevertheless, the c-Met/Fas signaling pathway ratio is so high that cells activate antiapoptotic signals through PI3K/AKT/Bad axis to prevent Fas-mediated apoptosis.

Mucin 1 (MUC1): MUC1 expression is increased during transformation from the normal liver to HCC, as was described by Bozkaya *et al*[35] MUC1 silencing in HCC cells leads to β-catenin activation and c-Myc expression. Under this condition, high levels of HGF in the microenvironment increase cellular motility and invasiveness[35]. However, there are also contradicting studies. For instance, Singh *et al*[36] reported that MUC1-induced c-Met activation by physical interaction decreased MMP-1 transcription and cell motility[36].

Neuropilin-1 and -2 (Nrp-1, -2): Neuropilins are a family of transmembrane glycoproteins involved in several processes, including axonal guidance, angiogenesis, tumorigenesis, and immunologic response. Nrp-1 can bind VEGF-A_{165}, VEGF-B, VEGF-E, and placental growth factor (PIGF). On the other hand, Nrp-2 can bind class III semaphorins and VEGF proteins (VEGF-A_{165}, VEGF-A_{145}, and VEGF-C). Nrp-2 binds VEGF proteins, and increases the VEGFR-2 phosphorylation threshold, promoting migration, and sprouting cells[37]. Nrp-2 and VEGFR2 can bind each other enhancing the signaling initiated by the HGF/c-Met axis. Moreover, Nrp-1 and Nrp-2 interact with other receptor tyrosine kinase, such as VEGFRs[37]. Neuropilins have a short cytoplasmic domain to act as catalytic domain. This evidence suggests that the intracellular domain may present a binding site involved in kinase signal transduction[37].

Focal adhesion kinase (FAK): Studies carried out in MEFs and HEK293 cells showed that FAK interacts directly with c-Met[38]. FAK is a non-receptor tyrosine kinase involved in several cell signaling pathways. Notably, it is well characterized for its role in formation and disassembly of focal adhesions, as well as cell protrusions[26]. However, FAK is also intimately involved in the regulation of cell proliferation because it is able to phosphorylate PI3K and ERK. Experiments in FAK knockout mice revealed suppressed hepatocarcinogenesis due to decreased PI3K and ERK signaling pathways[26].

Collectively, these pathways are responsible for promoting initiation and progression of HCC (Figure 1).

The expression of HGF is decreased in HCC, but it is increased in the surrounding tissue. On the other hand, c-Met is expressed in HCC at higher levels than in the surrounding tissue. These observations suggest that the overexpression of c-Met, together with additional oncogenes, is responsible of HCC aggressiveness[39].

MICROENVIRONMENT IN HCC

Microenvironment is created by extracellular matrix (ECM) and stromal cells. Stromal cells, such as endothelial cells, fibroblasts, and immune cells, increase the gene expression and release of chemokines, cytokines,

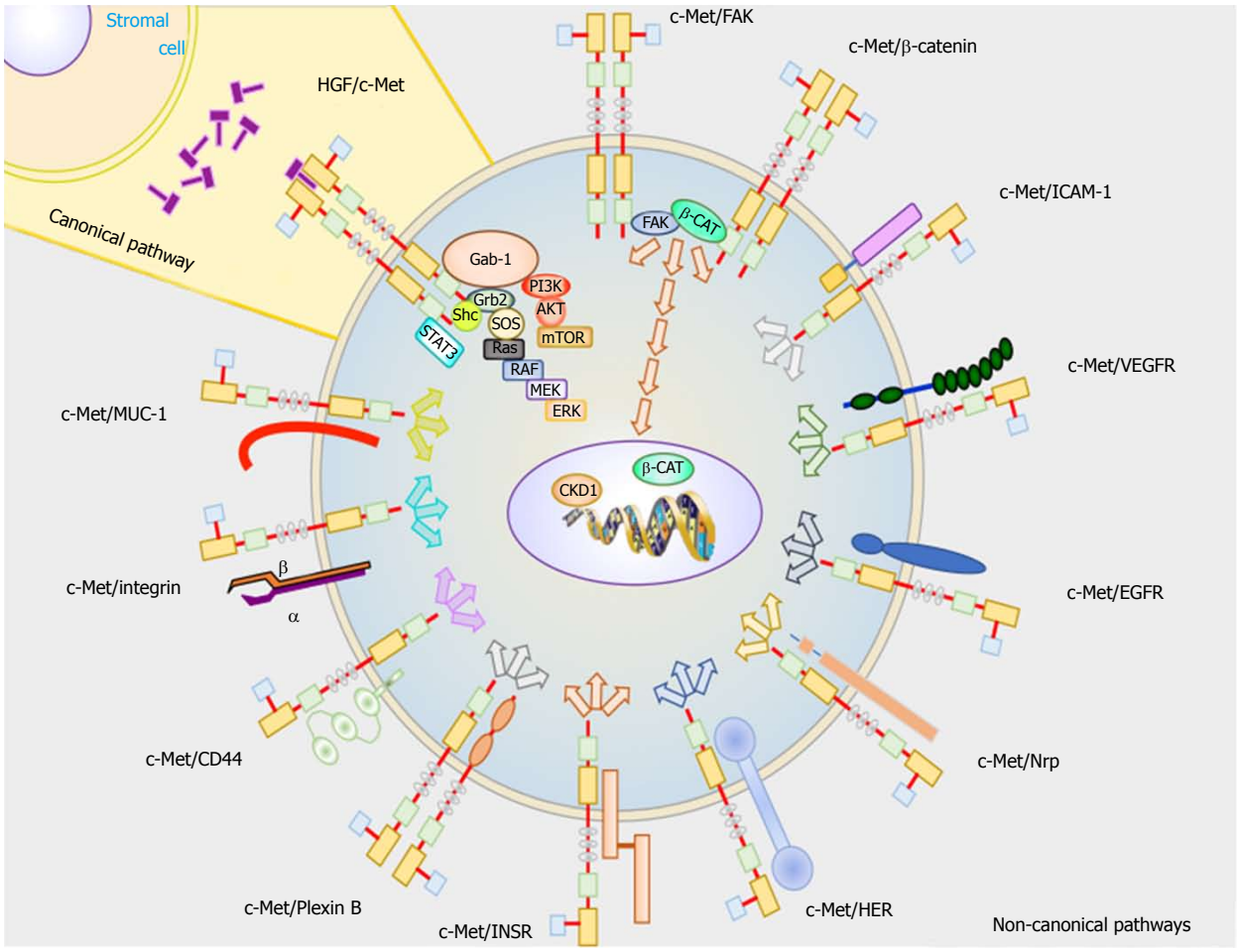


Figure 1 Representation of hepatocyte growth factor/c-Met canonical and non-canonical pathways in hepatocytes. Canonical pathway is activated by HGF release from stromal cells, and subsequent binding to the c-Met receptor inducing c-Met dimerization. Activated c-Met binds Gab-1, Grb-2, Shc, and STAT3. These proteins are involved in signal transduction regulating cell proliferation, migration, differentiation, or invasion, depending on the activated downstream proteins. The scheme represents the proteins described in the canonical pathway, but not all of the proteins involved in executing their cell activity. Non-canonical pathways are activated when a c-Met monomer binds to a monomer of another kind of receptor, or when a c-Met homodimer binds FAK or β-catenin on its cytoplasmic domain. The arrows represent different proteins performing different cell activities. HGF: Hepatocyte growth factor.

proteases and growth factors, such as HGF[40]. HGF released from stromal cells interacts with hepatocytes enhancing HGF/c-Met signaling transduction. Subsequently, hepatocytes intensify cell survival and cell proliferation. HGF may also modify cell-cell interaction, and cell-extracellular matrix interactions through activating proteolytic networks[41,42]. Furthermore, stromal cells increase the gene expression of HGF. Furthermore, HGF promotes angiogenesis from endothelial cells around the chronic liver disease.

Angiogenesis and lymphangiogenesis

The initial stages of liver tumor are characterized by hypoxia, a condition leading to the release of VEGF to increase tumor vascularization. In parallel, immune cells contribute to the activation of the vascularization process by releasing inflammatory mediators, including interleukin-1α (IL-1α), IL-1β, tumor necrosis factor-α, and prostaglandin E2. HCC is a highly vascularized tumor. However, the new blood vessels formed to irrigate the tumor mass present abnormalities, such as tortuous vessels and sinusoidal capillarization. Tumor blood vessels have incomplete basal membrane and incomplete pericyte coverage. Simultaneously, lymphangiogenesis, proliferation and sprouting of new lymphatic vessels from preexisting ones, also takes places. Lymphangiogenesis is tightly regulated by lymphoangiogenic growth factors, such as VEGF-C and VEGF-D, released from tumor and stromal cells. VEGF-C and VEGF-D interact with VEGFR-2 and VEGFR-3, respectively, in lymphatic cells. VEGFR-2 is responsible for vessel enlargement, whereas VEGFR-3 is critical for lymphangiogenic sprouting[43]. However, VEGF-A may interact with VEGFR-3. Further, venous and lymphatic endothelial cells express neuropilin-2 (Nrp-2), a protein that it has recently been associated to lymphatic cell survival and migration[37]. On the other hand, Nrp-2 is not involved in venous or lymphatic cell proliferation[44]. Tumor-associated lymphatic vessels express stromal cell-derived factor 1 (CXCL12), and tumor cells express the receptor for CXCL12, called C-X-C chemokine receptor 4 (CXCR4). Tumor cells have upregulated

CXCR4 under hypoxia conditions. However, hypoxia promotes migration of metastatic cells into the tumor-draining lymph node. Endothelial and lymphatic cell survival and cell proliferation promote tumor metastasis. In fact, high densities of vascular and lymphatic vessels are correlated with high patient lethality[44].

Inflammation

The role of immune cells in cancer is controversial, because they can eliminate tumor cells in their initial stages, but, due to their generation of oxidative stress and DNA damage, they may also promote cancer development.

A study carried out in a small cohort showed that patients with viral hepatitis presented high neutrophil levels and low lymphocyte levels. This has been proposed as a substitute parameter to determine initial development HCC stages, because neutrophils are the first kind of immune cells to interact with tumor cells[45]. Neutrophils located in the tumor mass are responsible for promoting inflammation and facilitating tumor progression. From the same study, the authors detected high platelet levels in circulation. This association might be caused because platelets are responsible for activating neutrophils, enabling neutrophils to migrate through blood vessels. A lower risk of HCC was shown with anti-platelet therapy from another independent study[46]. Additionally, platelets are key effectors to enhance the accumulation of $CD8^+$ T lymphocytes and mediate immune injury, intensifying the micro-inflammation.

Tumor-infiltrating T cells are regulated by VEGF-A and VEGF-C, as well as their release into the tumor microenvironment, cell proliferation phenotype, and activity[47].

Macrophages are responsible for digesting all damaged cells in the tissues including cancer cells. However, macrophages activated by inflammatory cytokines from the tumor microenvironment, express the PD-L1 protein. This results in them adopting a suppressive macrophage phenotype, avoiding tumor-specific T cell immunity and increasing HCC progression[48]. These macrophages activated by the tumor microenvironment express tumor necrosis factor α (TNF-α), which induces c-Met-expression on their surface. These macrophages also express MMP-9, which increases the remodeling of the tumor microenvironment of HCC[49].

Fibroblasts and hepatic progenitor stem cells

Fibroblasts are an important cell population in the microenvironment, and have a prominent role in tumor cell progression and metastasis. Fibroblasts have key functions in the tumor microenvironment: (1) synthesis and remodeling of the ECM; and (2) release of multiple cytokines and chemokines to promote inflammation, angiogenesis and epithelial differentiation. Tumor-associated fibroblasts acquire a modified phenotype induced by transforming growth factor-β (TGFβ), and become hepatic progenitor cancer cells (HPCs)[50].

HPCs have the ability to differentiate to several kinds of cells depending on the stimulus from their microenvironment. Under the influence of a tumor microenvironment, HPCs acquire genetic and epigenetic mutations, inducing their transformation from HPCs to cancer stem cells (CSCs)[51]. CSCs are able to perpetuate themselves through self-renewal, and generate mature cells of a particular tissue through differentiation, supporting tumor cell proliferation[52]. Likewise, CSCs have been proposed to be the clonogenic core of HCC, sustain the primary tumor, confer drug resistance, promote metastasis, and promote angiogenesis.

Fibrosis

All of the stages described above in the present section, cell transvasation, cell-cell interaction, oxidative stress, cytokines and proteases released into the tumor microenvironment, provoke changes and deregulation in the ECM. The deregulation of ECM is characterized by increasing deposition of fibronectin, fibrillar collagen types I and II into the liver. This ECM deregulation decreases liver plasticity, increasing ECM stiffness, cell survival and cell proliferation of tumor cells. These processes are mediated by integrin signaling pathways, such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Moreover, the ECM increases immune cell activation and differentiation, promotes angiogenesis, and activates tissue invasion[42].

Dapito *et al*[53] showed that translocation of components of intestinal bacterial, and Toll-like receptor 4 (TLR4), can reach the liver by the portal circulation. Both components promote liver inflammation, increasing the secretion of growth factor, such as epiregulin from hepatic stellate cells, and promoting the synthesis of ECM. This scenario enhances fibrosis and HCC development.

The microenvironment not only creates favorable conditions to develop the unregulated cell proliferation characteristic of cancer cells, but also influences their sensitivity to drugs and promotes metastasis. HGF delivered by stromal cells has been described as a key factor to confer resistance to molecular targeted drugs[54].

EPIGENETICS IN HCC

Only 2% of the human genome encodes proteins, and the majority of the transcriptome contains non-coding RNAs (ncRNAs). ncRNAs have regulatory functions and may be classified by their sequence length, in micro RNAs (miRNAs), small nucleolar RNAs (snoRNAs), small interfering RNAS (siRNAs), long non-coding RNAs (lncRNAs), and very long intergenic ncRNAs (vlincRNAs)[55,56]. However, there are other classifications based on association with annotated protein-coding genes, association with other DNA elements of known function, protein-coding RNA resemblance, association with repeats, with a biochemical pathway or stability, sequence and structure conservation, expression in

Table 1 MicroRNA involved in hepatocellular carcinoma and their function in the hepatocyte growth factor/c-Met axis

miRNA	Functions	Effect of miR in HGF/c-Met axis	Levels in HCC	Ref.
miR-34	Cell invasion, proliferation	Inhibits c-Met	Downregulated	[83]
miR-199	Proliferation, cell motility, cell invasion	Tumor-suppressor	Downregulated	[84]
miR-340	Cell invasion, cell migration	Inhibits c-Met	Downregulated	[85]
miR-126	Cell proliferation, cell invasion, inhibits angiogenesis	Inhibits c-Met	Downregulated	[86]
miR181-a	Cell motility and invasion	Inhibits c-Met	Upregulated	[87]
let-7 family	Represses cell proliferation, invasion, metastasis and resistance therapy	Inhibits c-Met signaling downstream	Downregulated	[88,89]
miR-148	Promotes apoptosis, suppress cell invasion	Tumor-suppressor	Downregulated	[90]
miR-1	Cell migration, cell proliferation	Tumor-suppressor	Downregulated	[91]
miR-26a	Cell proliferation, invasion, and migration	Inhibits c-Met signaling downstream	Downregulated	[92]
miR-122	Induces apoptosis	Inhibition of c-Met	Downregulated	[93]
miR-145	Cell viability, cell migration	Inhibits c-Met signaling downstream	Downregulated	[94]
miR-449	Promotes apoptosis, reduces proliferation	Decreases c-Met levels	Downregulated	[95]
miR-200	Cell migration and invasion	Decreases HGF-synthesis in fibroblasts	Upregulated	[96]
miR-101	Cell proliferation, migration and invasion	Inhibits c-Met signaling downstream	Downregulated	[97]

HCC: Hepatocellular carcinoma; HGF: Hepatocyte growth factor.

different biological states, subcellular structures, and based on function[56]. Independently of the classification criteria, the ncRNAs have physiological relevance in genetic and epigenetic regulation, such as control of chromatin remodeling, gene transcription, protein transport and metabolism[57].

Dysregulation of miRNAs has been associated with alterations in cell signaling pathways playing important roles in the control of cell invasion, proliferation, and metastasis, among many other pathophysiologic processes. For instance, miR-26a. behaves as a tumor suppressor in hepatocellular carcinoma (HCC), but it shows oncogenic properties in lung cancer. These observations suggest that microenvironment also determines the roles of miRNAs in cancer (Table 1). A RNA-sequencing study performed in 23 liver biopsies, between tumor and adjacent non-tumor tissue, showed 57 lncRNA with differential expression[58].

Histones acetylation, methylation, phosphorylation, sumoylation, and ubiquitylation, are post-translational modifications (PTMs) intimately related to epigenetic processes. These PTMs are responsible for the induction or repression of genes through modification in the level of DNA compaction or the recruitment of transcriptional machinery. Histones deacetylases (HDACs) are a family of proteins that remove acetyl groups from histone tail amino acids, decreasing gene expression. This family of proteins is overexpressed in HCC[59]. In fact, human HCC hallmarks are the loss of acetylation in H4K16[60], and increased methylation in H3K27 by EZH2 methylase[61]. Furthermore, EZH2 can inhibit the expression of miR-622, thus increasing the severity of HCC[62]. SET8 is a specific H4K20 methylase required for S phase progression by coupling to PCNA[63]. SET8 dysregulation has been described in human HCC[55]. Histone hypermethylation has also been related with decreased gene expression.

On the other hand, histones acetyltransferases (HAT) are a family of proteins that add acetyl groups to histone amino acids, inducing the relaxation of DNA strains, and subsequently up-regulating gene expression.

In the liver, xenobiotics such as alcohol can alter the epigenetic state by generating reactive oxygen species (ROS) and depleting S-adenosylmethionine (SAM) levels. This promotes H3K9 acetylation and alters the expression of several miRNAs[64]. Changes in the microbiota and viral liver infections can also modify DNA methylation, altering gene expression[65,66].

THERAPIES

HCC is difficult to diagnose by current methods with biopsy being the most validated method. However, it is difficult to obtain a representative biopsy sample because open tumor biopsies of HCC are not allowed[67]. This scenario makes difficult to perform an accurate diagnostic.

Chronic liver diseases caused by hepatitis virus B and/or C virus are the most prevalent causes of HCC. However, other factors have been related to increase the prevalence of HCC such alcohol-related liver diseases, obesity, and type 2 diabetes mellitus-related non-alcoholic fatty liver[45].

The HGF/c-Met axis has been proposed as a key target for clinical intervention. This is due to its relevance in cellular processes such as 3D morphogenesis, cell survival and metastasis. Due to their key role in HCC,

Table 2 Compounds used in clinical trials to treat hepatocellular carcinoma patients

Agent	Targets	Phase	Activity	HCC stage	Ref.
Sorafenib	Raf, MAPK, VEGFR, PDGFRβ	III	Anti-tumor Anti-angiogenesis	Advanced	[98]
Cabozanitinib	VEGFR2, KIT, RET, AXL	III	Anti-tumor	Advanced	[99]
Brivanib	FGFR, VEGFR	II	Anti-angiogenesis	Advanced	[100]
Foretinib	FLT1, PDGFRβ, c-Met, VEGFR-2, Tie-2 FLT4, RON, FLT3, KIT	II	Anti-tumor Anti-angiogenesis	Advanced	[101]
Everolimus	mTOR	III	Anti-tumor	Advanced	[102]
Cobazitinib	c-Met, VEGFR-2, RET	II	Anti-tumor Anti-angiogenesis	Advanced	[103]
Ramucirumab	VEGFR-2	III	Anti-angiogenesis	Advanced	[104]
MSC2156119J	c-Met	Ib/II	Anti-tumor, anti-mestastasis	Advanced	[105]
Gefitinib	EGFR, c-Met, HGF,	II	Anti-tumor	Advanced	[106]
Bevacizumab	VEGFRs	II	Anti-angiogenesis	Advanced	[107]
AZD6244	MEK1/2	Ib	Anti-tumor	Advanced	[108]
AZD4547	p-FGFR-1, p-FGFR-2 p-c-Met, p-AKT, p-ERK	I	Anti-tumor, anti-angiogenesis	Advanced	[109]
MK2461	c-Met, Ftl-1	I	Anti-tumor	Advanced	[110]
Crizotinib	p-c-Met ALK	Ib	Anti-angiogenesis	Advanced	[111]
Bortezomib	Proteasome inhibitor	II	Anti-tumor	Advanced	[102]
Docetaxel	EGFR	II	Anti-tumor	Advanced	
INC280	p-c-Met	II	Anti-tumor	Advanced	[111]

HCC: Hepatocellular carcinoma.

HGF and c-Met have been proposed as essential therapeutic targets (Table 2). A recent review on the therapeutic targeting of the HGF/c-Met signaling in HCC has focused on small c-Met kinase inhibitors[68]. This same review also comments problems as resistance to c-Met targeting drugs and side effects of c-Met targeting.

Although some improvements have been obtained in HCC therapy, this cancer still remains largely incurable. Nowadays, there is palliative therapy using a multikinase inhibitor, such as sorafenib, which limits cell proliferation, and tumor angiogenesis[69]. Many clinical trials have shown that sorafenib improves overall survival of patients with advanced HCC. However, there is no effective treatment for HCC based on conventional monotherapies using tyrosine kinase inhibitors. For example, a monotherapy with Tivantinib, a small molecule tyrosine kinase inhibitor exhibiting a high selectivity against c-Met, was clinically tested. Unfortunately, in a phase III clinical trial, this compound failed to meet the primary endpoint.

HCC develops resistance to the conventional chemotherapy and radiotherapy treatments[7]. Moreover, the tumor microenvironment blocks drug effects[70]. HGF confers resistance of HCC to inhibitors of EGFR. On the other hand, HGF decreases the expression of E-cadherin[71] and increases the expression of Snail 1, to protect cancer cells from apoptosis[72].

Novel strategies against HCC

Another difficulty in detecting HCC is the lack of a clinically relevant circulating biomarker. Current investigations are focused on discovering circulating biomarkers[45]. Sitia *et al*[73] showed that the $CD8^+$ T cell/platelet ratio could be a sign of HCC progression. Moreover, Sitia *et al*[73] verified that aspirin or clopidogrel, two anti-platelet drugs, decreased the number of $CD8^+$ T cells in liver infected with hepatitis B virus. After treatment with anti-platelet drugs, they observed a decrease in inflammation, fibrosis severity, and progression to HCC in a transgenic mouse model[73]. The effect was enhanced when mice were treated with aspirin and clopidogrel. The authors concluded that anti-platelet drugs diminished the amount of $CD8^+$ T cells into the liver, avoiding hepatocellular necrosis, hepatocyte regeneration, and inflammation. These events prevent or delay HCC in the mouse model used. On the other hand, this treatment does not eradicate the viral infection.

Preclinical studies in animals have shown that anti-lymphangiogenic strategies, sequestering VEGF-C and VEGF-D, or blocking VEGFR-3 with antibodies, are feasible therapies against HCC tumors[74]. Experimental therapy against Nrp-2 reduced tumor lymphangiogenesis, apparently delaying the departure of cells from the primary tumor[44]. Lymphatic cells release CXCL12, which can be blocked with antibodies and may provide therapeutics benefits[75]. Inhibiting the expression of Nrp-1revealed that Nrp-1 is required to activate VEGFR-2 signaling-dependent mitogenic[76].

Novel anticancer strategies are emerging based on ncRNA expression, because they have a myriad of cell functions, such as chromatin remodeling, protein transport, genes transcription and metabolism[57,77]. MicroRNAs (miRs) are being studied in cancer for their

Table 3 List of compounds analyzed in laboratories that show potential anti-tumor properties against hepatocellular carcinoma

Agent	Target	Activity	Ref.
LZ8	Inhibits the expression of c-Met, ERK, AKT Inhibits the phosphorylation of JNK, AKT, ERK, p-AKT Stabilizes p53	Induces apoptosis	[112]
Damnacanthal	Inhibits the phosphorylation of c-Met Decreases MMP-2 activity	Inhibits cell proliferation and cell invasion	[113]
GEN-203	Inhibits the phosphorylation of c-Met	Blocks cell proliferation	[114]
JNJ38877605	Inhibits phosphorylation of c-Met	Induces apoptosis	[70]
SRI31215	Blocks pro-HGF activation	Inhibits cell proliferation	[70]
Madecassoside	Inhibits the phosphorylation of ERK1/2 and PKC	Blocks cell proliferation	[115]
PHA665752	Inhibits phosphorylation of c-Met and FGFR	Blocks cell proliferation	[116]
EPZ011989	Inhibits histone methylation	Blocks cell proliferation	[82]
YC-1	Inhibits the DNA synthesis	Arrests cell cycle and induce apoptosis	[117]

key role as post-transcriptional regulators of gene expression, and for their potential to be used to classify tumors[78]. Recently studies are revealing that some miRNAs could be useful as prognostic biomarkers based on their presence in serum. They could be promising therapeutic targets as well.

Combinations of current drugs used for clinical treatments, such as sorafenib, tivantinib, 5-fluorouracil (5-FU), and potential miRs inhibitors, such as miR-93 inhibitor, are proposed as promising anti-HCC therapies[79]. Other studies demonstrated that miR26[80], miR-499, miR-30a, miR-122, and miR-148, among others, play a role in the development of normal physiological function in liver.

Some anti-cancer therapies against HCC are focused on HDACs as key targets. HDAC inhibitors (either miRNAs or small molecule HDAC inhibitors) have been shown to increase apoptosis and decrease cell proliferation. HCC treated with trichostatin A (TSA), a compound described as HDAC inhibitor, as well as with siRNA against HDACs (-1, -2, and -3) have increased apoptosis and decreased cell proliferation[60]. Sirtuins, vorinostat, romidepsin, belinostat, panobinastat, valproate and ITF2357 are HDAC inhibitors that have shown promising anti-cancer effects in clinical trials[55].

Histone methyltransferases, such as SET8, SUV39H1 and EZH2, are also promising targets for HCC therapy. Experiments performed in HCC cells showed that the silencing of EZH2 decreased the expression of CDKN2A, FOXO3, E2F1 and NOTCH2[81]. The silencing of EZH2 also repressed the expression of miR-622, increasing CXCR4 levels[61]. Small molecule inhibitors against EZH2, such as EPZ011989, may be potentially useful for the treatment of HCC patients[82].

The discovery of novel molecules related with HGF/c-Met axis signaling pathways presents a promising clinically avenue for predicting individual susceptibility and designing better specific personal therapies (Table 3).

CONCLUSION

HCC is a complex pathology with interconnected regulatory networks involved. This cancer is difficult to diagnose and the current treatments only produce modest therapeutic benefits. Moreover, the lack of biomarkers for early diagnosis contributes to making HCC one of the cancer types with the poorest prognosis. Novel approaches to elucidate the molecular etiology of HCC have shown that, in addition to the HGF/c-Met axis signaling, there are other receptors and ligands involved. Dysregulation in protein interactions, lncRNAs, post-translational modifications in histones, and DNA methylation levels can be responsible for cell proliferation, migration, invasion, and mestastasis. Novel treatments are being developed against HDACs, methyltransferases, and miRs, among other molecular targets. Therapies combining HGF/c-Met signaling pathway inhibitors with epigenetic inhibitors, such as histone methyltransferase or HDAC inhibitors, seem to be the most promising therapies to date. Additional experimental efforts will be needed to identify useful predictive biomarkers and to suggest new personal therapies.

ACKNOWLEDGMENTS

We thank Dr. Michael J Hendzel (Univerity of Alberta, Canada) for carefully reading the manuscript for English grammar and having made editing suggestions.

REFERENCES

1 **Waller LP**, Deshpande V, Pyrsopoulos N. Hepatocellular carcinoma: A comprehensive review. *World J Hepatol* 2015; **7**: 2648-2663 [PMID: 26609342 DOI: 10.4254/wjh.v7.i26.2648]

2 **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]

3 **Lau EY**, Lo J, Cheng BY, Ma MK, Lee JM, Ng JK, Chai S, Lin CH, Tsang SY, Ma S, Ng IO, Lee TK. Cancer-Associated Fibroblasts Regulate Tumor-Initiating Cell Plasticity in Hepatocellular Carcinoma through c-Met/FRA1/HEY1 Signaling. *Cell Rep* 2016; **15**: 1175-1189 [PMID: 27134167 DOI: 10.1016/j.celrep.2016.04.019]

4 **Donato F**, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int J Cancer* 1998; **75**:

347-354 [PMID: 9455792]
5 **Mohammed FF**, Khokha R. Thinking outside the cell: proteases regulate hepatocyte division. *Trends Cell Biol* 2005; **15**: 555-563 [PMID: 16150595 DOI: 10.1016/j.tcb.2005.08.009]
6 **Schmidt C**, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* 1995; **373**: 699-702 [PMID: 7854452 DOI: 10.1038/373699a0]
7 **Whittaker S**, Marais R, Zhu AX. The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* 2010; **29**: 4989-5005 [PMID: 20639898 DOI: 10.1038/onc.2010.236]
8 **Kawaguchi M**, Kataoka H. Mechanisms of hepatocyte growth factor activation in cancer tissues. *Cancers* (Basel) 2014; **6**: 1890-1904 [PMID: 25268161 DOI: 10.3390/cancers6041890]
9 **Bradley CA**, Salto-Tellez M, Laurent-Puig P, Bardelli A, Rolfo C, Tabernero J, Khawaja HA, Lawler M, Johnston PG, Van Schaeybroeck S; MErCuRIC consortium. Targeting c-MET in gastrointestinal tumours: rationale, opportunities and challenges. *Nat Rev Clin Oncol* 2017; **14**: 562-576 [PMID: 28374784 DOI: 10.1038/nrclinonc.2017.40]
10 **Nakayama M**, Sakai K, Yamashita A, Nakamura T, Suzuki Y, Matsumoto K. Met/HGF receptor activation is regulated by juxtamembrane Ser985 phosphorylation in hepatocytes. *Cytokine* 2013; **62**: 446-452 [PMID: 23618918 DOI: 10.1016/j.cyto.2013.04.006]
11 **Ghiso E**, Giordano S. Targeting MET: why, where and how? *Curr Opin Pharmacol* 2013; **13**: 511-518 [PMID: 23797036 DOI: 10.1016/j.coph.2013.05.018]
12 **Seruca R**, Suijkerbuijk RF, Gärtner F, Criado B, Veiga I, Olde-Weghuis D, David L, Castedo S, Sobrinho-Simões M. Increasing levels of MYC and MET co-amplification during tumor progression of a case of gastric cancer. *Cancer Genet Cytogenet* 1995; **82**: 140-145 [PMID: 7664243]
13 **Pawson T**, Nash P. Protein-protein interactions define specificity in signal transduction. *Genes Dev* 2000; **14**: 1027-1047 [PMID: 10809663]
14 **Viticchiè G**, Muller PAJ. c-Met and Other Cell Surface Molecules: Interaction, Activation and Functional Consequences. *Biomedicines* 2015; **3**: 46-70 [PMID: 28536399 DOI: 10.3390/biomedicines3010046]
15 **Boccaccio C**, Andò M, Tamagnone L, Bardelli A, Michieli P, Battistini C, Comoglio PM. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* 1998; **391**: 285-288 [PMID: 9440692 DOI: 10.1038/34657]
16 **Saucier C**, Khoury H, Lai KM, Peschard P, Dankort D, Naujokas MA, Holash J, Yancopoulos GD, Muller WJ, Pawson T, Park M. The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. *Proc Natl Acad Sci USA* 2004; **101**: 2345-2350 [PMID: 14983012 DOI: 10.1073/pnas.0308065101]
17 **Migliore C**, Giordano S. Molecular cancer therapy: can our expectation be MET? *Eur J Cancer* 2008; **44**: 641-651 [PMID: 18295476 DOI: 10.1016/j.ejca.2008.01.022]
18 **Scagliotti GV**, Novello S, von Pawel J. The emerging role of MET/HGF inhibitors in oncology. *Cancer Treat Rev* 2013; **39**: 793-801 [PMID: 23453860 DOI: 10.1016/j.ctrv.2013.02.001]
19 **Jeffers M**, Fiscella M, Webb CP, Anver M, Koochekpour S, Vande Woude GF. The mutationally activated Met receptor mediates motility and metastasis. *Proc Natl Acad Sci USA* 1998; **95**: 14417-14422 [PMID: 9826715 DOI: 10.1073/pnas.95.24.14417]
20 **Rong S**, Segal S, Anver M, Resau JH, Vande Woude GF. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. *Proc Natl Acad Sci USA* 1994; **91**: 4731-4735 [PMID: 8197126 DOI: 10.1073/pnas.91.11.4731]
21 **Ferracini R**, Di Renzo MF, Scotlandi K, Baldini N, Olivero M, Lollini P, Cremona O, Campanacci M, Comoglio PM. The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. *Oncogene* 1995; **10**: 739-749 [PMID: 7862451]
22 **Jo M**, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. *J Biol Chem* 2000; **275**: 8806-8811 [PMID: 10722725 DOI: 10.1074/jbc.275.12.8806]
23 **Fischer OM**, Giordano S, Comoglio PM, Ullrich A. Reactive oxygen species mediate Met receptor transactivation by G protein-coupled receptors and the epidermal growth factor receptor in human carcinoma cells. *J Biol Chem* 2004; **279**: 28970-28978 [PMID: 15123705 DOI: 10.1074/jbc.M402508200]
24 **Engelman JA**, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Jänne PA. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007; **316**: 1039-1043 [PMID: 17463250 DOI: 10.1126/science.1141478]
25 **Trusolino L**, Bertotti A, Comoglio PM. A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. *Cell* 2001; **107**: 643-654 [PMID: 11733063 DOI: 10.1016/S0092-8674(01)00567-0]
26 **Shang N**, Arteaga M, Zaidi A, Stauffer J, Cotler SJ, Zeleznik-Le NJ, Zhang J, Qiu W. FAK is required for c-Met/β-catenin-driven hepatocarcinogenesis. *Hepatology* 2015; **61**: 214-226 [PMID: 25163657 DOI: 10.1002/hep.27402]
27 **Monga SP**, Mars WM, Pediaditakis P, Bell A, Mulé K, Bowen WC, Wang X, Zarnegar R, Michalopoulos GK. Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. *Cancer Res* 2002; **62**: 2064-2071 [PMID: 11929826]
28 **van der Voort R**, Taher TE, Wielenga VJ, Spaargaren M, Prevo R, Smit L, David G, Hartmann G, Gherardi E, Pals ST. Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. *J Biol Chem* 1999; **274**: 6499-6506 [PMID: 10037743 DOI: 10.1074/jbc.274.10.6499]
29 **Olaku V**, Matzke A, Mitchell C, Hasenauer S, Sakkaravarthi A, Pace G, Ponta H, Orian-Rousseau V. c-Met recruits ICAM-1 as a coreceptor to compensate for the loss of CD44 in Cd44 null mice. *Mol Biol Cell* 2011; **22**: 2777-2786 [PMID: 21680714 DOI: 10.1091/mbc.E11-02-0134)]
30 **Giordano S**, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D, Tamagnone L, Comoglio PM. The semaphorin 4D receptor controls invasive growth by coupling with Met. *Nat Cell Biol* 2002; **4**: 720-724 [PMID: 12198496 DOI: 10.1038/ncb843]
31 **Corso S**, Comoglio PM, Giordano S. Cancer therapy: can the challenge be MET? *Trends Mol Med* 2005; **11**: 284-292 [PMID: 15949770 DOI: 10.1016/j.molmed.2005.04.005]
32 **Xin X**, Yang S, Ingle G, Zlot C, Rangell L, Kowalski J, Schwall R, Ferrara N, Gerritsen ME. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. *Am J Pathol*2001; **158**: 1111-1120 [PMID: 11238059 DOI: 10.1016/s0002-9440(10)64058-8]
33 **Fafalios A**, Ma J, Tan X, Stoops J, Luo J, Defrances MC, Zarnegar R. A hepatocyte growth factor receptor (Met)-insulin receptor hybrid governs hepatic glucose metabolism. *Nat Med* 2011; **17**: 1577-1584 [PMID: 22081023 DOI: 10.1038/nm.2531]
34 **Wang X**, DeFrances MC, Dai Y, Pediaditakis P, Johnson C, Bell A, Michalopoulos GK, Zarnegar R. A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. *Mol Cell* 2002; **9**: 411-421 [PMID: 11864613]
35 **Bozkaya G**, Korhan P, Cokaklı M, Erdal E, Sağol O, Karademir S, Korch C, Atabey N. Cooperative interaction of MUC1 with the HGF/c-Met pathway during hepatocarcinogenesis. *Mol Cancer* 2012; **11**: 64 [PMID: 22962849 DOI: 10.1186/1476-4598-11-64]
36 **Singh PK**, Behrens ME, Eggers JP, Cerny RL, Bailey JM,

Shanmugam K, Gendler SJ, Bennett EP, Hollingsworth MA. Phosphorylation of MUC1 by Met modulates interaction with p53 and MMP1 expression. *J Biol Chem*2008; **283**: 26985-26995 [PMID: 18625714 DOI: 10.1074/jbc.M805036200]

37 **Sulpice E**, Plouët J, Bergé M, Allanic D, Tobelem G, Merkulova-Rainon T. Neuropilin-1 and neuropilin-2 act as coreceptors, potentiating proangiogenic activity. *Blood* 2008; **111**: 2036-2045 [PMID: 18065694 DOI: 10.1182/blood-2007-04-084269]

38 **Chen SY**, Chen HC. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. *Mol Cell Biol* 2006; **26**: 5155-5167 [PMID: 16782899 DOI: 10.1128/MCB.02186-05]

39 **Tward AD**, Jones KD, Yant S, Cheung ST, Fan ST, Chen X, Kay MA, Wang R, Bishop JM. Distinct pathways of genomic progression to benign and malignant tumors of the liver. *Proc Natl Acad Sci USA* 2007; **104**: 14771-14776 [PMID: 17785413 DOI: 10.1073/pnas.0706578104]

40 **Matsumoto K**, Nakamura T. Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. *Kidney Int* 2001; **59**: 2023-2038 [PMID: 11380804 DOI: 10.1046/j.1523-1755.2001.00717.x]

41 **Kadono Y**, Shibahara K, Namiki M, Watanabe Y, Seiki M, Sato H. Membrane type 1-matrix metalloproteinase is involved in the formation of hepatocyte growth factor/scatter factor-induced branching tubules in madin-darby canine kidney epithelial cells. *Biochem Biophys Res Commun* 1998; **251**: 681-687 [PMID: 9790969 DOI: 10.1006/bbrc.1998.9531]

42 **Hernandez-Gea V**, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 2013; **144**: 512-527 [PMID: 23313965 DOI: 10.1053/j.gastro.2013.01.002]

43 **Wirzenius M**, Tammela T, Uutela M, He Y, Odorisio T, Zambruno G, Nagy JA, Dvorak HF, Ylä-Herttuala S, Shibuya M, Alitalo K. Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *J Exp Med* 2007; **204**: 1431-1440 [PMID: 17535974 DOI: 10.1084/jem.20062642]

44 **Caunt M**, Mak J, Liang WC, Stawicki S, Pan Q, Tong RK, Kowalski J, Ho C, Reslan HB, Ross J, Berry L, Kasman I, Zlot C, Cheng Z, Le Couter J, Filvaroff EH, Plowman G, Peale F, French D, Carano R, Koch AW, Wu Y, Watts RJ, Tessier-Lavigne M, Bagri A. Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell* 2008; **13**: 331-342 [PMID: 18394556 DOI: 10.1016/j.ccr.2008.01.029]

45 **Margetts J**, Ogle LF, Chan SL, Chan AWH, Chan KCA, Jamieson D, Willoughby CE, Mann DA, Wilson CL, Manas DM, Yeo W, Reeves HL. Neutrophils: driving progression and poor prognosis in hepatocellular carcinoma? *Br J Cancer* 2018; **118**: 248-257 [PMID: 29123264 DOI: 10.1038/bjc.2017.386]

46 **Lee M**, Chung GE, Lee JH, Oh S, Nam JY, Chang Y, Cho H, Ahn H, Cho YY, Yoo JJ, Cho Y, Lee DH, Cho EJ, Yu SJ, Lee DH, Lee JM, Kim YJ, Yoon JH. Antiplatelet therapy and the risk of hepatocellular carcinoma in chronic hepatitis B patients on antiviral treatment. *Hepatology* 2017; **66**: 1556-1569 [PMID: 28617992 DOI: 10.1002/hep.29318]

47 **Ziogas AC**, Gavalas NG, Tsiatas M, Tsitsilonis O, Politi E, Terpos E, Rodolakis A, Vlahos G, Thomakos N, Haidopoulos D, Antsaklis A, Dimopoulos MA, Bamias A. VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. *Int J Cancer* 2012; **130**: 857-864 [PMID: 21445972 DOI: 10.1002/ijc.26094]

48 **Kuang DM**, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, Zheng L. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med* 2009; **206**: 1327-1337 [PMID: 19451266 DOI: 10.1084/jem.20082173]

49 **Zhao L**, Wu Y, Xie XD, Chu YF, Li JQ, Zheng L. c-Met identifies a population of matrix metalloproteinase 9-producing monocytes in peritumoural stroma of hepatocellular carcinoma. *J Pathol* 2015; **237**: 319-329 [PMID: 26108200 DOI: 10.1002/path.4578]

50 **Kalluri R**, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006; **6**: 392-401 [PMID: 16572188 DOI: 10.1038/nrc1877]

51 **Yagci T**, Cetin M, Ercin PB. Cancer Stem Cells in Hepatocellular Carcinoma. *J Gastrointest Cancer* 2017; Epub ahead of print [PMID: 28643126 DOI: 10.1007/s12029-017-9960-7]

52 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955 DOI: 10.1038/35102167]

53 **Dapito DH**, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, Khiabanian H, Adeyemi A, Bataller R, Lefkowitch JH, Bower M, Friedman R, Sartor RB, Rabadan R, Schwabe RF. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* 2012; **21**: 504-516 [PMID: 22516259 DOI: 10.1016/j.ccr.2012.02.007]

54 **Straussman R**, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, Davis A, Mongare MM, Gould J, Frederick DT, Cooper ZA, Chapman PB, Solit DB, Ribas A, Lo RS, Flaherty KT, Ogino S, Wargo JA, Golub TR. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* 2012; **487**: 500-504 [PMID: 22763439 DOI: 10.1038/nature11183]

55 **Wilson CL**, Mann DA, Borthwick LA. Epigenetic reprogramming in liver fibrosis and cancer. *Adv Drug Deliv Rev* 2017; **121**: 124-132 [PMID: 29079534 DOI: 10.1016/j.addr.2017.10.011]

56 **St Laurent G**, Wahlestedt C, Kapranov P. The Landscape of long noncoding RNA classification. *Trends Genet* 2015; **31**: 239-251 [PMID: 25869999 DOI: 10.1016/j.tig.2015.03.007]

57 **Cao J**. The functional role of long non-coding RNAs and epigenetics. *Biol Proced Online* 2014; **16**: 11 [PMID: 25276098 DOI: 10.1186/1480-9222-16-11]

58 **Esposti DD**, Hernandez-Vargas H, Voegele C, Fernandez-Jimenez N, Forey N, Bancel B, Le Calvez-Kelm F, McKay J, Merle P, Herceg Z. Identification of novel long non-coding RNAs deregulated in hepatocellular carcinoma using RNA-sequencing. *Oncotarget* 2016; **7**: 31862-31877 [PMID: 26887054 DOI: 10.18632/oncotarget.7364]

59 **Quint K**, Agaimy A, Di Fazio P, Montalbano R, Steindorf C, Jung R, Hellerbrand C, Hartmann A, Sitter H, Neureiter D, Ocker M. Clinical significance of histone deacetylases 1, 2, 3, and 7: HDAC2 is an independent predictor of survival in HCC. *Virchows Arch* 2011; **459**: 129-139 [PMID: 21713366 DOI: 10.1007/s00428-011-1103-0)]

60 **Buurman R**, Gürlevik E, Schäffer V, Eilers M, Sandbothe M, Kreipe H, Wilkens L, Schlegelberger B, Kühnel F, Skawran B. Histone deacetylases activate hepatocyte growth factor signaling by repressing microRNA-449 in hepatocellular carcinoma cells. *Gastroenterology* 2012; **143**: 811-820.e15 [PMID: 22641068 DOI: 10.1002/hep.29519]

61 **Chen Y**, Lin MC, Yao H, Wang H, Zhang AQ, Yu J, Hui CK, Lau GK, He ML, Sung J, Kung HF. Lentivirus-mediated RNA interference targeting enhancer of zeste homolog 2 inhibits hepatocellular carcinoma growth through down-regulation of stathmin. *Hepatology* 2007; **46**: 200-208 [PMID: 17596871 DOI: 10.1002/hep.21668]

62 **Knutson SK**, Wigle TJ, Warholic NM, Sneeringer CJ, Allain CJ, Klaus CR, Sacks JD, Raimondi A, Majer CR, Song J, Scott MP, Jin L, Smith JJ, Olhava EJ, Chesworth R, Moyer MP, Richon VM, Copeland RA, Keilhack H, Pollock RM, Kuntz KW. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nat Chem Biol* 2012; **8**: 890-896 [PMID: 23023262 DOI: 10.1038/nchembio.1084]

63 **Huen MS**, Sy SM, van Deursen JM, Chen J. Direct interaction between SET8 and proliferating cell nuclear antigen couples H4-K20 methylation with DNA replication. *J Biol Chem* 2008; **283**: 11073-11077 [PMID: 18319261 DOI: 10.1074/jbc.C700242200]

64 **Shukla SD**, Lim RW. Epigenetic effects of ethanol on the liver and gastrointestinal system. *Alcohol Res* 2013; **35**: 47-55 [PMID: 24313164]

65 **Wijetunga NA**, Pascual M, Tozour J, Delahaye F, Alani M, Adeyeye M, Wolkoff AW, Verma A, Greally JM. A pre-neoplastic epigenetic field defect in HCV-infected liver at transcription factor binding sites and polycomb targets. *Oncogene* 2017; **36**: 2030-2044 [PMID: 27721404 DOI: 10.1038/onc.2016.340]

66 **Thaiss CA**, Levy M, Korem T, Dohnalová L, Shapiro H, Jaitin DA, David E, Winter DR, Gury-BenAri M, Tatirovsky E, Tuganbaev T, Federici S, Zmora N, Zeevi D, Dori-Bachash M, Pevsner-Fischer M, Kartvelishvily E, Brandis A, Harmelin A, Shibolet O, Halpern Z, Honda K, Amit I, Segal E, Elinav E. Microbiota Diurnal Rhythmicity Programs Host Transcriptome Oscillations. *Cell* 2016; **167**: 1495-1510.e12 [PMID: 27912059 DOI: 10.1016/j.cell.2016.11.003]

67 **Thelen A**, Jonas S, Benckert C, Weichert W, Schott E, Bötcher C, Dietz E, Wiedenmann B, Neuhaus P, Scholz A. Tumor-associated lymphangiogenesis correlates with prognosis after resection of human hepatocellular carcinoma. *Ann Surg Oncol* 2009; **16**: 1222-1230 [PMID: 19224279 DOI: 10.1245/s10434-009-0380-1]

68 **Hu CT**, Wu JR, Cheng CC, Wu WS. The Therapeutic Targeting of HGF/c-Met Signaling in Hepatocellular Carcinoma: Alternative Approaches. *Cancers* (Basel) 2017; **9**: [PMID: 28587113 DOI: 10.3390/cancers9060058]

69 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

70 **Owusu BY**, Galemmo R, Janetka J, Klampfer L. Hepatocyte Growth Factor, a Key Tumor-Promoting Factor in the Tumor Microenvironment. *Cancers* (Basel) 2017; **9**: [PMID: 28420162 DOI: 10.3390/cancers9040035]

71 **Witta SE**, Gemmill RM, Hirsch FR, Coldren CD, Hedman K, Ravdel L, Helfrich B, Dziadziuszko R, Chan DC, Sugita M, Chan Z, Baron A, Franklin W, Drabkin HA, Girard L, Gazdar AF, Minna JD, Bunn PA Jr. Restoring E-cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. *Cancer Res* 2006; **66**: 944-950 [PMID: 16424029 DOI: 10.1158/0008-5472.CAN-05-1988]

72 **Grotegut S**, von Schweinitz D, Christofori G, Lehembre F. Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail. *EMBO J* 2006; **25**: 3534-3545 [PMID: 16858414 DOI: 10.1038/sj.emboj.7601213]

73 **Sitia G**, Aiolfi R, Di Lucia P, Mainetti M, Fiocchi A, Mingozzi F, Esposito A, Ruggeri ZM, Chisari FV, Iannacone M, Guidotti LG. Antiplatelet therapy prevents hepatocellular carcinoma and improves survival in a mouse model of chronic hepatitis B. *Proc Natl Acad Sci USA* 2012; **109**: E2165-E2172 [PMID: 22753481 DOI: 10.1073/pnas.1209182109]

74 **Pytowski B**, Goldman J, Persaud K, Wu Y, Witte L, Hicklin DJ, Skobe M, Boardman KC, Swartz MA. Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *J Natl Cancer Inst* 2005; **97**: 14-21 [PMID: 15632376 DOI: 10.1093/jnci/dji003]

75 **Phillips RJ**, Burdick MD, Lutz M, Belperio JA, Keane MP, Strieter RM. The stromal derived factor-1/CXCL12-CXC chemokine receptor 4 biological axis in non-small cell lung cancer metastases. *Am J Respir Crit Care Med* 2003; **167**: 1676-1686 [PMID: 12626353 DOI: 10.1164/rccm.200301-071OC]

76 **Murga M**, Fernandez-Capetillo O, Tosato G. Neuropilin-1 regulates attachment in human endothelial cells independently of vascular endothelial growth factor receptor-2. *Blood* 2005; **105**: 1992-1999 [PMID: 15522955 DOI: 10.1182/blood-2004-07-2598]

77 **Saito Y**, Jones PA. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *Cell Cycle* 2006; **5**: 2220-2222 [PMID: 17012846 DOI: 10.4161/cc.5.19.3340]

78 **Calin GA**, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006; **66**: 7390-7394 [PMID: 16885332 DOI: 10.1158/0008-5472.CAN-06-0800]

79 **Ohta K**, Hoshino H, Wang J, Ono S, Iida Y, Hata K, Huang SK, Colquhoun S, Hoon DS. MicroRNA-93 activates c-Met/PI3K/Akt pathway activity in hepatocellular carcinoma by directly inhibiting PTEN and CDKN1A. *Oncotarget* 2015; **6**: 3211-3224 [PMID: 25633810 DOI: 10.18632/oncotarget.3085]

80 **Yang X**, Zhang XF, Lu X, Jia HL, Liang L, Dong QZ, Ye QH, Qin LX. MicroRNA-26a suppresses angiogenesis in human hepatocellular carcinoma by targeting hepatocyte growth factor-cMet pathway. *Hepatology* 2014; **59**: 1874-1885 [PMID: 24259426 DOI: 10.1002/hep.26941]

81 **Gao SB**, Xu B, Ding LH, Zheng QL, Zhang L, Zheng QF, Li SH, Feng ZJ, Wei J, Yin ZY, Hua X, Jin GH. The functional and mechanistic relatedness of EZH2 and menin in hepatocellular carcinoma. *J Hepatol* 2014; **61**: 832-839 [PMID: 24845612 DOI: 10.1016/j.jhep.2014.05.015]

82 **Campbell JE**, Kuntz KW, Knutson SK, Warholic NM, Keilhack H, Wigle TJ, Raimondi A, Klaus CR, Rioux N, Yokoi A, Kawano S, Minoshima Y, Choi HW, Porter Scott M, Waters NJ, Smith JJ, Chesworth R, Moyer MP, Copeland RA. EPZ011989, A Potent, Orally-Available EZH2 Inhibitor with Robust in Vivo Activity. *ACS Med Chem Lett* 2015; **6**: 491-495 [PMID: 26005520 DOI: 10.1021/acsmedchemlett.5b00037]

83 **Zhou JY**, Chen X, Zhao J, Bao Z, Chen X, Zhang P, Liu ZF, Zhou JY. MicroRNA-34a overcomes HGF-mediated gefitinib resistance in EGFR mutant lung cancer cells partly by targeting MET. *Cancer Lett* 2014; **351**: 265-271 [PMID: 24983493 DOI: 10.1016/j.canlet.2014.06.010]

84 **Kim S**, Lee UJ, Kim MN, Lee EJ, Kim JY, Lee MY, Choung S, Kim YJ, Choi YC. MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). *J Biol Chem*2008; **283**: 18158-18166 [PMID: 18456660 DOI: 10.1074/jbc.M800186200]

85 **Wu ZS**, Wu Q, Wang CQ, Wang XN, Huang J, Zhao JJ, Mao SS, Zhang GH, Xu XC, Zhang N. miR-340 inhibition of breast cancer cell migration and invasion through targeting of oncoprotein c-Met. *Cancer* 2011; **117**: 2842-2852 [PMID: 21692045 DOI: 10.1002/cncr.25860]

86 **Hu MH**, Ma CY, Wang XM, Ye CD, Zhang GX, Chen L, Wang JG. MicroRNA-126 inhibits tumor proliferation and angiogenesis of hepatocellular carcinoma by down-regulating EGFL7 expression. *Oncotarget* 2016; **7**: 66922-66934 [PMID: 27611944 DOI: 10.18632/oncotarget.11877]

87 **Meng F**, Glaser SS, Francis H, DeMorrow S, Han Y, Passarini JD, Stokes A, Cleary JP, Liu X, Venter J, Kumar P, Priester S, Hubble L, Staloch D, Sharma J, Liu CG, Alpini G. Functional analysis of microRNAs in human hepatocellular cancer stem cells. *J Cell Mol Med* 2012; **16**: 160-173 [PMID: 21352471 DOI: 10.1111/j.1582-4934.2011.01282.x]

88 **Shi W**, Zhang Z, Yang B, Guo H, Jing L, Liu T, Luo Y, Liu H, Li Y, Gao Y. Overexpression of microRNA let-7 correlates with disease progression and poor prognosis in hepatocellular carcinoma. *Medicine* (Baltimore) 2017; **96**: e7764 [PMID: 28796071 DOI: 10.1097/MD.0000000000007764]

89 **Jin B**, Wang W, Meng XX, Du G, Li J, Zhang SZ, Zhou BH, Fu ZH. Let-7 inhibits self-renewal of hepatocellular cancer stem-like cells through regulating the epithelial-mesenchymal transition and the Wnt signaling pathway. *BMC Cancer* 2016; **16**: 863 [PMID: 27821157 DOI: 10.1186/s12885-016-2904-y]

90 **Zheng B**, Liang L, Wang C, Huang S, Cao X, Zha R, Liu L, Jia D, Tian Q, Wu J, Ye Y, Wang Q, Long Z, Zhou Y, Du C, He X, Shi Y. MicroRNA-148a suppresses tumor cell invasion and metastasis by downregulating ROCK1 in gastric cancer. *Clin Cancer Res* 2011; **17**: 7574-7583 [PMID: 21994419 DOI: 10.1158/1078-0432.CCR-11-1714]

91 **Nasser MW**, Datta J, Nuovo G, Kutay H, Motiwala T, Majumder S, Wang B, Suster S, Jacob ST, Ghoshal K. Down-regulation of

micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1. *J Biol Chem* 2008; **283**: 33394-33405 [PMID: 18818206 DOI: 10.1074/jbc.M804788200]

92 **Yang X**, Liang L, Zhang XF, Jia HL, Qin Y, Zhu XC, Gao XM, Qiao P, Zheng Y, Sheng YY, Wei JW, Zhou HJ, Ren N, Ye QH, Dong QZ, Qin LX. MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-Stat3 pathway. *Hepatology* 2013; **58**: 158-170 [PMID: 23389848 DOI: 10.1002/hep.26305]

93 **Yang YM**, Lee CG, Koo JH, Kim TH, Lee JM, An J, Kim KM, Kim SG. Gα12 overexpressed in hepatocellular carcinoma reduces microRNA-122 expression via HNF4α inactivation, which causes c-Met induction. *Oncotarget* 2015; **6**: 19055-19069 [PMID: 25965999 DOI: 10.18632/oncotarget.3957]

94 **Ding W**, Tan H, Zhao C, Li X, Li Z, Jiang C, Zhang Y, Wang L. MiR-145 suppresses cell proliferation and motility by inhibiting ROCK1 in hepatocellular carcinoma. *Tumour Biol* 2016; **37**: 6255-6260 [PMID: 26615424 DOI: 10.1007/s13277-015-4462-3]

95 **Korhan P**, Erdal E, Atabey N. MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met. *Biochem Biophys Res Commun*2014; **450**: 1304-1312 [PMID: 25058462 DOI: 10.1016/j.bbrc.2014.06.142]

96 **Chen Y**, Du M, Wang J, Xing P, Zhang Y, Li F, Lu X. MiRNA-200a expression is inverse correlation with hepatocyte growth factor expression in stromal fibroblasts and its high expression predicts a good prognosis in patients with non-small cell lung cancer. *Oncotarget* 2016; **7**: 48432-48442 [PMID: 27374174 DOI: 10.18632/oncotarget.10302]

97 **Zhu Z**, Zhang X, Wang G, Zheng H. Role of MicroRNAs in Hepatocellular Carcinoma. *Hepat Mon* 2014; **14**: e18672 [PMID: 25337143 DOI: 10.5812/hepatmon.18672]

98 **Runge A**, Hu J, Wieland M, Bergeest JP, Mogler C, Neumann A, Géraud C, Arnold B, Rohr K, Komljenovic D, Schirmacher P, Goerdt S, Augustin HG. An inducible hepatocellular carcinoma model for preclinical evaluation of antiangiogenic therapy in adult mice. *Cancer Res* 2014; **74**: 4157-4169 [PMID: 24906623 DOI: 10.1158/0008-5472.CAN-13-2311]

99 **Xiang Q**, Chen W, Ren M, Wang J, Zhang H, Deng DY, Zhang L, Shang C, Chen Y. Cabozantinib suppresses tumor growth and metastasis in hepatocellular carcinoma by a dual blockade of VEGFR2 and MET. *Clin Cancer Res* 2014; **20**: 2959-2970 [PMID: 24700742 DOI: 10.1158/1078-0432.CCR-13-2620]

100 **Chan SL**, Yeo W. Targeted therapy of hepatocellular carcinoma: present and future. *J Gastroenterol Hepatol* 2012; **27**: 862-872 [PMID: 22369685 DOI: 10.1111/j.1440-1746.2012.07096.x]

101 **Yau TC**, Sukeepaisarnjaroen W, Chao Y, Yen CJ, Lausoontornsiri W, Chen PJ, Sanpajit T, Lencioni R, Camp AC, Cox DS. A phase I/II study of foretinib, an oral multikinase inhibitor targeting MET, RON, AXL, TIE-2, and VEGFR in advanced hepatocellular carcinoma (HCC). *J Clin Oncol* 2012; **30**: 4108-4108

102 **Zhu AX**, Kudo M, Assenat E, Cattan S, Kang YK, Lim HY, Poon RT, Blanc JF, Vogel A, Chen CL, Dorval E, Peck-Radosavljevic M, Santoro A, Daniele B, Furuse J, Jappe A, Perraud K, Anak O, Sellami DB, Chen LT. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial. *JAMA* 2014; **312**: 57-67 [PMID: 25058218 DOI: 10.1001/jama.2014.7189]

103 **Goyal L**, Muzumdar MD, Zhu AX. Targeting the HGF/c-MET pathway in hepatocellular carcinoma. *Clin Cancer Res* 2013; **19**: 2310-2318 [PMID: 23388504 DOI: 10.1158/1078-0432.CCR-12-2791]

104 **Zhu AX**, Baron AD, Malfertheiner P, Kudo M, Kawazoe S, Pezet D, Weissinger F, Brandi G, Barone CA, Okusaka T, Wada Y, Park JO, Ryoo BY, Cho JY, Chung HC, Li CP, Yen CJ, Lee KD, Chang SC, Yang L, Abada PB, Chau I. Ramucirumab as Second-Line Treatment in Patients With Advanced Hepatocellular Carcinoma: Analysis of REACH Trial Results by Child-Pugh Score. *JAMA Oncol* 2016 [PMID: 27657674 DOI: 10.1001/jamaoncol.2016.4115]

105 **Bladt F**, Friese-Hamim M, Ihling C, Wilm C, Blaukat A. The c-Met Inhibitor MSC2156119J Effectively Inhibits Tumor Growth in Liver Cancer Models. *Cancers* (Basel) 2014; **6**: 1736-1752 [PMID: 25256830 DOI: 10.3390/cancers6031736]

106 **Schiffer E**, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C, Clergue F, Poupon R, Barbu V, Rosmorduc O. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. *Hepatology* 2005; **41**: 307-314 [PMID: 15660382 DOI: 10.1002/hep.20538]

107 **Kaseb AO,** Morris JS, Iwasaki M, Al-Shamsi HO, Raghav KPS, Girard L, Cheung S, Nguyen V, Elsayes KM, Xiao L, Abdel-Wahab R, Shalaby AS, Hassan M, Hassabo HM, Wolff RA, Yao JC. Phase II trial of bevacizumab and erlotinib as a second-line therapy for advanced hepatocellular carcinoma. OncoTargets and therapy 2016; 9: 773-780 [PMID: 26929648 DOI: 10.2147/OTT.S91977]

108 **Tai WM**, Yong WP, Lim C, Low LS, Tham CK, Koh TS, Ng QS, Wang WW, Wang LZ, Hartano S, Thng CH, Huynh H, Lim KT, Toh HC, Goh BC, Choo SP. A phase Ib study of selumetinib (AZD6244, ARRY-142886) in combination with sorafenib in advanced hepatocellular carcinoma (HCC). *Ann Oncol* 2016; **27**: 2210-2215 [PMID: 27681866 DOI: 10.1093/annonc/mdw415]

109 **Gavine PR**, Mooney L, Kilgour E, Thomas AP, Al-Kadhimi K, Beck S, Rooney C, Coleman T, Baker D, Mellor MJ, Brooks AN, Klinowska T. AZD4547: an orally bioavailable, potent, and selective inhibitor of the fibroblast growth factor receptor tyrosine kinase family. *Cancer Res* 2012; **72**: 2045-2056 [PMID: 22369928 DOI: 10.1158/0008-5472.CAN-11-3034]

110 **Sun CY**, Zhu Y, Li XF, Tang LP, Su ZQ, Wang XQ, Li CY, Yang HM, Zheng GJ, Feng B. Norcantharidin alone or in combination with crizotinib induces autophagic cell death in hepatocellular carcinoma by repressing c-Met-mTOR signaling. *Oncotarget* 2017; **8**: 114945-114955 [PMID: 29383132 DOI: 10.18632/oncotarget.22935]

111 **Tanwandee T**, Sukeepaisarnjaroen W, Chan SL, Choo SP, Han GH, Sriuranpong V, Pan HM, Yau TC, Ren ZG, Xu JM. A phase (Ph) II study of the efficacy and safety of the cMET inhibitor capmatinib (INC280) in patients (pts) with advanced hepatocellular carcinoma (HCC). *J Clin Oncol* 2016; **34**: 4074-4074

112 **Li Z**, You K, Li J, Wang Y, Xu H, Gao B, Wang J. Madecassoside suppresses proliferation and invasiveness of HGF-induced human hepatocellular carcinoma cells via PKC-cMET-ERK1/2-COX-2-PGE2 pathway. *Int Immunopharmacol* 2016; **33**: 24-32 [PMID: 26851630 DOI: 10.1016/j.intimp.2016.01.027]

113 **García-Vilas JA**, Quesada AR, Medina MA. Damnacanthal, a noni anthraquinone, inhibits c-Met and is a potent antitumor compound against Hep G2 human hepatocellular carcinoma cells. *Sci Rep* 2015; **5**: 8021 [PMID: 25620570 DOI: 10.1038/srep08021]

114 **Diaz D**, Ford KA, Hartley DP, Harstad EB, Cain GR, Achilles-Poon K, Nguyen T, Peng J, Zheng Z, Merchant M, Sutherlin DP, Gaudino JJ, Kaus R, Lewin-Koh SC, Choo EF, Liederer BM, Dambach DM. Pharmacokinetic drivers of toxicity for basic molecules: strategy to lower pKa results in decreased tissue exposure and toxicity for a small molecule Met inhibitor. *Toxicol Appl Pharmacol* 2013; **266**: 86-94 [PMID: 23142475 DOI: 10.1016/j.taap.2012.10.026]

115 **Qu Z**, Wu J, Wu J, Luo D, Jiang C, Ding Y. Exosomes derived from HCC cells induce sorafenib resistance in hepatocellular carcinoma both in vivo and in vitro. *J Exp Clin Cancer Res* 2016; **35**: 159 [PMID: 27716356 DOI: 10.1186/s13046-016-0430-z]

116 **Jo JC**, Choi EK, Shin JS, Moon JH, Hong SW, Lee HR, Kim SM, Jung SA, Lee DH, Jung SH, Lee SH, Kim JE, Kim KP, Hong YS, Suh YA, Jang SJ, Choi EK, Lee JS, Jin DH, Kim TW. Targeting FGFR Pathway in Human Hepatocellular Carcinoma: Expressing pFGFR and pMET for Antitumor Activity. *Mol Cancer Ther*

2015; **14**: 2613-2622 [PMID: 26351320 DOI: 10.1158/1535-7163.MCT-14-0780]

117 **Wang SW**, Pan SL, Guh JH, Chen HL, Huang DM, Chang YL, Kuo SC, Lee FY, Teng CM. YC-1 [3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl Indazole] exhibits a novel antiproliferative effect and arrests the cell cycle in G0-G1 in human hepatocellular carcinoma cells. *J Pharmacol Exp Ther* 2005; **312**: 917-925 [PMID: 15525795 DOI: 10.1124/jpet.104.077230]

P- Reviewer: Aravinthan AD, Elalfy H, Roohvand F, Vento S, Zhu X
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3709-3723

DOI: 10.3748/wjg.v24.i33.3709

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

REVIEW

p21-activated kinase signalling in pancreatic cancer: New insights into tumour biology and immune modulation

Kai Wang, Graham S Baldwin, Mehrdad Nikfarjam, Hong He

Kai Wang, Graham S Baldwin, Mehrdad Nikfarjam, Hong He, Department of Surgery, University of Melbourne, Melbourne 3084, Australia

ORCID numbers: Kai Wang (0000-0003-4152-8752); Graham S Baldwin (0000-0002-0944-8747); Mehrdad Nikfarjam (0000-0003-4866-276X); Hong He (0000-0002-2342-8750).

Author contributions: Wang K reviewed the literature and drafted the manuscript; He H, Baldwin GS and Nikfarjam M revised the manuscript; all authors approved the final version of this review.

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Unsolicited manuscript

Correspondence to: Hong He, MD, PhD, Senior Research Fellow, Department of Surgery, University of Melbourne, Austin Health, 145 Studley Rd., Heidelberg 3084, Victoria, Australia. hong.he@unimelb.edu.au
Telephone: +61-3-94965468

Received: May 22, 2018
Peer-review started: May 23, 2018
First decision: June 11, 2018
Revised: June 22, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: September 7, 2018

Abstract

Pancreatic cancer is one of the most aggressive and lethal malignancies worldwide, with a very poor prognosis and a five-year survival rate less than 8%. This dismal outcome is largely due to delayed diagnosis, early distant dissemination and resistance to conventional chemo-therapies. Kras mutation is a well-defined hallmark of pancreatic cancer, with over 95% of cases harbouring Kras mutations that give rise to constitutively active forms of Kras. As important down-stream effectors of Kras, p21-activated kinases (PAKs) are involved in regulating cell proliferation, apoptosis, invasion/migration and chemo-resistance. Immunotherapy is now emerging as a promising treatment modality in the era of personalized anti-cancer therapeutics. In this review, basic knowledge of PAK structure and regulation is briefly summarised and the pivotal role of PAKs in Kras-driven pancreatic cancer is highlighted in terms of tumour biology and chemo-resistance. Finally, the involvement of PAKs in immune modulation in the tumour microenvironment is discussed and the potential advantages of targeting PAKs are explored.

Key words: Pancreatic cancer; Kras; p21-activated kinases; Cell signalling; Chemo-resistance; Immune response; Tumour microenvironment

Core tip: Pancreatic cancer is still one of the most lethal malignancies, with a five-year survival of less than 8%. The dismal prognosis is largely the result of reprogramming of the tumour microenvironment, which leads to chemo-resistance and high aggressiveness. So far, combination chemotherapies can only marginally improve patients' survival, but with high toxicity. Therefore, alternative treatment targeting protein kinase signalling has been proposed. As downstream effectors of Kras signalling, p21-activated kinases (PAKs) are positioned at the nexus of multiple oncogenic signalling pathways. Here, the importance of PAKs as therapeutic targets in Kras signalling is discussed, and their essential

role in tumour biology and immune modulation within the tumour microenvironment is highlighted.

Wang K, Baldwin GS, Nikfarjam M, He H. p21-activated kinase signalling in pancreatic cancer: New insights into tumour biology and immune modulation. *World J Gastroenterol* 2018; 24(33): 3709-3723 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3709.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3709

INTRODUCTION

Pancreatic cancer is a highly aggressive and lethal malignancy with a dismal prognosis. In contrast to the improvements in therapies and the consequent increasing long-term survival rate for most other cancers, few advances have been achieved in pancreatic cancer, for which the overall five-year survival rate is still less than 8%[1]. The death rate from pancreatic cancer continues to increase by 0.3% per annum, and it is estimated that this malignancy will become the second most common cause of cancer-related death in the United States by 2030[2].

Although surgery remains the only curative treatment, chemotherapy is still an important and indispensable treatment in maximizing the life span for both resectable and unresectable patients. Currently gemcitabine-based combination therapies and FOLFIRINOX (irinotecan, oxaliplatin, fluorouracil, and leucovorin) are the mainstream approaches for patients with local advanced and metastatic pancreatic cancer, with an increased survival compared to gemcitabine alone[3-6]. However, the modest improvement in survival, the highly toxic side effects and chemo-resistance have become major challenges in the clinical setting. Therefore, there is an urgent need to develop more effective and less toxic therapeutic strategies to treat this malignancy.

Progression of pancreatic cancer is marked by an accumulation of multiple genetic mutations, of which mutation in the Kras oncogene is the most frequent, with over 95% of pancreatic cancers harbouring a Kras mutation[7]. The presence of missense mutations at codons 12, 13 or 61 within the Kras gene disrupts the physiological inactivation cycle of the Kras protein, resulting in a constitutively activated state even in the presence of GTPase activating protein.

The Kras protein is notable for the absence of a well-defined drug-binding domain on its surface[8]. So far, despite over thirty years of intensive biomedical research, no drug directly targeting the Kras protein has proved to be an effective cancer treatment in the clinic[7,9]. While some exciting and promising results have appeared for treatments that targeted important downstream effectors of Kras such as PI3K, AKT and MEK, resistance developed rapidly in almost all cases, making these molecular targets less effective[10]. In order to overcome this challenge, approaches targeting novel downstream effectors of the Kras protein are urgently needed. Recently, the National Cancer Institute in the United States has proposed a new project to fight against Ras-driven cancers, with the stated aim that new therapeutic strategies interfering with Ras-dependent signalling pathways should be given priority in cancer research[11]. One such family of novel effectors is the p21-activated kinases (PAKs), which are activated by Kras and by other small GTPases like Cdc42 and Rac by both direct and indirect mechanisms. PAKs are positioned at the nexus of multiple oncogenic signalling pathways that mediate a variety of hallmark processes in pancreatic cancer.

Pancreatic cancer has its own unique immune response during tumour development. The Kras oncogene can mediate the inflammatory process and establish within the tumour microenvironment an immune-privileged condition, which is responsible for the suppression of effector cells and the stimulation of immunosuppressive cells[12]. Additionally, the extensive desmoplastic reaction in pancreatic cancer also functions as a physiological barrier against immune surveillance, leading to evasion of the anti-tumoural immune response and tumour progression[13].

In this review, basic knowledge of PAK structure and regulation is briefly summarised, and the importance of PAKs as a therapeutic target in Kras signalling is highlighted. The essential role of PAKs in regulating tumour biology and stromal re-programming, especially of the immune response within the tumour microenvironment, is also discussed.

STRUCTURE AND ACTIVATION OF PAKS

PAKs are a family of serine/threonine kinases that are the downstream effector proteins of Ras, and of other small GTPases such as Cdc42 and Rac. The six known members of the PAK family can be categorized by similarities in their sequence and structure into two groups: group I (PAK1-3) and group II (PAK4-6)[14]. All PAKs are characterized by an N-terminal regulatory domain and a conserved C-terminal serine/threonine kinase domain with a single phosphorylation site (Figure 1), but the activation of group I and group II PAKs is regulated through completely different mechanisms[15,16].

The group I PAKs share a high level of structural homology with over 88% identity in the GTPase-binding domain (GBD) that is responsible for binding Cdc42 or Rac, and more than 93% identity in the kinase domain[14]. However, their tissue specific distribution is quite different from each other. PAK1 can be found in various organs including brain, mammary gland, muscle, and spleen; PAK2 is ubiquitously expressed; whereas PAK3 is only expressed in the nervous system[17]. The N-terminal regulatory domain of group I PAKs contains an autoinhibitory domain (AID) that overlaps with the GBD. In the inactivated state, group

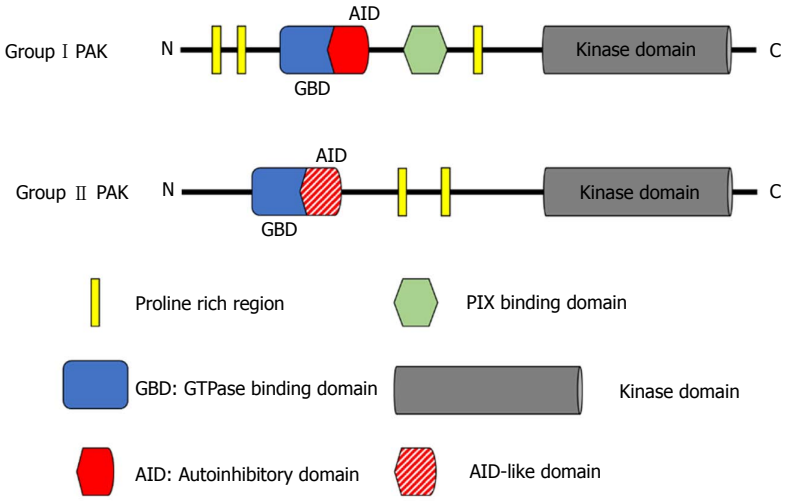


Figure 1 Structure of p21-activated kinases. The six members of the PAK family can be divided by sequence and structural differences into two groups: Group I (PAK1-3) and group II (PAK4-6). All PAKs have an N-terminal regulatory domain and a conserved C-terminal serine/threonine kinase domain. In group I PAKs, the regulatory domain contains an AID, whereas group II PAKs (with the possible exception of PAK5) do not have a well-defined AID, but instead an AID-like domain. PIX: PAK-interactive exchange factor; PAK: p21-activated kinases; AID: Autoinhibitory domain; GBD: GTPase-binding domain.

I PAKs form homodimers, with the AID domain of one PAK molecule binding to the kinase domain of its companion. When Cdc42/Rac binds to the GBD, binding of the AID to its partner PAK is disrupted. This critical process generates two PAK monomers, and allows the subsequent autophosphorylation at the Thr^{423} site, which is important for maintaining PAK1 activation[18-21].

Group II PAKs have a quite different structure from group I PAKs, but the three members are still similar to each other. They share at least 60% identity in the N-terminal GBD and over 75% identity in the kinase domain. However, the identities between Group I and Group II PAKs are less than 40% in the GBD and only about 54% in the kinase domain[14]. PAK4 is highly expressed throughout embryonic development, and ubiquitously expressed in all adult tissue at a low level[22,23]. PAK5 is specifically expressed in the brain[24]. PAK6 is not only found in the adult nervous system, but also in the male reproductive system (*e.g.,* testes and prostate). This distribution correlates with its important role in the androgen receptor signalling pathway[25,26]. Although group II PAKs (with the possible exception of PAK5) have no well-defined AID in the N-terminal domain, later studies have reported AID-like domains[27,28]. Unlike group I PAKs, group II PAKs are monomers and are constitutively phosphorylated, even in their inactivated state[15]. Although there is still much debate on the exact activation mechanism of group II PAKs, two different activation models have been proposed over the last decade. In the first model, the AID-like domain binds to the kinase domain of the same molecule, which results in an inactive confirmation regardless of the constitutive autophosphorylation. When Cdc42 binds to the GBD, binding of the AID-like domain to the kinase domain is disrupted, leading to an active conformation[15]. In the second model, an autoinhibitory pseudo-substrate domain, next to the GBD but distinct from the AID, interacts with the kinase domain, reducing activity. The binding of Cdc42 to GBD translocates the group II PAK to a subcellular region where a Src homology 3 domain-containing protein binds to the autoinhibitory pseudo-substrate domain, preventing its interaction with the kinase domain and hence increasing activity[28].

ROLE OF PAKS IN KRAS-DRIVEN ONCOGENIC PATHWAYS

Kras is the most frequently mutated isoform observed in all types of human cancer compared to NRas and Hras. Kras mutation is a key oncogenic driver in the development of pancreatic, colorectal and lung cancer[29]. It acts as a regulatory switch in diverse sub-cellular signal transduction networks, which are responsible for stem-cell like features, cell survival, proliferation, invasion and migration[30].

A study of a genetically engineered mouse model for pancreatic cancer, the KPC (LSL-$Kras^{G12D}$; LSL-$Trp53^{R172H}$; Pdx1-Cre) model, has revealed that expression of the $Kras^{G12D}$ mutation is sufficient to induce pancreatic intraepithelial neoplasia (PanIN), followed by advanced carcinoma[31]. Similarly, Ying and colleagues demonstrated that the $Kras^{G12D}$ mutation was necessary for the maintenance of pancreatic cancer as Kras depletion resulted in rapid tumoural regression and stromal degeneration in an oncogenic Kras-induced tumour model[32]. Mutated Kras can cause phosphorylation and activation of other p21 proteins such as Rac1 and Cdc42, through both canonical and alternative pathways[33]. Then the interaction between Rac1/Cdc42 and PAKs can increase PAK activity, leading to persistent activation of downstream signalling pathways such as the RAF/MEK/ERK and PI3K/PDK1/

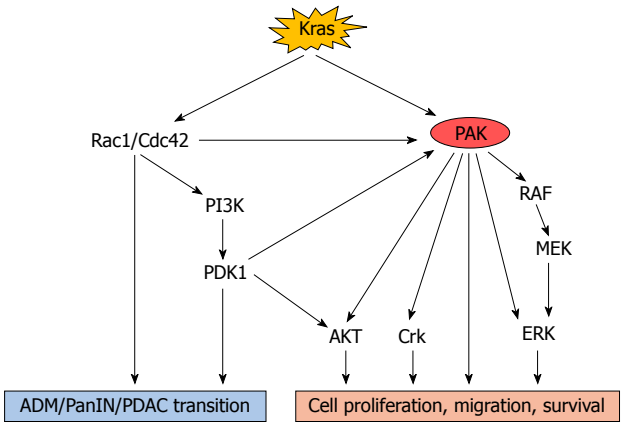


Figure 2 Role of p21-activated kinases in Kras-driven oncogenic signalling pathways. Rac1 is the 4th best validated effector in Kras signalling and is a well-defined upstream protein of PAKs. Rac1 plays an important role in the ADM/PanIN/PDAC transition. In addition, Rac1/Cdc42 mediates this pathological process *via* the PI3K-PDK1 signalling pathway. PDK1 can also interact with PAK1, leading to its phosphorylation. The Kras oncogene activates PAKs through direct and indirect pathways. Activated PAKs can increase cancer cell proliferation, migration and survival through activation of AKT, Crk and RAF-MEK-ERK pathways. PAK: p21-activated kinases; ADM: Acinar-ductal metaplasia; PDAC: Pancreatic ductal adenocarcinoma; PDK1: Phosphoinositide-dependent kinase-1.

AKT pathways[34-37] (Figure 2).

A recent study of non-small cell lung cancer reported that Kras-mutated tumours expressed more Thr^{423}-phosphorylated PAK1 than Kras-wild type tumours, and that the Kras/PAK1/Crk axis played an essential role in the oncogenesis of Kras-mutated lung cancer[38]. Activation of PAK1 could also be mediated by multiple Kras-dependent pathways *via* different cell surface receptors. Dominant-negative Ras, Rac, and Cdc42 suppressed PAK1 activation whereas activated Rac1 and Cdc42 were able to stimulate PAK1 even in the absence of any agonists[39]. As a potent activator of PAK1, Rac1 is the 4th best validated effector in the Kras-driven cell signalling cascade[40]. In an early study, Rac1 was found to be associated with pancreatic acinar plasticity and Rac1 inhibition reduced acinar cell damage induced by pathological inflammation[41]. The important role of Rac1 in early metaplasia and neoplasia-related actin rearrangements has been revealed in a Kras-driven mouse model of pancreatic cancer[42]. Rac1 ablation in this model reduced the incidence of acinar-ductal metaplasia (ADM), PanIN and tumour formation and significantly improved animal survival. Interestingly, this study also found that Rac1 was not indispensable in pancreas development[42]. Similarly, Zheng *et al*[43] reported that Rac1 and Cdc42 could mediate the activation of PI3K by interacting with its 85-kDa regulatory domain. Another study documented that PI3K together with PDK1 acted as critical downstream effectors of oncogenic Kras signalling in mediating ADM and formation of pancreatic cancer[44]. PDK1 was also reported to interact with PAK1 both *in vitro* and *in vivo*, leading to increased phosphorylation at the Thr^{423} site and hence activation of PAK1[45].

There is increasing evidence for a key role of PAK1 in regulating Kras-dependent signalling pathways. PAK1 can phosphorylate c-RAF at Ser^{338} in NIH3T3 cells (murine fibroblast cell line), and inhibition of group I PAK kinase activity significantly reduced the phosphorylation of MEK1 at Ser^{298} and the activation of ERK in response to different growth factors (*e.g.*, platelet-derived growth factor or epidermal growth factor) in NIH3T3 and HeLa cells (human cervical cancer cell line)[46]. Huynh *et al*[47] demonstrated that PAK1 stimulates colon cancer cell proliferation, migration/invasion, and survival *via* ERK- and AKT-dependent pathways. Inhibition of PAK1 effectively inhibits both ERK and AKT, to an extent which cannot be achieved by inhibition of either alone. Another study also showed that genetic deletion of PAK1, followed by decreased ERK and AKT activity, suppressed tumourigenesis and progression in a Kras-mediated skin cancer model[48]. In contrast, Tabusa and colleagues found that knockdown of PAK1 or PAK4 inhibited the proliferation of Kras-mutated colorectal cancer cells *via* non-canonical pathways independent of RAF/MEK/ERK and PI3K/AKT signalling[49].

A relationship between PAK4 and Kras has been identified through genetic analysis of human pancreatic cancer cell lines and patients' samples[50]. By sequencing the Kras gene in PAK4-amplified tumour samples, mutations in codon 12 were observed in 4 out of the 5 samples. Furthermore, genomic amplification and overexpression of Kras occurred in 3 samples. Interestingly, no mutations were detected in Kras or PAK4 in the fifth sample, but the observation of increased PAK4 expression suggests that PAK4 could be up-regulated and activated through some Kras-independent pathways. Taken together, the above evidence suggests that PAKs play an important role in interacting with and transmitting Kras-driven oncogenic signals in different kinds of human cancer.

PAK SIGNALLING IN PANCREATIC CANCER

Amplification of the PAK1 gene within chromosomal region 11q13 was reported to be linked to both tumourigenesis and poor prognosis of different human cancers[51,52]. Amplification of the PAK4 gene within chromosomal region 19q13.2 was also identified in a variety of human malignancies, especially pancreatic, breast, and ovarian cancer[50,53,54]. By using fluorescent in situ hybridization on tumour microarrays, Kimmelman *et al*[55] found PAK4 amplification occurred in 14 of 63 (22%) pancreatic cancer samples. In addition, RT-qPCR and Western blots showed increased PAK4 expression in multiple pancreatic cancer cell lines regardless of gene amplification, implying different underlying mechanisms mediating PAK4 expression. Interestingly, the observation that the CCND1 (Cyclin D1) and CCNE1 (Cyclin E1)

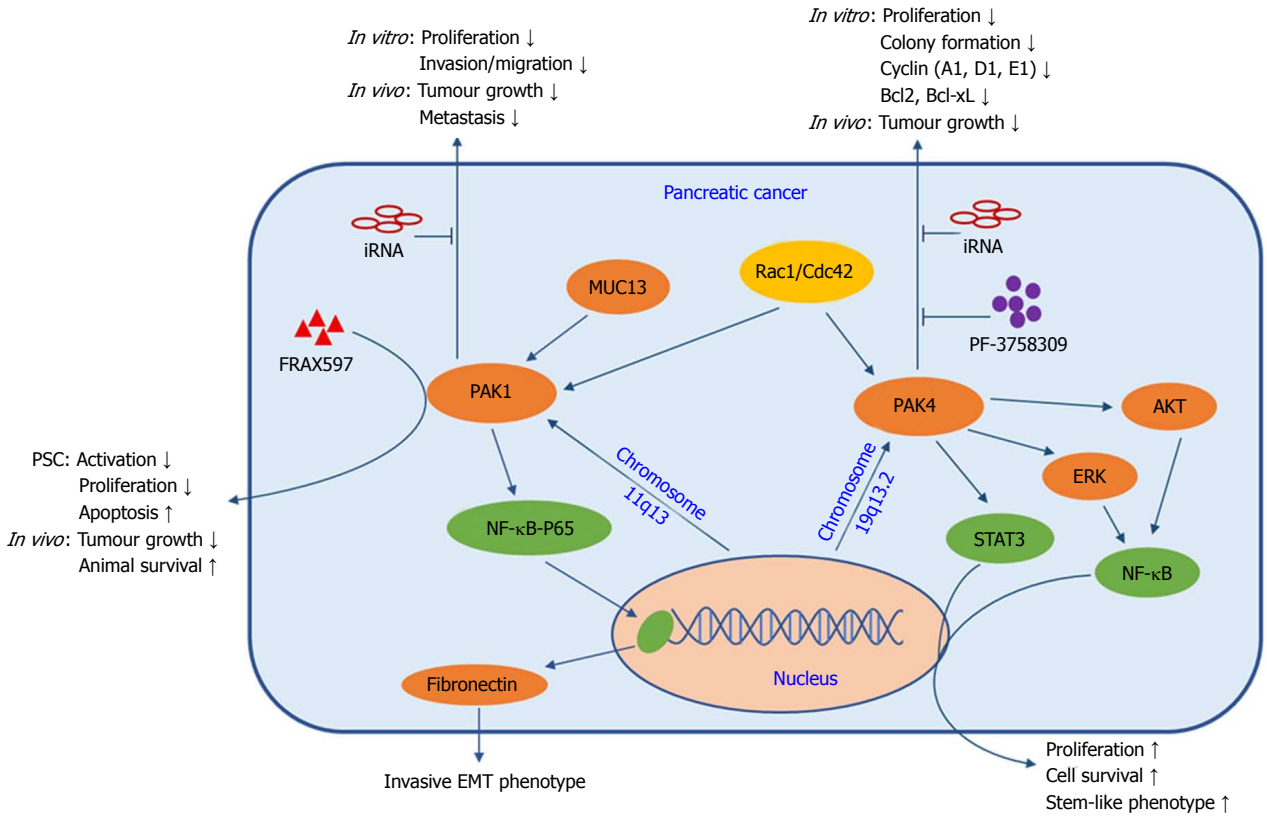


Figure 3 p21-activated kinases signalling in the development of pancreatic cancer. PAK signalling is involved in several pathobiological processes in pancreatic cancer, including proliferation, migration/invasion, apoptosis and maintenance of stem cell-like properties. Amplification of the PAK1 and PAK4 genes, present within the chromosomal regions 11q13 and 19q13.2, respectively, has been observed. Activated PAK1 regulates cell transformation and the invasive EMT phenotype of pancreatic cancer cells *via* the NF-κB-p65-fibronectin axis. Additionally, MUC13 promotes cancer cell growth and invasion/migration, and reduces animal survival, by up-regulating expression and phosphorylation of PAK1. Furthermore, PAK4 modulates proliferation and survival by mediating the activity of NF-κB *via* AKT- and ERK-dependent pathways, and cancer stem cell-like properties *via* STAT3 signalling. Pharmacological or genetic inhibition of PAK1 or PAK4 leads to decreased cancer cell proliferation, invasion/migration and PSC activation *in vitro*, and reduced tumour growth and metastasis, and increased animal survival *in vivo*. PAK: p21-activated kinases; PSC: Pancreatic stellate cells.

genes were co-amplified within the same chromosomal region as PAK1 and PAK4, respectively[16,56], suggests that co-amplification of PAK1 with CCND1 and PAK4 with CCNE1 may have synergistic effects on the initiation and progression of pancreatic cancer.

Role of PAK1 in tumour proliferation, migration and tumour-stroma interaction

A study of 304 human primary pancreatic cancer samples found that 262 (86%) cases were positive for cytoplasmic PAK1 staining, and approximately one-third of all samples showed moderate (2+) to strong (3+) intensity in the malignant cells and nuclear localization of PAK1[57]. Two more recent studies have also shown increased PAK1 expression in resected human pancreatic cancer tissues and cell lines, when compared to the adjacent normal pancreas and an immortalized normal pancreatic ductal epithelial cell line, respectively[58,59]. A PAK1 knock-down pancreatic cancer cell line failed to develop tumours in nude mice[58] and showed markedly reduced proliferation[59]. Furthermore, Jagadeeshan and colleagues demonstrated that fibronectin was a transcriptional target of PAK1 signalling *via* the NF-κB-p65-fibronectin axis, which modulates cell transformation and the invasive EMT phenotype of pancreatic cancer cells (Figure 3). Early studies also showed the localization of activated PAK1 to the cell nucleus[60] and its involvement in the activation of NF-κB, by demonstrating that active Ras or Rac1 stimulated NF-κB in a PAK1-dependent manner and that active PAK1 itself could stimulate NF-κB as well[61]. An important transmembrane mucin (MUC13) was also reported to be involved in PAK1 signalling in the development of pancreatic cancer[62]. Overexpression of MUC13 promoted cancer cell proliferation, invasion/migration and anchorage-dependent or -independent colony formation *in vitro* and led to increased xenograft tumour growth and decreased animal survival *in vivo*. These tumourigenic properties were closely associated with the up-regulated expression and phosphorylation of PAK1, ERK and AKT, and suppression of p53. Wei *et al*[57] screened a panel of pancreatic cancer cell lines and characterized PAK1 as a key downstream effector of cell motility triggered by multiple growth factors. In their study, PAK1 inhibition not only restored sensitivity to a hepatocyte growth factor/Met antagonist (onartuzumab) in the presence of exogenous growth factors or PAK1-amplification *in vitro*, but also suppressed tumour growth and metastasis *in vivo*.

In recent years, the interaction between pancreatic cancer cells and pancreatic stellate cells (PSCs) has become the focus of pancreatic cancer research. Activation of PSCs by cancer cells is predominately responsible for fibrosis and stromal remodelling. The role of PAK1 in modulating EMT markers (*e.g.*, fibronectin, E-cadherin, and vimentin) has been established in early studies[63]. A recent study revealed the role of PAK1 in PSC modulation for the first time by showing that inhibition of PAK1 by FRAX597 (a potent group I PAK inhibitor) reduced the activation and proliferation of PSC and increased apoptosis *in vitro*. In an orthotopic pancreatic cancer mouse model, survival in the PAK1 knockout group was significantly increased compared to the PAK1 wildtype group, and depletion of PAK1 in the pancreatic stroma also reduced PAK1 expression and activity in the tumour[64]. Similarly, survival was prolonged in the group treated with FRAX597 plus gemcitabine in the same orthotopic pancreatic cancer model[59]. These results pave the way to a detailed investigation of the role of PAK1 in tumour-stroma interactions in order to improve therapeutic response by using targeted inhibitors.

Role of PAK4 in tumour proliferation, migration, survival and stemness maintenance

PAK4 expression is reported to be correlated with pancreatic cancer pathology. Tyagi *et al*[65] found 54 out of 56 tumour samples from patients with pancreatic cancer had positive PAK4 staining, with no PAK4 positive staining in normal pancreatic tissue. Furthermore, PAK4 promoted cancer cell proliferation and survival by stimulating the nuclear accumulation and transcriptional activity of NF-κB *via* AKT- and ERK-dependent pathways (Figure 3). PAK4 knockdown in pancreatic cancer cells caused suppression of growth and colony formation associated with reduced expression of cell-cycle (cyclin A1, D1, E1) and anti-apoptosis (Bcl2, Bcl-xL) proteins. Similarly, inhibition of PAK4 by PF-3758309 (a potent pan-PAK inhibitor) suppressed cancer cell proliferation and migration both *in vitro* and *in vivo*[66]. Kimmelman *et al*[55] identified Rio Kinase 3 and PAK4 as amplified genes in highly recurrent and focal amplifications in pancreatic cancer. Rio Kinase 3 can activate the small GTPase protein Rac, which can subsequently promote cell motility and invasion *via* PAK4-mediated signalling. In addition, overexpression of activated PAK4 resulted in increased invasion/migration in a gain-of-function experiment, while PAK4 knockdown by shRNA significantly reduced anchorage independent growth in a loss-of-function experiment. Recently, PAK4 has been shown to modulate STAT3 signalling in the maintenance of pancreatic cancer stem cells, which are considered to be responsible for high aggressiveness and chemo-resistance. Pancreatic cancer stem-like cells ($CD24^+$/$CD44^+$/$EpCAM^+$) showed higher PAK4 expression as compared to triple negative cells ($CD24^-$/$CD44^-$/$EpCAM^-$). PAK4 expression enhanced the expression of stem cell-associated transcription factors (Oct4/Nanog/Sox2 and KLF4), whereas PAK4 silencing caused reduced nuclear accumulation and transcriptional activity of STAT3 and loss of stem cell phenotypes[67,68]. The accumulated evidence, which suggests that PAKs are positioned at the convergence point of numerous oncogenic pathways, highlights their potential as promising therapeutic targets in the treatment of pancreatic cancer.

PAKS AND CHEMO-RESISTANCE IN PANCREATIC CANCER

Currently, surgical resection is the only curative treatment for pancreatic cancer. However, due to the lack of biomarkers for early diagnosis, low surgical resectability (only 15%-20% of patients are considered to be eligible candidates)[69], and high recurrence rate (up to 60%), the overall median survival is still less than 20 mo in patients undergoing resection of pancreatic cancer with curative intent[70]. Therefore, chemotherapy remains a crucial alternative or adjuvant treatment for patients with resectable or unresectable tumours[71].

Two decades ago, gemcitabine emerged as the standard of care for pancreatic cancer patients[72]. So far, gemcitabine + nab-paclitaxel and FOLFIRINOX have been approved by the United States Food and Drug Administration as first-line therapies, especially for patients with locally advanced and metastatic pancreatic cancer[3,6]. Although previous clinical studies reported several advantages of gemcitabine over 5-FU, including prolonged median survival, improved tumour-related symptoms and lower systemic toxicity[73-75], the results were still unsatisfactory, with effective responses in less than 10% of patients. Therefore, various modifications of gemcitabine treatment have been designed to overcome resistance and increase drug delivery into the tumour. These modifications include CO-101[76] (a lipid-conjugated gemcitabine, which can be transported into tumour cells independently of the human equilibrative nucleoside transporter, and Acelarin[77] (an aryloxy phosphoramidate derivative of gemcitabine with greater lipophilicity, which accumulates in cancer cells by passive diffusion independently of the nucleoside transporter). However, intrinsic and acquired gemcitabine resistance occurs in most patients, and its underlying molecular mechanism is still not fully understood.

Mechanisms involved in chemo-resistance of cancer cells

In a prospective randomized clinical study, the human equilibrative nucleoside transporter 1(hENT1), which is the principal cellular transporter of gemcitabine, was found to be a valuable predictive marker of gemcitabine sensitivity in patients with resected pancreatic cancer[78]. In addition, a comparative study also indicated that decreased hENT1 expression was associated with gemcitabine resistance and poorer overall survival in

patients with pancreatic cancer[79]. However, an early study reported that up-regulated hENT1 expression was also observed in some gemcitabine-resistant pancreatic cancer cell lines[80]. This evidence implicated hENT1 as the predominant, but not the only, metabolic protein mediating resistance. To some extent, hENT2 and the human concentrative nucleoside transporters hCNT1 and hCNT3 may also contribute to the development of acquired and intrinsic gemcitabine resistance[81,82]. Moon and colleagues have shown that gemcitabine-resistant cell lines express more PAK4 and less hENT1. PAK4 knockdown in gemcitabine-resistant cell lines induces the up-regulation of hENT1 and restoration of sensitivity to gemcitabine[83]. In contrast, one recent retrospective clinical study, which analyzed 160 resected human pancreatic cancer samples by immunohistochemistry, reported that higher expression of PAK4 was correlated with higher expression of hENT1[84]. Therefore, the controversial role of PAK4 in regulating hENT1 should be further explored.

Furthermore, Jagadeeshan *et al*[85] revealed that PAK1 plays a pivotal role in mediating gemcitabine resistance by altering apoptosis and survival signals, and suppressing DNA damage *via* the NF-κB pathway. Phosphorylation of PAK1 and ribonucleotide reductase M1 was elevated in patient samples when compared with normal tissue. Combination treatment with a PAK1 inhibitor synergistically improved gemcitabine efficacy and led to tumour regression in animal models. In agreement with this finding, inhibition of PAK1 or/and PAK4 by shRNA knockdown or PAK inhibitors enhanced gemcitabine sensitivity both *in vitro* and *in vivo*[59,66].

Other potential PAK-associated signalling pathways also contribute to chemo-resistance. Higher expression of HIF-1α was observed in gemcitabine-resistant pancreatic cancer cell lines[86] and inhibition of HIF-1α can sensitize cancer cells to gemcitabine treatment[87]. The increased activity of HIF-1α was associated with inhibition of the transcription of hENT1 and hENT2, leading to reduced expression of transporter proteins followed by decreased gemcitabine uptake[88,89]. The critical role of PAKs in regulating HIF-1α has been demonstrated in our previous studies, which showed that PAK1 could enhance cancer cell survival by up-regulation of HIF-1α and that inhibition of PAK1 caused decreased expression of HIF-1α and tumour growth[59,90]. Another recent study also found that PAK4 inhibition reduced expression of HIF-1α *via* the AKT-mTOR-4E-BP1 axis[91].

Additionally, the important transcription factor NF-κB is also a critical regulator closely associated with gemcitabine resistance in pancreatic cancer. As discussed above, localization of active PAK1 to the nucleus is involved in activation of NF-κB[60,61], and both PAK1 and PAK4 contribute to cell transformation, proliferation and survival *via* NF-κB-dependent signalling pathways in pancreatic cancer[58,65]. Over a decade ago, Arlt and colleagues revealed that resistant cell lines (BxPC-3, Capan-1 and PancTu-1) showed higher expression of NF-κB, comparing to sensitive cell lines (PT45-P1 and T3M4). Treatment of these five pancreatic cancer cell lines with gemcitabine induced NF-κB activity in a dose-dependent manner[92]. Inhibition of the p65 subunit of NF-κB by siRNA can improve gemcitabine sensitivity to suppress proliferation and induce apoptosis both *in vitro* and *in vivo*[93]. In agreement with this conclusion, Skrypek *et al*[94] also showed that decreased activation of NF-κB pathway was correlated with an alteration of hCNT1 expression and increased gemcitabine sensitivity in MUC4-knockdown pancreatic cancer cell lines.

Furthermore, both expression and activity of PAK4 have also been reported to be up-regulated in cisplatin-resistant cancer cells as compared with parental cells. Inhibition of PAK4 diminished cisplatin resistance *via* PI3K/AKT and MEK/ERK-dependent signalling pathways[95].

Stromal remodelling

The extensive desmoplastic reaction, which is a hallmark of pancreatic cancer, is reported to result in a dense stroma, deficient vascularization and inefficient drug delivery, eventually leading to chemo-resistance[96,97]. As mentioned above, PAK1 is responsible for PSC activation, leading to stromal fibrosis[64] in pancreatic cancer similar to its pivotal role in liver fibrogenic pathways[98].

The importance of Hedgehog (Hh) signalling in tumourigenesis and desmoplasia has been well established. Hh can modify the extracellular matrix component *via* regulation of the differentiation and motility of PSCs and fibroblasts[99,100]. The observation, in an early global genomic analysis of 24 human pancreatic cancers, that Hh signalling was one of the core set of 12 most commonly altered cellular signalling pathways and was present in 100% of cases suggests a significant contribution of Hh signalling to the development of pancreatic cancer[101]. A number of previous studies also demonstrated that depletion of the Hh signalling pathway could partly diminish desmoplasia-associated resistance and synergistically enhance gemcitabine efficacy both *in vitro* and *in vivo*[102-104]. Hiroshi *et al*[105] found that activation of NF-κB resulted in the aberrant activation of Hh signalling *via* up-regulation of sonic Hh (a ligand of Hh signalling) in pancreatic cancer.

Interaction of the C-X-C motif chemokine 12 (CXCL12), which is also known as stromal cell-derived factor 1, with its receptor, the C-X-C motif chemokine receptor 4 (CXCR4), can induce activation of downstream signalling pathways related to tumour progression and metastasis[106]. Singh *et al*[107] have identified the essential role of the CXCL12/CXCR4 axis in stimulation of Hh signalling in a dose- and time-dependent manner. CXCL12-induced Hh up-regulation is due to the increased nuclear accumulation and activation of NF-κB mediated by AKT and ERK signalling pathways. The involvement of PAK1 and PAK4 in NF-κB signalling in pancreatic cancer has been clearly identified[58,61,65]. Although the interaction between PAKs and Hh-mediated chemo-resistance is still not clear, the above findings indicate that PAKs might

regulate Hh signalling in a NF-κB-dependent manner and further investigation is needed.

EMERGING ROLE OF PAKS IN IMMUNE MODULATION IN THE TUMOUR MICROENVIRONMENT

As an important component of the stromal microenvironment, infiltrating immune cells (IICs) have been characterized as valuable markers in predicting prognosis. Generally, IICs exhibit both pro-tumour and anti-tumour effects. The former class of IICs include regulatory T cells, myeloid-derived suppressor cells (MDSC), and tumour-associated macrophages (TAM) which suppress anti-tumour immunity and promote tumour growth, whereas the latter class include $CD8^+$ T cells, Th1-type $CD4^+$ T cells, and natural killer cells[108,109]. Immunosuppressive cells can ward off the host immune defence, prevent tumour cells from being recognized and further lead to immune evasion, even in pre-cancerous lesions such as PanINs and intraductal papillary mucinous neoplasm (IPMN)[110]. Therefore, a great deal of attention has been paid to targeting the aberrant immune regulation of the tumour microenvironment, with the intention of reversing the suppression of active anti-tumour immunity. A good example is the conversion of pancreatic cancer from "a non-immunogenic malignancy" into "an immunogenic malignancy" by treatment with a novel immunomodulatory vaccine, which blockaded the immune checkpoint (PD-1/PD-L1, CTLA-4) and made therapy more effective in vaccine-treated patients than in untreated patients[111].

Myeloid-derived suppressor cells

MDSCs, which include both granulocytic and monocytic subsets, are a heterogeneous mixture of activated immature myeloid cells, which can stimulate angiogenesis, promote tumour invasion and migration, and suppress T-cell activation[112]. Both circulating and tumour-infiltrating MDSCs, of the granulocytic subset (Lin-HLA-DR-$CD33^+CD11b^+CD15^+$), but not the monocytic subset (Lin-HLA-DR-$CD14^+$), are markedly elevated in patients with pancreatic cancer compared to the healthy population. Moreover, MDSCs can also serve as an independent prognostic factor for patients' survival as one unit increase in MDSC percentage leads to a 22% greater risk of mortality[113,114]. The report by Thomas *et al*[115] of a non-canonical role of the mammalian target of rapamycin (mTOR) protein in recruiting tumourigenic MDSC suggests that cancer cells can stimulate intra-tumoural MDSC accumulation by promoting granulocyte colony-stimulating factor (G-CSF) production *via* an mTOR-dependent pathway. He and colleagues demonstrated the critical role of PAK4 in regulating mTOR signalling through the PI3K/AKT axis in breast cancer[116]. In addition, PAK1 can be activated by the mTOR/p70 S6 kinase pathway, and treatment with rapamycin, a mTOR inhibitor, leads to reduced PAK1 expression[117]. Interestingly, an early study on vascular smooth muscle cells indicated that G-CSF was involved in activation of the GTPase Rac1, a potent upstream activator of PAK1, and inhibition of Rac1 suppressed G-CSF-driven migration of vascular smooth muscle cells[118]. Previous studies also found that pancreatic cancer cells or stellate cells can attract and transform peripheral blood monocytes into MDSCs *via* STAT3 activation, which in turn will increase the stem-cell properties and mesenchymal features of tumour cells[119,120]. The role of PAK1 and PAK4 in regulating STAT3 signalling in pancreatic cancer cells has been clearly identified[67,121]. Although there is as yet little direct evidence linking PAK to MDSC modulation, the above findings indicate that PAK might orchestrate multiple signalling pathways to mediate MDSC recruitment and activation.

Tumour-associated macrophages

TAMs can be divided into two subtypes: M1 (pro-inflammatory macrophages) and M2 (anti-inflammatory macrophages). Like MDSCs, the majority of TAMs are derived from circulating monocytes[122]. M1 TAMs can suppress tumour development by stimulating a T-cell-mediated anti-tumour response, whereas the crosstalk of M2 TAMs with tumour and stellate cells can stimulate secretion of various anti-inflammatory cytokines, and reprogram immune surveillance within the tumour microenvironment to facilitate tumour progression[123].

Stephen *et al*[124] have identified a role of PAK1 in regulating macrophage spreading and lamellipodial dynamics through the activation of ERK1/2. However, they also found that PAK1 knockout had no impact on migration or chemotaxis of macrophages, whereas another study reported that absence of Rac1 or Rac2 could promote macrophage migration[125]. These observations suggest either that PAK2 might compensate for the lack of PAK1, or that other Rac down-stream effectors are involved in regulating cell migration[126]. In addition, Gringel *et al*[127] found that PAK4 functioned as a physiological regulator of podosomes, which are involved in the migration of human macrophages. Up-regulated expression and activity of PAK4 and its regulator α-PIX (PAK-interacting exchange factor) enhanced the number and size of macrophage podosomes.

There are some additional potential mechanisms linking PAKs to macrophage migration and chemotaxis. The interaction between PAK and HIF-1α has been well established from previous studies[59,90,91]. Recently, HIF-1α was reported to be involved in the recruitment of TAMs in pancreatic cancer through promoting C-C motif chemokine ligand 2 (CCL2) secretion, which stimulated monocyte infiltration into the tumour microenvironment by binding to its receptor CCR2[128]. In agreement with this report, Sanford *et al*[129] revealed an important role of CCL2/CCR2 in TAM recruitment by showing that a CCR2 antagonist (PF-04136309) was able to block the migration of circulating $CCR2^+$ monocytes toward

the tumour with a consequent depletion of TAMs in a mouse model of pancreatic cancer. Their clinical data also indicated that pancreatic cancer patients with a higher level of CCL2 expression and greater infiltration of immunosuppressive $CCR2^+$ TAMs were significantly more likely to have decreased survival. Additionally, Allen and colleagues revealed the importance of Rho family proteins in regulating actin organization and cell adhesion in macrophages[130]. Using a colony-stimulating factor-1 -dependent murine macrophage cell line (Bac1.2F5), they demonstrated that constitutively activated Rac1 or Cdc42, which are both well-defined up-stream activators of PAKs, could stimulate formation of lamellipodia or filopodia, whereas dominant negative Rac1 or Cdc42 inhibited colony-stimulating factor-1-induced formation of lamellipodia or filopodia.

Macrophage polarization is induced by different stimuli *via* interferon-regulatory factor/signal transducer and activator of transcription (IRF/STAT) signalling pathways, NF-κB pathways and HIF stabilization. IRF/STAT factors (IRF3, IRF5, STAT1 and STAT5), HIF-1 and the active NF-κB heterodimer (p50-p65) contribute to M1 polarization, while IRF/STAT factors (IRF4, STAT3 and STAT6), HIF-2 and the inhibitory NF-κB heterodimer (p50-p50) trigger an M2 response[131]. The involvement of PAK1 in macrophage polarization has recently been characterized by Zhang and colleagues, who reported that pharmacological or genetic inhibition of PAK1 diminished M1 macrophage polarization. This observation suggested that the up-regulation of PAK1 induced by inflammatory stimuli may contribute to M1 polarization *via* NF-κB-mediated transcriptional activation. PAK1 was also observed to play a key role in suppressing M2 macrophage polarization[132]. Blockade of the M2 response is an important approach to treatment involving TAM reprogramming. As mentioned above, STAT3 and STAT6 have been reported to be important regulators of M2 polarization. Pharmacological inhibition of STAT3 and STAT6 with specific inhibitors resulted in suppression of M2 polarization, increased production of pro-inflammatory cytokines and stimulated a T cell response[133-135]. Since PAK1 and PAK4 are closely associated with the STAT3 and NF-κB signalling pathway[67,121], it is likely that PAK may interact with STAT3 or NF-κB signalling pathways to block M2 polarization.

Tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TILs), including $CD4^+$ T cells, $CD8^+$ T cells, regulatory T cells, B cells and natural killer cells, are another class of immune cells, which are critical in modulating the tumour microenvironment in pancreatic cancer[136]. Although $CD8^+$ T cells are also referred to as cytotoxic T cells, with the capability of recognizing and killing tumour cells, infiltration of $CD8^+$ T cells into the tumour microenvironment is very rare. In contrast, a large number of $CD4^+$ T cells, which can promote the development of PanINs *via* inhibition of the anti-tumour response, are observed in the stromal compartment[108,137]. Indeed, an increasing number of studies have revealed the predictive value of stromal TILs in patients with resectable pancreatic cancer. Two of the latest studies have demonstrated that negative stromal TIL patients had larger tumour at a more advanced stage and showed worse overall survival and liver metastasis[138], and that an increased number of tumour-infiltrated $CD8^+$ lymphocytes were significantly and independently related to improved disease-free survival and overall survival[139]. Recently, it was reported that pharmacological or genetic depletion of PAK1 up-regulated the immune response to tumours in a colorectal mouse model ($APC^{14/+}$ mouse)[140].Similarly, the role of PAK in regulating the tumour-associated immune response was investigated in an murine orthotopic pancreatic cancer model[66]. In agreement with the colorectal model, removal of PAK1 by knock-out or inhibition of PAK1 by PF-3758309 not only suppressed tumour growth *in vivo*, but also stimulated the immune response by increasing the numbers of splenic $CD3^+$ and $CD8^+$ T lymphocytes as well as by promoting tumour-infiltrating $CD3^+$ T lymphocytes. In contrast, gemcitabine did not significantly change the tumour-associated immune response. Furthermore, it has been reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by tumour cells can recruit and stimulate the development of stromal myeloid cells ($Gr\text{-}1^+$ $CD11b^+$ cells), which can suppress the anti-tumour effect of $CD8^+$ T cells[141]. So far, the mechanism by which PAK regulates the production of GM-CSF has not been fully elucidated. However, Kras activation is found to be positively associated with GM-CSF expression in cancer patients when compared to normal controls[142], and this observation is consistent with an early study indicating that oncogenic Kras-dependent secretion of GM-CSF can promote the development of pancreatic neoplasia *via* immunosuppression mediated by $Gr\text{-}1^+$ $CD11b^+$ myeloid cells[143]. As Kras is the most important oncogenic mutation in pancreatic cancer and a potent up-stream regulator of PAK, these studies provided possible evidence implicating the involvement of PAK in a pathway linking aberrantly activated Kras to GM-CSF-induced immuno-evasion. The mechanisms underlying the connection between PAKs and GM-CSF should be investigated further.

CONCLUSION

More than a decade ago, an expert consensus proposed precise targeting of protein kinase signalling pathways as a potential weapon against cancers[144,145]. As an important down-stream effector of Kras, PAK is overexpressed and hyperactivated in different types of cancer, especially pancreas, colorectal and lung cancer. This review highlights the key role of PAK in Kras signalling pathways in pancreatic cancer, and summarizes that PAK mediates the biological behaviour

of pancreatic cancer cells by orchestrating multiple oncogenic pathways, such as NF-κB, STAT3, RAF/MEK/ERK, PI3K/PDK1/AKT *etc*., PAK inhibition (FRAX597, PF-3758309 *etc.*), not only suppresses tumour growth and synergistically improves chemotherapeutic efficacy, but also plays a critical role in mediating tumour-stroma crosstalk. More importantly, immunotherapy is now emerging as a promising approach for cancer treatment and immune modulation within the tumour microenvironment has become a hot spot in pancreatic cancer research. The potential role of PAK in the anti-tumour immune response has been unveiled by showing that pharmacological and genetic depletion of PAK leads to an increased number of tumour infiltrated T cells in pancreatic and colorectal cancer. In this regard, PAK may become a novel target for reprogramming the tumour microenvironment.

Pancreatic cancer is still one of most lethal malignancies and, in contrast to other types of cancers (*e.g.*, melanoma, breast cancer, prostate cancer *etc.*), the poor survival of pancreatic cancer patients has been only marginally improved over past decades. Therapeutic breakthroughs in pancreatic cancer still require a more comprehensive understanding of its biology and of the intrinsic mechanisms involved in tumour progression. Further study of PAKs holds the promise of developing more effective and less toxic treatments for this devastating malignancy.

ACKNOWLEDGMENTS

The authors would like to acknowledge Pancare Foundation (https://www.pancare.org.au) for supporting the pancreatic cancer research program in the Department of Surgery, University of Melbourne. Kai Wang was supported by Melbourne International Fee Remission Scholarship (MIFRS), Melbourne International Research Scholarship (MIRS) and the Moshe Sambor Scholarship (Pancare Foundation).

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

2 **Rahib L**, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* 2014; **74**: 2913-2921 [PMID: 24840647 DOI: 10.1158/0008-5472.CAN-14-0155]

3 **Conroy T**, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817-1825 [PMID: 21561347 DOI: 10.1056/NEJMoa1011923]

4 **Cunningham D**, Chau I, Stocken DD, Valle JW, Smith D, Steward W, Harper PG, Dunn J, Tudur-Smith C, West J, Falk S, Crellin A, Adab F, Thompson J, Leonard P, Ostrowski J, Eatock M, Scheithauer W, Herrmann R, Neoptolemos JP. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. *J Clin Oncol* 2009; **27**: 5513-5518 [PMID: 19858379 DOI: 10.1200/JCO.2009.24.2446]

5 **Colucci G**, Giuliani F, Gebbia V, Biglietto M, Rabitti P, Uomo G, Cigolari S, Testa A, Maiello E, Lopez M. Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale. *Cancer* 2002; **94**: 902-910 [PMID: 11920457]

6 **Von Hoff DD**, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691-1703 [PMID: 24131140 DOI: 10.1056/NEJMoa1304369]

7 **Cox AD**, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Discov* 2014; **13**: 828-851 [PMID: 25323927 DOI: 10.1038/nrd4389]

8 **Lazo JS**, Sharlow ER. Drugging Undruggable Molecular Cancer Targets. *Annu Rev Pharmacol Toxicol* 2016; **56**: 23-40 [PMID: 26527069 DOI: 10.1146/annurev-pharmtox-010715-103440]

9 **Stephen AG**, Esposito D, Bagni RK, McCormick F. Dragging ras back in the ring. *Cancer Cell* 2014; **25**: 272-281 [PMID: 24651010 DOI: 10.1016/j.ccr.2014.02.017]

10 **Baines AT**, Xu D, Der CJ. Inhibition of Ras for cancer treatment: the search continues. *Future Med Chem* 2011; **3**: 1787-1808 [PMID: 22004085 DOI: 10.4155/fmc.11.121]

11 **Thompson H**. US National Cancer Institute's new Ras project targets an old foe. *Nat Med* 2013; **19**: 949-950 [PMID: 23921727 DOI: 10.1038/nm0813-949]

12 **Collins MA**, Bednar F, Zhang Y, Brisset JC, Galbán S, Galbán CJ, Rakshit S, Flannagan KS, Adsay NV, Pasca di Magliano M. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest* 2012; **122**: 639-653 [PMID: 22232209 DOI: 10.1172/JCI59227]

13 **Feig C**, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* 2012; **18**: 4266-4276 [PMID: 22896693 DOI: 10.1158/1078-0432.CCR-11-3114]

14 **Jaffer ZM**, Chernoff J. p21-activated kinases: three more join the Pak. *Int J Biochem Cell Biol* 2002; **34**: 713-717 [PMID: 11950587]

15 **Baskaran Y**, Ng YW, Selamat W, Ling FT, Manser E. Group I and II mammalian PAKs have different modes of activation by Cdc42. *EMBO Rep* 2012; **13**: 653-659 [PMID: 22653441 DOI: 10.1038/embor.2012.75]

16 **Radu M**, Semenova G, Kosoff R, Chernoff J. PAK signalling during the development and progression of cancer. *Nat Rev Cancer* 2014; **14**: 13-25 [PMID: 24505617]

17 **Manser E**, Leung T, Salihuddin H, Zhao ZS, Lim L. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 1994; **367**: 40-46 [PMID: 8107774 DOI: 10.1038/367040a0]

18 **Pirruccello M**, Sondermann H, Pelton JG, Pellicena P, Hoelz A, Chernoff J, Wemmer DE, Kuriyan J. A dimeric kinase assembly underlying autophosphorylation in the p21 activated kinases. *J Mol Biol* 2006; **361**: 312-326 [PMID: 16837009 DOI: 10.1016/j.jmb.2006.06.017]

19 **Lei M**, Lu W, Meng W, Parrini MC, Eck MJ, Mayer BJ, Harrison SC. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* 2000; **102**: 387-397 [PMID: 10975528]

20 **Buchwald G**, Hostinova E, Rudolph MG, Kraemer A, Sickmann A, Meyer HE, Scheffzek K, Wittinghofer A. Conformational switch and role of phosphorylation in PAK activation. *Mol Cell Biol* 2001; **21**: 5179-5189 [PMID: 11438672 DOI: 10.1128/MCB.21.15.5179-5189.2001]

21 **Parrini MC**, Lei M, Harrison SC, Mayer BJ. Pak1 kinase homodimers are autoinhibited in trans and dissociated upon

activation by Cdc42 and Rac1. *Mol Cell* 2002; **9**: 73-83 [PMID: 11804587]
22 **Qu J**, Li X, Novitch BG, Zheng Y, Kohn M, Xie JM, Kozinn S, Bronson R, Beg AA, Minden A. PAK4 kinase is essential for embryonic viability and for proper neuronal development. *Mol Cell Biol* 2003; **23**: 7122-7133 [PMID: 14517283]
23 **Callow MG**, Clairvoyant F, Zhu S, Schryver B, Whyte DB, Bischoff JR, Jallal B, Smeal T. Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines. *J Biol Chem* 2002; **277**: 550-558 [PMID: 11668177 DOI: 10.1074/jbc.M105732200]
24 **Pandey A**, Dan I, Kristiansen TZ, Watanabe NM, Voldby J, Kajikawa E, Khosravi-Far R, Blagoev B, Mann M. Cloning and characterization of PAK5, a novel member of mammalian p21-activated kinase-II subfamily that is predominantly expressed in brain. *Oncogene* 2002; **21**: 3939-3948 [PMID: 12032833 DOI: 10.1038/sj.onc.1205478]
25 **Yang F**, Li X, Sharma M, Zarnegar M, Lim B, Sun Z. Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6. *J Biol Chem* 2001; **276**: 15345-15353 [PMID: 11278661 DOI: 10.1074/jbc.M010311200]
26 **Schrantz N**, da Silva Correia J, Fowler B, Ge Q, Sun Z, Bokoch GM. Mechanism of p21-activated kinase 6-mediated inhibition of androgen receptor signaling. *J Biol Chem* 2004; **279**: 1922-1931 [PMID: 14573606 DOI: 10.1074/jbc.M311145200]
27 **Ching YP**, Leong VY, Wong CM, Kung HF. Identification of an autoinhibitory domain of p21-activated protein kinase 5. *J Biol Chem* 2003; **278**: 33621-33624 [PMID: 12860998 DOI: 10.1074/jbc.C300234200]
28 **Ha BH**, Davis MJ, Chen C, Lou HJ, Gao J, Zhang R, Krauthammer M, Halaban R, Schlessinger J, Turk BE, Boggon TJ. Type II p21-activated kinases (PAKs) are regulated by an autoinhibitory pseudosubstrate. *Proc Natl Acad Sci USA* 2012; **109**: 16107-16112 [PMID: 22988085 DOI: 10.1073/pnas.1214447109]
29 **Prior IA**, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. *Cancer Res* 2012; **72**: 2457-2467 [PMID: 22589270 DOI: 10.1158/0008-5472.CAN-11-2612]
30 **Morris JP 4th**, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010; **10**: 683-695 [PMID: 20814421 DOI: 10.1038/nrc2899]
31 **Hingorani SR**, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; **4**: 437-450 [PMID: 14706336]
32 **Ying H**, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, Locasale JW, Son J, Zhang H, Coloff JL, Yan H, Wang W, Chen S, Viale A, Zheng H, Paik JH, Lim C, Guimaraes AR, Martin ES, Chang J, Hezel AF, Perry SR, Hu J, Gan B, Xiao Y, Asara JM, Weissleder R, Wang YA, Chin L, Cantley LC, DePinho RA. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* 2012; **149**: 656-670 [PMID: 22541435 DOI: 10.1016/j.cell.2012.01.058]
33 **Yeo D**, He H, Baldwin GS, Nikfarjam M. The role of p21-activated kinases in pancreatic cancer. *Pancreas* 2015; **44**: 363-369 [PMID: 25760284 DOI: 10.1097/MPA.0000000000000276]
34 **Eser S**, Schnieke A, Schneider G, Saur D. Oncogenic KRAS signalling in pancreatic cancer. *Br J Cancer* 2014; **111**: 817-822 [PMID: 24755884 DOI: 10.1038/bjc.2014.215]
35 **Bryant KL**, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci* 2014; **39**: 91-100 [PMID: 24388967 DOI: 10.1016/j.tibs.2013.12.004]
36 **Réjiba S**, Wack S, Aprahamian M, Hajri A. K-ras oncogene silencing strategy reduces tumor growth and enhances gemcitabine chemotherapy efficacy for pancreatic cancer treatment. *Cancer Sci* 2007; **98**: 1128-1136 [PMID: 17489984 DOI: 10.1111/j.1349-7006.2007.00506.x]
37 **Pylayeva-Gupta Y**, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011; **11**: 761-774 [PMID: 21993244 DOI: 10.1038/nrc3106]
38 **Mortazavi F**, Lu J, Phan R, Lewis M, Trinidad K, Aljilani A, Pezeshkpour G, Tamanoi F. Significance of KRAS/PAK1/Crk pathway in non-small cell lung cancer oncogenesis. *BMC Cancer* 2015; **15**: 381 [PMID: 25956913 DOI: 10.1186/s12885-015-1360-4]
39 **Menard RE**, Mattingly RR. Cell surface receptors activate p21-activated kinase 1 via multiple Ras and PI3-kinase-dependent pathways. *Cell Signal* 2003; **15**: 1099-1109 [PMID: 14575865]
40 **Malliri A**, van der Kammen RA, Clark K, van der Valk M, Michiels F, Collard JG. Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours. *Nature* 2002; **417**: 867-871 [PMID: 12075356 DOI: 10.1038/nature00848]
41 **Binker MG**, Binker-Cosen AA, Gaisano HY, Cosen-Binker LI. Inhibition of Rac1 decreases the severity of pancreatitis and pancreatitis-associated lung injury in mice. *Exp Physiol* 2008; **93**: 1091-1103 [PMID: 18567599 DOI: 10.1113/expphysiol.2008.043141]
42 **Heid I**, Lubeseder-Martellato C, Sipos B, Mazur PK, Lesina M, Schmid RM, Siveke JT. Early requirement of Rac1 in a mouse model of pancreatic cancer. *Gastroenterology* 2011; **141**: 719-730, 730.e1-730.e7 [PMID: 21684285 DOI: 10.1053/j.gastro.2011.04.043]
43 **Zheng Y**, Bagrodia S, Cerione RA. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. *J Biol Chem* 1994; **269**: 18727-18730 [PMID: 8034624]
44 **Eser S**, Reiff N, Messer M, Seidler B, Gottschalk K, Dobler M, Hieber M, Arbeiter A, Klein S, Kong B, Michalski CW, Schlitter AM, Esposito I, Kind AJ, Rad L, Schnieke AE, Baccarini M, Alessi DR, Rad R, Schmid RM, Schneider G, Saur D. Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer. *Cancer Cell* 2013; **23**: 406-420 [PMID: 23453624 DOI: 10.1016/j.ccr.2013.01.023]
45 **King CC**, Gardiner EM, Zenke FT, Bohl BP, Newton AC, Hemmings BA, Bokoch GM. p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1). *J Biol Chem* 2000; **275**: 41201-41209 [PMID: 10995762 DOI: 10.1074/jbc.M006553200]
46 **Beeser A**, Jaffer ZM, Hofmann C, Chernoff J. Role of group A p21-activated kinases in activation of extracellular-regulated kinase by growth factors. *J Biol Chem* 2005; **280**: 36609-36615 [PMID: 16129686 DOI: 10.1074/jbc.M502306200]
47 **Huynh N**, Liu KH, Baldwin GS, He H. P21-activated kinase 1 stimulates colon cancer cell growth and migration/invasion via ERK- and AKT-dependent pathways. *Biochim Biophys Acta* 2010; **1803**: 1106-1113 [PMID: 20595063 DOI: 10.1016/j.bbamcr.2010.05.007]
48 **Chow HY**, Jubb AM, Koch JN, Jaffer ZM, Stepanova D, Campbell DA, Duron SG, O'Farrell M, Cai KQ, Klein-Szanto AJ, Gutkind JS, Hoeflich KP, Chernoff J. p21-Activated kinase 1 is required for efficient tumor formation and progression in a Ras-mediated skin cancer model. *Cancer Res* 2012; **72**: 5966-5975 [PMID: 22983922 DOI: 10.1158/0008-5472.CAN-12-2246]
49 **Tabusa H**, Brooks T, Massey AJ. Knockdown of PAK4 or PAK1 inhibits the proliferation of mutant KRAS colon cancer cells independently of RAF/MEK/ERK and PI3K/AKT signaling. *Mol Cancer Res* 2013; **11**: 109-121 [PMID: 23233484 DOI: 10.1158/1541-7786.MCR-12-0466]
50 **Chen S**, Auletta T, Dovirak O, Hutter C, Kuntz K, El-ftesi S, Kendall J, Han H, Von Hoff DD, Ashfaq R, Maitra A, Iacobuzio-Donahue CA, Hruban RH, Lucito R. Copy number alterations in pancreatic cancer identify recurrent PAK4 amplification. *Cancer Biol Ther* 2008; **7**: 1793-1802 [PMID: 18836286]
51 **Brown LA**, Kalloger SE, Miller MA, Shih IeM, McKinney SE, Santos JL, Swenerton K, Spellman PT, Gray J, Gilks CB, Huntsman DG. Amplification of 11q13 in ovarian carcinoma. *Genes Chromosomes Cancer* 2008; **47**: 481-489 [PMID: 18314909 DOI: 10.1002/gcc.20549]
52 **Rane CK**, Minden A. P21 activated kinases: structure, regulation, and functions. *Small GTPases* 2014; **5** [PMID: 24658305 DOI:

10.4161/sgtp.28003]
53 **Shrestha Y**, Schafer EJ, Boehm JS, Thomas SR, He F, Du J, Wang S, Barretina J, Weir BA, Zhao JJ, Polyak K, Golub TR, Beroukhim R, Hahn WC. PAK1 is a breast cancer oncogene that coordinately activates MAPK and MET signaling. *Oncogene* 2012; **31**: 3397-3408 [PMID: 22105362 DOI: 10.1038/onc.2011.515]
54 **Mahlamäki EH**, Kauraniemi P, Monni O, Wolf M, Hautaniemi S, Kallioniemi A. High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer. *Neoplasia* 2004; **6**: 432-439 [PMID: 15548351 DOI: 10.1593/neo.04130]
55 **Kimmelman AC**, Hezel AF, Aguirre AJ, Zheng H, Paik JH, Ying H, Chu GC, Zhang JX, Sahin E, Yeo G, Ponugoti A, Nabioullin R, Deroo S, Yang S, Wang X, McGrath JP, Protopopova M, Ivanova E, Zhang J, Feng B, Tsao MS, Redston M, Protopopov A, Xiao Y, Futreal PA, Hahn WC, Klimstra DS, Chin L, DePinho RA. Genomic alterations link Rho family of GTPases to the highly invasive phenotype of pancreas cancer. *Proc Natl Acad Sci USA* 2008; **105**: 19372-19377 [PMID: 19050074 DOI: 10.1073/pnas.0809966105]
56 **Lundgren K**, Holm K, Nordenskjöld B, Borg A, Landberg G. Gene products of chromosome 11q and their association with CCND1 gene amplification and tamoxifen resistance in premenopausal breast cancer. *Breast Cancer Res* 2008; **10**: R81 [PMID: 18823530 DOI: 10.1186/bcr2150]
57 **Zhou W**, Jubb AM, Lyle K, Xiao Q, Ong CC, Desai R, Fu L, Gnad F, Song Q, Haverty PM, Aust D, Grützmann R, Romero M, Totpal K, Neve RM, Yan Y, Forrest WF, Wang Y, Raja R, Pilarsky C, de Jesus-Acosta A, Belvin M, Friedman LS, Merchant M, Jaffee EM, Zheng L, Koeppen H, Hoeflich KP. PAK1 mediates pancreatic cancer cell migration and resistance to MET inhibition. *J Pathol* 2014; **234**: 502-513 [PMID: 25074413 DOI: 10.1002/path.4412]
58 **Jagadeeshan S**, Krishnamoorthy YR, Singhal M, Subramanian A, Mavuluri J, Lakshmi A, Roshini A, Baskar G, Ravi M, Joseph LD, Sadasivan K, Krishnan A, Nair AS, Venkatraman G, Rayala SK. Transcriptional regulation of fibronectin by p21-activated kinase-1 modulates pancreatic tumorigenesis. *Oncogene* 2015; **34**: 455-464 [PMID: 24561527 DOI: 10.1038/onc.2013.576]
59 **Yeo D**, He H, Patel O, Lowy AM, Baldwin GS, Nikfarjam M. FRAX597, a PAK1 inhibitor, synergistically reduces pancreatic cancer growth when combined with gemcitabine. *BMC Cancer* 2016; **16**: 24 [PMID: 26774265 DOI: 10.1186/s12885-016-2057-z]
60 **Singh RR**, Song C, Yang Z, Kumar R. Nuclear localization and chromatin targets of p21-activated kinase 1. *J Biol Chem* 2005; **280**: 18130-18137 [PMID: 15749698 DOI: 10.1074/jbc.M412607200]
61 **Frost JA**, Swantek JL, Stippec S, Yin MJ, Gaynor R, Cobb MH. Stimulation of NFkappa B activity by multiple signaling pathways requires PAK1. *J Biol Chem* 2000; **275**: 19693-19699 [PMID: 10779525 DOI: 10.1074/jbc.M909860199]
62 **Chauhan SC**, Ebeling MC, Maher DM, Koch MD, Watanabe A, Aburatani H, Lio Y, Jaggi M. MUC13 mucin augments pancreatic tumorigenesis. *Mol Cancer Ther* 2012; **11**: 24-33 [PMID: 22027689 DOI: 10.1158/1535-7163.MCT-11-0598]
63 **Jagadeeshan S**, Venkatraman G, Rayala SK. Targeting p21 activated kinase 1 (Pak1) to PAKup Pancreatic Cancer. *Expert Opin Ther Targets* 2016; **20**: 1283-1285 [PMID: 27654702 DOI: 10.1080/14728222.2016.1239719]
64 **Yeo D**, Phillips P, Baldwin GS, He H, Nikfarjam M. Inhibition of group 1 p21-activated kinases suppresses pancreatic stellate cell activation and increases survival of mice with pancreatic cancer. *Int J Cancer* 2017; **140**: 2101-2111 [PMID: 28109008 DOI: 10.1002/ijc.30615]
65 **Tyagi N**, Bhardwaj A, Singh AP, McClellan S, Carter JE, Singh S. p-21 activated kinase 4 promotes proliferation and survival of pancreatic cancer cells through AKT- and ERK-dependent activation of NF-κB pathway. *Oncotarget* 2014; **5**: 8778-8789 [PMID: 25238288 DOI: 10.18632/oncotarget.2398]
66 **Wang K**, Huynh N, Wang X, Baldwin G, Nikfarjam M, He H. Inhibition of p21 activated kinase enhances tumour immune response and sensitizes pancreatic cancer to gemcitabine. *Int J Oncol* 2018; **52**: 261-269 [PMID: 29115428 DOI: 10.3892/ijo.2017.4193]
67 **Tyagi N**, Marimuthu S, Bhardwaj A, Deshmukh SK, Srivastava SK, Singh AP, McClellan S, Carter JE, Singh S. p-21 activated kinase 4 (PAK4) maintains stem cell-like phenotypes in pancreatic cancer cells through activation of STAT3 signaling. *Cancer Lett* 2016; **370**: 260-267 [PMID: 26546043 DOI: 10.1016/j.canlet.2015.10.028]
68 **Nuche-Berenguer B**, Ramos-Álvarez I, Jensen RT. The p21-activated kinase, PAK2, is important in the activation of numerous pancreatic acinar cell signaling cascades and in the onset of early pancreatitis events. *Biochim Biophys Acta* 2016; **1862**: 1122-1136 [PMID: 26912410 DOI: 10.1016/j.bbadis.2016.02.008]
69 **Konstantinidis IT**, Warshaw AL, Allen JN, Blaszkowsky LS, Castillo CF, Deshpande V, Hong TS, Kwak EL, Lauwers GY, Ryan DP, Wargo JA, Lillemoe KD, Ferrone CR. Pancreatic ductal adenocarcinoma: is there a survival difference for R1 resections versus locally advanced unresectable tumors? What is a "true" R0 resection? *Ann Surg* 2013; **257**: 731-736 [PMID: 22968073 DOI: 10.1097/SLA.0b013e318263da2f]
70 **Oettle H**, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; **297**: 267-277 [PMID: 17227978 DOI: 10.1001/jama.297.3.267]
71 **Hammel P**, Huguet F, van Laethem JL, Goldstein D, Glimelius B, Artru P, Borbath I, Bouché O, Shannon J, André T, Mineur L, Chibaudel B, Bonnetain F, Louvet C; LAP07 Trial Group. Effect of Chemoradiotherapy vs Chemotherapy on Survival in Patients With Locally Advanced Pancreatic Cancer Controlled After 4 Months of Gemcitabine With or Without Erlotinib: The LAP07 Randomized Clinical Trial. *JAMA* 2016; **315**: 1844-1853 [PMID: 27139057 DOI: 10.1001/jama.2016.4324]
72 **Burris HA 3rd**, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413 [PMID: 9196156 DOI: 10.1200/JCO.1997.15.6.2403]
73 **Rothenberg ML**, Moore MJ, Cripps MC, Andersen JS, Portenoy RK, Burris HA 3rd, Green MR, Tarassoff PG, Brown TD, Casper ES, Storniolo AM, Von Hoff DD. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* 1996; **7**: 347-353 [PMID: 8805925]
74 **Di Costanzo F**, Carlini P, Doni L, Massidda B, Mattioli R, Iop A, Barletta E, Moscetti L, Recchia F, Tralongo P, Gasperoni S. Gemcitabine with or without continuous infusion 5-FU in advanced pancreatic cancer: a randomised phase II trial of the Italian oncology group for clinical research (GOIRC). *Br J Cancer* 2005; **93**: 185-189 [PMID: 15986036 DOI: 10.1038/sj.bjc.6602640]
75 **Min YJ**, Joo KR, Park NH, Yun TK, Nah YW, Nam CW, Park JH. Gemcitabine therapy in patients with advanced pancreatic cancer. *Korean J Intern Med* 2002; **17**: 259-262 [PMID: 12647642]
76 **Poplin E**, Wasan H, Rolfe L, Raponi M, Ikdahl T, Bondarenko I, Davidenko I, Bondar V, Garin A, Boeck S, Ormanns S, Heinemann V, Bassi C, Evans TR, Andersson R, Hahn H, Picozzi V, Dicker A, Mann E, Voong C, Kaur P, Isaacson J, Allen A. Randomized, multicenter, phase II study of CO-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. *J Clin Oncol* 2013; **31**: 4453-4461 [PMID: 24220555 DOI: 10.1200/JCO.2013.51.0826]
77 **Adamska A**, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. *Int J Mol Sci* 2017; **18** [PMID: 28640192 DOI: 10.3390/ijms18071338]

78 **Farrell JJ**, Elsaleh H, Garcia M, Lai R, Ammar A, Regine WF, Abrams R, Benson AB, Macdonald J, Cass CE, Dicker AP, Mackey JR. Human equilibrative nucleoside transporter 1 levels predict response to gemcitabine in patients with pancreatic cancer. *Gastroenterology* 2009; **136**: 187-195 [PMID: 18992248 DOI: 10.1053/j.gastro.2008.09.067]

79 **Giovannetti E**, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del Chiaro M, Iannopollo M, Bevilacqua G, Mosca F, Danesi R. Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res* 2006; **66**: 3928-3935 [PMID: 16585222 DOI: 10.1158/0008-5472.CAN-05-4203]

80 **Nakano Y**, Tanno S, Koizumi K, Nishikawa T, Nakamura K, Minoguchi M, Izawa T, Mizukami Y, Okumura T, Kohgo Y. Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells. *Br J Cancer* 2007; **96**: 457-463 [PMID: 17224927 DOI: 10.1038/sj.bjc.6603559]

81 **Ritzel MW**, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, Hyde RJ, Karpinski E, Cass CE, Baldwin SA, Young JD. Recent molecular advances in studies of the concentrative Na+-dependent nucleoside transporter (CNT) family: identification and characterization of novel human and mouse proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *Mol Membr Biol* 2001; **18**: 65-72 [PMID: 11396613]

82 **Mackey JR**, Mani RS, Selner M, Mowles D, Young JD, Belt JA, Crawford CR, Cass CE. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. *Cancer Res* 1998; **58**: 4349-4357 [PMID: 9766663]

83 **Moon SU**, Kim JW, Sung JH, Kang MH, Kim SH, Chang H, Lee JO, Kim YJ, Lee KW, Kim JH, Bang SM, Lee JS. p21-Activated Kinase 4 (PAK4) as a Predictive Marker of Gemcitabine Sensitivity in Pancreatic Cancer Cell Lines. *Cancer Res Treat* 2015; **47**: 501-508 [PMID: 25672581 DOI: 10.4143/crt.2014.054]

84 **Park S**, Kim JW, Kim H, Kim JW, Kim YJ, Lee KW, Kim JH, Kim JH, Hwang JH, Choi YR, Cho JY, Yoon YS, Han HS. Prognostic value of p21-activated kinase 4 in resected pancreatic cancer. *APMIS* 2017; **125**: 699-707 [PMID: 28556956 DOI: 10.1111/apm.12705]

85 **Jagadeeshan S**, Subramanian A, Tentu S, Beesetti S, Singhal M, Raghavan S, Surabhi RP, Mavuluri J, Bhoopalan H, Biswal J, Pitani RS, Chidambaram S, Sundaram S, Malathi R, Jeyaraman J, Nair AS, Venkatraman G, Rayala SK. P21-activated kinase 1 (Pak1) signaling influences therapeutic outcome in pancreatic cancer. *Ann Oncol* 2016; **27**: 1546-1556 [PMID: 27117533 DOI: 10.1093/annonc/mdw184]

86 **Wang R**, Cheng L, Xia J, Wang Z, Wu Q, Wang Z. Gemcitabine resistance is associated with epithelial-mesenchymal transition and induction of HIF-1α in pancreatic cancer cells. *Curr Cancer Drug Targets* 2014; **14**: 407-417 [PMID: 24575976]

87 **Zhao T**, Ren H, Jia L, Chen J, Xin W, Yan F, Li J, Wang X, Gao S, Qian D, Huang C, Hao J. Inhibition of HIF-1α by PX-478 enhances the anti-tumor effect of gemcitabine by inducing immunogenic cell death in pancreatic ductal adenocarcinoma. *Oncotarget* 2015; **6**: 2250-2262 [PMID: 25544770 DOI: 10.18632/oncotarget.2948]

88 **Eltzschig HK**, Abdulla P, Hoffman E, Hamilton KE, Daniels D, Schönfeld C, Löffler M, Reyes G, Duszenko M, Karhausen J, Robinson A, Westerman KA, Coe IR, Colgan SP. HIF-1-dependent repression of equilibrative nucleoside transporter (ENT) in hypoxia. *J Exp Med* 2005; **202**: 1493-1505 [PMID: 16330813 DOI: 10.1084/jem.20050177]

89 **Morote-Garcia JC**, Rosenberger P, Nivillac NM, Coe IR, Eltzschig HK. Hypoxia-inducible factor-dependent repression of equilibrative nucleoside transporter 2 attenuates mucosal inflammation during intestinal hypoxia. *Gastroenterology* 2009; **136**: 607-618 [PMID: 19105964 DOI: 10.1053/j.gastro.2008.10.037]

90 **Liu KH**, Huynh N, Patel O, Shulkes A, Baldwin G, He H. P21-activated kinase 1 promotes colorectal cancer survival by up-regulation of hypoxia-inducible factor-1α. *Cancer Lett* 2013; **340**: 22-29 [PMID: 23811286 DOI: 10.1016/j.canlet.2013.06.024]

91 **Kim H**, Woo DJ, Kim SY, Yang EG. p21-activated kinase 4 regulates HIF-1α translation in cancer cells. *Biochem Biophys Res Commun* 2017; **486**: 270-276 [PMID: 28288786 DOI: 10.1016/j.bbrc.2017.03.024]

92 **Arlt A**, Gehrz A, Müerköster S, Vorndamm J, Kruse ML, Fölsch UR, Schäfer H. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. *Oncogene* 2003; **22**: 3243-3251 [PMID: 12761494 DOI: 10.1038/sj.onc.1206390]

93 **Kong R**, Sun B, Jiang H, Pan S, Chen H, Wang S, Krissansen GW, Sun X. Downregulation of nuclear factor-kappaB p65 subunit by small interfering RNA synergizes with gemcitabine to inhibit the growth of pancreatic cancer. *Cancer Lett* 2010; **291**: 90-98 [PMID: 19880242 DOI: 10.1016/j.canlet.2009.10.001]

94 **Skrypek N**, Duchêne B, Hebbar M, Leteurtre E, van Seuningen I, Jonckheere N. The MUC4 mucin mediates gemcitabine resistance of human pancreatic cancer cells via the Concentrative Nucleoside Transporter family. *Oncogene* 2013; **32**: 1714-1723 [PMID: 22580602 DOI: 10.1038/onc.2012.179]

95 **Fu X**, Feng J, Zeng D, Ding Y, Yu C, Yang B. PAK4 confers cisplatin resistance in gastric cancer cells via PI3K/Akt- and MEK/ERK-dependent pathways. *Biosci Rep* 2014; **34** [PMID: 27919028 DOI: 10.1042/BSR20130102]

96 **Neesse A**, Michl P, Frese KK, Feig C, Cook N, Jacobetz MA, Lolkema MP, Buchholz M, Olive KP, Gress TM, Tuveson DA. Stromal biology and therapy in pancreatic cancer. *Gut* 2011; **60**: 861-868 [PMID: 20966025 DOI: 10.1136/gut.2010.226092]

97 **Vonlaufen A**, Joshi S, Qu C, Phillips PA, Xu Z, Parker NR, Toi CS, Pirola RC, Wilson JS, Goldstein D, Apte MV. Pancreatic stellate cells: partners in crime with pancreatic cancer cells. *Cancer Res* 2008; **68**: 2085-2093 [PMID: 18381413 DOI: 10.1158/0008-5472.CAN-07-2477]

98 **Martin K**, Pritchett J, Llewellyn J, Mullan AF, Athwal VS, Dobie R, Harvey E, Zeef L, Farrow S, Streuli C, Henderson NC, Friedman SL, Hanley NA, Piper Hanley K. PAK proteins and YAP-1 signalling downstream of integrin beta-1 in myofibroblasts promote liver fibrosis. *Nat Commun* 2016; **7**: 12502 [PMID: 27535340 DOI: 10.1038/ncomms12502]

99 **Thayer SP**, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, Qi YP, Gysin S, Fernández-del Castillo C, Yajnik V, Antoniu B, McMahon M, Warshaw AL, Hebrok M. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 2003; **425**: 851-856 [PMID: 14520413 DOI: 10.1038/nature02009]

100 **Bailey JM**, Swanson BJ, Hamada T, Eggers JP, Singh PK, Caffery T, Ouellette MM, Hollingsworth MA. Sonic hedgehog promotes desmoplasia in pancreatic cancer. *Clin Cancer Res* 2008; **14**: 5995-6004 [PMID: 18829478 DOI: 10.1158/1078-0432.CCR-08-0291]

101 **Jones S**, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; **321**: 1801-1806 [PMID: 18772397 DOI: 10.1126/science.1164368]

102 **Olive KP**, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Rückert F, Grützmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, Tuveson DA. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic

cancer. *Science* 2009; **324**: 1457-1461 [PMID: 19460966 DOI: 10.1126/science.1171362]
103 **Huang FT**, Zhuan-Sun YX, Zhuang YY, Wei SL, Tang J, Chen WB, Zhang SN. Inhibition of hedgehog signaling depresses self-renewal of pancreatic cancer stem cells and reverses chemoresistance. *Int J Oncol* 2012; **41**: 1707-1714 [PMID: 22923052 DOI: 10.3892/ijo.2012.1597]
104 **Xu M**, Li L, Liu Z, Jiao Z, Xu P, Kong X, Huang H, Zhang Y. ABCB2 (TAP1) as the downstream target of SHH signaling enhances pancreatic ductal adenocarcinoma drug resistance. *Cancer Lett* 2013; **333**: 152-158 [PMID: 23340176 DOI: 10.1016/j.canlet.2013.01.002]
105 **Nakashima H**, Nakamura M, Yamaguchi H, Yamanaka N, Akiyoshi T, Koga K, Yamaguchi K, Tsuneyoshi M, Tanaka M, Katano M. Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res* 2006; **66**: 7041-7049 [PMID: 16849549 DOI: 10.1158/0008-5472.CAN-05-4588]
106 **Teicher BA**, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res* 2010; **16**: 2927-2931 [PMID: 20484021 DOI: 10.1158/1078-0432.CCR-09-2329]
107 **Singh AP**, Arora S, Bhardwaj A, Srivastava SK, Kadakia MP, Wang B, Grizzle WE, Owen LB, Singh S. CXCL12/CXCR4 protein signaling axis induces sonic hedgehog expression in pancreatic cancer cells via extracellular regulated kinase- and Akt kinase-mediated activation of nuclear factor κB: implications for bidirectional tumor-stromal interactions. *J Biol Chem* 2012; **287**: 39115-39124 [PMID: 22995914 DOI: 10.1074/jbc.M112.409581]
108 **Clark CE**, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res* 2007; **67**: 9518-9527 [PMID: 17909062 DOI: 10.1158/0008-5472.CAN-07-0175]
109 **Roghanian A**, Fraser C, Kleyman M, Chen J. B Cells Promote Pancreatic Tumorigenesis. *Cancer Discov* 2016; **6**: 230-232 [PMID: 26951836 DOI: 10.1158/2159-8290.CD-16-0100]
110 **Inman KS**, Francis AA, Murray NR. Complex role for the immune system in initiation and progression of pancreatic cancer. *World J Gastroenterol* 2014; **20**: 11160-11181 [PMID: 25170202 DOI: 10.3748/wjg.v20.i32.11160]
111 **Lutz ER**, Wu AA, Bigelow E, Sharma R, Mo G, Soares K, Solt S, Dorman A, Wamwea A, Yager A, Laheru D, Wolfgang CL, Wang J, Hruban RH, Anders RA, Jaffee EM, Zheng L. Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation. *Cancer Immunol Res* 2014; **2**: 616-631 [PMID: 24942756 DOI: 10.1158/2326-6066.CIR-14-0027]
112 **Zhan HX**, Zhou B, Cheng YG, Xu JW, Wang L, Zhang GY, Hu SY. Crosstalk between stromal cells and cancer cells in pancreatic cancer: New insights into stromal biology. *Cancer Lett* 2017; **392**: 83-93 [PMID: 28189533 DOI: 10.1016/j.canlet.2017.01.041]
113 **Gabitass RF**, Annels NE, Stocken DD, Pandha HA, Middleton GW. Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. *Cancer Immunol Immunother* 2011; **60**: 1419-1430 [PMID: 21644036 DOI: 10.1007/s00262-011-1028-0]
114 **Khaled YS**, Ammori BJ, Elkord E. Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients. *J Immunol Res* 2014; **2014**: 879897 [PMID: 24741628 DOI: 10.1155/2014/879897]
115 **Welte T**, Kim IS, Tian L, Gao X, Wang H, Li J, Holdman XB, Herschkowitz JI, Pond A, Xie G, Kurley S, Nguyen T, Liao L, Dobrolecki LE, Pang L, Mo Q, Edwards DP, Huang S, Xin L, Xu J, Li Y, Lewis MT, Wang T, Westbrook TF, Rosen JM, Zhang XH. Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation. *Nat Cell Biol* 2016; **18**: 632-644 [PMID: 27183469 DOI: 10.1038/ncb3355]
116 **He LF**, Xu HW, Chen M, Xian ZR, Wen XF, Chen MN, Du CW, Huang WH, Wu JD, Zhang GJ. Activated-PAK4 predicts worse prognosis in breast cancer and promotes tumorigenesis through activation of PI3K/AKT signaling. *Oncotarget* 2017; **8**: 17573-17585 [PMID: 28407679 DOI: 10.18632/oncotarget.7466]
117 **Khare V**, Dammann K, Asboth M, Krnjic A, Jambrich M, Gasche C. Overexpression of PAK1 promotes cell survival in inflammatory bowel diseases and colitis-associated cancer. *Inflamm Bowel Dis* 2015; **21**: 287-296 [PMID: 25569743 DOI: 10.1097/MIB.0000000000000281]
118 **Chen X**, Kelemen SE, Autieri MV. Expression of granulocyte colony-stimulating factor is induced in injured rat carotid arteries and mediates vascular smooth muscle cell migration. *Am J Physiol Cell Physiol* 2005; **288**: C81-C88 [PMID: 15385271 DOI: 10.1152/ajpcell.00322.2004]
119 **Panni RZ**, Sanford DE, Belt BA, Mitchem JB, Worley LA, Goetz BD, Mukherjee P, Wang-Gillam A, Link DC, Denardo DG, Goedegebuure SP, Linehan DC. Tumor-induced STAT3 activation in monocytic myeloid-derived suppressor cells enhances stemness and mesenchymal properties in human pancreatic cancer. *Cancer Immunol Immunother* 2014; **63**: 513-528 [PMID: 24652403 DOI: 10.1007/s00262-014-1527-x]
120 **Mace TA**, Ameen Z, Collins A, Wojcik S, Mair M, Young GS, Fuchs JR, Eubank TD, Frankel WL, Bekaii-Saab T, Bloomston M, Lesinski GB. Pancreatic cancer-associated stellate cells promote differentiation of myeloid-derived suppressor cells in a STAT3-dependent manner. *Cancer Res* 2013; **73**: 3007-3018 [PMID: 23514705 DOI: 10.1158/0008-5472.CAN-12-4601]
121 **Gan J**, Ke X, Jiang J, Dong H, Yao Z, Lin Y, Lin W, Wu X, Yan S, Zhuang Y, Chu WK, Cai R, Zhang X, Cheung HS, Block NL, Pang CP, Schally AV, Zhang H. Growth hormone-releasing hormone receptor antagonists inhibit human gastric cancer through downregulation of PAK1-STAT3/NF-κB signaling. *Proc Natl Acad Sci USA* 2016; **113**: 14745-14750 [PMID: 27930339 DOI: 10.1073/pnas.1618582114]
122 **Engblom C**, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. *Nat Rev Cancer* 2016; **16**: 447-462 [PMID: 27339708 DOI: 10.1038/nrc.2016.54]
123 **Hu H**, Jiao F, Han T, Wang LW. Functional significance of macrophages in pancreatic cancer biology. *Tumour Biol* 2015; **36**: 9119-9126 [PMID: 26411672 DOI: 10.1007/s13277-015-4127-2]
124 **Smith SD**, Jaffer ZM, Chernoff J, Ridley AJ. PAK1-mediated activation of ERK1/2 regulates lamellipodial dynamics. *J Cell Sci* 2008; **121**: 3729-3736 [PMID: 18940914 DOI: 10.1242/jcs.027680]
125 **Wheeler AP**, Wells CM, Smith SD, Vega FM, Henderson RB, Tybulewicz VL, Ridley AJ. Rac1 and Rac2 regulate macrophage morphology but are not essential for migration. *J Cell Sci* 2006; **119**: 2749-2757 [PMID: 16772332 DOI: 10.1242/jcs.03024]
126 **Weiss-Haljiti C**, Pasquali C, Ji H, Gillieron C, Chabert C, Curchod ML, Hirsch E, Ridley AJ, Hooft van Huijsduijnen R, Camps M, Rommel C. Involvement of phosphoinositide 3-kinase gamma, Rac, and PAK signaling in chemokine-induced macrophage migration. *J Biol Chem* 2004; **279**: 43273-43284 [PMID: 15292195 DOI: 10.1074/jbc.M402924200]
127 **Gringel A**, Walz D, Rosenberger G, Minden A, Kutsche K, Kopp P, Linder S. PAK4 and alphaPIX determine podosome size and number in macrophages through localized actin regulation. *J Cell Physiol* 2006; **209**: 568-579 [PMID: 16897755 DOI: 10.1002/jcp.20777]
128 **Li N**, Li Y, Li Z, Huang C, Yang Y, Lang M, Cao J, Jiang W, Xu Y, Dong J, Ren H. Hypoxia Inducible Factor 1 (HIF-1) Recruits Macrophage to Activate Pancreatic Stellate Cells in Pancreatic Ductal Adenocarcinoma. *Int J Mol Sci* 2016; **17** [PMID: 27271610 DOI: 10.3390/ijms17060799]
129 **Sanford DE**, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, Mitchem JB, Plambeck-Suess SM, Worley LA, Goetz BD, Wang-Gillam A, Eberlein TJ, Denardo DG, Goedegebuure SP, Linehan DC. Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res* 2013; **19**: 3404-3415 [PMID: 23653148 DOI: 10.1158/1078-0432.CCR-13-0525]
130 **Allen WE**, Jones GE, Pollard JW, Ridley AJ. Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J*

Cell Sci 1997; **110**: 707-720 [PMID: 9099945]
131 **Biswas SK**, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 2010; **11**: 889-896 [PMID: 20856220 DOI: 10.1038/ni.1937]
132 **Zhang W**, Liu H, Liu W, Liu Y, Xu J. Polycomb-mediated loss of microRNA let-7c determines inflammatory macrophage polarization via PAK1-dependent NF-κB pathway. *Cell Death Differ* 2015; **22**: 287-297 [PMID: 25215948 DOI: 10.1038/cdd.2014.142]
133 **Hussain SF**, Kong LY, Jordan J, Conrad C, Madden T, Fokt I, Priebe W, Heimberger AB. A novel small molecule inhibitor of signal transducers and activators of transcription 3 reverses immune tolerance in malignant glioma patients. *Cancer Res* 2007; **67**: 9630-9636 [PMID: 17942891 DOI: 10.1158/0008-5472.CAN-07-1243]
134 **Dong R**, Gong Y, Meng W, Yuan M, Zhu H, Ying M, He Q, Cao J, Yang B. The involvement of M2 macrophage polarization inhibition in fenretinide-mediated chemopreventive effects on colon cancer. *Cancer Lett* 2017; **388**: 43-53 [PMID: 27913199 DOI: 10.1016/j.canlet.2016.11.029]
135 **Sun L**, Chen B, Jiang R, Li J, Wang B. Resveratrol inhibits lung cancer growth by suppressing M2-like polarization of tumor associated macrophages. *Cell Immunol* 2017; **311**: 86-93 [PMID: 27825563 DOI: 10.1016/j.cellimm.2016.11.002]
136 **Nielsen MF**, Mortensen MB, Detlefsen S. Key players in pancreatic cancer-stroma interaction: Cancer-associated fibroblasts, endothelial and inflammatory cells. *World J Gastroenterol* 2016; **22**: 2678-2700 [PMID: 26973408 DOI: 10.3748/wjg.v22.i9.2678]
137 **Neesse A**, Algül H, Tuveson DA, Gress TM. Stromal biology and therapy in pancreatic cancer: a changing paradigm. *Gut* 2015; **64**: 1476-1484 [PMID: 25994217 DOI: 10.1136/gutjnl-2015-309304]
138 **Lianyuan T**, Dianrong X, Chunhui Y, Zhaolai M, Bin J. The predictive value and role of stromal tumor-infiltrating lymphocytes in pancreatic ductal adenocarcinoma (PDAC). *Cancer Biol Ther* 2018; **19**: 296-305 [PMID: 29313457 DOI: 10.1080/15384047.2017.1416932]
139 **Lohneis P**, Sinn M, Bischoff S, Jühling A, Pelzer U, Wislocka L, Bahra M, Sinn BV, Denkert C, Oettle H, Bläker H, Riess H, Jöhrens K, Striefler JK. Cytotoxic tumour-infiltrating T lymphocytes influence outcome in resected pancreatic ductal adenocarcinoma. *Eur J Cancer* 2017; **83**: 290-301 [PMID: 28772128 DOI: 10.1016/j.ejca.2017.06.016]
140 **Huynh N**, Wang K, Yim M, Dumesny CJ, Sandrin MS, Baldwin GS, Nikfarjam M, He H. Depletion of p21-activated kinase 1 up-regulates the immune system of APC Δ14/+ mice and inhibits intestinal tumorigenesis. *BMC Cancer* 2017; **17**: 431 [PMID: 28629331 DOI: 10.1186/s12885-017-3432-0]
141 **Bayne LJ**, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ, Vonderheide RH. Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer Cell* 2012; **21**: 822-835 [PMID: 22698406 DOI: 10.1016/j.ccr.2012.04.025]
142 **Petanidis S**, Anestakis D, Argyraki M, Hadzopoulou-Cladaras M, Salifoglou A. Differential expression of IL-17, 22 and 23 in the progression of colorectal cancer in patients with K-ras mutation: Ras signal inhibition and crosstalk with GM-CSF and IFN-γ. *PLoS One* 2013; **8**: e73616 [PMID: 24040001 DOI: 10.1371/journal.pone.0073616]
143 **Pylayeva-Gupta Y**, Lee KE, Hajdu CH, Miller G, Bar-Sagi D. Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. *Cancer Cell* 2012; **21**: 836-847 [PMID: 22698407 DOI: 10.1016/j.ccr.2012.04.024]
144 **Keen N**, Taylor S. Aurora-kinase inhibitors as anticancer agents. *Nat Rev Cancer* 2004; **4**: 927-936 [PMID: 15573114 DOI: 10.1038/nrc1502]
145 **Melnikova I**, Golden J. Targeting protein kinases. *Nat Rev Drug Discov* 2004; **3**: 993-994 [PMID: 15645605 DOI: 10.1038/nrd1600]

P- Reviewer: Chandra D, Fusai G, Guo JC **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3724-3737

DOI: 10.3748/wjg.v24.i33.3724

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

REVIEW

Long non-coding RNAs involved in metastasis of gastric cancer

Meng-Ting Lin, Hao-Jun Song, Xiao-Yun Ding

Meng-Ting Lin, Medical School of Ningbo University, Ningbo 315000, Zhejiang Province, China

Hao-Jun Song, Xiao-Yun Ding, Gastroenterology Department, Ningbo First Hospital, Ningbo 315000, Zhejiang Province, China

ORCID number: Meng-Ting Lin (0000-0002-3632-3325); Hao-Jun Song (0000-0001-5796-0967); Xiao-Yun Ding (0000-0002-3731-3508).

Author contributions: Lin MT wrote the paper; Song HJ and Ding XY revised this manuscript; all of the authors gave their approval of the final version.

Supported by the Natural Science Foundation of Ningbo, No. 2014A610226 and No.2016A610158; the Scientific Benefit for People Project of Ningbo, No. 2014C51001.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Manuscript source: Invited manuscript

Correspondence to: Xiao-Yun Ding, MD, PhD, Chief Doctor, Occupational Physician, Gastroenterology Department, Ningbo First Hospital, No. 59 Liu Ting Street, Haishu District, Ningbo 315000, Zhejiang Province, China. dyyyding@126.com
Telephone: +86-574-87085588
Fax: +86-574-87291583

Received: April 25, 2018
Peer-review started: April 25, 2018
First decision: May 16, 2018
Revised: May 22, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: September 7, 2018

Abstract

Gastric cancer (GC) is one of the most frequently diagnosed malignant diseases. The molecular mechanisms of metastasis remain unclear. Recently, studies have shown that long non-coding RNAs (lncRNAs) play critical roles in metastasis. Therefore, deeper understanding of this mechanism could provide potential diagnostic tools and therapeutic targets for metastatic GC. This review focuses on dysregulated lncRNAs in GC metastases. Due to the identification of multiple diverse mechanisms involved in GC metastasis, we classified them into seven categories, including lncRNAs related to epithelial-mesenchymal transition, regulation of degradation of extracellular matrix, angiopoiesis, vasculogenic mimicry, and immunologic escape. As the TNM stage is pivotal for evaluating the severity and prognosis of GC patients, we summarize the lncRNAs relevant to lymphatic metastasis, distant metastasis and TNM classification. This review summarizes the lncRNAs related to metastasis, which may provide insight into the mechanisms, and provide potential markers for prognostic prediction and monitoring the relapse of GC.

Key words: Long noncoding RNAs; Stomach neoplasms; Metastasis

Core tip: This review summarizes the long noncoding RNAs (lncRNAs) that influence metastasis of gastric cancer. We classified lncRNAs according to their molecular mechanism, which included epithelial-mesenchymal transition, epigenetic regulation, degradation of the

extracellular matrix, angiopoiesis, vasculogenic mimicry, and immunologic escape. Finally, we summarized the lncRNAs that have stable expression in serum and describe their clinical value. A table lists the clinical correlation of the lncRNAs in details.

Lin MT, Song HJ, Ding XY. Long non-coding RNAs involved in metastasis of gastric cancer. *World J Gastroenterol* 2018; 24(33): 3724-3737 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3724.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3724

INTRODUCTION

Gastric cancer (GC) is a major public health problem across the life span of human beings and is one of the top two leading causes of cancer-related death worldwide. Eastern Asia has the highest incidence rates of GC, which is particularly prevalent in China[1]. According to statistical analysis, lung cancer is the only cancer with higher rates of incidence and mortality compared to stomach neoplasms[2]. Approximately 28000 cases of gastric neoplasms are expected to be diagnosed in 2017, and 10960 of them are expected to result in death[3]. Patients are usually diagnosed with GC after metastasis has occurred or in an advanced stage due to limitations in early noninvasive detection techniques. Even when diagnosed at an early stage and endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) are successfully performed, the local recurrence rate is still high, ranging from 2.8%-12.5%[4,5]. Despite multiple post-operative monitoring tools, including endoscopic monitoring, CT, MRI, PET, and serological monitoring (CA19-9, CA153, CA125, and CA724), the sensitivity has not met expectations yet. Recently, circulating tumor DNA (ctDNA) has been studied as GC relapse predictive markers[6,7]. Because of the unsatisfactory prognosis in advanced stage GC patients who have undergone surgery, chemotherapy or radiotherapy, measures should be taken to intensively monitor GC patients[8]. In recent years, significant advances have been made in understanding the molecular mechanisms involved in GC metastasis, however, the overall view of the mechanism map is limited and ambiguous[9,10]. Therefore, clarification of the pathogenesis and corresponding molecular alterations in GC is imperative in seeking diagnostic biomarkers and therapeutic targets.

Noncoding RNAs (ncRNAs) longer than 200 nucleotides are defined as long noncoding RNAs (lncRNAs). ncRNAs are emerging elements that are recognized to play critical roles in cancer development and progression. lncRNAs do not perform transcriptional tasks, but they can affect gene expression at the transcriptional or post-transcriptional levels[11-13].

Increasingly, lncRNAs have been found to participate in GC metastasis. lncRNAs function by impacting embryogenesis, epigenetic regulation, imprinting, angiopoiesis, and vasculogenic mimicry[14-18]. This article reviews the lncRNAs that regulate certain critical steps of GC metastasis, with particular emphasis on epithelial-mesenchymal transition (EMT), vascularization, and vasculogenic mimicry.

LNCRNAS AFFECT EMT

EMT is a vital process involved in embryonic development and cancer metastasis[19]. EMT is the process by which epithelial cells gain increasing migratory potential and mesenchymal characteristics[20]. It has been shown to play an important role in GC metastasis. There are many lncRNAs that facilitate GC metastasis *via* EMT (Figure 1).

Chen *et al*[14] showed that metastasis associated lung adenocarcinoma 1 (MALAT1) is downregulated in GC cells, and that E-cadherin expression is increased while vimentin expression is decreased at both the mRNA and protein levels. Li *et al*[21] detected UPF1, a key part of the nonsense-mediated mRNA decay (NMD) pathway, which alters mRNA transcription, and showed that it negatively correlated with MALAT1 expression. Subsequent experiments showed that increased UPF1 expression inhibited migration, invasion and EMT of GC cells. Increased MALAT1 expression decreased the influence of UPF1 in GC cells, including UPF1's ability to inhibit cell proliferation, EMT and facilitate apoptosis. Taken together, Li *et al*[22] postulated that UPF1 directly binds MALAT1 to downregulate MALAT1 (UPF1/MALAT1), thus, inhibiting GC progression. Lee *et al*[23] further confirmed that MALAT1 regulates mesenchymal maker Snail, N-cadherin and ZEB1 to influence EMT.

Another classic lncRNA, HOX transcript antisense intergenic RNA (HOTAIR), has been shown to be elevated in GC cells and promote gastric tumor metastasis *via* enhancement of EMT. E-cadherin expression was higher in cells with HOTAIR knockdown compared to cells with HOTAIR overexpression, while expression of N-cadherin and vimentin were decreased. The detailed mechanism is believed to involve HOTAIR recruitment and binding of PRC2 to epigenetically silence miR-34a, which activates the HGF/c-Met/Snail pathway, thus facilitating EMT in tumor cells[24].

FRLnc1 is also upregulated in GC cell lines. *In vitro* functional analysis and a pulmonary metastasis model demonstrated that FRLnc1 enhanced the migration capacity of GC cells. Hui *et al*[25] discovered that FRLnc1 functions as an EMT promoter to affect the migration of GC cells by upregulating the downstream elements TGFβ-1 and Twist.

lncRNA activated by TGF-β (lncRNA-ATB), also known as lncRNA-AL (ENST00000493038), was overexpressed in TGF-β treated cancer cells, with the cells exhibiting a spindle-like morphology. lncRNA-ATB

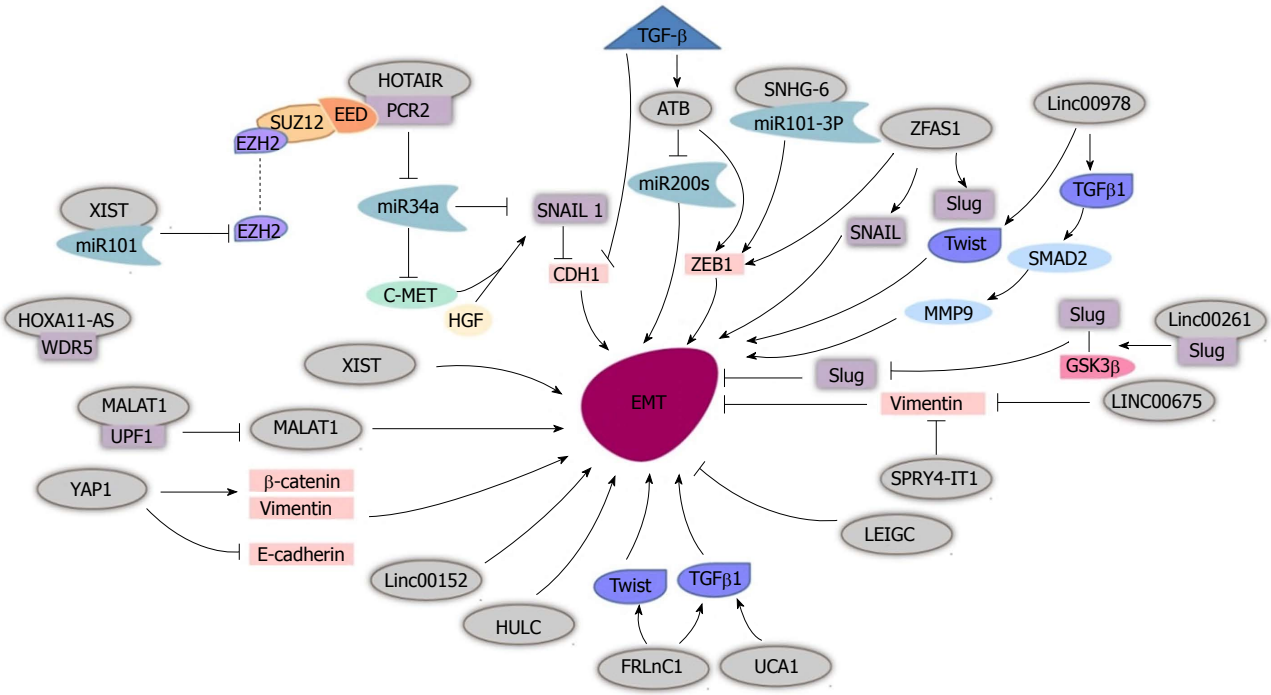


Figure 1 Long non-coding RNAs affect epithelial-mesenchymal transition in gastric cancer cells. HOTAIR recruits PRC2 to silence miR-34a, and then activates the HGF/c-Met/Snail pathway to promote EMT. TGF-β induces lncATB, which inhibits miR-200s and provokes ZEB1 to promote EMT. SNHG6 binds miR-101-3P to activate ZEB1 and then promotes EMT. lncRNA ZFAS1 induces EMT by activating SNAIL, Slug, ZEB1 and Twist. Linc00978 induces Twist and TGFβ1, and TGFβ1 then activates SMAD2 and MMP9 to facilitate EMT. MALAT1 binds UPF1 to reduce its level and activate EMT. FRLnC1 induces EMT by activating Twist and TGFβ1. HUCA1 induces EMT *via* TGFβ1 activation. YAP1 promotes EMT by increasing vimentin and β-catenin, and decreasing E-cadherin. lncRNA XIST, Linc00152 and HULC promote EMT. Linc00261 binds Slug resulting in reduced Slug levels and decreased EMT. LINC00675 and SPRY4-IT1 restrain EMT by reducing vimentin. LEIGC inhibits EMT. lncRNAs: Long non-coding RNAs; HOTAIR: HOX transcript antisense intergenic RNA; EMT: Epithelial-mesenchymal transition; YAP1: Yes-associated protein 1; MALAT1: Metastasis associated lung adenocarcinoma 1.

induced ZEB1 expression and inhibited miR-200s in tumor cells to affect EMT in stomach neoplasm cells. Saito *et al*[26] uncovered a positive correlation between TGF-β, ZEB1 and lncRNA-ATB, while miR-200c inversely correlated with lncRNA-ATB expression. Saito *et al*[26] demonstrated that lncRNA-ATB participate in the EMT process in GC *via* the TGF-β/miR-200/ZEB axis.

It has been reported that the lncRNA X-inactive specific transcript (lncRNA XIST) regulates activation of tumor cell migration and initiates EMT *via* upregulation of vimentin and fibronectin and downregulation of E-cadherin and α-catenin in stomach cancer cells. lncRNA XIST negatively correlates with miR-101 and decreased lncRNA XIST expression led to downregulation of EZH2 at both the mRNA and protein levels and was reversed with an miR-101 inhibitor. Thus, lncRNA XIST functions by sponging miR-101 and regulating EZH2 in GC cells[27]. The lncRNA small nucleolar RNA host gene 6 (SNHG6) is overexpressed in GC cell lines and facilitates EMT as a competing endogenous (ce) RNA *via* sponging miR-101-3p, which leads to an increase in ZEB1, thus boosting tumor cell migration at the post-transcriptional level[28].

The lncRNA zinc finger antisense 1 (ZFAS1) expression level is elevated in GC tissues, serum and exosomes and ZFAS1 also activates ZEB1 to affect EMT. Lei *et al*[29] showed that ZFAS1 promotes the transformation from mesenchymal-epithelial transition (MET) to EMT by increasing the expression of N-cadherin, Slug, Snail, Twist and ZEB1 and decreasing the expression of E-cadherin. Exosomes that originate from GC cells might promote the GC metastasis by producing ZFAS1.

lncRNA urothelial carcinoma associated 1 (UCA1) is induced by TGFβ-1 and expedites EMT. As UCA1 knockdown partly mitigates the impact of TGFβ-1 on EMT, the specific role of TGFβ-1 in accelerating EMT requires further investigation[30]. Silencing UCA1 inhibits resistance to adriamycin in GC, which suggests that UCA1 may be a novel therapeutic target[31].

LincRNA00978 is reportedly elevated in GC tissues and plasma. It could induce EMT by activating the TGFβ/SMAD2/MMP9 pathway. Another potential pathway is composed of downregulated LincRNA00978, leading to decreased Twist1 and Slug, followed by a decrease in downstream molecules, such as N-cadherin and vimentin and an increase in E-cadherin[32]. Yes-associated protein1 (YAP1) also promotes EMT by upregulating vimentin and β-catenin and downregulating E-cadherin[33]. lncRNAs highly upregulated in liver cancer(HULC) and Linc00152 also increase tumor cell's migration through acceleration of EMT in GC[34,35].

The lncRNAs mentioned above function by promoting EMT in GC cells, but there are also numerous

lncRNAs that function by repressing EMT progression.

Linc00261, which is repressed in GC cells, suppresses E-cadherin and promotes N-cadherin, FN1 and vimentin expression, reverses EMT in gastric tumor cells, and increases the malignant phenotype[36]. Yu *et al*[37] deduced that Linc00261 reverses EMT by binding Slug. As mass experiments indicated that GSK3β affects the ubiquitin-proteasome pathway to degrade Slug in breast cancer cells[38,39], additional experiments demonstrated that Linc00261 attenuates the stability of Slug proteins through strengthening the interaction between GSK3β and Slug.

Linc00675, also found to be significantly downregulated in GC tissues, suppresses the migration of GC both *in vitro* and *in vivo* (pulmonary and hepatic metastases). Mechanistic studies showed that Linc00675 directly interacts with vimentin, resulting in increased phosphorylation of vimentin on Ser83 rather than on Ser39, thereby causing the degradation of vimentin filaments[40,41]. Since vimentin is considered to be a master regulator of EMT, Linc00675 was deduced to be a tumor repressor that inhibits metastasis *via* reversing EMT[42].

lncRNA SPRY4 intronic transcript 1 (lncRNA SPRY4-IT1), prevents cancer cell migration partly through its role in the regulation of EMT. Xie *et al*[43] found that SPRY4-IT1 increases the expression of E-cadherin and decreases the expression of vimentin, resulting in EMT inhibition.

After observing significantly decreased lncRNA: chr2:118381039-118383698 levels in GC tissue, Han *et al*[44] named this lncRNA LEIGC and assessed its role in regulating tumor cell migration. In monolayer cultures, cells with downregulated LEIGC showed a dramatic change in morphology and transitioned from a cobblestone-like-shape to a spindle-like fibroblastic status, whereas LEIGC-overexpressing cells maintained a cobblestone-like morphology. In addition, mRNA and protein levels illustrated that LEIGC could reverse EMT by lowering the expression of vimentin, Snail, Slug, Zeb, and Twist and increasing the expression of E-cadherin. Furthermore, LEIGC overexpression enhances the GC cells sensitivity to 5-fluorouracil, and this characteristic enables LEIGC to be a potential therapeutic target.

LNCRNAS AFFECT EPIGENETIC REGULATION IN GC

Epigenetic processes include the recruitment of histone-modifying enzymes and DNA methyltransferases, and chromatin remodeling. It has been reported that lncRNAs interact with DNA to control gene expression[45]. Given that promoter CpG island hypermethylation, an abnormal DNA modification, is involved in pivotal cellular pathways and is characteristically a hallmark of cancer cells[46], several lncRNAs have been found to play roles in controlling the DNA modification system in GC cells.

Sun *et al*[15] evaluated the genome-wide expression profile of lncRNAs and discovered BC041951, designating it as gastric cancer-associated lncRNA 1 (GClnc1). Because mice injected with GClnc1 had an increased overall survival time and more metastatic lung nodules than control mice, GClnc1 was determined to enhance the metastatic capability of tumor cells. The mechanism behind GClnc1's carcinogenesis stems from its ability to function as a molecular scaffold for the WDR5/KAT2A complex, which leads to trimethylation of H3K4 and acetylation of H3K9 in the transcription promoter region of mitochondrial superoxide dismutase (SOD), which upregulates the transcription of mitochondrial superoxide dismutase 2 (SOD2).

LOC100130476, which is dysregulated in gastric cardia adenocarcinoma, is considered to be a tumor suppressor due to the tumor specific hypermethylation of region 1 near the transcription start site. Methylation of region 1 in peripheral white blood cells had a similar effect and may play key roles in gene silencing. Advanced gastric carcinoma patients with low hypermethylation of region 1 preferentially developed metastases, leading to poor prognosis[47].

Xie *et al*[43] also determined that lncRNA SPRY4-IT1 is downregulated in gastric tumor cells and tissues. Furthermore, Sun *et al*[48] identified a canonical CpG island in the SPRY4-IT1 loci promoter region. DNMT1 inhibits expression of SPRY4-IT1 in GC cells by altering the DNA methylation level. After treatment with 3.7- and 2.8-fold 5-aza-CdR, the expression of SPRY4-IT1 was significantly higher than in controls. Therefore, SPRY4-IT1 could be a potential therapeutic target[43].

LNCRNAS INVOLVED IN REGULATION OF DEGRADATION OF THE EXTRACELLULAR MATRIX

Tumor cells are exposed to a multitude of abnormal situations due to changes in the ECM that significantly impact cancer cell behavior. Dysregulated ECM cross-linking and repressed stiffness jointly contribute to cancer metastasis and progression[49,50]. Metalloproteases (MMPs) typically participate in adjusting the ECM and vascularization[51].

The lncRNA UCA1 facilitates GC cell migration both *in vitro* and *in vivo via* the UCA1/GRK2/ERK/MMP9 axis. Meanwhile, the lncRNA UCA1 increases the degradation of GRK2 *via* Cbl-c-mediated ubiquitination following the activation of the ERK-MMP9 pathway, which may be involved in vascularization[52]. Xu *et al*[16] found that FENDRR negatively correlated with FN1 mRNA and that the induction of FENDRR strongly inhibits the activity of MMP2/MMP9, which corroborates FENDRR's role in preventing GC cell metastasis. Then, Park *et al*[53] determined that overexpression of BM742401 decreased the B95kDa band, which corresponds to MMP9, *via* a zymography assay. The reduced concentration of MMP9

in BM74240-induced cells further verified these findings. However, BM742401 did not alter the expression level of intracellular MMP9. Therfore, BM742401 may diminish MMP9 secretion to inhibit cancer metastasis.

lncRNA olfactory receptor, family 3, subfamily A, member 4 (OR3A4), contributes to GC metastasis as it was found to be overexpressed in primary tumor tissue, metastatic tissue and in the peripheral blood. Upregulated OR3A4 induced MMP9, which is involved in the breakdown of the ECM[54]. LINC00052 plays an oncogenous role in GC cells. It promotes GC cell migration and invasion through promoting the SMYD2 related β-catenin methylation to stabilize its expression and activating the Wnt/β-catenin pathway. When upregulating LINC00052 level in GC cells, MMP2, MMP9, and Cyclin D1 expression were upregulated while E-cadherin and P21 were downregulated. The downstream MMP2 and MMP9 are related to the breakdown of the ECM.

Degradation of the extracellular matrix is one way to modulate the tumor microenvironment. Hypoxia is another key change in the tumor microenvironment that promotes tumor metastasis[55]. AK058003, a lncRNA that is induced by hypoxia, is positively associated with γ-synuclein (SNCG) in GC cells. AK058003 and SNCG are both upregulated in hypoxic environments, and SNCG facilitates hypoxia-induced GC cell metastasis, which is regulated by AK058003. Thus, a novel hypoxia/lncRNA-AK058003/SNCG pathway that is related to metastasis was identified[56]. Wang *et al*[56] found that lncRNA AK058003 is increased in hypoxia-induced GC cells, where it facilitates GC cell migration and invasion *in vivo* and *in vitro*. AK058003 positively altered SNCG, a member of the synuclein family, by decreasing methylation of the SNCG gene CpG island. Elevated SNCG expression can also be induced by hypoxia, which in turn induces GC cell metastasis in primary tumor tissue. lncRNA BC005927 is induced by hypoxia and hypoxia inducible factor-1α (HIF-1α), which is a factor involved in hypoxia induced GC metastasis through directly binding the HIF-1 response element to promote GC metastasis and invasion. This hypoxia-induced auxo-action is partially regulated by BPHB4[57].

LNCRNAS INVOLVED IN ANGIOPOIESIS AND VASCULOGENIC MIMICRY

Ample evidence has shown that the development of endothelial vessels (EVs) and vasculogenic mimicry (VM) supply nutrition to tumors and sustain tumor growth. Highly vascular tumors show an increased ability to develop metastases compared to tumors that lack adequate vascularization[58,59]. VM involves the formation of *de novo* channels by pluripotent embryonic-like and highly invasive tumor cells, mimicking tumor feeding[60]. VM has already been reported in melanoma, soft tissue sarcomas, GIST and hepatocellular carcinoma[61-64].

MALAT1, an oncogenic lncRNA, can increase tumorigenicity and metastasis in GC by facilitating VM and angiogenesis. MALAT1 induces the expression of β-catenin and E-cadherin and increases the p-ERK, p-FAK, and p-paxillin levels. MT1-MMP and MMP2 and MMP9, which are downstream of p-ERK, are consequently altered. MALAT1 functions as an active regulator of VM and EV through the E-cadherin/β-catenin complex and *via* the ERK/MMP and FAK/paxillin signaling pathways[17]. Another mechanism involving MALAT1 was discovered by which MLAT1 regulates the acetylation level of H3 histone in the EGFL7 promoter region to boost the EGFL7 expression level[65]. An intron of the EGFL7 gene, miR-126, is pivotal in alterations of H3 histone acetylation but not methylation in the EGFL7 promoter in colorectal cancer and non-small cell lung cancer cells and cooperates with MALAT1 to alter angiogenesis[66,67].

Another lncRNA, C21orF96, which is upregulated in gastric tumor tissues, was found to be significantly higher in metastatic tissues compared to histologically normal lymph node tissues. Yang *et al*[68] determined that ectopic expression of C21orF96 promotes lymphangiogenesis of stomach neoplasms. With respect to VM, C21orF96 increases the number of tubulars, intersecting nodes, and the length of the tubes in human umbilical vein endothelial cells (HUVECs).

Likewise, OR3A4, an oncogenic lncRNA, was found to facilitate the formation of tubules in HUVECs. Upregulated OR3A4 induces vascular endothelial growth factor C (VEGF-C), which is a known promoter of angiogenesis and vascular permeability[69]. Furthermore, the chicken embryo chorioallantoic membrane (CAM) assay demonstrated that OR3A4 promotes angiogenesis. OR3A4 may exert its effects by inhibiting PDLIM2, promoting MACC1 and GNB2L1, and directly targeting NTN4 to enhance metastasis and tumorigenesis in GC[54].

LNCRNAS RELATED TO IMMUNE ESCAPE OF GC CELLS

Immune escape, the third step of cancer immunoediting[70], reduces the immunogenicity of tumor cells, creating an immunosuppressive tumor microenvironment in which cancer cells can survive and grow[71]. Evading immune destruction has been deemed as a hallmark of cancer[72].

The classical lncRNA, HOTAIR, has been reported to promote GC progression and metastasis[73-76]. Song *et al*[18] determined that upregulated HOTAIR in GC cells positively correlates with human leukocyte antigen (HLA)-G levels both in tissue and peripheral blood samples. Furthermore, HOTAIR was also found to induce the expression of HLA-G at both the mRNA and protein secretion levels. HOTAIR directly interacts with miR-152 and decreases miR-152 expression level, which reverses the miR-152 induced dysregulated activity of HLA-G 30UTR, while, Mut-HOTAIR fails

Table 1 Mechanistic analysis of long non-coding RNAs involved in gastric cancer metastasis and clinical correlations

LncRNA ID	Dysregulation	Upstream regulators	Downstream targets	Metastasis processes	Clinical correlation	Univariate analysis (HR 95%CI) $P < 0.05$		Multivariate analysis (HR 95%CI) $P < 0.05$		Ref.
						OS	DFS	OS	DFS	
MALAT1	Up	JMJD1A	UPF1, Snail, N-cadherin, ZEB1, VE-cadherin/β-catenin complex, ERK/MMP, FAK/paxillin, EGFL7, miR-122	EMT, Angiopoiesis, VM	Lymphatic metastasis, distant metastasis, TNM stage	1.38 (1.03-1.85)	1.40 (1.01-1.94)			[14,17,23,89,90]
HOTAIR	Up		PCR2, miR-34a, c-MET, SNAIL1, CDH1, miR-152, HLA-G	EMT, immune escape	Lymphatic metastasis, distant metastasis, TNM stage					[73-75,77,91-93]
FRLnC	Up	FOXM1	Twist, TGFβ-1	EMT						[25]
UCA1	Up		TGFβ-1, GRK2/ERK/MMP9	EMT, degradation of the ECM	Lymphatic metastasis, TNM stage	3.909 (1.592-9.599)		2.917 (1.069-7.962)		[30,73]
ATB	Up	TGFβ-1	miR-200s, ZEB1	EMT				3.50 (1.73-7.44)		[26]
XIST	Up		miR-101	EMT	Lymphatic metastasis, distant metastasis, TNM stage					[27]
SNHG-6	Up		miR-101-3P, ZEB1	EMT	Lymphatic metastasis, distant metastasis, TNM stage					[28]
ZFAS1	Up		ZEB1, SNAIL, Slug, Twist	EMT	Lymphatic metastasis, TNM stage					[29]
LINC00152	Up			EMT	Lymphatic metastasis, TNM stage	2.162 (1.327-3.524)		1.659 (1.008-2.731)		[94]
HULC	Up			EMT	Lymphatic metastasis, distant metastasis, TNM stage					[35]
Linc00978	Up		TGFβ/SMAD,Twist1, Slug	EMT	Lymphatic metastasis, TNM stage					[32]
YAP1	Up		vimentin, β-catenin, E-cadherin	EMT	Lymphatic metastasis, distant metastasis, TNM stage					[33]
Linc00261	Down		Slug, GSK3β	EMT	Lymphatic, metastasis		0.494 (0.300-0.812)		0.551 (0.323-0.940)	[36]
Linc00675	Down		Vimentin	EMT						[40]
SPRY4-IT1	Down		Vimentin	EMT, epigenetic regulation	Lymphatic metastasis, distant metastasis, TNM stage	1.247 (1473-1.996)	2.223 (1.806-2.59)	0.818 (0.314-1.567)	1.741 (1.324-2477)	[43,95]
LEIGC	Down			EMT						[44]
GClnc1	Up		WDR5/KAT2, H3K4, H3K9, SOD2	Epigenetic regulation		2.21 (1.46-3.33)		1.93 (1.24-3.00)		[15]
LOC100130476	Down	DNMT1		Epigenetic regulation	Lymphatic metastasis, distant metastasis, TNM stage					[47]
AK058003	Up		SNCG	Epigenetic regulation, Hypoxia	Lymphatic metastasis, TNM stage					[56]
BC005927	Up	HIF-1α	BPHB4	Hypoxia	Lymphatic metastasis, TNM stage					[57]
SNHG15	Up		MMP2, MMP9	Degradation of the ECM	Lymphatic metastasis, TNM stage					[96]
FENDRR	Down		MMP2, MMP9	Degradation of the ECM	Lymphatic metastasis	0.539 (0.337-0.862)	0.563 (0.370-0.856)	0.569 (0.321-0.960)	0.555 (0.344-0.897)	[16]

BM742401	Down		MMP9	Degradation of the ECM					[53]
C21orF96	Up			Lymphangiogenesis, VM	Lymphatic metastasis, distant metastasis				[68]
LINC00052	Up		Wnt/β-catenin pathway		TNM stage				[97]
AA174084	Down								[80]
RMRP	Down				Lymphatic metastasis				[81]
SNHG1	Up				Lymphatic metastasis, TNM stage				[98]
SNHG5	Down				TNM stage				[99]
MSTO2P	Up		miR-335		Lymphatic metastasis, distant metastasis				[100]
ZEB1-AS1	Up		miR-335-5p		Lymphatic metastasis, TNM stage				[101]
PTENP1	Down		miR-106b, miR-93		Lymphatic metastasis, TNM stage				[102]
RP11-19P22.6-001	Down		Nitric oxide synthase 2 (NOS2)		Lymphatic metastasis, TNM stage				[103]
PCAT-1	Up				distant metastasis				[104]
HOXD-ASI	Up				Lymphatic metastasis, distant metastasis, TNM stage				[105]
CARLo-5	Up				Lymphatic metastasis, distant metastasis				[106]
LINC00673	Down				Lymphatic metastasis				[107]
LINC00982	Down				Lymphatic metastasis, TNM stage				[108]
HMlincRNA717	Down				distant metastasis				[109]
PVT1	Up				Lymphatic metastasis				[110]
GACAT3	Up	IL-6/STAT3			Distant metastasis, TNM stage				[111]
Sox2ot	Down				Distant metastasis	3.241 (1.239-6.428)		3.844 (1.873-7.332)		[85]
HOTTIP	Up		HOXA13		Lymphatic metastasis, TNM stage				[112]
NEAT1	Up				Lymphatic metastasis, distant metastasis				[113,114]
OTUB1-isoform2	Up		N-cadherin, MMP2, MMP9, E-cadherin		Lymphatic metastasis, TNM stage	1,538 (1.044-2.265)	1.615 (1.111-2.348)		1.498 (1.021-2.200)	[86]
PANDAR					Lymphatic metastasis, TNM stage	4.612 (1.59-13.825)	3.113 (1.591-6.093)	3.683 (1.125-12.058)	2.359 (1.153-4.830)	[87]
ZMAT1 transcript variant 2	Down				Lymphatic metastasis, distant metastasis, TNM stage					[115]
JMJD1A	Up		MALAT1, MAPK		Lymphatic metastasis, TNM stage	8.446 (4.480-15.923)		3.988 (1.948-8.167)		[116]
OR3A4	Up		PDLIM2, MACC1, NTN4, GNB2L1	Degradation of the ECM, angiopoiesis, VM	Lymphatic metastasis distant metastasis					[54]
HNF1A-AS1	Down				Lymphatic metastasis					[117]
BANCR	Up				Lymphatic metastasis, distant metastasis	2.457 (1.715-3.521)		1.511 (1.02-2.227)		[118]

DQ786243	Up		Lymphatic metastasis, TNM stage					[119]
XLOC_010235	Up		distant metastasis, TNM stage					[120]
CCAT2	Up		Lymphatic metastasis, distant metastasis, TNM stage	2.631 (1.348-5.672)	2.574 (1.201-5.476)	2.405 (1.194-5.417)	2.315 (1.097-5.283)	[121,122]
Linc-UBC1	Up		Lymphatic metastasis, TNM stage					[123]
HIF1A-AS2	Up		Lymphatic metastasis, TNM stage	2.346 (1.379-3.991)		1.724 (1.002-2.964)		[124]
LET	Down		Lymphatic metastasis, distant metastasis, TNM stage	2.513 (1.414-5.847)		2.275 (1.301-5.176)		[125]
LSINCT5	Up		Lymphatic metastasis, TNM stage		2.501 (1.326-4.719)		1.081 (1.286-3.564)	[126]
AC130710	Up		distant metastasis, TNM stage					[127]
FER1L4	Down		Lymphatic metastasis, distant metastasis, TNM stage					[88]
RuPAR	Down		Lymphatic metastasis, distant metastasis, TNM stage					[128]
H19	Up	miR-675	Lymphatic metastasis, TNM stage	1.170 (1.050-1.304)		1.137 (1.005-1.287)		[129,130]
AC096655.1-002	Down		Lymphatic metastasis, distant metastasis, TNM stage					[131]
SUMO1P3	Up		Lymphatic metastasis					[132]
IGF2	Up		Lymphatic metastasis					[133]
CCAT1	Up		Lymphatic metastasis, TNM stage					[134]

EMT: Epithelial-mesenchymal transition; VM: Vasculogenic mimicry; TNM: Tumor, node, metastasis; ECM: Extracellular matrix.

to have the same effect. Thus, HOTAIR overexpression might play roles in tumor immune escape. Furthermore, polymerase chain reaction-restriction fragment length polymorphism (PCRRFLP) was used to detect three htSNPs of the HOTAIR gene (rs12826786 C > T, rs4759314 A > G, and rs10783618 C > T). In normal and GCA tumor tissues, rs12826786 presented higher HOTAIR expression levels than the CC genotype, and the sore T allele of rs12826786 increased the GCA risk and reduced the five-year survival rates[77].

LNCRNAS DYSREGULATED IN PERIPHERAL BLOOD AND IN GASTRIC ACID

Given that patients are usually asymptomatic and that relapsed GC patients have poor prognosis, many doctors recognize the importance of surveillance in detecting recurrence[78]. Studies have shown that hematogenous metastasis is the most frequent recurrence pattern during the first year following resection[79], and identification of a simple method to monitor patients, for example, using serum lncRNAs, is a top priority. Identification of a noninvasive approach with a high degree of sensitivity and specificity is urgently needed to predict and monitor the prognosis of GC patients and the relapse of patients post-operation.

OR3A4 is upregulated in both metastatic tissue and serum[54], as is ZFAS1 and exosomal ZFAS1. The level of circulating ZFAS1 correlates with lymphatic metastasis and the TNM stage, when the area under the ROC curve is up to 0.792 (95%CI: 0.703-0.881, $P < 0.001$)[29].

AA174084 is not only ectopically expressed in GC tissue but is also expressed in plasma and in gastric acid. The expression of AA174084 in GC patients' gastric acid is significantly higher than that in control groups. In addition, the amount of AA174084 in plasma decreases after patients undergo surgery and is positively correlated with

invasion and lymphatic metastasis. Thus, AA174084 could serve as a potential biomarker to predict a patient's prognosis[80].

The lncRNA RNA component of mitochondrial RNA processing endoribonuclease (RMRP) has been reported to be decreased in GC tissues, but increased in the plasma and gastric acid of GC patients. After subtotal gastrectomy, this aberrant expression dramatically declines. Importantly, the RMRP level in gastric acid or in plasma is not only sufficient for clinical detection but that method for RMRP detection are also more sensitive and specific than that for carcinoembryonic antigen (CEA) and carbohydrate antigen19-9 (CA199). These results could provide a new method for GC detection, and the postoperative decline of RMRP implies that this lncRNA has appropriate characteristics for prognostic prediction[81].

Five novel plasma lncRNAs (TINCR, CCAT2, AOC4P, BANCR and LINC00857) demonstrate excellent stability and show little to no change in hostile environments. The diagnostic significance of lncRNA-based Index I, established by logistic regression, is better than that of the CEA-based Index II. Because the lncRNA based index declined dramatically two weeks post-operation, this index is highly effective in monitoring tumor recurrence. The lncRNA based index significantly correlates with tumor size, depth of invasion, lymphatic metastasis and TNM stages[82].

Currently, the majority of GC research focuses on the expression level of lncRNAs in GC tissue, while many of them are stably expressed in plasma. Though systematic evaluation of the lncRNAs mentioned above is lacking, those that are stable in circulation could be useful for predicting metastasis of primary tumors, but this hypothesis must be confirmed. Individual markers, such as a single lncRNA, may not be adequate for determining prognosis in GC, but interested readers could refer to the analysis by Zhang *et al*[82] and Shao *et al*[80]. The combination of several lncRNAs known to participate in GC progression may overcome these existing issues.

LNCRNAS AND CLINICAL CORRELATION

Recently, the seventh edition of tumor, node, metastasis (TNM) classification has been widely accepted[83]. Gu *et al*[84] identified patients diagnosed with GC in the first hospital of the China Medical University and the Liaoning Cancer Hospital from January 1980 to December 2009 and systematically reviewed the data. These authors found that according to the 7th edition of the TNM classification, classification of stage T4b and N0 as stage IIIA had statistical significance in regard to the survival outcome and in predicting prognoses in Chinese GC patients. Given that lymph node and distant metastasis were found to be key factors in the prognosis of GC patients, we identified the lncRNAs that correlated with lymph node, distant metastasis and the TNM stage, as shown in Table 1[14-17,23,25-30,32,33,35,36,40,43,44,53,57,58,69,74-78,80-132].

CONCLUSION

Utilizing a variety of techniques, including RT-PCR, computer-assisted microscopic image analysis, bioinformatics methods, ChIP assays, *etc.,* a myriad of lncRNAs have been found to participate in the proliferation, growth, invasion, metastasis, motility, and phenotype of GC cells, with dozens of them correlating with the invasion depth, size, lymph node metastasis, TNM stage, OS and DFS of GC tumors. In this review, we emphasized epithelial-mesenchymal transition, epigenetic regulation, and degradation of the extracellular matrix, angiopoiesis, vasculogenic mimicry, and immune escape in examining the ectopic expression of lncRNAs. lncRNAs involved in specific mechanisms of GC progression could be helpful in GC treatment. Those lncRNAs that are considered as independent prognostic factors by survival analysis such as MALAT1[17], Sox2ot[85], OTUB1-isoform 2[86], PANDAR[87], *etc*., and those lncRNAs dramatically altered in postoperative GC patients such as FERI4[88], may be utilized as prognosis evaluation markers. Some lncRNAs increased in metastatic tissue compared to primary focus may be beneficial in predicting metastasis.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; **66**: 7-30 [PMID: 26742998 DOI: 10.3322/caac.21332]

2 **Chen W**, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]

3 **Siegel RL**, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin* 2017; **67**: 7-30 [PMID: 28055103 DOI: 10.3322/caac.21387]

4 **Tanabe S**, Koizumi W, Mitomi H, Nakai H, Murakami S, Nagaba S, Kida M, Oida M, Saigenji K. Clinical outcome of endoscopic aspiration mucosectomy for early stage gastric cancer. *Gastrointest Endosc* 2002; **56**: 708-713 [PMID: 12397280 DOI: 10.1067/mge.2002.129085]

5 **Tanabe S**, Ishido K, Higuchi K, Sasaki T, Katada C, Azuma M, Naruke A, Kim M, Koizumi W. Long-term outcomes of endoscopic submucosal dissection for early gastric cancer: a retrospective comparison with conventional endoscopic resection in a single center. *Gastric Cancer* 2014; **17**: 130-136 [PMID: 23576197 DOI: 10.1007/s10120-013-0241-2]

6 **Diehl F**, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA, Kinzler KW, Vogelstein B, Diaz LA Jr. Circulating mutant DNA to assess tumor dynamics. *Nat Med* 2008; **14**: 985-990 [PMID: 18670422 DOI: 10.1038/nm.1789]

7 **Hamakawa T**, Kukita Y, Kurokawa Y, Miyazaki Y, Takahashi T, Yamasaki M, Miyata H, Nakajima K, Taniguchi K, Takiguchi S, Mori M, Doki Y, Kato K. Monitoring gastric cancer progression with circulating tumour DNA. *Br J Cancer* 2015; **112**: 352-356 [PMID: 25490524 DOI: 10.1038/bjc.2014.609]

8 **Ferro A**, Peleteiro B, Malvezzi M, Bosetti C, Bertuccio P, Levi F, Negri E, La Vecchia C, Lunet N. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. *Eur J Cancer* 2014; **50**: 1330-1344 [PMID: 24650579 DOI: 10.1016/j.ejca.2014.01.029]

9 **Djebali S**, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi

A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J, Williams BA, Zaleski C, Rozowsky J, Röder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Bar NS, Batut P, Bell K, Bell I, Chakrabortty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Duttagupta R, Falconnet E, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena H, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Luo OJ, Park E, Persaud K, Preall JB, Ribeca P, Risk B, Robyr D, Sammeth M, Schaffer L, See LH, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Ruan X, Hayashizaki Y, Harrow J, Gerstein M, Hubbard T, Reymond A, Antonarakis SE, Hannon G, Giddings MC, Ruan Y, Wold B, Carninci P, Guigó R, Gingeras TR. Landscape of transcription in human cells. *Nature* 2012; **489**: 101-108 [PMID: 22955620 DOI: 10.1038/nature11233]

10 **Criscione SW**, Zhang Y, Thompson W, Sedivy JM, Neretti N. Transcriptional landscape of repetitive elements in normal and cancer human cells. *BMC Genomics* 2014; **15**: 583 [PMID: 25012247 DOI: 10.1186/1471-2164-15-583]

11 **Novikova IV**, Hennelly SP, Tung CS, Sanbonmatsu KY. Rise of the RNA machines: exploring the structure of long non-coding RNAs. *J Mol Biol* 2013; **425**: 3731-3746 [PMID: 23467124 DOI: 10.1016/j.jmb.2013.02.030]

12 **Iyer MK**, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, Barrette TR, Prensner JR, Evans JR, Zhao S, Poliakov A, Cao X, Dhanasekaran SM, Wu YM, Robinson DR, Beer DG, Feng FY, Iyer HK, Chinnaiyan AM. The landscape of long noncoding RNAs in the human transcriptome. *Nat Genet* 2015; **47**: 199-208 [PMID: 25599403 DOI: 10.1038/ng.3192]

13 **Grammatikakis I**, Panda AC, Abdelmohsen K, Gorospe M. Long noncoding RNAs(lncRNAs) and the molecular hallmarks of aging. *Aging* (Albany NY) 2014; **6**: 992-1009 [PMID: 25543668 DOI: 10.18632/aging.100710]

14 **Chen D**, Liu L, Wang K, Yu H, Wang Y, Liu J, Guo Y, Zhang H. The role of MALAT-1 in the invasion and metastasis of gastric cancer. *Scand J Gastroenterol* 2017; **52**: 790-796 [PMID: 28276823 DOI: 10.1080/00365521.2017.1280531]

15 **Sun TT**, He J, Liang Q, Ren LL, Yan TT, Yu TC, Tang JY, Bao YJ, Hu Y, Lin Y, Sun D, Chen YX, Hong J, Chen H, Zou W, Fang JY. LncRNA GClnc1 Promotes Gastric Carcinogenesis and May Act as a Modular Scaffold of WDR5 and KAT2A Complexes to Specify the Histone Modification Pattern. *Cancer Discov* 2016; **6**: 784-801 [PMID: 27147598 DOI: 10.1158/2159-8290.cd-15-0921]

16 **Xu TP**, Huang MD, Xia R, Liu XX, Sun M, Yin L, Chen WM, Han L, Zhang EB, Kong R, De W, Shu YQ. Decreased expression of the long non-coding RNA FENDRR is associated with poor prognosis in gastric cancer and FENDRR regulates gastric cancer cell metastasis by affecting fibronectin1 expression. *J Hematol Oncol* 2014; **7**: 63 [PMID: 25167886 DOI: 10.1186/s13045-014-0063-7]

17 **Li Y**, Wu Z, Yuan J, Sun L, Lin L, Huang N, Bin J, Liao Y, Liao W. Long non-coding RNA MALAT1 promotes gastric cancer tumorigenicity and metastasis by regulating vasculogenic mimicry and angiogenesis. *Cancer Lett* 2017; **395**: 31-44 [PMID: 28268166 DOI: 10.1016/j.canlet.2017.02.035]

18 **Song B**, Guan Z, Liu F, Sun D, Wang K, Qu H. Long non-coding RNA HOTAIR promotes HLA-G expression via inhibiting miR-152 in gastric cancer cells. *Biochem Biophys Res Commun* 2015; **464**: 807-813 [PMID: 26187665 DOI: 10.1016/j.bbrc.2015.07.040]

19 **Bonnomet A**, Brysse A, Tachsidis A, Waltham M, Thompson EW, Polette M, Gilles C. Epithelial-to-mesenchymal transitions and circulating tumor cells. *J Mammary Gland Biol Neoplasia* 2010; **15**: 261-273 [PMID: 20449641 DOI: 10.1007/s10911-010-9174-0]

20 **Guarino M**. Epithelial-mesenchymal transition and tumour invasion. *Int J Biochem Cell Biol* 2007; **39**: 2153-2160 [PMID: 17825600 DOI: 10.1016/j.biocel.2007.07.011]

21 **Hurt JA**, Robertson AD, Burge CB. Global analyses of UPF1 binding and function reveal expanded scope of nonsense-mediated mRNA decay. *Genome Res* 2013; **23**: 1636-1650 [PMID: 23766421 DOI: 10.1101/gr.157354.113]

22 **Li L**, Geng Y, Feng R, Zhu Q, Miao B, Cao J, Fei S. The Human RNA Surveillance Factor UPF1 Modulates Gastric Cancer Progression by Targeting Long Non-Coding RNA MALAT1. *Cell Physiol Biochem* 2017; **42**: 2194-2206 [PMID: 28942451 DOI: 10.1159/000479994]

23 **Lee NK**, Lee JH, Ivan C, Ling H, Zhang X, Park CH, Calin GA, Lee SK. MALAT1 promoted invasiveness of gastric adenocarcinoma. *BMC Cancer* 2017; **17**: 46 [PMID: 28077118 DOI: 10.1186/s12885-016-2988-4]

24 **Liu YW**, Sun M, Xia R, Zhang EB, Liu XH, Zhang ZH, Xu TP, De W, Liu BR, Wang ZX. LincHOTAIR epigenetically silences miR34a by binding to PRC2 to promote the epithelial-to-mesenchymal transition in human gastric cancer. *Cell Death Dis* 2015; **6**: e1802 [PMID: 26136075 DOI: 10.1038/cddis.2015.150]

25 **Cai H**, Chen J, He B, Li Q, Li Y, Gao Y. A FOXM1 related long non-coding RNA contributes to gastric cancer cell migration. *Mol Cell Biochem* 2015; **406**: 31-41 [PMID: 25907137 DOI: 10.1007/s11010-015-2421-3]

26 **Saito T**, Kurashige J, Nambara S, Komatsu H, Hirata H, Ueda M, Sakimura S, Uchi R, Takano Y, Shinden Y, Iguchi T, Eguchi H, Ehata S, Murakami K, Sugimachi K, Mimori K. A Long Non-coding RNA Activated by Transforming Growth Factor-β is an Independent Prognostic Marker of Gastric Cancer. *Ann Surg Oncol* 2015; **22 Suppl 3**: S915-S922 [PMID: 25986864 DOI: 10.1245/s10434-015-4554-8]

27 **Chen DL**, Ju HQ, Lu YX, Chen LZ, Zeng ZL, Zhang DS, Luo HY, Wang F, Qiu MZ, Wang DS, Xu DZ, Zhou ZW, Pelicano H, Huang P, Xie D, Wang FH, Li YH, Xu RH. Long non-coding RNA XIST regulates gastric cancer progression by acting as a molecular sponge of miR-101 to modulate EZH2 expression. *J Exp Clin Cancer Res* 2016; **35**: 142 [PMID: 27620004 DOI: 10.1186/s13046-016-0420-1]

28 **Yan K**, Tian J, Shi W, Xia H, Zhu Y. LncRNA SNHG6 is Associated with Poor Prognosis of Gastric Cancer and Promotes Cell Proliferation and EMT through Epigenetically Silencing p27 and Sponging miR-101-3p. *Cell Physiol Biochem* 2017; **42**: 999-1012 [PMID: 28683446 DOI: 10.1159/000478682]

29 **Pan L**, Liang W, Fu M, Huang ZH, Li X, Zhang W, Zhang P, Qian H, Jiang PC, Xu WR, Zhang X. Exosomes-mediated transfer of long noncoding RNA ZFAS1 promotes gastric cancer progression. *J Cancer Res Clin Oncol* 2017; **143**: 991-1004 [PMID: 28285404 DOI: 10.1007/s00432-017-2361-2]

30 **Zuo ZK**, Gong Y, Chen XH, Ye F, Yin ZM, Gong QN, Huang JS. TGFβ1-Induced LncRNA UCA1 Upregulation Promotes Gastric Cancer Invasion and Migration. *DNA Cell Biol* 2017; **36**: 159-167 [PMID: 28075173 DOI: 10.1089/dna.2016.3553]

31 **Gao J**, Cao R, Mu H. Long non-coding RNA UCA1 may be a novel diagnostic and predictive biomarker in plasma for early gastric cancer. *Int J Clin Exp Pathol* 2015; **8**: 12936-12942 [PMID: 26722487]

32 **Fu M**, Huang Z, Zang X, Pan L, Liang W, Chen J, Qian H, Xu W, Jiang P, Zhang X. Long noncoding RNA LINC00978 promotes cancer growth and acts as a diagnostic biomarker in gastric cancer. *Cell Prolif* 2018; **51** [PMID: 29271006 DOI: 10.1111/cpr.12425]

33 **Sun D**, Li X, He Y, Li W, Wang Y, Wang H, Jiang S, Xin Y. YAP1 enhances cell proliferation, migration, and invasion of gastric cancer in vitro and in vivo. *Oncotarget* 2016; **7**: 81062-81076 [PMID: 27835600 DOI: 10.18632/oncotarget.13188]

34 **Zhao J**, Liu Y, Zhang W, Zhou Z, Wu J, Cui P, Zhang Y, Huang G. Long non-coding RNA Linc00152 is involved in cell cycle arrest, apoptosis, epithelial to mesenchymal transition, cell migration and invasion in gastric cancer. *Cell Cycle* 2015; **14**: 3112-3123 [PMID: 26237576 DOI: 10.1080/15384101.2015.1078034]

35 **Zhao Y**, Guo Q, Chen J, Hu J, Wang S, Sun Y. Role of long non-coding RNA HULC in cell proliferation, apoptosis and tumor metastasis of gastric cancer: a clinical and in vitro investigation. *Oncol Rep* 2014; **31**: 358-364 [PMID: 24247585 DOI: 10.3892/or.2013.2850]

36 **Fan Y**, Wang YF, Su HF, Fang N, Zou C, Li WF, Fei ZH. Decreased expression of the long noncoding RNA LINC00261 indicate poor prognosis in gastric cancer and suppress gastric cancer metastasis

by affecting the epithelial-mesenchymal transition. *J Hematol Oncol* 2016; **9**: 57 [PMID: 27439973 DOI: 10.1186/s13045-016-0288-8]
37 **Yu Y**, Li L, Zheng Z, Chen S, Chen E, Hu Y. Long non-coding RNA linc00261 suppresses gastric cancer progression via promoting Slug degradation. *J Cell Mol Med* 2017; **21**: 955-967 [PMID: 27878953 DOI: 10.1111/jcmm.13035]
38 **Wu ZQ**, Li XY, Hu CY, Ford M, Kleer CG, Weiss SJ. Canonical Wnt signaling regulates Slug activity and links epithelial-mesenchymal transition with epigenetic Breast Cancer 1, Early Onset (BRCA1) repression. *Proc Natl Acad Sci U S A* 2012; **109**: 16654-16659 [PMID: 23011797 DOI: 10.1073/pnas.1205822109]
39 **Raja**, Vidhya G. GSK3B regulates epithelial-mesenchymal transition and cancer stem cell properties and is a novel drug target for triple-negative breast cancer. 2017
40 **Zeng S**, Xie X, Xiao YF, Tang B, Hu CJ, Wang SM, Wu YY, Dong H, Li BS, Yang SM. Long noncoding RNA LINC00675 enhances phosphorylation of vimentin on Ser83 to suppress gastric cancer progression. *Cancer letters* 2017; **412**: 179
41 **Zhang X**, Liu W, Yang H, Tan L, Ao L, Liu J, Cao J, Cui Z. Inhibition of PPARα attenuates vimentin phosphorylation on Ser-83 and collapse of vimentin filaments during exposure of rat Sertoli cells in vitro to DBP. *Reprod Toxicol* 2014; **50**: 11-18 [PMID: 25291543 DOI: 10.1016/j.reprotox.2014.09.015]
42 **Dave JM**, Bayless KJ. Vimentin as an integral regulator of cell adhesion and endothelial sprouting. *Microcirculation* 2014; **21**: 333–344
43 **Xie M**, Nie FQ, Sun M, Xia R, Liu YW, Zhou P, De W, Liu XH. Decreased long noncoding RNA SPRY4-IT1 contributing to gastric cancer cell metastasis partly via affecting epithelial-mesenchymal transition. *J Transl Med* 2015; **13**: 250 [PMID: 26238992 DOI: 10.1186/s12967-015-0595-9]
44 **Han Y**, Ye J, Wu D, Wu P, Chen Z, Chen J, Gao S, Huang J. LEIGC long non-coding RNA acts as a tumor suppressor in gastric carcinoma by inhibiting the epithelial-to-mesenchymal transition. *BMC Cancer* 2014; **14**: 932 [PMID: 25496320 DOI: 10.1186/1471-2407-14-932]
45 **Beckedorff FC**, Amaral MS, Deocesanopereira C, Verjovskialmeida S. Long non-coding RNAs and their implications in cancer epigenetics. *Bioscience Reports* 2013; **33**: 667-675
46 Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. Human Molecular Genetics 2007; 16 Spec No 1(1): R50
47 **Guo W**, Dong Z, Shi Y, Liu S, Liang J, Guo Y, Guo X, Shen S, Wang G. Methylation-mediated downregulation of long noncoding RNA LOC100130476 in gastric cardia adenocarcinoma. *Clin Exp Metastasis* 2016; **33**: 497-508 [PMID: 27189370 DOI: 10.1007/s10585-016-9794-x]
48 **Sun M**, Liu XH, Lu KH, Nie FQ, Xia R, Kong R, Yang JS, Xu TP, Liu YW, Zou YF, Lu BB, Yin R, Zhang EB, Xu L, De W, Wang ZX. EZH2-mediated epigenetic suppression of long noncoding RNA SPRY4-IT1 promotes NSCLC cell proliferation and metastasis by affecting the epithelial-mesenchymal transition. *Cell Death Dis* 2014; **5**: e1298 [PMID: 24967960 DOI: 10.1038/cddis.2014.256]
49 **Buchheit CL**, Weigel KJ, Schafer ZT. Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression. *Nature Reviews Cancer* 2014; **14**: 632
50 **Jodele S**, Blavier L, Yoon JM, Declerck YA. Modifying the soil to affect the seed: role of stromal-derived matrix metalloproteinases in cancer progression. *Cancer Metasta Revie* 2006; **25**: 35
51 **Hadler-Olsen E**, Winberg JO, Uhlin-Hansen L. Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets. *Bio J of Inter Soci Biol Medic* 2013; **34**: 2041-2051
52 **Wang ZQ**, He CY, Hu L, Shi HP, Li JF, Gu QL, Su LP, Liu BY, Li C, Zhu Z. Long noncoding RNA UCA1 promotes tumour metastasis by inducing GRK2 degradation in gastric cancer. *Cancer Lett* 2017; **408**: 10-21 [PMID: 28843497 DOI: 10.1016/j.canlet.2017.08.013]
53 **Park SM**, Park SJ, Kim HJ, Kwon OH, Kang TW, Sohn HA, Kim SK, Moo Noh S, Song KS, Jang SJ, Sung Kim Y, Kim SY. A known expressed sequence tag, BM742401, is a potent lincRNA inhibiting cancer metastasis. *Exp Mol Med* 2013; **45**: e31 [PMID: 23846333 DOI: 10.1038/emm.2013.59]
54 **Guo X**, Yang Z, Zhi Q, Wang D, Guo L, Li G, Miao R, Shi Y, Kuang Y. Long noncoding RNA OR3A4 promotes metastasis and tumorigenicity in gastric cancer. *Oncotarget* 2016; **7**: 30276-30294 [PMID: 26863570 DOI: 10.18632/oncotarget.7217]
55 **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]
56 **Wang Y**, Liu X, Zhang H, Sun L, Zhou Y, Jin H, Zhang H, Zhang H, Liu J, Guo H, Nie Y, Wu K, Fan D, Zhang H, Liu L. Hypoxia-inducible lncRNA-AK058003 promotes gastric cancer metastasis by targeting γ-synuclein. *Neoplasia* 2014; **16**: 1094-1106 [PMID: 25499222 DOI: 10.1016/j.neo.2014.10.008]
57 **Liu X**, Wang Y, Sun L, Min J, Liu J, Chen D, Zhang H, Zhang H, Zhang H, Zhou Y, Liu L. Long noncoding RNA BC005927 upregulates EPHB4 and promotes gastric cancer metastasis under hypoxia. *Cancer Sci* 2018; **109**: 988-1000 [PMID: 29383777 DOI: 10.1111/cas.13519]
58 **Koliopanos A**, Avgerinos C, Paraskeva C, Touloumis Z, Kelgiorgi D, Dervenis C. *Molecu aspe carcinogene pancre cancer* 2008; **7**: 345-356
59 **Coomber BL**, Yu JL, Fathers KE, Plumb C, Rak JW. Angiogenesis and the role of epigenetics in metastasis. *Clini experimen metasta* 2003; **20**: 215
60 **Chen X**, Maniotis AJ, Majumdar D, Pe'Er J, Folberg R. Uveal melanoma cell staining for CD34 and assessment of tumor vascularity. *Investigat Ophthalmol Visu Scie* 2002; **43**: 2533-2539
61 **Sun B**, Zhang S, Zhang D, Du J, Guo H, Zhao X, Zhang W, Hao X. Vasculogenic mimicry is associated with high tumor grade, invasion and metastasis, and short survival in patients with hepatocellular carcinoma. *Oncol repor* 2006; **16**: 693-698
62 **Maniotis AJ**, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'Er J, Trent JM, Meltzer PS, Hendrix MJ. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Americ J Pathol* 1999; **155**: 739
63 **Sun B**, Zhang S, Zhao X, Zhang W, Hao X, Sun, B, Zhang, S, Zhao, X, Zhang W, Hao X. Vasculogenic mimicry is associated with poor survival in patients with mesothelial sarcomas and alveolar rhabdomyosarcomas. *Int J Oncol* 2005; **25**: 1609-1614
64 **Sun B**, Qie S, Zhang S, Sun T, Zhao X, Gao S, Ni C, Wang X, Liu Y, Zhang L. Role and mechanism of vasculogenic mimicry in gastrointestinal stromal tumors. *Hum Pathol* 2008; **39**: 444
65 **Deng QJ**, Xie LQ, Li H. Overexpressed MALAT1 promotes invasion and metastasis of gastric cancer cells via increasing EGFL7 expression. *Life Sci* 2016; **157**: 38-44 [PMID: 27259812 DOI: 10.1016/j.lfs.2016.05.041]
66 **Hansen TF**, Christensen RD, Andersen RF, Sørensen FB, Johnsson A, Jakobsen A. MicroRNA-126 and epidermal growth factor-like domain 7|[ndash]|an angiogenic couple of importance in metastatic colorectal cancer. Results from the Nordic ACT trial. *Bri J Cancer* 2013; **109**: 1243
67 **Sun Y**, Bai Y, Zhang F, Wang Y, Guo Y, Guo L. miR-126 inhibits non-small cell lung cancer cells proliferation by targeting EGFL7. *Biophyl Resea Communica* 2010; **391**: 1483-1489
68 **Yang Z**, Zhi Q, Wang D, Zhang L, Preston B, Brandon C, Kuang Y, Miao R, Shi Y, Guo X. Long Noncoding RNA C21orF96 Promotes the Migration, Invasion and Lymph Node Metastasis in Gastric Cancer. *Anticancer Agents Med Chem* 2016; **16**: 1101-1108 [PMID: 26567621]
69 **Kamei S**, Kono K, Amemiya H, Takahashi A, Sugai H, Ichihara F, Fujii H, Matsumoto Y. Evaluation of VEGF and VEGF-C expression in gastric cancer cells producing alpha-fetoprotein. *J Gastroenterol* 2003; **38**: 540-547 [PMID: 12825129 DOI: 10.1007/s00535-002-1099-y]
70 **Koebel CM**, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 2007; **450**: 903-907 [PMID: 18026089 DOI: 10.1038/nature06309]
71 **Khong HT**, Restifo NP. Natural selection of tumor variants in the

generation of "tumor escape" phenotypes. *Nat Immunol* 2002; **3**: 999-1005 [PMID: 12407407 DOI: 10.1038/ni1102-999]

72 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

73 **Li CY**, Liang GY, Yao WZ, Sui J, Shen X, Zhang YQ, Peng H, Hong WW, Ye YC, Zhang ZY, Zhang WH, Yin LH, Pu YP. Integrated analysis of long non-coding RNA competing interactions reveals the potential role in progression of human gastric cancer. *Int J Oncol* 2016; **48**: 1965-1976 [PMID: 26935047 DOI: 10.3892/ijo.2016.3407]

74 **Zhang ZZ**, Shen ZY, Shen YY, Zhao EH, Wang M, Wang CJ, Cao H, Xu J. HOTAIR Long Noncoding RNA Promotes Gastric Cancer Metastasis through Suppression of Poly r(C)-Binding Protein (PCBP) 1. *Mol Cancer Ther* 2015; **14**: 1162-1170 [PMID: 25612617 DOI: 10.1158/1535-7163.mct-14-0695]

75 **Lee NK**, Lee JH, Park CH, Yu D, Lee YC, Cheong JH, Noh SH, Lee SK. Long non-coding RNA HOTAIR promotes carcinogenesis and invasion of gastric adenocarcinoma. *Biochem Biophys Res Commun* 2014; **451**: 171-178 [PMID: 25063030 DOI: 10.1016/j.bbrc.2014.07.067]

76 **Okugawa Y**, Toiyama Y, Hur K, Toden S, Saigusa S, Tanaka K, Inoue Y, Mohri Y, Kusunoki M, Boland CR, Goel A. Metastasis-associated long non-coding RNA drives gastric cancer development and promotes peritoneal metastasis. *Carcinogenesis* 2014; **35**: 2731-2739 [PMID: 25280565 DOI: 10.1093/carcin/bgu200]

77 **Guo W**, Dong Z, Bai Y, Guo Y, Shen S, Kuang G, Xu J. Associations between polymorphisms of HOTAIR and risk of gastric cardia adenocarcinoma in a population of north China. *Tumour Biol* 2015; **36**: 2845-2854 [PMID: 25476857 DOI: 10.1007/s13277-014-2912-y]

78 **Hondo FY**, Kishi H, Safatle-Ribeiro AV, Pessorrusso FCS, Ribeiro U, Maluf-Filho F. Characterization of the mucin phenotype can predict gastric cancer recurrence after endoscopic mucosal resection. *Arq Gastroenterol* 2017; **54**: 308-314 [PMID: 28954038 DOI: 10.1590/s0004-2803.201700000-38]

79 **Eom BW**, Yoon H, Ryu KW, Lee JH, Cho SJ, Lee JY, Kim CG, Choi IJ, Lee JS, Kook MC, Park SR, Nam BH, Kim YW. Predictors of timing and patterns of recurrence after curative resection for gastric cancer. *Dig Surg* 2010; **27**: 481-486 [PMID: 21063125 DOI: 10.1159/000320691]

80 **Shao Y**, Ye M, Jiang X, Sun W, Ding X, Liu Z, Ye G, Zhang X, Xiao B, Guo J. Gastric juice long noncoding RNA used as a tumor marker for screening gastric cancer. *Cancer* 2014; **120**: 3320-3328 [PMID: 24986041 DOI: 10.1002/cncr.28882]

81 **Shao Y**, Ye M, Li Q, Sun W, Ye G, Zhang X, Yang Y, Xiao B, Guo J. LncRNA-RMRP promotes carcinogenesis by acting as a miR-206 sponge and is used as a novel biomarker for gastric cancer. *Oncotarget* 2016; **7**: 37812-37824 [PMID: 27192121 DOI: 10.18632/oncotarget.9336]

82 **Zhang K**, Shi H, Xi H, Wu X, Cui J, Gao Y, Liang W, Hu C, Liu Y, Li J, Wang N, Wei B, Chen L. Genome-Wide lncRNA Microarray Profiling Identifies Novel Circulating lncRNAs for Detection of Gastric Cancer. *Theranostics* 2017; **7**: 213-227 [PMID: 28042329 DOI: 10.7150/thno.16044]

83 **Montes HZ**. TNM Classification of Malignant Tumors, 7th edition. *Inter J Radia Oncol Biol Phys* 2010; **78**: 1278

84 **Gu H**, Li D, Zhu H, Zhang H, Yu Y, Qin D, Yi M, Li X, Lu P. The prognostic efficacy and improvements of the 7th edition Union for International Cancer Control tumor-node-metastasis classifications for Chinese patients with gastric cancer: Results based on a retrospective three-decade population study. *Tumour Biol* 2017; **39**: 1010428317694548 [PMID: 28351302 DOI: 10.1177/1010428317694548]

85 **Zou JH**, Li CY, Bao J, Zheng GQ. High expression of long noncoding RNA Sox2ot is associated with the aggressive progression and poor outcome of gastric cancer. *Eur Rev Med Pharmacol Sci* 2016; **20**: 4482-4486 [PMID: 27874951]

86 **Wang YQ**, Zhang QY, Weng WW, Wu Y, Yang YS, Shen C, Chen XC, Wang L, Liu KJ, Xu MD, Sheng WQ. Upregulation of the Non-Coding RNA OTUB1-isoform 2 Contributes to Gastric Cancer Cell Proliferation and Invasion and Predicts Poor Gastric Cancer Prognosis. *Int J Biol Sci* 2016; **12**: 545-557 [PMID: 27019636 DOI: 10.7150/ijbs.13540]

87 **Ma P**, Xu T, Huang M, Shu Y. Increased expression of LncRNA PANDAR predicts a poor prognosis in gastric cancer. *Biomed Pharmacother* 2016; **78**: 172-176 [PMID: 26898439 DOI: 10.1016/j.biopha.2016.01.025]

88 **Liu Z**, Shao Y, Tan L, Shi H, Chen S, Guo J. Clinical significance of the low expression of FER1L4 in gastric cancer patients. *Tumour Biol* 2014; **35**: 9613-9617 [PMID: 24961353 DOI: 10.1007/s13277-014-2259-4]

89 **Zhang Y**, Chen Z, Li MJ, Guo HY, Jing NC. Long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 regulates the expression of Gli2 by miR-202 to strengthen gastric cancer progression. *Biomed Pharmacother* 2017; **85**: 264-271 [PMID: 27887846 DOI: 10.1016/j.biopha.2016.11.014]

90 **Xia H**, Chen Q, Chen Y, Ge X, Leng W, Tang Q, Ren M, Chen L, Yuan D, Zhang Y, Liu M, Gong Q, Bi F. The lncRNA MALAT1 is a novel biomarker for gastric cancer metastasis. *Oncotarget* 2016; **7**: 56209-56218 [PMID: 27486823 DOI: 10.18632/oncotarget.10941]

91 **Liu XH**, Sun M, Nie FQ, Ge YB, Zhang EB, Yin DD, Kong R, Xia R, Lu KH, Li JH, De W, Wang KM, Wang ZX. Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer. *Mol Cancer* 2014; **13**: 92 [PMID: 24775712 DOI: 10.1186/1476-4598-13-92]

92 **Endo H**, Shiroki T, Nakagawa T, Yokoyama M, Tamai K, Yamanami H, Fujiya T, Sato I, Yamaguchi K, Tanaka N, Iijima K, Shimosegawa T, Sugamura K, Satoh K. Enhanced expression of long non-coding RNA HOTAIR is associated with the development of gastric cancer. *PLoS One* 2013; **8**: e77070 [PMID: 24130837 DOI: 10.1371/journal.pone.0077070]

93 **Hajjari M**, Behmanesh M, Sadeghizadeh M, Zeinoddini M. Up-regulation of HOTAIR long non-coding RNA in human gastric adenocarcinoma tissues. *Med Oncol* 2013; **30**: 670 [PMID: 23888369 DOI: 10.1007/s12032-013-0670-0]

94 **Chen WM**, Huang MD, Sun DP, Kong R, Xu TP, Xia R, Zhang EB, Shu YQ. Long intergenic non-coding RNA 00152 promotes tumor cell cycle progression by binding to EZH2 and repressing p15 and p21 in gastric cancer. *Oncotarget* 2016; **7**: 9773-9787 [PMID: 26799422 DOI: 10.18632/oncotarget.6949]

95 **Peng W**, Wu G, Fan H, Wu J, Feng J. Long noncoding RNA SPRY4-IT1 predicts poor patient prognosis and promotes tumorigenesis in gastric cancer. *Tumour Biol* 2015; **36**: 6751-6758 [PMID: 25835973 DOI: 10.1007/s13277-015-3376-4]

96 **Chen SX**, Yin JF, Lin BC, Su HF, Zheng Z, Xie CY, Fei ZH. Upregulated expression of long noncoding RNA SNHG15 promotes cell proliferation and invasion through regulates MMP2/MMP9 in patients with GC. *Tumour Biol* 2016; **37**: 6801-6812 [PMID: 26662309 DOI: 10.1007/s13277-015-4404-0]

97 **Shan Y**, Ying R, Jia Z, Kong W, Wu Y, Zheng S, Jin H. LINC00052 Promotes Gastric Cancer Cell Proliferation and Metastasis via Activating the Wnt/β-Catenin Signaling Pathway. *Oncol Res* 2017; **25**: 1589-1599 [PMID: 28337962 DOI: 10.3727/096504017x14897896412027]

98 **Hu Y**, Ma Z, He Y, Liu W, Su Y, Tang Z. LncRNA-SNHG1 contributes to gastric cancer cell proliferation by regulating DNMT1. *Biochem Biophys Res Commun* 2017; **491**: 926-931 [PMID: 28754593 DOI: 10.1016/j.bbrc.2017.07.137]

99 **Zhao L**, Guo H, Zhou B, Feng J, Li Y, Han T, Liu L, Li L, Zhang S, Liu Y, Shi J, Zheng D. Long non-coding RNA SNHG5 suppresses gastric cancer progression by trapping MTA2 in the cytosol. *Oncogene* 2016; **35**: 5770-5780 [PMID: 27065326 DOI: 10.1038/onc.2016.110]

100 **Li H**, Zhu H, Zhou Y, Wang H, Niu Z, Shen Y, Lv L. Long non-coding RNA MSTO2P promotes the proliferation and colony formation in gastric cancer by indirectly regulating miR-335 expression. *Tumour Biol* 2017; **39**: 1010428317705506 [PMID: 28618927 DOI: 10.1177/1010428317705506]

101 **Zhang LL**, Zhang LF, Guo XH, Zhang DZ, Yang F, Fan YY.

Downregulation of miR-335-5p by Long Noncoding RNA ZEB1-AS1 in Gastric Cancer Promotes Tumor Proliferation and Invasion. *DNA Cell Biol* 2018; **37**: 46-52 [PMID: 29215918 DOI: 10.1089/dna.2017.3926]

102 **Zhang R**, Guo Y, Ma Z, Ma G, Xue Q, Li F, Liu L. Long non-coding RNA PTENP1 functions as a ceRNA to modulate PTEN level by decoying miR-106b and miR-93 in gastric cancer. *Oncotarget* 2017; **8**: 26079-26089 [PMID: 28212532 DOI: 10.18632/oncotarget.15317]

103 **Sun W**, Mo X, Li T, Xie Y, Guo J. Clinical significance of the long noncoding RNA RP11-19P22.6-001 in gastric cancer. *Cancer Biomark* 2017; **18**: 397-403 [PMID: 28128738 DOI: 10.3233/cbm-160264]

104 **Bi M**, Yu H, Huang B, Tang C. Long non-coding RNA PCAT-1 over-expression promotes proliferation and metastasis in gastric cancer cells through regulating CDKN1A. *Gene* 2017; **626**: 337-343 [PMID: 28571676 DOI: 10.1016/j.gene.2017.05.049]

105 **Zheng L**, Chen J, Zhou Z, He Z. Knockdown of long non-coding RNA HOXD-AS1 inhibits gastric cancer cell growth via inactivating the JAK2/STAT3 pathway. *Tumour Biol* 2017; **39**: 1010428317705335 [PMID: 28475004 DOI: 10.1177/1010428317705335]

106 **Liu JN**, Shangguan YM. Long non-coding RNA CARLo-5 upregulation associates with poor prognosis in patients suffering gastric cancer. *Eur Rev Med Pharmacol Sci* 2017; **21**: 530-534 [PMID: 28239816]

107 **Huang M**, Hou J, Wang Y, Xie M, Wei C, Nie F, Wang Z, Sun M. Long Noncoding RNA LINC00673 Is Activated by SP1 and Exerts Oncogenic Properties by Interacting with LSD1 and EZH2 in Gastric Cancer. *Mol Ther* 2017; **25**: 1014-1026 [PMID: 28214253 DOI: 10.1016/j.ymthe.2017.01.017]

108 **Fei ZH**, Yu XJ, Zhou M, Su HF, Zheng Z, Xie CY. Upregulated expression of long non-coding RNA LINC00982 regulates cell proliferation and its clinical relevance in patients with gastric cancer. *Tumour Biol* 2016; **37**: 1983-1993 [PMID: 26334618 DOI: 10.1007/s13277-015-3979-9]

109 **Shao Y**, Chen H, Jiang X, Chen S, Li P, Ye M, Li Q, Sun W, Guo J. Low expression of lncRNA-HMlincRNA717 in human gastric cancer and its clinical significances. *Tumour Biol* 2014; **35**: 9591-9595 [PMID: 24961350 DOI: 10.1007/s13277-014-2243-z]

110 **Huang T**, Liu HW, Chen JQ, Wang SH, Hao LQ, Liu M, Wang B. The long noncoding RNA PVT1 functions as a competing endogenous RNA by sponging miR-186 in gastric cancer. *Biomed Pharmacother* 2017; **88**: 302-308 [PMID: 28122299 DOI: 10.1016/j.biopha.2017.01.049]

111 **Shen W**, Yuan Y, Zhao M, Li J, Xu J, Lou G, Zheng J, Bu S, Guo J, Xi Y. Novel long non-coding RNA GACAT3 promotes gastric cancer cell proliferation through the IL-6/STAT3 signaling pathway. *Tumour Biol* 2016; **37**: 14895-14902 [PMID: 27644247 DOI: 10.1007/s13277-016-5372-8]

112 **Chang S**, Liu J, Guo S, He S, Qiu G, Lu J, Wang J, Fan L, Zhao W, Che X. HOTTIP and HOXA13 are oncogenes associated with gastric cancer progression. *Oncol Rep* 2016; **35**: 3577-3585 [PMID: 27108607 DOI: 10.3892/or.2016.4743]

113 **Fu JW**, Kong Y, Sun X. Long noncoding RNA NEAT1 is an unfavorable prognostic factor and regulates migration and invasion in gastric cancer. *J Cancer Res Clin Oncol* 2016; **142**: 1571-1579 [PMID: 27095450 DOI: 10.1007/s00432-016-2152-1]

114 **Ma Y**, Liu L, Yan F, Wei W, Deng J, Sun J. Enhanced expression of long non-coding RNA NEAT1 is associated with the progression of gastric adenocarcinomas. *World J Surg Oncol* 2016; **14**: 41 [PMID: 26911892 DOI: 10.1186/s12957-016-0799-3]

115 **Lai Y**, Xu P, Li Q, Ren D, Wang J, Xu K, Gao W. Downregulation of long noncoding RNA ZMAT1 transcript variant 2 predicts a poor prognosis in patients with gastric cancer. *Int J Clin Exp Pathol* 2015; **8**: 5556-5562 [PMID: 26191264]

116 **Yang H**, Liu Z, Yuan C, Zhao Y, Wang L, Hu J, Xie D, Wang L, Chen D. Elevated JMJD1A is a novel predictor for prognosis and a potential therapeutic target for gastric cancer. *Int J Clin Exp Pathol* 2015; **8**: 11092-11099 [PMID: 26617828]

117 **Dang Y**, Lan F, Ouyang X, Wang K, Lin Y, Yu Y, Wang L, Wang Y, Huang Q. Expression and clinical significance of long non-coding RNA HNF1A-AS1 in human gastric cancer. *World J Surg Oncol* 2015; **13**: 302 [PMID: 26472090 DOI: 10.1186/s12957-015-0706-3]

118 **Li L**, Zhang L, Zhang Y, Zhou F. Increased expression of LncRNA BANCR is associated with clinical progression and poor prognosis in gastric cancer. *Biomed Pharmacother* 2015; **72**: 109-112 [PMID: 26054683 DOI: 10.1016/j.biopha.2015.04.007]

119 **Shan T**, Fan J, Zhao Q, Deng K, Xia J. Upregulation of long non-coding RNA DQ786243 promotes the progression of gastric cancer. *Mol Med Rep* 2017; **16**: 3761-3768 [PMID: 28731138 DOI: 10.3892/mmr.2017.7062]

120 **Song W**, Liu YY, Peng JJ, Liang HH, Chen HY, Chen JH, He WL, Xu JB, Cai SR, He YL. Identification of differentially expressed signatures of long non-coding RNAs associated with different metastatic potentials in gastric cancer. *J Gastroenterol* 2016; **51**: 119-129 [PMID: 26045391 DOI: 10.1007/s00535-015-1091-y]

121 **Wang CY**, Hua L, Yao KH, Chen JT, Zhang JJ, Hu JH. Long non-coding RNA CCAT2 is up-regulated in gastric cancer and associated with poor prognosis. *Int J Clin Exp Pathol* 2015; **8**: 779-785 [PMID: 25755774]

122 **Wu SW**, Hao YP, Qiu JH, Zhang DB, Yu CG, Li WH. High expression of long non-coding RNA CCAT2 indicates poor prognosis of gastric cancer and promotes cell proliferation and invasion. *Minerva Med* 2017; **108**: 317-323 [PMID: 28248065 DOI: 10.23736/s0026-4806.17.04703-6]

123 **Hu Y**, Pan J, Wang Y, Li L, Huang Y. Long noncoding RNA linc-UBC1 is negative prognostic factor and exhibits tumor pro-oncogenic activity in gastric cancer. *Int J Clin Exp Pathol* 2015; **8**: 594-600 [PMID: 25755750]

124 **Chen WM**, Huang MD, Kong R, Xu TP, Zhang EB, Xia R, Sun M, De W, Shu YQ. Antisense Long Noncoding RNA HIF1A-AS2 Is Upregulated in Gastric Cancer and Associated with Poor Prognosis. *Dig Dis Sci* 2015; **60**: 1655-1662 [PMID: 25686741 DOI: 10.1007/s10620-015-3524-0]

125 **Zhou B**, Jing XY, Wu JQ, Xi HF, Lu GJ. Down-regulation of long non-coding RNA LET is associated with poor prognosis in gastric cancer. *Int J Clin Exp Pathol* 2014; **7**: 8893-8898 [PMID: 25674261]

126 **Xu MD**, Qi P, Weng WW, Shen XH, Ni SJ, Dong L, Huang D, Tan C, Sheng WQ, Zhou XY, Du X. Long non-coding RNA LSINCT5 predicts negative prognosis and exhibits oncogenic activity in gastric cancer. *Medicine* (Baltimore) 2014; **93**: e303 [PMID: 25526476 DOI: 10.1097/md.0000000000000303]

127 **Xu C**, Shao Y, Xia T, Yang Y, Dai J, Luo L, Zhang X, Sun W, Song H, Xiao B, Guo J. lncRNA-AC130710 targeting by miR-129-5p is upregulated in gastric cancer and associates with poor prognosis. *Tumour Biol* 2014; **35**: 9701-9706 [PMID: 24969565 DOI: 10.1007/s13277-014-2274-5]

128 **Liu L**, Yan B, Yang Z, Zhang X, Gu Q, Yue X. ncRuPAR inhibits gastric cancer progression by down-regulating protease-activated receptor-1. *Tumour Biol* 2014; **35**: 7821-7829 [PMID: 24817013 DOI: 10.1007/s13277-014-2042-6]

129 **Li H**, Yu B, Li J, Su L, Yan M, Zhu Z, Liu B. Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. *Oncotarget* 2014; **5**: 2318-2329 [PMID: 24810858 DOI: 10.18632/oncotarget.1913]

130 **Zhang EB**, Han L, Yin DD, Kong R, De W, Chen J. c-Myc-induced, long, noncoding H19 affects cell proliferation and predicts a poor prognosis in patients with gastric cancer. *Med Oncol* 2014; **31**: 914 [PMID: 24671855 DOI: 10.1007/s12032-014-0914-7]

131 **Sun W**, Wu Y, Yu X, Liu Y, Song H, Xia T, Xiao B, Guo J. Decreased expression of long noncoding RNA AC096655.1-002 in gastric cancer and its clinical significance. *Tumour Biol* 2013; **34**: 2697-2701 [PMID: 23645148 DOI: 10.1007/s13277-013-0821-0]

132 **Mei D**, Song H, Wang K, Lou Y, Sun W, Liu Z, Ding X, Guo J. Up-regulation of SUMO1 pseudogene 3 (SUMO1P3) in gastric cancer and its clinical association. *Med Oncol* 2013; **30**: 709 [PMID: 23996296 DOI: 10.1007/s12032-013-0709-2]

133 **Lu Y**, Lu P, Zhu Z, Xu H, Zhu X. Loss of imprinting of insulin-

like growth factor 2 is associated with increased risk of lymph node metastasis and gastric corpus cancer. *J Exp Clin Cancer Res* 2009; **28**: 125 [PMID: 19737423 DOI: 10.1186/1756-9966-28-125]

134 **Li X**, Zhou Y, Qian H. CCAT1 expressed in malignant and pre-malignant human gastric tissues. *Cell Mol Biol* (Noisy-le-grand) 2017; **63**: 89-93 [PMID: 28719351 DOI: 10.14715/cmb/2017.63.5.16]

P- Reviewer: Das U, Mavridis K **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3738-3748

DOI: 10.3748/wjg.v24.i33.3738

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

MINIREVIEWS

Antiangiogenic therapy for portal hypertension in liver cirrhosis: Current progress and perspectives

Dmitry Victorovich Garbuzenko, Nikolay Olegovich Arefyev, Evgeniy Leonidovich Kazachkov

Dmitry Victorovich Garbuzenko, Department of Faculty Surgery, South Ural State Medical University, Chelyabinsk 454092, Russia

Nikolay Olegovich Arefyev, Evgeniy Leonidovich Kazachkov, Department of Pathological Anatomy and Forensic Medicine, South Ural State Medical University, Chelyabinsk 454092, Russia

ORCID number: Dmitry Victorovich Garbuzenko (0000-0001-9809-8015); Nikolay Olegovich Arefyev (0000-0002-1770-064X); Evgeniy Leonidovich Kazachkov (0000-0002-2008-7671).

Author contributions: Garbuzenko DV contributed to the conception, design, acquisition, analysis, interpretation of data, wrote the manuscript and approved the final version; Arefyev NO contributed to analysis of data, wrote and revised the manuscript, generated the figures and tables, and approved the final version; Kazachkov EL contributed to analysis of data, revised the manuscript, and approved the final version.

Supported by RFBR according to the research project, No. 18-315-00434.

Conflict-of-interest statement: Dr. Arefyev reports a grant from RFBR (Russian Foundation for Basic Research), during the conduct of the study. The information included in this manuscript is not related to the interests of RFBR. Prof. Garbuzenko and Prof. Kazachkov have nothing to disclose.

Manuscript source: Invited manuscript

Correspondence to: Dmitry Victorovich Garbuzenko, MD, PhD, Professor, Department of Faculty Surgery, South Ural State Medical University, PO Box 12317, Chelyabinsk 454080, Russia. garb@inbox.ru
Telephone: +7-909-7459826
Fax: +7-351-2687772

Received: May 27, 2018
Peer-review started: May 27, 2018
First decision: July 4, 2018
Revised: July 9, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 7, 2018

Abstract

Developing medicines for hemodynamic disorders that are characteristic of cirrhosis of the liver is a relevant problem in modern hepatology. The increase in hepatic vascular resistance to portal blood flow and subsequent hyperdynamic circulation underlie portal hypertension (PH) and promote its progression, despite the formation of portosystemic collaterals. Angiogenesis and vascular bed restructurization play an important role in PH pathogenesis as well. In this regard, strategic directions in the therapy for PH in cirrhosis include selectively decreasing hepatic vascular resistance while preserving or increasing portal blood flow, and correcting hyperdynamic circulation and pathological angiogenesis. The aim of this review is to describe the mechanisms of angiogenesis in PH and the methods of antiangiogenic therapy. The PubMed database, the Google Scholar retrieval system, and the reference lists from related articles were used to search for relevant publications. Articles corresponding to the aim of the review were selected for 2000-2017 using the keywords: "liver cirrhosis", "portal hypertension", "pathogenesis", "angiogenesis", and "antiangiogenic therapy". Antiangiogenic therapy for PH was the inclusion criterion. In this review, we have described angiogenesis inhibitors and their mechanism of action in relation to PH. Although most of them were studied

only in animal experiments, this selective therapy for abnormally growing newly formed vessels is pathogenetically reasonable to treat PH and associated complications.

Key words: Liver cirrhosis; Portal hypertension; Pathogenesis; Angiogenesis; Antiangiogenic therapy

Core tip: This review describes the role of angiogenesis in the pathogenesis of portal hypertension in liver cirrhosis and the prospects of antiangiogenic therapy. The analysis of the data showed that angiogenesis plays an important role in the pathogenesis of cirrhosis and accompanies portal hypertension, underlying its development and causing related complications. Although most of angiogenesis inhibitors were studied only in animal experiments, this selective therapy for abnormally growing newly formed vessels is pathogenetically reasonable to treat portal hypertension and associated complications.

Garbuzenko DV, Arefyev NO, Kazachkov EL. Antiangiogenic therapy for portal hypertension in liver cirrhosis: Current progress and perspectives. *World J Gastroenterol* 2018; 24(33): 3738-3748 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3738.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3738

INTRODUCTION

Developing medicines to treat hemodynamic disorders that are characteristic of liver cirrhosis and promote portal hypertension (PH) and related complications is a relevant problem in modern hepatology. In accordance with the current clinical recommendations, nonselective β-adrenoblockers are the drugs of choice[1]. However, their influence on portal pressure is variable. A number of studies showed that they did not lead to a clinically significant decrease in portal pressure, and the weakening of their therapeutic effect was noted in 50%-70% of cases in the long-term period. Also, the question of the appropriateness of using nonselective β-adrenergic blockers in patients with decompensated cirrhosis has not been finally resolved[2].

Ideally, the pharmacotherapy of PH should lessen the severity of morphofunctional disorders in the liver, contributing to the reduction of the vascular resistance to portal blood flow. Also, it should successfully correct a hyperdynamic circulatory state. As a result, the hepatic venous pressure gradient (HVPG), the most accurate equivalent of portal pressure, should be reduced to less than 12 mmHg or be 20% lower than an original value. In addition, it is necessary to avoid arterial hypotension and at the same time reduce the influx of splanchnic blood into the portal vein, keeping unchanged the portal blood flow, which participates in liver perfusion[3].

Angiogenesis plays an important role in the pathogenesis of many chronic liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma[4]. It can also accompany PH, underlying its development and causing related complications. Indeed, the newly formed blood vessels, which bypass sinusoids in response to the gross morphofunctional rearrangement of the liver in cirrhosis, fail to provide oxygen and nutrients to the tissues, which worsens the course of the disease and increases hepatic vascular resistance to portal blood flow[5]. Further progression of PH is a consequence of complex processes including angiogenesis, vascular remodeling, and endothelial dysfunction, which contribute to splanchnic congestion, portosystemic shunt formation, and a hyperdynamic circulatory state[6] (Figure 1). From this, it can be inferred that antiangiogenic therapy, which is selectively aimed at suppressing newly formed vessels' formation and growth, is a pathogenetically grounded method of treating PH and associated complications[7].

The efforts to develop angiogenesis inhibitors began in the 1970s at Harvard University under the guidance of Judah Folkman. The drugs were actively introduced into clinical practice a decade after the first were developed[8].

INHIBITORS OF INTRAHEPATIC ANGIOGENESIS

One of the two main mechanisms in the formation of new blood vessels in the liver in cirrhosis is associated with an increased expression of pro-angiogenic growth factors, cytokines, and matrix metalloproteinases in the presence of chronic inflammation. Proinflammatory mediators produced by Kupffer cells, mast cells, and leukocytes may manifest an angiogenic response at the expense of hypoxia-inducible factor-1α (HIF-1α) induction and increased transcription activity[9]. HIF-1α activates hepatic stellate cells (HSC), which leads to the development of various angiogenic and fibrogenic factors, promoting both angiogenesis and liver fibrosis[10]. At the same time, diffuse fibrosis, the formation of regenerative nodules, and also the capillarization of sinusoids cause an increase in hepatic vascular resistance and impair oxygen delivery to liver cells[11]. Accumulation of HIFs, in particular HIF-1α, increases the expression of vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang1), and their related receptors on activated HSC. This leads to recruitment and stimulation of sinusoidal endothelial cells (SEC), which stabilizes the newly formed vessels and ensures their strength. In turn, SEC produce platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1), contributing to the recruitment and migration of HSC, a process that involves reactive oxygen species-dependent activation of the extracellular

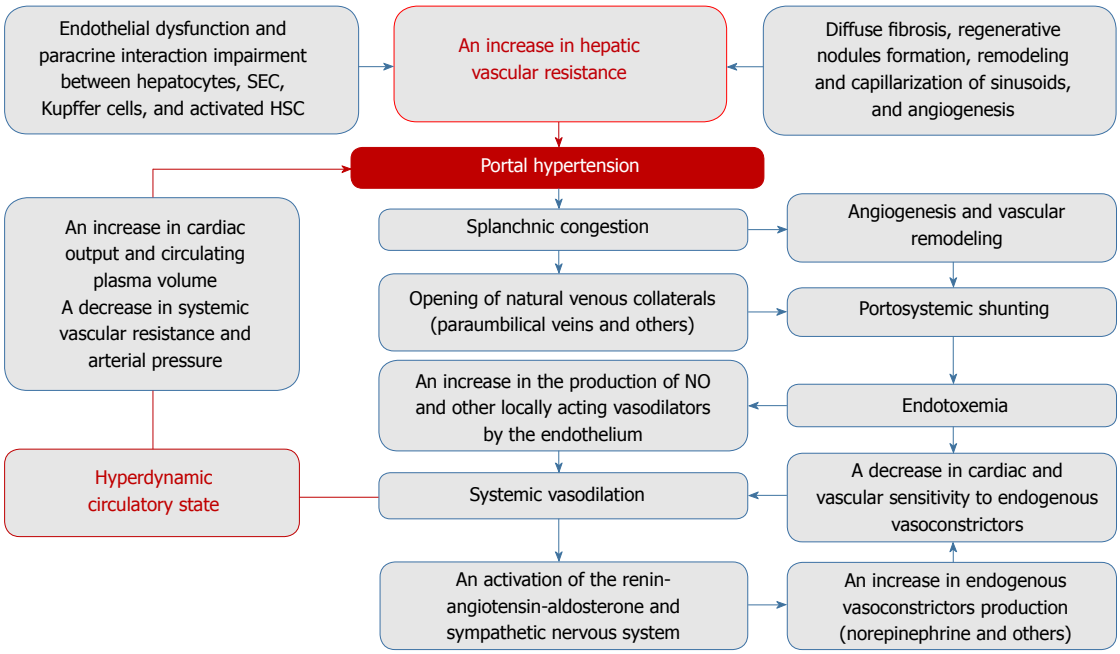


Figure 1 Potential mechanisms of portal hypertension pathogenesis in cirrhosis. The newly formed blood vessels, which bypass sinusoids in response to the gross morphofunctional rearrangement of the liver in cirrhosis, fail to provide oxygen and nutrients to the tissues. With endothelial dysfunction and impaired paracrine interaction between hepatocytes, sinusoidal endothelial cells (SEC), Kupffer cells, and activated hepatic stellate cells (HSC), this increases hepatic vascular resistance to portal blood flow. Further progression of portal hypertension is a consequence of complex processes including angiogenesis, vascular remodeling, and endothelial dysfunction, which contribute to splanchnic congestion, systemic vasodilation, and portosystemic shunt formation. The subsequent hyperdynamic circulatory state worsens the course of the disease.

signal-regulated kinase (ERK) pathway and c-Jun NH2-terminal kinases (JNK) followed by HIF-1α-dependent synthesis of VEGF[12].

Tyrosine kinase inhibitors

The introduction of antiangiogenic therapy into hepatological practice began with the treatment of hepatocellular carcinoma, a well-vascularized tumor that needs intense angiogenic activity for its development[13]. The most studied drug used for this purpose is sorafenib, a multi-targeted inhibitor of receptor and non-receptor tyrosine kinases, which are responsible for transmitting various signals to cells, including proliferative stimuli. The antitumor and antiangiogenic effect of sorafenib is achieved mainly through the suppression of the Raf/MEK/ERK signaling pathway and blockade of signaling from the receptors of VEGF (VEGFR), PDGF (PDGFR), and c-kit (SCFR)[14].

Experimental studies have shown the antiangiogenic effect of sorafenib during the early stage of hepatic fibrosis[15]. In animals with various models of cirrhosis, it had positive effects on some pathogenetic pathways of fibrogenesis and angiogenesis in the liver by blocking the receptor tyrosine kinases located on the surface of HSC, the expression of which, especially VEGFR and PDGFR, was increased[16] (Figure 2): (1) The suppression of activated HSC proliferation and the activation of apoptosis; (2) the inhibition of cyclin D1 and cyclin-dependent kinase 4 (Cdk-4) with a simultaneous increase in the expression of Fas, Fas-L, and Caspase-3, and a decrease in the ratio of Bcl-2 to Bax; (3) an increase in the ratio of matrix metalloproteinases to the tissue inhibitor of matrix metalloproteinases, and also a decrease in the synthesis of collagen by HSC; (4) the inhibition of phosphorylation of ERK, Akt, and ribosomal protein kinase S6 with a molecular mass of 70 kDa (p70S6K)[17]; and (5) the disturbance of the Kruppel-like factor 6–Ang1–fibronectin molecular triad functioning[18]. Sorafenib decreased the severity of inflammation, fibrogenesis, and angiogenesis in rats with biliary cirrhosis, which led to a reduction in hepatic vascular resistance to portal blood flow[19].

Another multi-targeted tyrosine kinase inhibitor sunitinib is less studied but known to block VEGFR1/2/3, PDGFR-α/β, fibroblast growth factor receptor (FGFR), and c-kit signaling[20]. In addition, an *in vitro* study by Majumder *et al*[21] showed that sunitinib can slow HSC collagen synthesis by 47%, reduce HSC contractility by 65%, and decrease cellular migration by 28%, as well as inhibit the angiogenic capacity of SEC.

Branivib is a double inhibitor of VEGFR and FGFR signaling. It significantly suppressed intrahepatic angiogenesis and reduced PH in rats with biliary cirrhosis[22]. Additionally, it improved blood circulation in the liver and hindered the formation of ascites in rats with liver cirrhosis caused by nonalcoholic steatohepatitis[23].

Statins

The positive effect of statins on hepatic fibro- and angiogenesis in cirrhosis is associated with the induction

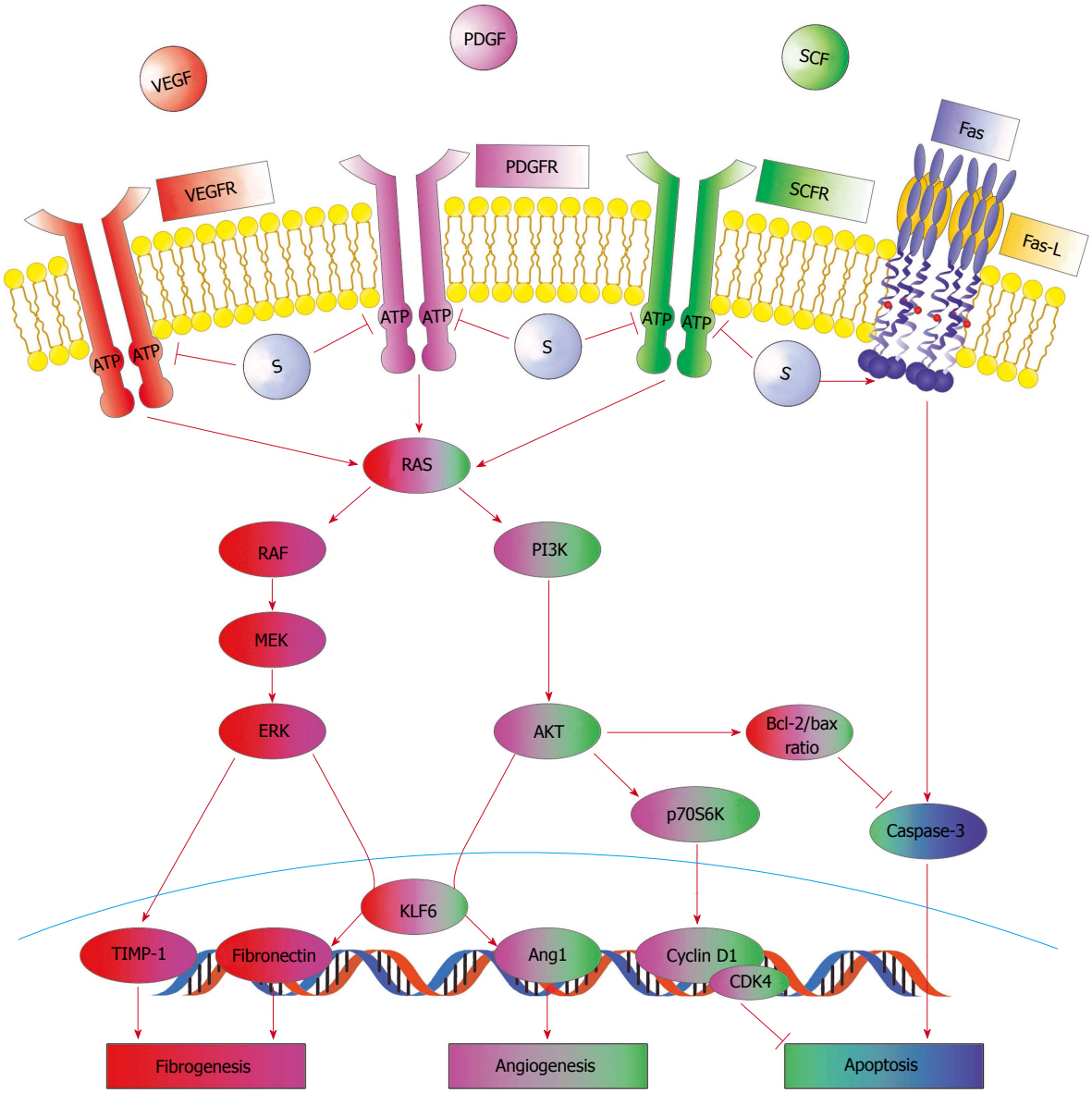


Figure 2 Positive effects of sorafenib on some pathogenetic pathways of fibrogenesis and angiogenesis in the liver. Sorafenib (S) blocks the ATP-binding site of the vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and stem cell growth factor receptor (SCFR) tyrosine kinases located on the surface of hepatic stellate cells (HSC), inhibiting the two main cellular pathways of the RAS protein. At the same time, sorafenib increases the expression of Fas and its ligand. This decreases the severity of fibrogenesis and angiogenesis and increases apoptosis, leading to a reduction in hepatic vascular resistance to portal blood flow.

of KLF2 in SEC[24]. KLF2 is a member of a family of widely expressed transcription factors that regulate cell and tissue growth. KLF2 is well represented in the vascular endothelium and is necessary for the normal development of vessels; in addition, it is a well-known antiangiogenic factor that modulates the severity of many endothelial vasoprotective genes[25]. KLF2 can effectively inhibit HIF-1α, reducing the expression of such proangiogenic factors as VEGF and Ang2[26].

The mechanical stimuli generated by shear stress are the main physiological impulse for triggering and maintaining endothelial KLF2 expression[27]. In the cirrhotic liver, KLF2 expression was elevated in both SEC[28] and activated HSC[29]. This serves as a compensatory mechanism aimed at eliminating vascular dysfunction and preventing angiogenesis by suppressing the proliferation and migration of SEC, as well as downregulating the ERK1/2 signaling pathway to inhibit the formation of tubular structures[30].

In an *in-vitro* study conducted by Miao *et al*[31], simvastatin eliminated the pro-angiogenic environment for TGF-β-activated HSC as a result of the following processes: (1) The reduction of cell migration and proliferation; (2) the inhibition of the α-smooth muscle actin expression, and the elevation of mRNA and KLF2 levels in HSC; (3) an increase in the production of endothelial nitric oxide synthase (eNOS) and suppression of the various proangiogenic proteins expression in HSC, such as VEGF, HIF-1α, and pro-inflammatory nuclear factor kappa B (NF-κB); and (4) the reduction

of the hyperactivity of interferon γ, which participates in angiogenesis. In rats with CCL_4-induced liver cirrhosis, it was noted that statins (atorvastatin, mevastatin, simvastatin, and lovastatin) enhanced the effect of KLF2. By doing this, they deactivated SEC and reduced the severity of fibrosis and associated angiogenesis, thereby exerting a positive effect on PH[32].

Rifaximin

Endotoxemia, which is due to the translocation of gram-negative bacteria from the intestine, plays an important role in the pathogenesis of both cirrhosis and associated complications[33]. During the development of cirrhosis, bacterial lipopolysaccharide influences Kupffer cells and HSC. Nevertheless, SEC is affected first. Toll-like receptors 4 (TLR4), which are located on their surface and capable of binding bacterial lipopolysaccharide, is involved in fibrosis-associated angiogenesis. These receptors manifest such properties through the related cytosolic adapter protein MyD88, which is involved in the production of extracellular protease regulating the invasive ability of SEC[34].

In mice with biliary cirrhosis, it was shown that rifaximin, a nonabsorbable antibiotic with broad antimicrobial activity against aerobic and anaerobic gram-negative bacteria, reduced the severity of fibrosis and angiogenesis in the liver by inhibiting bacterial lipopolysaccharide binding to TLR4. As a consequence, it reduced PH[35]. This drug is already used to treat hepatic encephalopathy. It has an acceptable safety profile when applied in patients with chronic liver diseases and is approved by the US Food and Drug Administration[36]. The experimental study[35] may be a basis for evaluation of rifaximin in other complications of cirrhosis.

Largazole

The histone deacetylase inhibitor largazole is a natural compound derived from marine cyanobacteria Symploca sp. With a strong antiproliferative and cytotoxic effect, it has a wide spectrum but differential activity against several different lines of cancer cells[37]. In addition, in experimental studies *in vitro* and *in vivo*, largazole attenuated the severity of liver fibrosis and associated angiogenesis through numerous independent mechanisms: (1) The reduction of VEGF production by HSC; (2) the inhibition of VEGF-stimulated HSC proliferation; (3) the downregulation of TGF-β1- and VEGF-induced Akt phosphorylation in activated HSC, as well as the downregulation of VEGFR2-dependent p38MAPK phosphorylation in SEC; and (4) the suppression of CD34, VEGF, and VEGFR2 expression[38]. The ability of largazole to affect the main fibrogenic and angiogenic pathways in the cirrhotic liver can be used to test its effectiveness in PH.

Ribavirin

In addition to antiviral activity against certain DNA- and RNA-containing viruses, ribavirin may have a positive effect on the morphological changes underlying the development of cirrhosis[39]. In addition, at therapeutic concentrations, it is able to inhibit angiogenesis both *in vitro* and *in vivo*, which is due to the inhibition of inosine-5'-monophosphate dehydrogenase 1 activity and a decrease in tetrahydrobiopterin, NO, and cGMP levels in SEC[40].

INHIBITORS OF EXTRAHEPATIC ANGIOGENESIS

Disturbances of organ and systemic hemodynamics and the development of portosystemic collateral circulation in PH begin with splanchnic vasodilation and neovascularization caused by hypoxia of intestinal mucosa and pro-inflammatory cytokines, chemokines, and angiogenic factors, such as VEGF, PDGF, the placental growth factor (PlGF), and others[41]. It was traditionally thought that portosystemic shunts are formed when increased portal pressure "opens" pre-existing vessels in the areas of embryonic connection between the portal and systemic circulation. This paradigm was challenged by Fernandez, who first reported that portosystemic collaterals in PH are formed due to active angiogenesis. It was shown in an animal model of prehepatic PH induced by partial portal vein ligation that the blockade of VEGFR-2 with anti-VEGFR-2 monoclonal antibody for 5-7 d and inhibition of VEGF/VEGFR-2 signalization using autophosphorylation inhibitor VEGFR-2 for 5 d after the operation resulted in 50% reduction of portosystemic collateral vessel formation[42,43]. Blockade of NAD(P)H also contributed to this owing to the reduced splanchnic expression of VEGF, VEGFR-2, and CD31[44].

It should be noted that VEGF is of the greatest importance only at the initial stages of angiogenesis, when it activates endothelial cell proliferation and the subsequent formation of endothelial tubules. Vascular maturation is modulated mainly by PDGF, which regulates the introduction of endothelial tubules into the population of intramural cells and pericytes, thus stabilizing the newly formed vasculature[45]. The simultaneous suppression of the signaling caused by both VEGF and PDGF appears more promising than suppressing them individually.

Tyrosine kinase inhibitors

Fernandez *et al*[46] studied the combined effect of rapamycin (mTOR inhibitor) and glivec (tyrosine protein kinase inhibitor) on VEGF and PDGF signaling, respectively, in rats with extrahepatic PH caused by partial portal vein ligation and with well-developed portosystemic collateral circulation. It was noted that rapamycin and glivec in combination markedly reduced the splanchnic neovascularization and pericyte coverage of new vessels through the decreased expression of VEGF, VEGFR2, CD31, PDGF, PDGFR-β, and α-smooth muscle actin. In addition, there was a reduction of

portal pressure and blood flow along the superior mesenteric artery by 40% and 30% from the baseline level, respectively.

Similar results were obtained by Mejias *et al*[19], who found that multi-kinase inhibitor sorafenib triggered blockade of VEGF and PDGF signaling transduction and the Raf/MEK/ERK signaling pathways. Sorafenib significantly reduced intraorgan and systemic blood flow, and increased splanchnic neovascularization by 80% and portosystemic shunting by 18%. This led to a reduction in hepatic vascular resistance and decrease in portal pressure by 25% from the baseline. It was also noted that the positive effect of sorafenib on PH was more significant when it was combined with propranolol[47].

Somatostatin and its synthetic analogs

Somatostatin is a cyclic 14-amino acid peptide, which is secreted by nerve, endocrine, and enteroendocrine cells in the hypothalamus and digestive system (in the stomach, intestine, and pancreatic δ-cells). Somatostatin and its synthetic analogs (octreotide, vapreotide, and others) are used in patients with cirrhosis to treat bleeding from esophageal varices by affecting both intra- and extrahepatic mechanisms of PH[48].

The ability of octreotide to inhibit cell proliferation and neovascularization through the high-affinity somatostatin subtype receptor 2 (SSTR2) was an impetus for studying its antiangiogenic properties in various diseases[49]. In studies involving rats with extrahepatic PH caused by partial portal vein ligation, octreotide significantly weakened the expression of VEGF and CD31 in the internal organs, reduced the development of splanchnic neovascularization by 64%, and lessened the severity of a portosystemic collateral circulation by 16%. At the same time, its angioinhibitory effect manifested only in the first four days of the experiment and completely disappeared after a week, as PH progressed. This is possibly due to a decrease in SSTR2 expression in mucosa, intestinal vessels, and portosystemic collaterals[50].

Spironolactone

Pathophysiological disturbances inherent to PH underlie the occurrence of ascites in cirrhosis. Systemic arterial vasodilation and the activation of various neurohormonal pathways, including the renin-angiotensin-aldosterone system, caused renal dysfunction. This decreases Na^+ and water excretion and reduces the glomerular filtration rate. The drug of choice for treatment is spironolactone, an antagonist of aldosterone, a mineralocorticoid, that mediates the reabsorption of Na^+ and water in the distal part of the nephron[51]. In addition to the important role in maintaining water-salt metabolism, aldosterone has angiogenic properties. In particular, it enhances ischemia-induced neovascularization[52], stimulates pathological angiogenesis in the retina[53], and promotes the proliferation of endothelial cells of the heart[54] by activating angiotensin Ⅱ signaling. At the same time, its antagonist spironolactone inhibits these processes both *in vitro* and *in vivo*[55]. In rats with biliary cirrhosis, spironolactone significantly reduced the degree of mesenteric angiogenesis and portosystemic shunting by suppressing the VEGF signal transduction pathway[56].

N-acetylcysteine

Because hypoxia serves as the main inducer of angiogenesis both under physiological and pathological conditions, angiogenesis inhibitors may be drugs with antioxidant properties. One of them is N-acetylcysteine, which is a derivative of amino acid cysteine, the thiol groups of which directly interact with electrophilic groups of free radicals. N-acetylcysteine can also enhance the activity of glutathione-S-transferase, glutathione peroxidase, glutathione reductase, and a number of other enzymes involved in maintaining the oxidant/antioxidant balance[57].

Long-term application of N-acetylcysteine in rats with biliary cirrhosis lessened oxidative stress in the mesentery of the small intestine, reduced the level of circulating inflammatory cytokines, and inhibited mesenteric angiogenesis by decreasing angiogenic marker expression (VEGF, VEGFR2, Ang1, and CD31). This eventually improved splanchnic and systemic hemodynamics.

In addition, N-acetylcysteine inhibited VEGF-induced endothelial tubule formation and endothelial cell migration by suppressing tumor necrosis factor-α (TNF-α) and Akt/eNOS/NO angiogenic signaling cascade *in vitro*. It also reduced the number of reactive oxygen species (including reactive compounds of thiobarbituric acid and malondialdehyde) and inflammatory cytokines in the human umbilical vein endothelial cell supernatant[58].

Endothelin receptor blockers

Endothelin-1 (ET-1) is one of the mediators whose synthesis is enhanced in conditions of tissue hypoxia. It belongs to the endothelin family, which includes two more homologous oligopeptides (ET-2 and ET-3). Endothelins are the products of the proteolysis of their precursor "large endothelin", which is driven by the endothelin-converting enzyme. They act through two types of G-protein-coupled receptors: type A (ET_A) and type B (ET_B). Type B (ET_B) has two isoforms: ET_{B1} and ET_{B2}. ET_A are located primarily on membranes of vascular smooth muscle cells, whereas ET_B are present on both endothelial and smooth muscle cells.

ET-1, the most studied potent vasoconstrictor, is produced by vascular endothelial and smooth muscle cells. It is directly involved in intra- and extrahepatic mechanisms of PH pathogenesis, and its circulating level is increased in cirrhosis because of "large endothelin" hyperproduction and increased expression of endothelin-converting enzyme[59]. Experimental studies have shown that ET-1 induces angiogenic responses in

cultured endothelial cells through endothelial ET_B-type receptors and, in combination with VEGF, stimulates neovascularization *in vivo*[60]. The nonselective endothelin receptor blocker bosentan and the selective ET_A receptor blocker ambrisentan reduced the degree of mesenteric angiogenesis and portosystemic shunting in rats with biliary cirrhosis by suppressing inducible nitric oxide synthase (iNOS), cyclooxygenase 2, VEGF and VEGFR2, and Akt signaling[61].

Pioglitazone

Pioglitazone, a potent selective agonist of peroxisome proliferator-activated receptors-γ (PPAR-γ), is able to reduce the level of systemic inflammation in patients with a high cardiovascular disease risk. It blocks the activity of pro-inflammatory genes by post-transcriptional modification of their products (by attaching small SUMO proteins to them) and suppresses NF-κB expression by transrepression. All PPAR isomers (PPAR-α, PPAR-β/-δ, and PPAR-γ) are anti-inflammatory nuclear transcription factors and NF-κB antagonists. Dominant negative mutation of PPAR-γ leads to systemic inflammation and rapid development of related diseases: arterial hypertension, atherosclerosis, type 2 diabetes, nonalcoholic steatohepatitis, psoriasis, and premature aging[62].

In addition to systemic inflammation reduction, PPAR-γ agonists are also capable of inhibiting oxidative stress and angiogenesis[63]. In rats with models of biliary cirrhosis and extrahepatic PH caused by partial portal vein ligation, pioglitazone reduced the degree of portosystemic shunting by 22%-30% by suppressing angiogenic and pro-inflammatory cytokines, chemokines, and growth factors (VEGF, PDGF, and PIGF)[64].

Thalidomide

Thalidomide, a glutamic acid derivative with anti-angiogenic, anti-inflammatory, and immunomodulatory properties, is able to hinder TNF-α/interleukin-1β production for which activated immune cells are responsible[65]. It was also shown in rats with biliary cirrhosis that thalidomide blocked the TNFα-VEGF-NOS-NO pathway by downregulating elevated inflammasome expression in the intestinal and mesenteric tissues, which weakened mesenteric angiogenesis and portosystemic shunting[66].

Polyphenols

The possibility of influencing the pathogenetic mechanisms of extrahepatic angiogenesis was found in polyphenols, the chemicals of plant origin with a strong antioxidant effect.

The tea catechins extracted from the dried leaves of *Camellia sinensis* reduced the severity of mesenteric angiogenesis and portosystemic shunting in rats with biliary cirrhosis by reducing HIF-1α expression, Akt signaling, and VEGF synthesis[67].

2′-hydroxyflavonoid, which is contained in citruses, prevented the formation of new splanchnic vessels and portosystemic collaterals in rats with thioacetamide-induced liver cirrhosis by downregulating apoptosis[68].

The long-term use of curcumin, a polyphenol extracted from turmeric roots, improved the course of PH in liver cirrhosis by positively affecting liver fibrosis and reducing portal influx. These effects were achieved through inhibiting mesenteric angiogenesis and restoring mesenteric vessel contractility, as well as decreasing the degree of portosystemic collateral circulation and hyperdynamic circulatory state. Moreover, its favorable effects on the splanchnic and systemic blood flow included the suppression of VEGF, cyclooxygenase 2, and eNOS[69].

CLINICAL EXPERIENCE OF ANTIANGIOGENIC THERAPY FOR PH

The effect of the drugs described above was studied only in experiments involving animals (Tables 1 and 2), and only tyrosine kinase inhibitors were tested as an antiangiogenic therapy in patients with cirrhosis and PH.

Coriat *et al*[70] were the first to assess the effect of sorafenib on the portal and systemic hemodynamics, in seven patients with cirrhosis and hepatocellular carcinoma. Five of them had Child–Turcotte–Pugh (CTP) class A, and two had CTP class B. Sorafenib was administered for one month at 400 mg twice a day. In one patient, this was first reduced to 400 mg once a day and then to 400 mg every two days because side effects appeared. A decrease in portal blood flow by at least 36% was noted, while no changes in blood flow were found in the azygos vein and abdominal aorta.

In a pilot study, Pinter *et al*[71] investigated the effects of sorafenib on HVPG and systemic hemodynamics, as well as the expression of mRNA genes involved in fibrogenesis, angiogenesis, and inflammation in the liver in 13 patients suffering from cirrhosis and hepatocellular carcinoma (10 patients had CTP class A and three patients had CTP class B). The drug was administered at 400 mg twice a day for two weeks. Four of the 11 patients with PH (eight of whom had clinically significant PH) had a reduction in HVPG by more than 20% from the baseline values and, in addition, a decrease in the levels of mRNA, VEGF, PDGF, PIGF, RhoA kinase, and TNF-α.

However, the results were not as optimistic in a randomized double-blind placebo-controlled study that assessed the effects of sorafenib administered at 400 mg twice a day on HVPG in nine patients with cirrhosis and hepatocellular carcinoma[72].

The main drawback of tyrosine kinase inhibitors is hepatotoxicity. A study of possibilities of their selective delivery to target cells, in particular, HSC, seems to be a promising direction in solving this problem.

CONCLUSION

Advances in understanding the pathogenesis of PH in cirrhosis stimulated the development of new methods

Table 1 Drugs that can inhibit intrahepatic angiogenesis in portal hypertension

Ref.	Drugs	Experimental models	Effects
Liu *et al*[15], Qu *et al*[16], Wang *et al*[17], Thabut *et al*[18], Mejias *et al*[19]	Sorafenib	Biliary cirrhosis, non-alcoholic steatohepatitis, thioacetamide-, diethylnitrosamine-, dimethylnitrosamine-, and CCl_4-induced cirrhosis	Suppresses the Raf/MEK/ERK signaling pathway and blocks the signaling from the VEGFR, PDGFR, and SCFR; therefore, increases apoptosis and decreases inflammation, fibrogenesis, angiogenesis, and hepatic vascular resistance
Tugues *et al*[20], Majumder *et al*[21]	Sunitinib	CCl_4-induced cirrhosis and cell cultures (immortalized human activated HSC cell line, human HSC, and isolated primary human liver sinusoidal endothelial cells)	Blocks VEGFR1/2/3, PDGFR-α/β, FGFR, and SCFR; reduces HSC collagen synthesis, contractility, cellular migration, and SEC angiogenic capacity
Lin *et al*[22], Yang *et al*[23]	Brivanib	Biliary cirrhosis, non-alcoholic steatohepatitis	Inhibits VEGFR and FGFR; therefore, suppresses intrahepatic angiogenesis and portal hypertension, improves blood circulation, and hinders ascites formation
Miao *et al*[31], Marrone *et al*[32]	Simvastatin	CCl_4-induced cirrhosis and LX-2 cell line	Enhances KLF2, through which deactivates SEC and reduces the severity of fibrosis and associated angiogenesis
Zhu *et al*[35]	Rifaximin	Biliary cirrhosis	Downregulates bacterial lipopolysaccharide binding to TLR4; therefore, reduces the severity of fibrosis and associated angiogenesis
Liu *et al*[37], Liu *et al*[38]	Largazole	Human colorectal carcinoma cells (HCT116, HT29, and HCT15), human HSC, and CCl_4-induced cirrhosis	Suppresses the effects of CD34, VEGF, TGF-β1, and VEGFR2, blocking the main fibrogenic and angiogenic pathways
Michaelis *et al*[40]	Ribavirin	Human umbilical vein endothelial cells	Hinders angiogenesis by inhibiting inosine-5'-monophosphate dehydrogenase 1, tetrahydrobiopterin, NO, and cGMP

CCl_4: Carbon tetrachloride; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; SCFR: Stem cell growth factor receptor; FGFR: Fibroblast growth factor receptor; HSC: Hepatic stellate cells; SEC: Sinusoidal endothelial cells; KLF2: Krüppel-like factor 2; TLR4: Toll-like receptor 4; TGF-β1: Transforming growth factor beta 1; NO: Nitric oxide; cGMP: Cyclic guanosine monophosphate.

Table 2 Drugs that can inhibit extrahepatic angiogenesis in portal hypertension

Ref.	Drugs	Experimental models	Effects
Fernandez *et al*[46]	Rapamycin and glivec	Partial portal vein ligation	Downregulates VEGF, VEGFR2, CD31, PDGF, PDGFR-β, and α-SMA
Mejias *et al*[19]	Sorafenib	Partial portal vein ligation and CCl_4-induced cirrhosis	Blocks VEGF, PDGF, and Raf/MEK/ERK signaling pathway; therefore, reduces intraorgan and systemic blood flow, splanchnic neovascularization, portosystemic shunting, hepatic vascular resistance, and portal pressure
Woltering *et al*[49], Mejias *et al*[50]	Somatostatin and its synthetic analogs	Partial portal vein ligation	Reduces VEGF and CD31 expression, splanchnic neovascularization, and portosystemic collateral circulation by blocking SSTR2
Miternique-Grosse *et al*[55]	Spironolactone	Biliary cirrhosis	Suppresses the effects of aldosterone and the VEGF signal transduction pathway
Lee *et al*[58]	N-acetylcysteine	Biliary cirrhosis	Reduces oxidative stress, inflammatory cytokine levels, TNF-α, VEGF, VEGFR2, Ang1, CD31 expression, and suppresses Akt/eNOS/NO pathway
Hsu *et al*[61]	Bosentan and ambrisentan	Biliary cirrhosis	Block endothelin receptors and suppress iNOS, cyclooxygenase 2, VEGF, VEGFR2, and Akt signaling
Schwabl *et al*[64]	Pioglitazone	Biliary cirrhosis	Downregulates inflammatory genes and NF-κB expression, suppresses angiogenic and pro-inflammatory cytokines, chemokines, and growth factors (VEGF, PDGF, and PIGF)
Li *et al*[66]	Thalidomide	Biliary cirrhosis	Hinders TNF-α/interleukin-1β production and blocks the TNFα-VEGF-NOS-NO pathway
Hsu *et al*[67]	Catechins of *Camellia sinensis*	Biliary cirrhosis	Reduce HIF-1α expression, Akt signaling, and VEGF synthesis
Hsin *et al*[68]	2′-hydroxyflavonoid	Thioacetamide-induced liver cirrhosis	Downregulates apoptosis
Hsu *et al*[69]	Curcumin	Biliary cirrhosis	Suppresses VEGF, cyclooxygenase 2, and eNOS

CCl_4: Carbon tetrachloride; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; α-SMA: Alpha smooth muscle actin; SSTR2: Somatostatin receptor type 2; TNF-α: Tumor necrosis factor alpha; Ang1: Angiopoietin 1; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; iNOS: Inducible nitric oxide synthase; NF-κB: Factor kappa-light-chain-enhancer of activated B cells; PIGF: Placental growth factor; HIF-1α: Hypoxia-inducible factor-1 alpha.

for its pharmacotherapy. Currently, the drugs of choice are nonselective β-blockers. Nevertheless, their use is not recommended during the subclinical stage of the disease, when the most justified treatment is etiotropic and pathogenetic and aimed at, for example, affecting fibro- and angiogenesis in the liver, as well as angiogenesis underlying the formation of portosystemic shunts. Etiogenic approach, as part of a complex correction of pathophysiological disorders that contribute to the development of PH, may be the key to

success in preventing related complications.

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Jean Kollantai, MSW from Tomsk State University Center for Academic Writing for proofreading this article.

REFERENCES

1 **de Franchis R**; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]

2 **Garbuzenko DV**. Contemporary concepts of the medical therapy of portal hypertension under liver cirrhosis. *World J Gastroenterol* 2015; **21**: 6117-6126 [PMID: 26034348 DOI: 10.3748/wjg.v21.i20.6117]

3 **Berzigotti A**, Bosch J. Pharmacologic management of portal hypertension. *Clin Liver Dis* 2014; **18**: 303-317 [PMID: 24679496 DOI: 10.1016/j.cld.2013.12.003]

4 **Coulon S**, Heindryckx F, Geerts A, Van Steenkiste C, Colle I, Van Vlierberghe H. Angiogenesis in chronic liver disease and its complications. *Liver Int* 2011; **31**: 146-162 [PMID: 21073649 DOI: 10.1111/j.1478-3231.2010.02369.x]

5 **Garbuzenko DV**, Arefyev NO, Belov DV. Mechanisms of adaptation of the hepatic vasculature to the deteriorating conditions of blood circulation in liver cirrhosis. *World J Hepatol* 2016; **8**: 665-672 [PMID: 27326313 DOI: 10.4254/wjh.v8.i16.665]

6 **Garbuzenko DV**, Arefyev NO, Belov DV. Restructuring of the vascular bed in response to hemodynamic disturbances in portal hypertension. *World J Hepatol* 2016; **8**: 1602-1609 [PMID: 28083082 DOI: 10.4254/wjh.v8.i36.1602]

7 **Rosmorduc O**. Antiangiogenic therapies in portal hypertension: a breakthrough in hepatology. *Gastroenterol Clin Biol* 2010; **34**: 446-449 [PMID: 20630674 DOI: 10.1016/j.gcb.2010.05.007]

8 **Folkman J**. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007; **6**: 273-286 [PMID: 17396134 DOI: 10.1038/nrd2115]

9 **Chaparro M**, Sanz-Cameno P, Trapero-Marugan M, Garcia-Buey L, Moreno-Otero R. Mechanisms of angiogenesis in chronic inflammatory liver disease. *Ann Hepatol* 2007; **6**: 208-213 [PMID: 18007549]

10 **Lemoinne S**, Cadoret A, El Mourabit H, Thabut D, Housset C. Origins and functions of liver myofibroblasts. *Biochim Biophys Acta* 2013; **1832**: 948-954 [PMID: 23470555 DOI: 10.1016/j.bbadis.2013.02.019]

11 **Yokomori H**, Oda M, Yoshimura K, Hibi T. Enhanced expressions of apelin on proliferative hepatic arterial capillaries in human cirrhotic liver. *Hepatol Res* 2012; **42**: 508-514 [PMID: 22502744 DOI: 10.1111/j.1872-034X.2011.00945.x]

12 **Novo E**, Povero D, Busletta C, Paternostro C, di Bonzo LV, Cannito S, Compagnone A, Bandino A, Marra F, Colombatto S, David E, Pinzani M, Parola M. The biphasic nature of hypoxia-induced directional migration of activated human hepatic stellate cells. *J Pathol* 2012; **226**: 588-597 [PMID: 21959987 DOI: 10.1002/path.3005]

13 **Dufour JF**. Anti-angiogenic therapy for HCC. *Minerva Gastroenterol Dietol* 2012; **58**: 81-86 [PMID: 22419006]

14 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

15 **Liu L**, You Z, Yu H, Zhou L, Zhao H, Yan X, Li D, Wang B, Zhu L, Xu Y, Xia T, Shi Y, Huang C, Hou W, Du Y. Mechanotransduction-modulated fibrotic microniches reveal the contribution of angiogenesis in liver fibrosis. *Nat Mater* 2017; **16**: 1252-1261 [PMID: 29170554 DOI: 10.1038/nmat5024]

16 **Qu K**, Huang Z, Lin T, Liu S, Chang H, Yan Z, Zhang H, Liu C. New Insight into the Anti-liver Fibrosis Effect of Multitargeted Tyrosine Kinase Inhibitors: From Molecular Target to Clinical Trials. *Front Pharmacol* 2016; **6**: 300 [PMID: 26834633 DOI: 10.3389/fphar.2015.00300]

17 **Wang Y**, Gao J, Zhang D, Zhang J, Ma J, Jiang H. New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. *J Hepatol* 2010; **53**: 132-144 [PMID: 20447716 DOI: 10.1016/j.jhep.2010.02.027]

18 **Thabut D**, Routray C, Lomberk G, Shergill U, Glaser K, Huebert R, Patel L, Masyuk T, Blechacz B, Vercnocke A, Ritman E, Ehman R, Urrutia R, Shah V. Complementary vascular and matrix regulatory pathways underlie the beneficial mechanism of action of sorafenib in liver fibrosis. *Hepatology* 2011; **54**: 573-585 [PMID: 21567441 DOI: 10.1002/hep.24427]

19 **Mejias M**, Garcia-Pras E, Tiani C, Miquel R, Bosch J, Fernandez M. Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology* 2009; **49**: 1245-1256 [PMID: 19137587 DOI: 10.1002/hep.22758]

20 **Tugues S**, Fernandez-Varo G, Muñoz-Luque J, Ros J, Arroyo V, Rodés J, Friedman SL, Carmeliet P, Jiménez W, Morales-Ruiz M. Antiangiogenic treatment with sunitinib ameliorates inflammatory infiltrate, fibrosis, and portal pressure in cirrhotic rats. *Hepatology* 2007; **46**: 1919-1926 [PMID: 17935226 DOI: 10.1002/hep.21921]

21 **Majumder S**, Piguet AC, Dufour JF, Chatterjee S. Study of the cellular mechanism of Sunitinib mediated inactivation of activated hepatic stellate cells and its implications in angiogenesis. *Eur J Pharmacol* 2013; **705**: 86-95 [PMID: 23454556 DOI: 10.1016/j.ejphar.2013.02.026]

22 **Lin HC**, Huang YT, Yang YY, Lee PC, Hwang LH, Lee WP, Kuo YJ, Lee KC, Hsieh YC, Liu RS. Beneficial effects of dual vascular endothelial growth factor receptor/fibroblast growth factor receptor inhibitor brivanib alaninate in cirrhotic portal hypertensive rats. *J Gastroenterol Hepatol* 2014; **29**: 1073-1082 [PMID: 24325631 DOI: 10.1111/jgh.12480]

23 **Yang YY**, Liu RS, Lee PC, Yeh YC, Huang YT, Lee WP, Lee KC, Hsieh YC, Lee FY, Tan TW, Lin HC. Anti-VEGFR agents ameliorate hepatic venous dysregulation/microcirculatory dysfunction, splanchnic venous pooling and ascites of NASH-cirrhotic rat. *Liver Int* 2014; **34**: 521-534 [PMID: 23998651 DOI: 10.1111/liv.12299]

24 **Bieker JJ**. Krüppel-like factors: three fingers in many pies. *J Biol Chem* 2001; **276**: 34355-34358 [PMID: 11443140 DOI: 10.1074/jbc.R100043200]

25 **Taniguchi H**, Jacinto FV, Villanueva A, Fernandez AF, Yamamoto H, Carmona FJ, Puertas S, Marquez VE, Shinomura Y, Imai K, Esteller M. Silencing of Kruppel-like factor 2 by the histone methyltransferase EZH2 in human cancer. *Oncogene* 2012; **31**: 1988-1994 [PMID: 21892211 DOI: 10.1038/onc.2011.387]

26 **Kawanami D**, Mahabeleshwar GH, Lin Z, Atkins GB, Hamik A, Haldar SM, Maemura K, Lamanna JC, Jain MK. Kruppel-like factor 2 inhibits hypoxia-inducible factor 1alpha expression and function in the endothelium. *J Biol Chem* 2009; **284**: 20522-20530 [PMID: 19491109 DOI: 10.1074/jbc.M109.025346]

27 **Doddaballapur A**, Michalik KM, Manavski Y, Lucas T, Houtkooper RH, You X, Chen W, Zeiher AM, Potente M, Dimmeler S, Boon RA. Laminar shear stress inhibits endothelial cell metabolism via KLF2-mediated repression of PFKFB3. *Arterioscler Thromb Vasc Biol* 2015; **35**: 137-145 [PMID: 25359860 DOI: 10.1161/ATVBAHA.114.304277]

28 **Gracia-Sancho J**, Russo L, García-Calderó H, García-Pagán JC, García-Cardeña G, Bosch J. Endothelial expression of transcription factor Kruppel-like factor 2 and its vasoprotective target genes in the normal and cirrhotic rat liver. *Gut* 2011; **60**: 517-524 [PMID: 21112949 DOI: 10.1136/gut.2010.220913]

29 **Das A**, Shergill U, Thakur L, Sinha S, Urrutia R, Mukhopadhyay

D, Shah VH. Ephrin B2/EphB4 pathway in hepatic stellate cells stimulates Erk-dependent VEGF production and sinusoidal endothelial cell recruitment. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G908-G915 [PMID: 20338920 DOI: 10.1152/ajpgi.00510.2009]

30 **Zeng XQ**, Li N, Pan DY, Miao Q, Ma GF, Liu YM, Tseng YJ, Li F, Xu LL, Chen SY. Kruppel-like factor 2 inhibit the angiogenesis of cultured human liver sinusoidal endothelial cells through the ERK1/2 signaling pathway. *Biochem Biophys Res Commun* 2015; **464**: 1241-1247 [PMID: 26212440 DOI: 10.1016/j.bbrc.2015.07.113]

31 **Miao Q**, Zeng X, Ma G, Li N, Liu Y, Luo T, Lian J, Chen S. Simvastatin suppresses the proangiogenic microenvironment of human hepatic stellate cells via the Kruppel-like factor 2 pathway. *Rev Esp Enferm Dig* 2015; **107**: 63-71 [PMID: 25659387]

32 **Marrone G**, Russo L, Rosado E, Hide D, García-Cardeña G, García-Pagán JC, Bosch J, Gracia-Sancho J. The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial-stellate cell deactivation induced by statins. *J Hepatol* 2013; **58**: 98-103 [PMID: 22989565 DOI: 10.1016/j.jhep.2012.08.026]

33 **Garbuzenko DV**. [The role of intestinal microflora in the development of complications of hepatic cirrhosis-associated portal hypertension.] *Klin Med* (Mosk) 2007; **85**: 15-19 [PMID: 17926483]

34 **Jagavelu K**, Routray C, Shergill U, O'Hara SP, Faubion W, Shah VH. Endothelial cell toll-like receptor 4 regulates fibrosis-associated angiogenesis in the liver. *Hepatology* 2010; **52**: 590-601 [PMID: 20564354 DOI: 10.1002/hep.23739]

35 **Zhu Q**, Zou L, Jagavelu K, Simonetto DA, Huebert RC, Jiang ZD, DuPont HL, Shah VH. Intestinal decontamination inhibits TLR4 dependent fibronectin-mediated cross-talk between stellate cells and endothelial cells in liver fibrosis in mice. *J Hepatol* 2012; **56**: 893-899 [PMID: 22173161 DOI: 10.1016/j.jhep.2011.11.013]

36 **DuPont HL**. Biologic properties and clinical uses of rifaximin. *Expert Opin Pharmacother* 2011; **12**: 293-302 [PMID: 21226639 DOI: 10.1517/14656566.2011.546347]

37 **Liu Y**, Salvador LA, Byeon S, Ying Y, Kwan JC, Law BK, Hong J, Luesch H. Anticolon cancer activity of largazole, a marine-derived tunable histone deacetylase inhibitor. *J Pharmacol Exp Ther* 2010; **335**: 351-361 [PMID: 20739454 DOI: 10.1124/jpet.110.172387]

38 **Liu Y**, Wang Z, Wang J, Lam W, Kwong S, Li F, Friedman SL, Zhou S, Ren Q, Xu Z, Wang X, Ji L, Tang S, Zhang H, Lui EL, Ye T. A histone deacetylase inhibitor, largazole, decreases liver fibrosis and angiogenesis by inhibiting transforming growth factor-β and vascular endothelial growth factor signalling. *Liver Int* 2013; **33**: 504-515 [PMID: 23279742 DOI: 10.1111/liv.12034]

39 **Fontaine H**, Vallet-Pichard A, Equi-Andrade C, Nalpas B, Verkarre V, Chaix ML, Lebray P, Sobesky R, Serpaggi J, Kreis H, Pol S. Histopathologic efficacy of ribavirin monotherapy in kidney allograft recipients with chronic hepatitis C. *Transplantation* 2004; **78**: 853-857 [PMID: 15385804 DOI: 10.1097/01.TP.0000128911.87538.AA]

40 **Michaelis M**, Michaelis R, Suhan T, Schmidt H, Mohamed A, Doerr HW, Cinatl J Jr. Ribavirin inhibits angiogenesis by tetrahydrobiopterin depletion. *FASEB J* 2007; **21**: 81-87 [PMID: 17135367 DOI: 10.1096/fj.06-6779com]

41 **Bosch J**, Groszmann RJ, Shah VH. Evolution in the understanding of the pathophysiological basis of portal hypertension: How changes in paradigm are leading to successful new treatments. *J Hepatol* 2015; **62**: S121-S130 [PMID: 25920081 DOI: 10.1016/j.jhep.2015.01.003]

42 **Fernandez M**, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. *Gastroenterology* 2004; **126**: 886-894 [PMID: 14988842 DOI: 10.1053/j.gastro.2003.12.012]

43 **Fernandez M**, Mejias M, Angermayr B, Garcia-Pagan JC, Rodés J, Bosch J. Inhibition of VEGF receptor-2 decreases the development of hyperdynamic splanchnic circulation and portal-systemic collateral vessels in portal hypertensive rats. *J Hepatol* 2005; **43**: 98-103 [PMID: 15893841 DOI: 10.1016/j.jhep.2005.02.022]

44 **Angermayr B**, Fernandez M, Mejias M, Gracia-Sancho J, Garcia-Pagan JC, Bosch J. NAD(P)H oxidase modulates angiogenesis and the development of portosystemic collaterals and splanchnic hyperaemia in portal hypertensive rats. *Gut* 2007; **56**: 560-564 [PMID: 16854998 DOI: 10.1136/gut.2005.088013]

45 **Fernández M**, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol* 2009; **50**: 604-620 [PMID: 19157625 DOI: 10.1016/j.jhep.2008.12.011]

46 **Fernandez M**, Mejias M, Garcia-Pras E, Mendez R, Garcia-Pagan JC, Bosch J. Reversal of portal hypertension and hyperdynamic splanchnic circulation by combined vascular endothelial growth factor and platelet-derived growth factor blockade in rats. *Hepatology* 2007; **46**: 1208-1217 [PMID: 17654489 DOI: 10.1002/hep.21785]

47 **D'Amico M**, Mejías M, García-Pras E, Abraldes JG, García-Pagán JC, Fernández M, Bosch J. Effects of the combined administration of propranolol plus sorafenib on portal hypertension in cirrhotic rats. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G1191-G1198 [PMID: 22403792 DOI: 10.1152/ajpgi.00252.2011]

48 **Garbuzenko DV**. Current approaches to the management of patients with liver cirrhosis who have acute esophageal variceal bleeding. *Curr Med Res Opin* 2016; **32**: 467-475 [PMID: 26804426 DOI: 10.1185/03007995.2015.1124846]

49 **Woltering EA**. Development of targeted somatostatin-based antiangiogenic therapy: a review and future perspectives. *Cancer Biother Radiopharm* 2003; **18**: 601-609 [PMID: 14503956 DOI: 10.1089/108497803322287691]

50 **Mejias M**, Garcia-Pras E, Tiani C, Bosch J, Fernandez M. The somatostatin analogue octreotide inhibits angiogenesis in the earliest, but not in advanced, stages of portal hypertension in rats. *J Cell Mol Med* 2008; **12**: 1690-1699 [PMID: 18194463 DOI: 10.1111/j.1582-4934.2008.00218.x]

51 **Garbuzenko DV**. The principles of management of patients with liver cirrhosis comlicated by ascites. *Klin Med* (Mosk) 2017; **95**: 789-796

52 **Michel F**, Ambroisine ML, Duriez M, Delcayre C, Levy BI, Silvestre JS. Aldosterone enhances ischemia-induced neovascularization through angiotensin Ⅱ-dependent pathway. *Circulation* 2004; **109**: 1933-1937 [PMID: 15078792 DOI: 10.1161/01.CIR.0000127112.36796.9B]

53 **Wilkinson-Berka JL**, Tan G, Jaworski K, Miller AG. Identification of a retinal aldosterone system and the protective effects of mineralocorticoid receptor antagonism on retinal vascular pathology. *Circ Res* 2009; **104**: 124-133 [PMID: 19038868 DOI: 10.1161/CIRCRESAHA.108.176008]

54 **Gravez B**, Tarjus A, Pelloux V, Ouvrard-Pascaud A, Delcayre C, Samuel J, Clément K, Farman N, Jaisser F, Messaoudi S. Aldosterone promotes cardiac endothelial cell proliferation in vivo. *J Am Heart Assoc* 2015; **4**: e001266 [PMID: 25564371 DOI: 10.1161/JAHA.114.001266]

55 **Miternique-Grosse A**, Griffon C, Siegel L, Neuville A, Weltin D, Stephan D. Antiangiogenic effects of spironolactone and other potassium-sparing diuretics in human umbilical vein endothelial cells and in fibrin gel chambers implanted in rats. *J Hypertens* 2006; **24**: 2207-2213 [PMID: 17053542 DOI: 10.1097/01.hjh.0000249698.26983.4e]

56 **Hsu SJ**, Wang SS, Huo TI, Lee FY, Huang HC, Chang CC, Hsin IF, Ho HL, Lin HC, Lee SD. The Impact of Spironolactone on the Severity of Portal-Systemic Collaterals and Hepatic Encephalopathy in Cirrhotic Rats. *J Pharmacol Exp Ther* 2015; **355**: 117-124 [PMID: 26260462 DOI: 10.1124/jpet.115.225516]

57 **Chikina SY**. Antioxidant effects of N-acetylcysteine in modern clinical practice. *Jeffektivnaja farmakoterapija* 2011; **32**: 19-24

58 **Lee PC**, Yang YY, Huang CS, Hsieh SL, Lee KC, Hsieh YC, Lee TY, Lin HC. Concomitant inhibition of oxidative stress and angiogenesis by chronic hydrogen-rich saline and N-acetylcysteine treatments improves systemic, splanchnic and hepatic hemodynamics of cirrhotic rats. *Hepatol Res* 2015; **45**: 578-588 [PMID: 24961937 DOI: 10.1111/hepr.12379]

59 **Garbuzenko DV**. Pathophysiological mechanisms and new directions of therapy of portal hypertension at liver cirrhosis. *Klin persp gastrojenterol gepatol* 2010; **6**: 11-20

60 **Salani D**, Taraboletti G, Rosanò L, Di Castro V, Borsotti P, Giavazzi R, Bagnato A. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am J Pathol* 2000; **157**: 1703-1711 [PMID: 11073829 DOI: 10.1016/S0002-9440(10)64807-9]

61 **Hsu SJ**, Lin TY, Wang SS, Chuang CL, Lee FY, Huang HC, Hsin IF, Lee JY, Lin HC, Lee SD. Endothelin receptor blockers reduce shunting and angiogenesis in cirrhotic rats. *Eur J Clin Invest* 2016; **46**: 572-580 [PMID: 27091078 DOI: 10.1111/eci.12636]

62 **Gerasimenko ND**, Degtyar NI, Racin MS. Systemic inflammation and aging: the role of nuclear transcription factors and therapeutic possibilities (Review of the literature). *Probl starenija i dolgoletija* 2016; **25**: 554-561

63 **Dana N**, Javanmard SH, Rafiee L. Role of peroxisome proliferator-activated receptor alpha and gamma in antiangiogenic effect of pomegranate peel extract. *Iran J Basic Med Sci* 2016; **19**: 106-110 [PMID: 27096071]

64 **Schwabl P**, Payer BA, Grahovac J, Klein S, Horvatits T, Mitterhauser M, Stift J, Boucher Y, Trebicka J, Trauner M, Angermayr B, Fuhrmann V, Reiberger T, Peck-Radosavljevic M. Pioglitazone decreases portosystemic shunting by modulating inflammation and angiogenesis in cirrhotic and non-cirrhotic portal hypertensive rats. *J Hepatol* 2014; **60**: 1135-1142 [PMID: 24530596 DOI: 10.1016/j.jhep.2014.01.025]

65 **Enomoto N**, Takei Y, Hirose M, Ikejima K, Miwa H, Kitamura T, Sato N. Thalidomide prevents alcoholic liver injury in rats through suppression of Kupffer cell sensitization and TNF-alpha production. *Gastroenterology* 2002; **123**: 291-300 [PMID: 12105857 DOI: 10.1053/gast.2002.34161]

66 **Li TH**, Huang CC, Yang YY, Lee KC, Hsieh SL, Hsieh YC, Alan L, Lin HC, Lee SD, Tsai CY. Thalidomide Improves the Intestinal Mucosal Injury and Suppresses Mesenteric Angiogenesis and Vasodilatation by Down-Regulating Inflammasomes-Related Cascades in Cirrhotic Rats. *PLoS One* 2016; **11**: e0147212 [PMID: 26820153 DOI: 10.1371/journal.pone.0147212]

67 **Hsu SJ**, Wang SS, Hsin IF, Lee FY, Huang HC, Huo TI, Lee WS, Lin HC, Lee SD. Green tea polyphenol decreases the severity of portosystemic collaterals and mesenteric angiogenesis in rats with liver cirrhosis. *Clin Sci* (Lond) 2014; **126**: 633-644 [PMID: 24063570 DOI: 10.1042/CS20130215]

68 **Hsin IF**, Lee JY, Huo TI, Lee FY, Huang HC, Hsu SJ, Wang SS, Ho HL, Lin HC, Lee SD. 2'-Hydroxyflavanone ameliorates mesenteric angiogenesis and portal-systemic collaterals in rats with liver fibrosis. *J Gastroenterol Hepatol* 2016; **31**: 1045-1051 [PMID: 26474184 DOI: 10.1111/jgh.13197]

69 **Hsu SJ**, Lee JY, Lin TY, Hsieh YH, Huang HC, Lee FY, Lin HC, Hou MC, Lee SD. The beneficial effects of curcumin in cirrhotic rats with portal hypertension. *Biosci Rep* 2017; **37**: pii: BSR2017101 [PMID: 29162665 DOI: 10.1042/BSR20171015]

70 **Coriat R**, Gouya H, Mir O, Ropert S, Vignaux O, Chaussade S, Sogni P, Pol S, Blanchet B, Legmann P, Goldwasser F. Reversible decrease of portal venous flow in cirrhotic patients: a positive side effect of sorafenib. *PLoS One* 2011; **6**: e16978 [PMID: 21340026 DOI: 10.1371/journal.pone.0016978]

71 **Pinter M**, Sieghart W, Reiberger T, Rohr-Udilova N, Ferlitsch A, Peck-Radosavljevic M. The effects of sorafenib on the portal hypertensive syndrome in patients with liver cirrhosis and hepatocellular carcinoma--a pilot study. *Aliment Pharmacol Ther* 2012; **35**: 83-91 [PMID: 22032637 DOI: 10.1111/j.1365-2036.2011.04896.x]

72 **Garcia-Tsao G**, Fallon M, Reddy K, Loo N, Bari K, Augustin S, Ciarleglio M, Deng Y, Taddei TH, Strazzabosco M. Placebo-controlled, randomized, pilot study of the effect of sorafenib on portal pressure in patients with cirrhosis, portal hypertension and ablated hepatocellular carcinoma (HCC). *Hepatology* 2015; **62**: 580A

P- Reviewer: Kamimura K **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3749-3759

DOI: 10.3748/wjg.v24.i33.3749

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

ORIGINAL ARTICLE

Basic Study

Establishment, functional and genetic characterization of a colon derived large cell neuroendocrine carcinoma cell line

Michael Gock, Christina S Mullins, Christine Harnack, Friedrich Prall, Robert Ramer, Anja Göder, Oliver H Krämer, Ernst Klar, Michael Linnebacher

Michael Gock, Ernst Klar, Department of General Surgery, University of Rostock, Rostock 18055, Germany

Christina S Mullins, Christine Harnack, Michael Linnebacher, Department of General Surgery, Section of Molecular Oncology and Immunotherapy, University of Rostock, Rostock 18055, Germany

Friedrich Prall, Institute of Pathology, University of Rostock, Rostock 18055, Germany

Robert Ramer, Institute of Pharmacology, University of Rostock, Rostock 18055, Germany

Anja Göder, Oliver H Krämer, Institute of Toxicology, University Medical Center Mainz, Mainz 55131, Germany

ORCID number: Michael Gock (0000-0001-6393-1097); Christina S Mullins (0000-0003-2296-2027); Christine Harnack (0000-0003-0067-4921); Friedrich Prall (0000-0001-8103-5544); Robert Ramer (0000-0003-1778-8829); Anja Göder (0000-0001-9743-1656); Oliver H Krämer (0000-0003-3973-045X); Ernst Klar (0000-0002-1159-5797); Michael Linnebacher (0000-0001-8054-1402).

Author contributions: Linnebacher M and Klar E designed research; Mullins CS, Harnack C, Prall F, Ramer R and Göder A performed research; Gock M, Krämer OH and Linnebacher M analyzed data; Gock M, Mullins CS, Krämer OH and Linnebacher M wrote the paper.

Institutional review board statement: This study was reviewed and approved by Ethikkommission an der Medizinischen Fakultät der Universität Rostock, St.-Georg-Str. 108, Rostock 18055, Germany.

Informed consent statement: All procedures were approved by Ethikkommission an der Medizinischen Fakultät der Universität Rostock (reference number II HV 43/2004) in accordance with generally accepted guidelines for the use of human material.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Michael Linnebacher, PhD, Postdoc, Research Scientist, Department of General Surgery, Section of Molecular Oncology and Immunotherapy, University of Rostock, Schillingallee 35, Rostock 18055, Germany. michael.linnebacher@med.uni-rostock.de
Telephone: +49-381-4946043
Fax: +49-381-4946002

Received: February 28, 2018
Peer-review started: March 1, 2018
First decision: April 18, 2018
Revised: June 14, 2018
Accepted: June 16, 2018
Article in press: June 16, 2018
Published online: September 7, 2018

Abstract

AIM

To establish cell line and patient-derived xenograft (PDX) models for neuroendocrine carcinomas (NEC) which is highly desirable for gaining insight into tumor development as well as preclinical research including

biomarker testing and drug response prediction.

METHODS

Cell line establishment was conducted from direct *in vitro* culturing of colonic NEC tissue (HROC57). A PDX could also successfully be established from vitally frozen tumor samples. Morphological features, invasive and migratory behavior of the HROC57 cells as well as expression of neuroendocrine markers were vastly analyzed. Phenotypic analysis was done by microscopy and multicolor flow cytometry. The extensive molecular-pathological profiling included mutation analysis, assessment of chromosomal and microsatellite instability; and in addition, fingerprinting (*i.e.*, STR analysis) was performed from the cell line in direct comparison to primary patient-derived tissues and the PDX model established. Drug responsiveness was examined for a panel of chemotherapeutics in clinical use for the treatment of solid cancers.

RESULTS

The established cell line HROC57 showed distinct morphological and molecular features of a poorly differentiated large-cell NEC with KI-67 > 50%. Molecular-pathological analysis revealed a CpG island promoter methylation positive cell line with microsatellite instability being absent. The following mutation profile was observed: *KRAS* (wt), *BRAF* (mut). A high sensitivity to etoposide, cisplatin and 5-FU could be demonstrated while it was more resistant towards rapamycin.

CONCLUSION

We successfully established and characterized a novel patient-derived NEC cell line in parallel to a PDX model as a useful tool for further analysis of the biological characteristics and for development of novel diagnostic and therapeutic options for NEC.

Key words: Patient-derived tumor model; Large cell neuroendocrine carcinoma; Individualized medicine

Core tip: Since incidence of G3 poorly differentiated neuroendocrine carcinomas (NEC) is very low, data is substantially scarcer than on G1 or G2 neuroendocrine tumors. Herein we describe an ultra-low passage NEC cell line and corresponding patient-derived xenograft model established directly from patient derived colonic tumor samples. We characterized our model according to phenotype, molecular, morphological and growth characteristics, as well as drug response and radiation response profiles. We present a useful tool for further analysis of the biological characteristics and for development of novel diagnostic and therapeutic options for NEC. The model is available on request.

Gock M, Mullins CS, Harnack C, Prall F, Ramer R, Göder A, Krämer OH, Klar E, Linnebacher M. Establishment, functional and genetic characterization of a colon derived large cell neuroendocrine carcinoma cell line. *World J Gastroenterol* 2018; 24(33): 3749-3759 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3749.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3749

INTRODUCTION

Neuroendocrine tumors (NET) are a heterogeneous group of tumors arising from cells which are derived from the embryonic neural crest, neuroectoderm, and endoderm[1]. The age-adjusted incidence of all NETs increased from 1.9 to 5.25 cases per 100000 people[2] which is probably due to improvements in surveillance and diagnostic endoscopy[3]. Gastroenteropancreatic neuroendocrine neoplasms (GEP-NEN) are a subgroup of NET that derive from neuroectodermal cells, such as the enterochromaffin-like cells of the intestine[4]. These tumors can produce a multitude of different markers and peptide hormones; *i.e.*, neuron-specific enolase, synaptophysin and chromogranin A[5,6]. Based on the actual revised 2010 WHO classification, NEN are classified according to their differentiation and proliferation. In addition, the European Neuroendocrine Tumor Society has proposed a tumor-node-metastasis staging and grading system for various types of GEP-NET[7]. Well-differentiated NEN are classified together as NET G1 or G2 (G1: < 2 mitoses/10 high power fields; Ki-67 index ≤ 2%, G2: 2-20 mitoses/10 high power fields; Ki-67 index 3%-20%)[8,9]. While NET G1 can be equated with carcinoid, all poorly differentiated G3 NEN are termed neuroendocrine carcinoma (NEC). This NEC group is further subdivided into a small-cell and a large-cell variant. Regarding their proliferation activity, all NEC are poorly differentiated actively proliferating G3 tumors (NEC; G3: > 20 mitoses/10 high power fields; Ki-67 index > 20%)[7,8]. Recently, a proportion of NET could be identified presenting a high proliferation with either mitoses or Ki-67 index cutoff above 20% and most important a well-differentiated morphology. This novel category was called well-differentiated grade 3 NET (NET G-3)[10].

In local or locoregional disease all patients should be considered for curative resection of the primary tumor and locoregional lymph nodes according to actual guidelines[11]. In advanced or metastatic disease, cytoreductive surgery should be considered when metastatic disease is localized or if > 70% of tumor load is thought to be resectable[11]. In metastatic disease involving high-grade G3 NEC combination chemotherapy consisting of cisplatinum and etoposide is suggested[12]. Nonetheless response rates to chemotherapy are low and there is no established second line therapy[11].

For further research on the biological characteristics of these tumors, for development and preclinical testing of new treatment modalities and potential molecular therapeutical targets, establishment of new *in vitro* and

in vivo models is mandatory. In the last decades, only a very few GEP-NEN cell lines have been established and characterized[13] but pathological terminology is very heterogeneous especially regarding the actual revised 2010 WHO classification[13]. For colorectal adenocarcinoma, many patient-individual tumor models, which have been generated in the last decade by us and others[14-16], have proven extremely helpful in deciphering colorectal cancers' molecular heterogeneity[16] and in the identification of novel druggable targets and combinatorial treatment strategies[17,18].

In this study, we describe the establishment and functional characterization of a novel NEC colon derived cell line with corresponding patient-derived xenograft (PDX) model. A broad analysis of tumor biology, genetic alterations and assessment of chemosensitivity towards an extensive range of chemotherapeutic drugs and of radiosensitivity has been performed. Considering these aspects, such characterized matched *in vitro* and *in vivo* tumor models represent excellent tools for further development of individual therapy regimens and are a valuable tool to gain additional insight in the tumor biology of NEC.

MATERIALS AND METHODS

Tumor preparation and cell line establishment

Primary NEC resection specimens of HROC57 were received fresh from surgery, with informed written patient consent. All procedures were approved by the Ethics Committee of the University of Rostock (reference number II HV 43/2004) in accordance with generally accepted guidelines for the use of human material. Tumor samples were cut into small pieces. Parts of the tumor were immediately frozen in freezing medium [foetal calf serum (FCS) containing 10% DMSO] at -80 ℃ for subsequent xenografting. Other pieces were frozen in liquid nitrogen for molecular analysis. Cell line establishment protocol was adapted according to Maletzki *et al*[19], 2012.

For *in vivo* engraftment, six-week-old female NMRI nu/nu mice were used as recipients. Mice were bred in the university's animal facility and maintained in specified pathogen-free conditions. All surgical interventions were performed under Ketamin/Xylazin anaesthesia (dose: 90/25 mg/kg body weight), and all efforts were made to minimize suffering. Subcutaneous tumour implantation was performed as previously described[19]. Established xenografts (> 1.500 mm^3) were removed and underwent *in vitro* culture protocols as described above. All *in vivo* experimental procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Rostock (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern; Thierfelder Str. 18, Rostock 18059, Germany; permit number: LALLF M-V/TSD/7221.3-1.1-071-10).

Histology and immunohistochemistry of original tumors and PDX

Histopathological examination of HE-stained primary tumors and corresponding PDX was done according to standard protocols for clinicopathological tumor staging[20], and additional staging information was compiled from patients' clinical charts. Supplementary, immunostainings from paraffin-embedded primary tumors were done for cytokeratin 20, cytokeratin MNF116, CEA, synaptophysin and chromogranin.

Mutational and methylation profile of tumor-associated target genes and determining the level of chromosomal instability

Molecular classification was done according to Ostwald *et al*[21]. Mutations in tumor-associated *APC*, *P53*, *KRAS*, and $BRAF^{V600E}$ genes were analyzed as described. DNA-methylation in CIMP-sensitive promoters was traced by the MethyLight technology with a modified marker panel originally published by Ogino *et al*[22]. The degree of chromosomal instability was assessed using the SNP Array 6.0 from Affymetrix (Cleveland, OH) according to manufacturer's instructions.

DNA typing

Genomic DNA was isolated from cell lines at different passages, matched tumor and normal tissue, as well as corresponding B cells using Wizard® Genomic DNA Purification Kit provided by Promega (Mannheim, Germany). Highly polymorphic short tandem repeat (STR) DNA marker (CSF1PO, TPOX, THO1, vWA, D16S539, D13S317, D5S818 and D7S820) and the gender marker amelogenin were amplified in standard PCR reactions and analyzed on an ABI Prism 3100 system. PCR primers were based on the original publication[23].

Generation of peripheral Bc cultures

Peripheral blood lymphocytes were purified by Ficoll density-gradient centrifugation. B-lymphoid cell lines (B-LCLs) were generated by Epstein-Barr virus (EBV)-transformation as described before[24]. Outgrowing B-LCL cultures were harvested, expanded, characterized, and frozen.

In vitro growth kinetics, ploidy and cell cycle analysis

Doubling time of HROC57 cells was determined from serial passages. Therefore, 5×10^5 viable cells were seeded into 25 cm^2 flasks and viable cells (defined by trypan blue exclusion) were daily counted for seven days. Cultures were fed every 3 or 4 d. Ploidy and cell cycle analysis was performed by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany) using fixed cells (70% ethanol), after RNase A digestion (100 μg/mL; Sigma Aldrich, Munich, Germany) and propidium iodide (10 μg/mL) addition. 10000 events

were measured for each sample and cell cycle analysis was done by applying Modfit software (Verity Software House, Topsham, United States). Matched B-LCLs were used as diploid controls.

Flow cytometric phenotyping of primary cell line

Cell surface marker expression on NEC line was traced by flow cytometry with and without IFN-γ pre-treatment using a panel of FITC-, PE- or APC-conjugated Abs: CD44, CD56, CD71, CD90, CD326, chromogranin A, synaptophysin, neuron specific enolase, Ki-67, MHC I, MHC II, and HLA-A2 (cell culture supernatant clone BB7.2). For HLA-A2, a secondary FITC-conjugated anti-mouse Ab was applied. Samples were analyzed using CellQuest software (BD Biosciences).

Mycoplasma and human viral infection

Mycoplasma contamination was tested by the 16S-rRNA-gene-based polymerase chain reaction (PCR) amplification method from whole cell lysates. For determining potential polyomavirus infection (JC/BK and SV40) gDNA was isolated using Wizard genomic DNA purification kit (Promega). All procedures were done as described in[19].

Migration and invasion assay

Analysis of tumour cell invasion was performed using a classical Boyden chamber test (8 μm pore size in a 24-well plate format) with Matrigel-coating (BD Biosciences) according to the manufacturer's instructions. Cells were suspended to yield a cell number of 2×10^5 cells per upper Boyden chamber in 500 μL serum-free medium. Medium in the lower Boyden chamber was supplemented with 10% heat-inactivated FCS serving as chemo-attractant. After a 72 h incubation period, the non-invading cells on the upper surface of the inserts were removed with a cotton swab, and viability of cells on the lower surface was measured by the colorimetric 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,6-benzene disulfonate (WST-1) test (Roche Diagnostics, Mannheim, Germany)[25]. Quantification of migration was performed in parallel using the same protocol but with uncoated upper Boyden chambers.

In vitro chemo-and radiosensitivity analysis

For chemosensitivity, cells were seeded into 96-well microtiter plates at 5×10^3 or 1×10^4 cells/well. When cells reached 30%-40% confluency, cultures were exposed to increasing concentrations of etoposide, cisplatin, 5-FU, oxaliplatin, irinotecan and rapamycin (pharmacy of the University Hospital Rostock). After three days of exposure, media were removed and replaced by fresh medium supplemented with therapeutics. Following another three days, medium was removed; plates were carefully washed and stained with crystal violet (0.2%, 10 min). Finally, drug effects from triplicate wells were determined at the level of 50% inhibition (IC_{50}) in comparison to control, measured at 570 nm (reference wavelength: 620 nm). For radiosensitivity analysis, cells radiated with 50 Gy using a ^{137}Cs-source were seeded into 96-well microtiter plates in triplicates (1×10^5 cells per well and six serial two-fold dilutions). Control cells were not radiated. After 4 and 7 d, duplicate plates were analyzed for total cell growth using crystal violet as described above.

Western blot

Western blot was done as previously described[26]. Antibodies specific for the following targets were from Santa Cruz (Heidelberg, Germany): Histone deacetylase (HDAC) 2 (sc-7899), p53 (sc-126) and p21 (sc-6246). Anti-HDAC1 (05-100) was obtained from Millipore (Burlington, MA, United Atates). Anti-HSP90 (ADI-SPA-830) was purchased from Enzo Life Sciences (Farmingdale, NY, United States). Anti-survivin (NB500-201) was obtained from Novus Biologicals (Cambridge, United Kingdom). Anti-acetyl-K382-p53 (ab75754) was from Abcam (Cambridge, United Kingdom), while phospho-S15-p53 (9284) was purchased from Cell Signaling Technology (Cambridge, United Kingdom). Western Blots were detected with the Odyssey Infrared Imaging System (Licor) using IRDye® 680RD- or IRDye® 800CW-coupled secondary antibodies.

Statistical analysis

Values are reported as the mean ± SD. After proving the assumption of normality, differences were determined by using the unpaired Student's *t*-test. If normality failed, the nonparametric Mann-Whitney *U*-Test was applied. The tests were performed by using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, United States). The criterion for significance was set to $P < 0.05$.

RESULTS

Clinical case of a colonic large cell neuroendocrine carcinoma and origin of cell line

We report on a 43-year-old patient with a history of weight loss of 12 kg and upper abdominal pain. CT scan showed a tumor of the ascending colon with diffuse liver metastasis and colonoscopy revealed a tumor of 5 cm length with tumor stenosis underneath the right flexure. First tumor biopsies showed an undifferentiated carcinoma. A right hemicolectomy was performed and intraoperatively several liver metastases in both lobes could be confirmed. Macroscopically, the tumor size was 9 cm × 5 cm × 5 cm. Microscopically, a poorly differentiated large cell neuroendocrine carcinoma with deep infiltration of pericolic fatty tissue, lymphatic tract invasion and major lymph node involvement was revealed [UICC G3 pT3 pN2 (16/28) L1 V1 cM1 (HEP)]. The patient was dismissed 8 d after the operation and received several courses of chemotherapy with cisplatin etoposide at last but died one year after first diagnosis due to tumor progression with lung and liver metastases.

The tumor showed a poorly differentiated large cell

Table 1 Immunohistochemically analysis of primary tumor

Immunohistochemistry	
Cytokeratin 20	Positive
Pan cytokeratin MNF116	Positive
Synaptophysin	Positive
Chromogranin	Negative
CEA	Negative

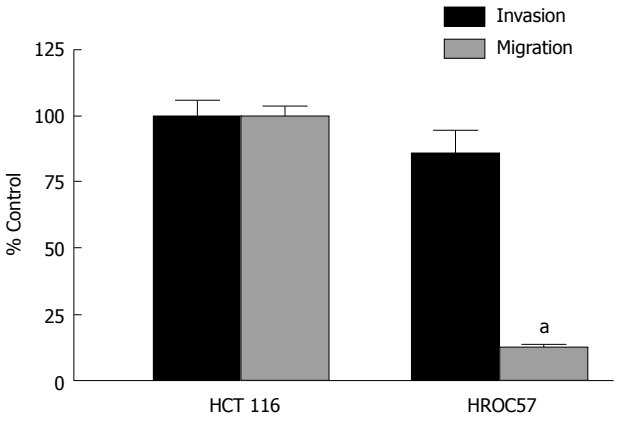


Figure 1 Migratory potential and invasiveness of HROC 57 cells. Invasion and migration of the cell line HROC57 in comparison to the reference cell line HCT116 was analyzed. Cells were subjected to migration assay (Migration, grey bars) and matrigel invasion assay (Invasion, black bars). Values are means ± SEM of $n = 3$. $^{a}P < 0.05$ *vs* HCT116.

neuroendocrine cytology and distinct neuroendocrine and epithelial markers stained positive (Table 1).

Establishment of permanent cell lines and PDX model

In vitro and *in vivo* approaches were combined as described previously for establishment of a permanent cell line growing 2D[19] and a subcutaneous PDX[27]. Outgrowth of cells in culture occurred immediately and doubling time of the outgrowing cell line was 26.13 h.

Tumor formation in immunodeficient mice could be observed 37 d after the tumor engraftment. Similar to previous findings with adenocarcinomas, histological analysis revealed preserved tumor architecture in the PDX compared to the original patient tumor architecture (data not shown).

Analysis of invasion and migration revealed a slight non-significant difference in the infiltrative activity of the patient derived HROC57 compared to HCT116, which served as positive control. In contrast, patient-derived HROC57 revealed a significantly lower migratory activity (*t*-test, $P < 0.0001$) compared to HCT116 (Figure 1).

Phenotyping

To investigate whether or not HROC57 cells recapitulate a neuroendocrine phenotype, expression of neuroendocrine markers was analyzed by flow cytometry. Chromogranin A, synaptophysin and neuron specific enolase were highly expressed by HROC57, whereas CD56 (neural cell adhesion molecule, NCAM) was only expressed to a very low extend (Figure 2). In addition to neuroendocrine markers, HROC57 expressed several epithelial and so-called stem-cell markers. We found a very high expression of CD326 (EpCAM) reflecting an epithelial origin of the tumor, moderate expression of the cellular migration and adhesion marker CD44 but almost no expression of CD166. CD26 and CD29, which have been described as stem cell and metastasis-promoting surface receptors[28,29], were highly expressed; CD90 only marginally.

Further characterization showed a high expression of the proliferation markers KI-67 and CD71 (Figure 2) reflecting the high proliferative activity of the tumor.

Regarding HLA molecules which play an important role in specific immune recognition and tumor cell defense, expression of HLA class I (ß2M and pan-HLA-ABC) could be observed, but no expression of HLA class II (Figure 2). The latter could also not be reversed by interferon pretreatment (data not shown). HROC57 cells additionally express high levels of CD73, which has been described as immune-evasion molecule[30]. Lower levels were observed for the other immune evasion molecules CD278 (ICOS), CD275 (B7-H2) and CD152 (CTLA-4) (Figure 2).

Molecular characterization

For further characterization, a comprehensive molecular analysis of the parental tumor and the HROC57 cell line was performed.

The tumor and the cell line showed a distinct degree of aneuploidy, moderate CpG island methylation and absent microsatellite instability. Mutational analysis revealed a wild-type *KRAS* status and, of note, a mutant *BRAF* status.

For assessment of larger genomic aberrations, single nucleotide polymorphisms and general gene expression, microarray analyses were performed. While the correspondent HROC57 B cell line showed no distinctive features, the HROC57 tumor cell line showed several aberrations. The tip of chromosome 8p showed losses, whereas gains in 7q and 19p were observed. Thus, HROC57 can be classified as only mildly chromosomal instable.

For further discrimination of HROC57, we performed an RNA expression analysis and compared HROC57 with 23 cell lines from colorectal adenocarcinomas also established in our lab. Within the 20 most over expressed genes, two neuroendocrine markers could readily be identified (CHGB and CPLX2). In addition, three genes involved in tumor immune modulation could be recognized (TRBC1, CTLA4 and LAT). Contrary to that, HROC57 did not express NOX1 and OLFM4, genes known for promoting colorectal adenocarcinoma.

Morphology and viral contamination

As determined by phase contrast microscopy, cells adhered tightly to the cell culture flask. The cell line was growing as monolayers on conventional tissue culture

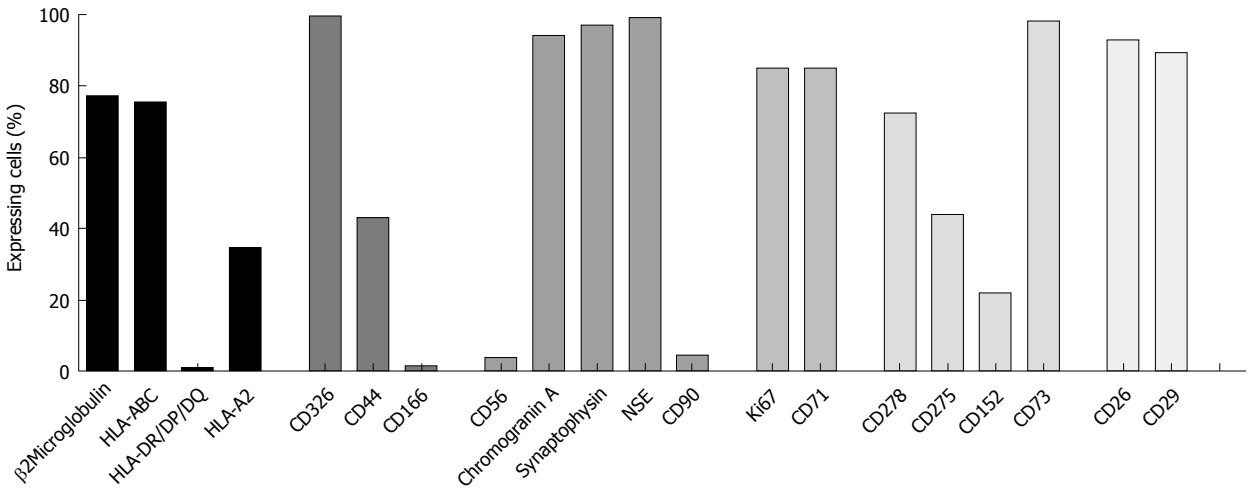
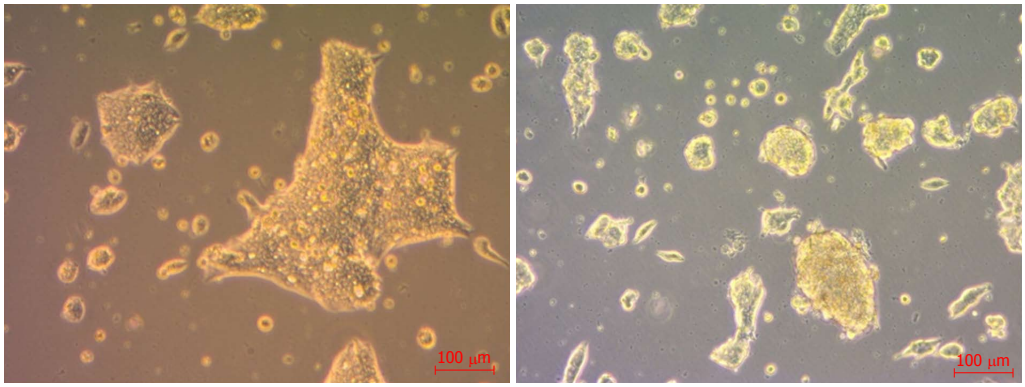


Figure 2 Expression of human lymphocyte antigen molecules (ß2Microglobulin, HLA-ABC, HLA-DR/DP/DQ, HLA-A2, epithelial cell markers (CD26, CD29, CD44, CD166, CD326), neuroendocrine markers (CD56, Chromogranin A, Synapthophysin, NSE, CD90), proliferation markers (Ki67, CD71) and immune evasion molecules (CD73, CD152, CD275, CD278) were assessed by flow cytometry using a BD FACSARIA II.



HROC57 P10 HROC57 P22

Figure 3 Light microscopy of HROC57 after direct establishment (P10) and medium-term *in vitro* culture (P22). Cell lines were established directly from patients' tumor material as described in material and methods. Original magnification: × 100.

plastic and showed a stable outgrowth as defined by passaging > 50 times. HROC57 cells proliferated as polygonal cell colonies forming small grape-like cell clusters (Figure 3). Morphology did not change during long term passage (up to 50 passages). As determined by semi-quantitative PCR, HROC57 cell line was free of mycoplasma and several other potential contaminants (JC/BK and SV40) which have been described for colorectal cell lines (data not shown)

Chemo- and radiosensitivity of HROC57 cells

To assess the sensitivity of HROC57 cells to a variety of current chemotherapeutic drugs, standard proliferation and cytotoxicity assays were performed. In particular, sensitivity to etoposide and cisplatin was analyzed as actual guidelines suggest the combination of etoposide and cisplatin as first line chemotherapy for advanced NEC[11].

HROC57 showed a high sensitivity towards etoposide and cisplatin with a distinct increase of sensitivity after combination of cisplatin and etoposide (Figure 4) especially if patient plasma drug levels are set for reference (Table 2). Examination of the sensitivity of HROC57 cells to further chemotherapeutic drugs showed their high sensitivity to 5-FU, oxaliplatin, irinotecan, the histone deacetylase inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA), the mTOR inhibitor rapamycin and the drug combinations Folfox and Folfiri (Table 2). Again, when patient plasma levels are set for reference, measured IC_{50} concentrations were below those plasma concentrations (Table 2).

HROC57 cells were not completely resistant towards γ-radiation but even a radiation with 50Gy did not completely abolish further growth of the cell line (Figure 5). In comparison to several other patient-derived HROC cell lines established in our lab, they can be ranked as intermediate radiation sensitive (data not shown).

Remarkably, the sensitivity of HROC57 cells to chemotherapeutics is independent from the presence of wild-type p53 (Figure 6), since HROC57 cells (and p53-negative HCT116 cells used as controls) lack p53 protein expression as analyzed by Western-blot (Figure

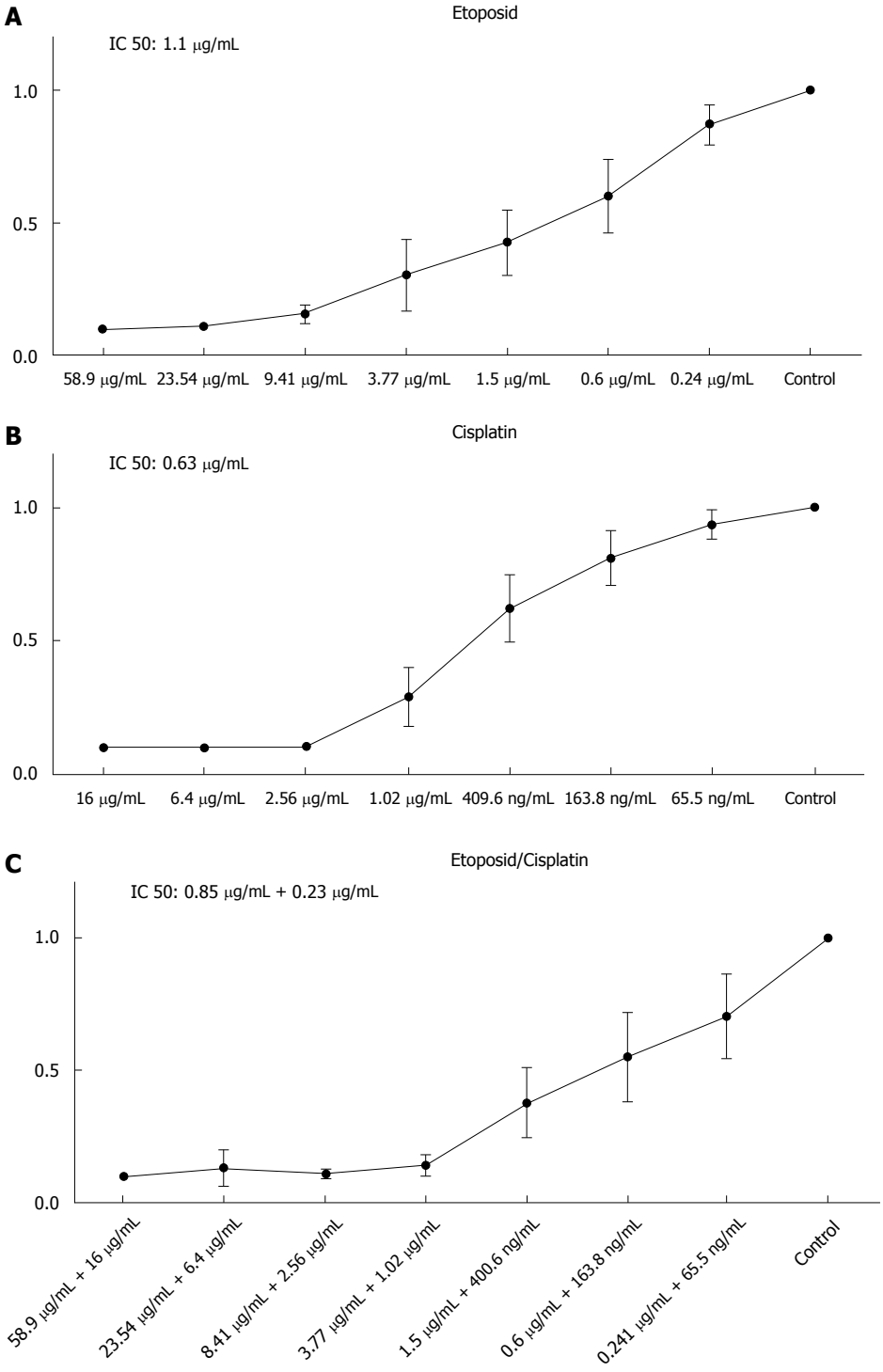


Figure 4 Sensitivity of HROC57 to conventional chemotherapeutics. HROC57was treated for 72 h with etoposide (A), cisplatin (B) and combination of etoposide and cisplatin (C). Cell viability was measured using the crystal violet assay as described in materials and methods. Values represent the mean absorbance at 570 nm ± SD of *n* = 4 analyses.

4A). P53-negative tumor cells typically express less of the p53-activated factor p21 and more of the p53-repressed factor survivin[31] - as can easily be depicted in direct comparison to p53-positive HCT116 cells (Figure 4B). Moreover, HDAC1 and HDAC2 are both expressed by HROC57 cells; and this correlates with their sensitivity to SAHA (Table 2).

DISCUSSION

Currently, only a small number of GEP-NEN cell lines have been established and most of them are insufficiently characterized, especially with regard to the latest WHO classification and its proliferation based grading system[13].

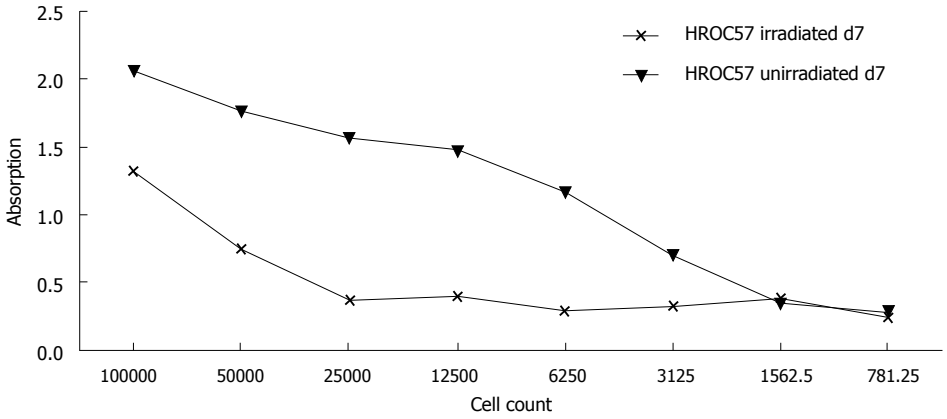


Figure 5 Radiosensitivity of HROC57. HROC57 was irradiated with 50 Gy using a ^{137}CS-source. Control cells were not irradiated. Cell viability was measured using the crystal violet assay as described in materials and methods. *n* = 2 analyses.

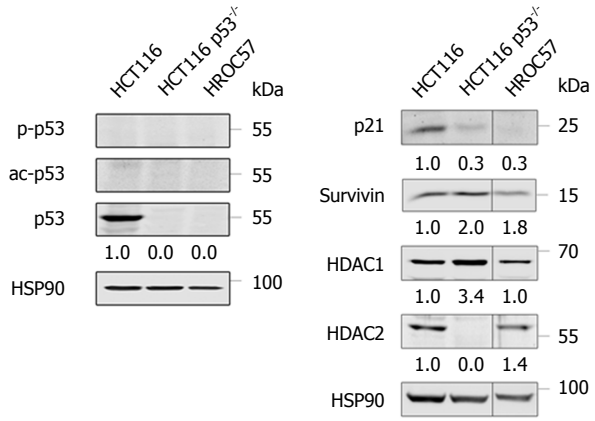


Figure 6 Expression of p53 and some of its targets in HROC57 cells. Western Blot analysis of untreated HROC57 cells compared to HCT116 (p53 wildtype) and HCT116 p53$^{-/-}$ was performed. Acetylation (K382) and phosphorylation (S15) of p53 (A) and expression of its targets p21, survivin and HDAC 1 and 2 (B) were assessed using specific antibodies. HSP90 served as loading control. Numbers indicate densitometric analysis of respective signals normalized to loading control and relative to HCT116 cells.

Here, we report on the first characterization of a poorly differentiated large cell NEC that could be established directly from a primary tumor in the ascending colon. HROC57 cells showed a typical neuroendocrine cytology and a profile of neuro-endocrine markers that are commonly used for diagnosis of neuroendocrine tumors. Notably, flow cytometry revealed high expression of chromogranin A, synaptophysin and neuron specific enolase, which are common markers of neuroendocrine tumors. Only CD56 was expressed to a very low extend. Reflecting the high proliferative activity of the tumor cells, the proliferation markers Ki-67 and CD71 were highly expressed. Gene expression analysis unveiled the upregulation of CPLX2, which was recently identified as a new prognostic biomarker in human lung high grade neuroendocrine tumors[32] and thus might be worth being tested as a prognostic biomarker in GEP-NET as well.

As therapeutical interference with tumor immune escape mechanisms plays a more and more important role in clinical oncologic, we analyzed several key molecules of immune escape. HROC57 cells express high levels of CD73, which has been described as immune-evasion molecule and overexpression was observed in various tumor tissues[30]. CD73 functions as a rate-limiting enzyme in the generation of extracellular adenosine[30] and recent studies could show that increased adenosine levels result in immune tolerance by accumulating in the tumor environment and contribute to a cancer growth promoting environment. Adenosine not only functions in the tumor microenvironment, but is also involved in the regulation of proliferation, differentiation and apoptosis of cancer cells[33]. Additionally, we could demonstrate an upregulation of CTLA-4 on HROC57 cells. CTLA-4 is an immunoglobulin superfamily receptor and was the first immune-checkpoint receptor that was clinically targeted using blocking antibodies[34]. PD-1 is another member of this superfamily. This finding might open new therapeutical opportunities for NET/NEC. Tumor models like HROC57 could also help to develop immune checkpoint inhibiting strategies and subsequently optimize therapeutical applications.

In addition to the neuroendocrine markers, HROC57 cells highly express EpCAM (CD326), thus reflecting a possible epithelial, rather than neural crest provenance as was thought of these tumors[35]. Khan *et al*[36] analyzed EpCAM expression in several neuroendocrine tumors and found high expression of EpCAM in all analyzed midgut NET (ileal, pancreatic and gastric) but no colonic NET were included in this study[36]. Hence, this is the first report of high EpCAM expression in a colon-derived NEC. EpCAM plays an important role in promoting cell cycling and proliferation by upregulating c-myc and cyclins[37]. Therefore, EpCAM directed therapy might be an additional option in this tumor entity[38]. Moreover, expression of EpCAM in NET/NEC opens up opportunities to be further developed as biomarker and relapse prediction by screening for circulating tumor cells[39].

Molecular analysis of HROC57 revealed mild chromosomal instability with several chromosomal gains

Table 2 Measured IC_{50} values and selected human plasma reference levels of analyzed chemotherapeutic agents

Drug	IC_{50}	Plasma reference
5-FU	10.53 μg/mL	50 μg/mL[44]
SAHA	0.249 μg/mL	
Oxaliplatin	701 ηg/mL	2 μg/mL[45]
Folfox	5.48 μg/mL + 137.1 ηg/mL	
Irinotecan	0.745 μg/mL	1 μg/mL[46]
Folfiri	1 μg/mL + 0.674 μg/mL	
Rapamycin	4.8 μg/mL	5-100 mg/L[47]

References for human plasma levels are set in brackets. SAHA: Suberoylanilide hydroxamic acid.

and losses. In particular, gains in 7q und 19p and a prominent loss in the tip of 8q were found. Little is known of the prevalence of these events in colonic NEC. Krieg *et al*[13] also described gains in 7q but not in 19p in their lymph node metastasis-derived NEC cell line NEC-DUE2, while gains in 19p are reported up to 50% in small intestine NEN[40]. Interestingly, we could not observe the reported deletions of 18q that are characteristic of midgut NEN[41]. Mutational analysis revealed wild-type *KRAS* but a mutant *BRAF* status. Karkouche and coworkers analyzed these mutations in a consecutive series of 12 colonic NECs[42]. They demonstrated a *KRAS*/*BRAF* mutation in six out of twelve tumors. They concluded that these *KRAS*/*BRAF* mutations might indicate that colorectal NEC may correspond to a high-grade transformation of colorectal carcinoma[42].

Generally, gastrointestinal NEC shows an aggressive and chemoresistant phenotype that often results in a rapid progressive course of disease[11]. A basic requirement for determining initial drug sensitivity and for predicting response is the maintenance of the original tumors' signature[14]. With this principal condition fulfilled by the freshly established HROC57 cell line, the sensitivity towards a large panel of chemotherapeutic drugs was tested. Etoposide and cisplatin were included as actual guidelines suggest this combination as first line chemotherapy for advanced NEC[11]. Of note, HROC57 cells showed high sensitivity towards etoposide and cisplatin alone and even a distinct increase in sensitivity when both drugs were combined. While this sensitivity does not require the expression and activity of wild-type p53, the poor radiation sensitivity may be attributable to the lack of wild-type p53[43]. We should though mention that the *ex vivo* sensitivity of HROC57 cells does not recapitulate the clinical course of the patient who died within one year after diagnosis and operation due to rapid progressive disease. This discrepancy can best be explained by the fact that this potentially beneficial chemotherapeutic regimen was only applied in a situation with vast tumor recurrence, diffuse peritoneal and lung metastases and could not prevent the death of the patient one month later.

Examination of further chemotherapeutic drugs showed interestingly a likewise high sensitivity to 5-FU, SAHA, oxaliplatin, irinotecan, rapamycin and the drug combinations Folfox and Folfiri. This finding is in contrast to Krieg *et al*[13] who reported a high chemoresistance of their colonic NEC-cell line. A detailed analysis of these differences in chemosensitivity is recommended in the near future in order to identify similarities and disparities between primary and metastatic NEC cells.

At last, analysis of radiosensitivity revealed that HROC57 cells were relatively resistant towards γ-radiation but even a radiation with 50 Gy did not completely abolish further growth of the cell line. This observation is well in line with actual treatment algorithms of NEC which no longer recommend radiotherapy[11].

Our data emphasize the use of the HROC57 models for further basic and preclinical research to gain insight into the tumor biology of large cell neuroendocrine carcinoma, to develop and to optimize therapy regimens or to identify novel biomarkers. Albeit not emphasized in this manuscript, a non-tumorous B cell line of HROC57 is available beside the tumor models. Thus, comparative analyses of NET cells and matching normal cells are easily possible.

ARTICLE HIGHLIGHTS

Research background

Only a few gastrointestinal neuroendocrine tumor lines have been published in the last decades. A major problem is the very heterogeneous pathological terminology when trying to classify these cell lines according to the lately revised WHO classification with regard to their original tumors. Particularly, reports of poorly differentiated neuroendocrine carcinoma (NEC) are very scarce mainly due to their low incidence.

Research motivation

Gaining insights in the biology of large cell NEC is essential for the identification of potentially therapeutic molecular targets of this highly malignant neoplasia. Individual tumor models deliver exceptional tools for further research of these objectives. However, well characterized and low passage NEC models are still rare.

Research objectives

Main objective of the study was the establishing and profound characterization of an patient derived ultra-low passage NEC cell line and corresponding patient-derived xenograft (PDX) model that allows drug response testing and prediction.

Research methods

Cell line establishment could be realized from direct *in vitro* culturing of colonic NEC tissue. In addition, a PDX model could be established from frozen tumor samples. Profound analysis of morphological features, invasive and migratory behavior as well as expression of neuroendocrine markers was done. Detailed phenotypic analysis was performed by microscopy and multicolor flow cytometry. Chromosomal aberrations were mapped by array comparative genomic hybridization and DNA profiling was analyzed by DNA fingerprinting. At last drug responsiveness was evaluated and the sensitivity against chemotherapeutic agents assessed.

Research results

The cell line displayed characteristic morphological and molecular features of large cell NEC with KI-67 > 50%. *In vitro* and *in vivo* experiments demonstrated that the cell line retained their malignant properties. Molecular-pathological analysis revealed a CpG island promoter methylation positive cell line with

microsatellite instability being absent. The KRAS gene was not mutated whereas a BRAF V600E mutation was detected. A high sensitivity to such drugs as etoposide, cisplatin and 5-FU could be observed with a more resistant phenotype to rapamycin.

Research conclusions

Taken together, this study describes the development and basic characterization of powerful matched *in vitro* and *in vivo* patient-derived models not only to perform basic research to better understand the biology of NECs, but also to establish novel therapeutic options.

Research perspectives

This descriptive study exemplifies the methodology and characterization of a large cell NEC cell line directly from original patients' tumor material. This will help to improve the ability for personalizing tumor therapy in the near future.

REFERENCES

1 **Van Buren G 2nd**, Rashid A, Yang AD, Abdalla EK, Gray MJ, Liu W, Somcio R, Fan F, Camp ER, Yao JC, Ellis LM. The development and characterization of a human midgut carcinoid cell line. *Clin Cancer Res* 2007; **13**: 4704-4712[PMID: 17699847 DOI: 10.1158/1078-0432.CCR-06-2723]

2 **Yao JC**, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, Abdalla EK, Fleming JB, Vauthey JN, Rashid A, Evans DB. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J Clin Oncol* 2008; **26**: 3063-3072 [PMID: 18565894 DOI: 10.1200/JCO.2007.15.4377]

3 **Modlin IM**, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A. Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008; **9**: 61-72 [PMID: 18177818 DOI: 10.1016/S1470-2045(07)70410-2]

4 **Schnirer II**, Yao JC, Ajani JA. Carcinoid--a comprehensive review. *Acta Oncol* 2003; **42**: 672-692 [PMID: 14690153]

5 **Wiedenmann B**, John M, Ahnert-Hilger G, Riecken EO. Molecular and cell biological aspects of neuroendocrine tumors of the gastroenteropancreatic system. *J Mol Med* (Berl) 1998; **76**: 637-647 [PMID: 9725766]

6 **Bishop AE**, Polak JM, Facer P, Ferri GL, Marangos PJ, Pearse AG. Neuron specific enolase: a common marker for the endocrine cells and innervation of the gut and pancreas. *Gastroenterology* 1982; **83**: 902-915 [PMID: 7106520]

7 **Schott M**, Klöppel G, Raffel A, Saleh A, Knoefel WT, Scherbaum WA. Neuroendocrine neoplasms of the gastrointestinal tract. *Dtsch Arztebl Int* 2011; **108**: 305-312 [PMID: 21629514 DOI: 10.3238/arztebl.2011.0305]

8 **Bosman FT**, Caneiro F. WHO classification of tumours of the digestive system. Lyon: IARC Press 2010

9 **Klöppel G**, Couvelard A, Perren A, Komminoth P, McNicol AM, Nilsson O, Scarpa A, Scoazec JY, Wiedenmann B, Papotti M, Rindi G, Plöckinger U; Mallorca Consensus Conference participants; European Neuroendocrine Tumor Society. ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: towards a standardized approach to the diagnosis of gastroenteropancreatic neuroendocrine tumors and their prognostic stratification. *Neuroendocrinology* 2009; **90**: 162-166 [PMID: 19060454 DOI: 10.1159/000182196]

10 **Öberg K**, Knigge U, Kwekkeboom D, Perren A; ESMO Guidelines Working Group. Neuroendocrine gastro-entero-pancreatic tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2012; **23** Suppl 7: vii124-vii130 [PMID: 22997445 DOI: 10.1093/annonc/mds295]

11 **Coriat R**, Walter T, Terris B, Couvelard A, Ruszniewski P. Gastroenteropancreatic Well-Differentiated Grade 3 Neuroendocrine Tumors: Review and Position Statement. *Oncologist* 2016; **21**: 1191-1199 [PMID: 27401895 DOI: 10.1634/theoncologist.2015-0476]

12 **Fazio N**, Spada F, Giovannini M. Chemotherapy in gastroenteropancreatic (GEP) neuroendocrine carcinomas (NEC): a critical view. *Cancer Treat Rev* 2013; **39**: 270-274 [PMID: 22819619 DOI: 10.1016/j.ctrv.2012.06.009]

13 **Krieg A**, Mersch S, Boeck I, Dizdar L, Weihe E, Hilal Z, Krausch M, Möhlendick B, Topp SA, Piekorz RP, Huckenbeck W, Stoecklein NH, Anlauf M, Knoefel WT. New model for gastroenteropancreatic large-cell neuroendocrine carcinoma: establishment of two clinically relevant cell lines. *PLoS One* 2014; **9**: e88713 [PMID: 24551139 DOI: 10.1371/journal.pone.0088713]

14 **Maletzki C**, Gock M, Randow M, Klar E, Huehns M, Prall F, Linnebacher M. Establishment and characterization of cell lines from chromosomal instable colorectal cancer. *World J Gastroenterol* 2015; **21**: 164-176 [PMID: 25574089 DOI: 10.3748/wjg.v21.i1.164]

15 **Kuehn F**, Mullins CS, Krohn M, Harnack C, Ramer R, Krämer OH, Klar E, Huehns M, Linnebacher M. Establishment and characterization of HROC69 - a Crohn´s related colonic carcinoma cell line and its matched patient-derived xenograft. *Sci Rep* 2016; **6**: 24671 [PMID: 27087592 DOI: 10.1038/srep24671]

16 **van de Wetering M**, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, McLaren-Douglas A, Blokker J, Jaksani S, Bartfeld S, Volckman R, van Sluis P, Li VS, Seepo S, Sekhar Pedamallu C, Cibulskis K, Carter SL, McKenna A, Lawrence MS, Lichtenstein L, Stewart C, Koster J, Versteeg R, van Oudenaarden A, Saez-Rodriguez J, Vries RG, Getz G, Wessels L, Stratton MR, McDermott U, Meyerson M, Garnett MJ, Clevers H. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 2015; **161**: 933-945 [PMID: 25957691 DOI: 10.1016/j.cell.2015.03.053]

17 **Wiegering A**, Matthes N, Mühling B, Koospal M, Quenzer A, Peter S, Germer CT, Linnebacher M, Otto C. Reactivating p53 and Inducing Tumor Apoptosis (RITA) Enhances the Response of RITA-Sensitive Colorectal Cancer Cells to Chemotherapeutic Agents 5-Fluorouracil and Oxaliplatin. *Neoplasia* 2017; **19**: 301-309 [PMID: 28284059 DOI: 10.1016/j.neo.2017.01.007]

18 **Medico E**, Russo M, Picco G, Cancelliere C, Valtorta E, Corti G, Buscarino M, Isella C, Lamba S, Martinoglio B, Veronese S, Siena S, Sartore-Bianchi A, Beccuti M, Mottolese M, Linnebacher M, Cordero F, Di Nicolantonio F, Bardelli A. The molecular landscape of colorectal cancer cell lines unveils clinically actionable kinase targets. *Nat Commun* 2015; **6**: 7002 [PMID: 25926053 DOI: 10.1038/ncomms8002]

19 **Maletzki C**, Stier S, Gruenert U, Gock M, Ostwald C, Prall F, Linnebacher M. Establishment, characterization and chemosensitivity of three mismatch repair deficient cell lines from sporadic and inherited colorectal carcinomas. *PLoS One* 2012; **7**: e52485 [PMID: 23300683 DOI: 10.1371/journal.pone.0052485]

20 **Fielding LP**, Arsenault PA, Chapuis PH, Dent O, Gathright B, Hardcastle JD, Hermanek P, Jass JR, Newland RC. Clinicopathological staging for colorectal cancer: an International Documentation System (IDS) and an International Comprehensive Anatomical Terminology (ICAT). *J Gastroenterol Hepatol* 1991; **6**: 325-344 [PMID: 1912440]

21 **Ostwald C**, Linnebacher M, Weirich V, Prall F. Chromosomally and microsatellite stable colorectal carcinomas without the CpG island methylator phenotype in a molecular classification. *Int J Oncol* 2009; **35**: 321-327 [PMID: 19578746]

22 **Ogino S**, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009; **58**: 90-96 [PMID: 18832519 DOI: 10.1136/gut.2008.155473]

23 **Goodenough DJ**. The use of ROC curves in testing the proficiency of individuals in classifying pneumoconiosis. *Radiology* 1975; **114**: 472-473 [PMID: 1111020]

24 **Klier U**, Maletzki C, Klar E, Linnebacher M. Generation of highly pure fusions of colorectal carcinoma and antigen-presenting cells. *Langenbecks Arch Surg* 2010; **395**: 365-371 [PMID: 20309577

DOI: 10.1007/s00423-010-0598-1]
25 **Ramer R**, Hinz B. Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1. *J Natl Cancer Inst* 2008; **100**: 59-69 [PMID: 18159069 DOI: 10.1093/jnci/djm268]
26 **Beyer M**, Kiweler N, Mahboobi S, Krämer OH. How to Distinguish Between the Activity of HDAC1-3 and HDAC6 with Western Blot. *Methods Mol Biol* 2017; **1510**: 355-364 [PMID: 27761834]
27 **Gock M**, Kühn F, Mullins CS, Krohn M, Prall F, Klar E, Linnebacher M. Tumor Take Rate Optimization for Colorectal Carcinoma Patient-Derived Xenograft Models. *Biomed Res Int* 2016; **2016**: 1715053 [PMID: 27999790 DOI: 10.1155/2016/1715053]
28 **Davies S**, Beckenkamp A, Buffon A. CD26 a cancer stem cell marker and therapeutic target. *Biomed Pharmacother* 2015; **71**: 135-138 [PMID: 25960228 DOI: 10.1016/j.biopha.2015.02.031]
29 **Vassilopoulos A**, Chisholm C, Lahusen T, Zheng H, Deng CX. A critical role of CD29 and CD49f in mediating metastasis for cancer-initiating cells isolated from a Brca1-associated mouse model of breast cancer. *Oncogene* 2014; **33**: 5477-5482 [PMID: 24317509 DOI: 10.1038/onc.2013.516]
30 **Jin D**, Fan J, Wang L, Thompson LF, Liu A, Daniel BJ, Shin T, Curiel TJ, Zhang B. CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. *Cancer Res* 2010; **70**: 2245-2255 [PMID: 20179192 DOI: 10.1158/0008-5472.CAN-09-3109]
31 **Brandl A**, Wagner T, Uhlig KM, Knauer SK, Stauber RH, Melchior F, Schneider G, Heinzel T, Krämer OH. Dynamically regulated sumoylation of HDAC2 controls p53 deacetylation and restricts apoptosis following genotoxic stress. *J Mol Cell Biol* 2012; **4**: 284-293 [PMID: 22493095]
32 **Komatsu H**, Kakehashi A, Nishiyama N, Izumi N, Mizuguchi S, Yamano S, Inoue H, Hanada S, Chung K, Wei M, Suehiro S, Wanibuchi H. Complexin-2 (CPLX2) as a potential prognostic biomarker in human lung high grade neuroendocrine tumors. *Cancer Biomark* 2013; **13**: 171-180 [PMID: 23912489 DOI: 10.3233/CBM-130336]
33 **Antonioli L**, Blandizzi C, Pacher P, Haskó G. Immunity, inflammation and cancer: a leading role for adenosine. *Nat Rev Cancer* 2013; **13**: 842-857 [PMID: 24226193 DOI: 10.1038/nrc3613]
34 **Pardoll DM**. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; **12**: 252-264 [PMID: 22437870 DOI: 10.1038/nrc3239]
35 **Rindi G**, Leiter AB, Kopin AS, Bordi C, Solcia E. The "normal" endocrine cell of the gut: changing concepts and new evidences. *Ann NY Acad Sci* 2004; **1014**: 1-12 [PMID: 15153415]
36 **Khan MS**, Tsigani T, Rashid M, Rabouhans JS, Yu D, Luong TV, Caplin M, Meyer T. Circulating tumor cells and EpCAM expression in neuroendocrine tumors. *Clin Cancer Res* 2011; **17**: 337-345 [PMID: 21224371 DOI: 10.1158/1078-0432.CCR-10-1776]
37 **Maetzel D**, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M, Gires O. Nuclear signalling by tumour-associated antigen EpCAM. *Nat Cell Biol* 2009; **11**: 162-171 [PMID: 19136966 DOI: 10.1038/ncb1824]
38 **Raffel A**, Eisenberger CF, Cupisti K, Schott M, Baldus SE, Hoffmann I, Aydin F, Knoefel WT, Stoecklein NH. Increased EpCAM expression in malignant insulinoma: potential clinical implications. *Eur J Endocrinol* 2010; **162**: 391-398 [PMID: 20097833 DOI: 10.1530/EJE-08-0916]
39 **Schulze K**, Gasch C, Staufer K, Nashan B, Lohse AW, Pantel K, Riethdorf S, Wege H. Presence of EpCAM-positive circulating tumor cells as biomarker for systemic disease strongly correlates to survival in patients with hepatocellular carcinoma. *Int J Cancer* 2013; **133**: 2165-2171 [PMID: 23616258 DOI: 10.1002/ijc.28230]
40 **Tönnies H**, Toliat MR, Ramel C, Pape UF, Neitzel H, Berger W, Wiedenmann B. Analysis of sporadic neuroendocrine tumours of the enteropancreatic system by comparative genomic hybridisation. *Gut* 2001; **48**: 536-541 [PMID: 11247899]
41 **Löllgen RM**, Hessman O, Szabo E, Westin G, Akerström G. Chromosome 18 deletions are common events in classical midgut carcinoid tumors. *Int J Cancer* 2001; **92**: 812-815 [PMID: 11351300 DOI: 10.1002/ijc.1276]
42 **Karkouche R**, Bachet JB, Sandrini J, Mitry E, Penna C, Côté JF, Blons H, Penault-Llorca F, Rougier P, Saint André JP, Emile JF. Colorectal neuroendocrine carcinomas and adenocarcinomas share oncogenic pathways. A clinico-pathologic study of 12 cases. *Eur J Gastroenterol Hepatol* 2012; **24**: 1430-1437 [PMID: 23114745 DOI: 10.1097/MEG.0b013e3283583c87]
43 **Tomicic MT**, Christmann M, Kaina B. Topotecan triggers apoptosis in p53-deficient cells by forcing degradation of XIAP and survivin thereby activating caspase-3-mediated Bid cleavage. *J Pharmacol Exp Ther* 2010; **332**: 316-325 [PMID: 19812371]
44 **Bocci G**, Danesi R, Di Paolo AD, Innocenti F, Allegrini G, Falcone A, Melosi A, Battistoni M, Barsanti G, Conte PF, Del Tacca M. Comparative pharmacokinetic analysis of 5-fluorouracil and its major metabolite 5-fluoro-5,6-dihydrouracil after conventional and reduced test dose in cancer patients. *Clin Cancer Res* 2000; **6**: 3032-3037 [PMID: 10955781]
45 **Culy CR**, Clemett D, Wiseman LR. Oxaliplatin. A review of its pharmacological properties and clinical efficacy in metastatic colorectal cancer and its potential in other malignancies. *Drugs* 2000; **60**: 895-924 [PMID: 11085200]
46 **Chabot GG**. Clinical pharmacokinetics of irinotecan. *Clin Pharmacokinet* 1997; **33**: 245-259 [PMID: 9342501]
47 **Widmer N**, Bardin C, Chatelut E, Paci A, Beijnen J, Levêque D, Veal G, Astier A. Review of therapeutic drug monitoring of anticancer drugs part two--targeted therapies. *Eur J Cancer* 2014; **50**: 2020-2036 [PMID: 24928190 DOI: 10.1016/j.ejca.2014.04.015]

P- Reviewer: Adachi Y **S- Editor**: Wang XJ **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3760-3769

DOI: 10.3748/wjg.v24.i33.3760

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

ORIGINAL ARTICLE

Basic Study

Metabolomic alterations and chromosomal instability status in gastric cancer

Cheng-Kun Tsai, Ta-Sen Yeh, Ren-Chin Wu, Ying-Chieh Lai, Meng-Han Chiang, Kuan-Ying Lu, Cheng-Yu Hung, Hung-Yao Ho, Mei-Ling Cheng, Gigin Lin

Cheng-Kun Tsai, Meng-Han Chiang, Hung-Yao Ho, Mei-Ling Cheng, Gigin Lin, Clinical Metabolomics Core Lab, Chang Gung Memorial Hospital at Linkou and Chang Gung University, Taoyuan 333, Taiwan

Cheng-Kun Tsai, Ying-Chieh Lai, Meng-Han Chiang, Kuan-Ying Lu, Cheng-Yu Hung, Gigin Lin, Department of Medical Imaging and Intervention, Imaging Core Lab, Institute for Radiological Research, Chang Gung Memorial Hospital at Linkou and Chang Gung University, Taoyuan 333, Taiwan

Ta-Sen Yeh, Department of Surgery, Chang Gung Memorial Hospital at Linkou and Chang Gung University, Taoyuan 333, Taiwan

Ren-Chin Wu, Department of Pathology, Chang Gung Memorial Hospital at Linkou and Chang Gung University, Taoyuan 333, Taiwan

Hung-Yao Ho, Department of Medical Biotechnology and Laboratory Science, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

Mei-Ling Cheng, Department of Biomedical Science, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

ORCID number: Cheng-Kun Tsai (0000-0002-9214-2801); Ta-Sen Yeh (0000-0002-1830-9466); Ren-Chin Wu (0000-0003-1439-0874); Ying-Chieh Lai (0000-0003-0148-9488); Meng-Han Chiang (0000-0002-2697-4757); Kuan-Ying Lu (0000-0002-4677-9000); Cheng-Yu Hung (0000-0003-4158-5079); Hung-Yao Ho (0000-0001-5990-6622); Mei-Ling Cheng (0000-0003-2006-133X); Gigin Lin (0000-0001-7246-1058).

Author contributions: Lin G conceived and designed the experiments; Yeh TS, Chiang MH, Lu KY and Hung CY performed the experiments; Tsai CK and Chiang MH analyzed the data; Wu RC, Lai YC, Ho HY and Cheng ML contributed reagents/materials/analysis tools; Tsai CK and Lin G wrote the paper.

Supported by the Ministry of Science and Technology Taiwan grant, No. MOST 106-2314-B-182A-019-MY3; the Chang Gung Foundation, No. CMRPG3E1321-2, No. IRB201601916B0, and No. IRB103-7448B.

Institutional review board statement: All procedures in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Unsolicited manuscript

Correspondence to: Gigin Lin, MD, PhD, Associate Professor, Department of Medical Imaging and Intervention, Imaging Core Lab, Institute for Radiological Research, Chang Gung Memorial Hospital at Linkou and Chang Gung University, No. 5, Fuxing St., Guishan Dist., Taoyuan 333, Taiwan. giginlin@cgmh.org.tw
Telephone: +886-3-3281200
Fax: +886-3-3971936

Received: May 22, 2018
Peer-review started: May 23, 2018
First decision: June 13, 2018
Revised: June 27, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 7, 2018

Abstract

AIM

To explore the correlation of metabolomics profiles of

gastric cancer (GC) with its chromosomal instability (CIN) status.

METHODS

Nineteen GC patients were classified as CIN and non-CIN type by The Cancer Genome Atlas Research Group system, based on 409 oncogenes and tumor suppressor genes sequenced. The aqueous metabolites of the GC tumor and its surrounding adjacent healthy tissues were identified through liquid chromatography-mass spectrometry. Groups were compared by defining variable importance in projection score of > 1.2, a fold change value or its reciprocal of > 1.2, and a *P* value of < 0.05 as a significant difference.

RESULTS

In total, twelve men and seven women were enrolled, with a median age of 66 years (range, 47-87 years). The numbers of gene alterations in the CIN GC group were significantly higher than those in the non-CIN GC (32-218 *vs* 2-17; $P < 0.0005$). Compared with the adjacent healthy tissues, GC tumors demonstrated significantly higher aspartic acid, citicoline, glutamic acid, oxidized glutathione, succinyladenosine, and uridine diphosphate-N-acetylglucosamine levels, but significantly lower butyrylcarnitine, glutathione hydroxyhexanoycarnitine, inosinic acid, isovalerylcarnitine, and threonine levels (all $P < 0.05$). CIN tumors contained significantly higher phosphocholine and uridine 5′-monophosphate levels but significantly lower beta-citryl-L-glutamic acid levels than did non-CIN tumors (all $P < 0.05$). CIN GC tumors demonstrated additional altered pathways involving alanine, aspartate, and glutamate metabolism, glyoxylate and dicarboxylate metabolism, histidine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis.

CONCLUSION

Metabolomic profiles of GC tumors and the adjacent healthy tissue are distinct, and the CIN status is associated with downstream metabolic alterations in GC.

Key words: Gastric cancer; Metabolomics; Oncogene; Copy-number; Chromosomal instability; Liquid chromatography-mass spectrometry

Core tip: We studied the correlation of the comprehensive metabolomic profiles of gastric cancer with its chromosomal instability (CIN) status. In this disease landscape study with no pre-specified hypothesis, we combined a gene molecule classification method with a metabolomics method to discover metabolic information for accurate tumor classification. CIN status-based metabolomic profiling has demonstrated translational potential in biomarker discovery and novel therapeutics development.

Tsai CK, Yeh TS, Wu RC, Lai YC, Chiang MH, Lu KY, Hung CY, Ho HY, Cheng ML, Lin G. Metabolomic alterations and chromosomal instability status in gastric cancer. *World J Gastroenterol* 2018; 24(33): 3760-3769 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3760.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3760

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide[1,2], with the highest incidence rates in Asia[3]. Most patients with GC are diagnosed in the advanced stage and thus have poor prognosis and limited treatment options[4]. GC can be histologically classified using the Lauren classification system, developed in 1965, which divides GC into diffuse and intestinal subtypes[5]. Although this classification system can suggest surgical choices, it cannot provide precise information of treatments suitable for individual patients[6]. In addition to histological subtypes, the clinicopathological characteristics of GC vary from case to case, making it difficult to identify detailed subtypes and to choose a subtype-optimized therapeutic approach[7].

The Cancer Genome Atlas (TCGA) Research Group has developed molecular classification systems based on gene expression profiling[8]. The TCGA network has provided sequencing- and array-based approaches to investigate exome sequences, copy-number alterations, gene expression, DNA methylation, and protein activities in GC[7,9] and classified GC into four subtypes: Epstein Barr Virus positive, microsatellite unstable, chromosomal instability (CIN), and genomically stable (GS)[8]. The GS and CIN subtypes are the most common and are distinguished by low-*vs*-high somatic copy-number variation. In general, GS tumors are of the Lauren diffuse subtype; they are typically diagnosed at a young age, and thus have insufficient time to accumulate mutations[10]. By definition, CIN tumors have a high degree of somatic copy-number variation, and they account for nearly half of all GCs, making them the predominant cancer subtype in the gastroesophageal junction or cardia[11]. CIN GCs are the most common Lauren intestinal subtype; however, molecularly and histologically, CIN GCs comprise a highly heterogeneous group of tumors. In addition, an easy-to-use biomarker for CIN is still not available[12].

Metabolomics may offer practical solutions to the traditional methods for GC detection and treatment[13]. Metabolites are not merely the end product of gene expression; they are the result of the interaction of the system's genome with its environment. They are an integral part of any cellular regulatory system[9]. Liquid chromatography-mass spectrometry (LC-MS) is the most common method of analysis. Several biomarkers have been proposed for GC diagnosis, prognosis, and surveillance[14-16]. Systematic reviews have demonstrated

variation in the relative abundance of the metabolites of glycolysis, lactic acid fermentation, de novo lipid, and amino acid synthesis in biological samples of patients with GC compared with controls[13,17,18]. Glutamine is the most consistent biomarker, showing upregulation in the serum, urine, and tumor tissues of patients with GC[18]. Thus far, numerous biomarkers discovered from metabolomic studies may play a noteworthy role in GC with regard to early-stage detection, diagnosis, prognosis, drug development, and chemosensitivity predictions[13], but the evidence of metabolomics' association with CIN status remains lacking.

We hypothesize that metabolic alternations reflect the CIN or GS status of GC and aim to study the comprehensive metabolomic profiles of GC and correlate them to CIN status.

MATERIALS AND METHODS

Patients and histopathology

This was a disease landscape study with no pre-specified hypothesis. The institutional review board approved the protocol of this prospective study (IRB103-7448B), and informed consents were obtained in a tertiary referral center with a dedicated GC interdisciplinary team to screen patient enrollment. From May 2015 to April 2017, we screened a consecutive cohort of patients with GC. The inclusion criteria were (1) histologically confirmed adenocarcinoma of the stomach, (2) surgical resection of primary GC, (3) age of 20-80 years, and complete pathological, surgical, treatment, and follow-up data. Exclusion criteria were (1) patients received neoadjuvant chemotherapy or chemoradiation therapy, (2) tumor size < 1 cm on computed tomography (CT), (3) prior gastric surgery, (4) anti-*Helicobacter pylori* eradication therapy, and (5) taking nonsteroidal anti-inflammatory drugs within 1 wk prior to surgery. A gastrointestinal pathologist (RCW) reviewed hematoxylin and eosin stain slides to select cases with estimated carcinoma. We used primary GC tissues for the genomic analysis and reevaluated the pathological diagnosis, histological Lauren subtype, invasion depth, and lymphovascular invasion in all tumors.

Genomics

Patient samples were classified as CIN or non-CIN type by the TCGA system. Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), and quantified using the Quant-iT dsDNA HS Assay (Invitrogen, Waltham, MA, United States). Genomic DNA (80 ng) was amplified using four pools of 15992 primer pairs (Ion AmpliSeq Comprehensive Cancer Panel, Life Technologies, Carlsbad, CA, United States) to target the coding exon regions of 409 cancer-related genes, which covered TP53/cell cycle, JAK/STAT, Ras/PI3K, Wnt, receptor tyrosine kinase, chromatin remodeling, DNA repair, TGF, and cadherin signaling. We classified patients with GC by tumor using the high and low proportion of the altered genes. The 409 oncogene and tumor suppressor genes in the GC tumor tissue were sequenced (Supplementary Table 1).

Hydrophilic metabolite extraction

Here, a modified Folch's method was employed[19]. In brief, 50 mg of homogenized tissue was transferred to a glass tube, to which 6 mL of chloroform/methanol (2:1, v/v) solution and 1.5 mL of water were added. The sample was vortexed four times for 30 s each and subsequently centrifuged at 700 × *g* for 30 min at 4 ℃. The upper phase (the hydrophilic phase and water soluble phase) and lower phase (the hydrophobic phase and lipid layer) were transferred to new glass tubes and then dried using nitrogen gas. The dried samples were stored at -80 ℃. Prior to analysis, the sample was dissolved in 200 μL of 40% methanol.

Global analysis of hydrophilic metabolites by LC-TOF-MS

Liquid chromatographic separation was achieved on a 100 × 2.1 mm^2 Acquity 1.7 μm C8 column (Waters, Milford, MA, United States) using an ACQUITY Ultra Performance Liquid Chromatography system (Waters, Milford, MA, United States). The column was maintained at 45 ℃ and at a flow rate of 0.5 mL/min. Samples were eluted from the liquid chromatography column using a linear gradient of: 0-2 min: 1%-80% B; 2-6.5 min: 80%-99% B; 6.5-8.0 min: 99% B; 8.1-10 min: 1% B for re-equilibration. Solvent A was water and solvent B was acetonitrile, and both contained 0.1% formic acid. The lyophilized sample was diluted with 200 μL of water/acetonitrile (95:5, v/v). Each sample was analyzed six times. MS was performed on Waters Q TOF-MS (SYNAPT HDMS; Waters MS Technologies, United Kingdom) operated in electrospray ionization (ESI)-positive ion mode. The desolvation gas was set to 700 L/h at 300 ℃, the cone gas at 25 L/h, and the source temperature at 80 ℃. The capillary and cone voltages were set to 3000 and 35 V, respectively. The micro-channel plate detector voltage was set to 1700 V. All analyses were acquired using a lock spray to ensure accuracy and reproducibility; sulfadimethoxine was used as the lock mass.

Metabolite identification

We analyzed the aqueous metabolites of the GC tumor and its surrounding adjacent healthy tissues through ESI+/− LC-MS by applying an untargeted metabolic approach to screen all potential metabolomic biomarkers[20]. Identified metabolites demonstrating notable differences between the control and test groups were searched against the METLIN database and the Human Metabolome Database (HMDB) by the m/z of their features. Potential metabolites were confirmed by local database according to the m/z and retention time under identical chromatographic conditions. MS and MS/MS analyses were performed under identical conditions.

Table 1 Clinical characteristics of the study

Term	TCGA system		*P* value
	CIN	non-CIN	(CIN *vs* non-CIN)
Number	9	10	
Age (median yr, range)	68.1 (56-79)	64.7 (47-87)	0.485
Sex (male/female)	7/2	5/5	0.233
Size (cm)	4.0 (1.8-6.9)	5.4 (2.3-11.6)	0.297
Stage			
Ⅰ	1	0	
Ⅱ	2	1	
Ⅲ	5	8	
Ⅳ	1	1	

TCGA: The Cancer Genome Atlas; CIN: Chromosomal instability.

MS/MS spectra were collected at 10 spectra/s, with a medium isolation window of approximately 4 m/z. The collision energy was set from 5 to 35 V. Several metabolites for the MS/MS analysis were confirmed through chemical standards under chromatographic conditions identical to those of the profiling experiment.

Data processing and statistical analysis

All MS data, namely retention times, m/z, and ion intensities, were extracted using MarkerLynx XS software (Waters, Milford, MA, United States) and inserted into a matrix. Metabolites were searched against the HMDB (http://www.hmdb.ca) or confirmed by in-house data (standards based on both retention times and MS spectra). The data were then analyzed through principal component analysis and partial least squares discriminate analysis (PLS-DA) using Markerlyn XS (Waters, Milford, MA, United States) and Metaboanalyst 3 (http://www.metaboanalyst.ca).

The variable importance in the projection (VIP) value of each variable in the model was calculated to indicate its contribution to the classification. A higher VIP value represented a stronger contribution to discrimination among the groups. VIP values > 1.2 were considered significant. The results are expressed as the mean ± standard deviation for continuous variables and as the number (percent) for categorical variables. Data were compared by 2-sample or paired Student's *t* test, analysis of variance, or chi-square test, when appropriate. A *P* value of < 0.05 was considered significant (Supplementary Tables 2 and 3).

RESULTS

Patient demographics

In total, 19 patients with GC enrolled in this study (median age, 66 years; range, 47-87 years) were divided into CIN and non-CIN types by using a 5% frequency of genetic variation as a demarcation point. We prospectively enrolled these 19 patients in a continuous cohort. No noteworthy difference in demographics was observed between the two groups (Table 1).

Metabolic alterations of GC tumors vs adjacent healthy tissues

The PLS-DA results of metabolite concentration distribution in the GC tumors and their surrounding healthy tissues are illustrated in Figure 1A and B. Compared with adjacent healthy tissue, GC tumors demonstrated significantly higher levels of butyrylcarnitine, hydroxyhexanoycarnitine, inosinic acid, isovalerylcarnitine, and threonine, but significantly lower levels of aspartic acid, citicoline, glutamic acid, glutamine, isoleucine, oxidized glutathione, proline, succinyladenosine, and xanthine (all $P < 0.05$; Table 2).

Metabolomic profiling of CIN vs non-CIN GC tumors

Among the 409 genes, GC patients exhibited had the highest proportion (53%) of mutations in *TP53*, followed by *JAK2*, *PSIP1*, and *PTPRO* (47%; Table 3). In the TCGA classification, the proportion of *TP53* mutations in the CIN-type population was 1.6-fold higher than that in the non-CIN type population. In the CIN GC group, all patients had alterations in *JAK2*, *PSIP1*, and *PTPRD* (Table 3).

The numbers of gene alterations in the CIN GC group were significantly higher than those in the non-CIN GC (32-218 *vs* 2-17; $P < 0.0005$). CIN GC tumors contained significantly higher levels of phosphocholine and uridine 5′-monophosphate ($P < 0.05$) and a significantly lower level of beta-citryl-L-glutamic acid ($P < 0.05$) than non-CIN GC tumors (Table 4). Compared with adjacent healthy tissues, 12 and six metabolites in CIN and non-CIN GC were significantly different, respectively (Table 5). CIN and non-CIN tumors demonstrated changes in glutamic acid, oxidized glutathione, and succinyladenosine levels, indicating alterations in aminoacyl-tRNA biosynthesis, arginine and proline metabolism, glutamine and glutamate metabolism, and glutathione metabolism. The metabolite distributions in GC tumors between CIN and non-CIN GC could be clearly distinguished (Figure 1C and D). CIN GC tumors demonstrated additional altered pathways involving alanine, aspartate, and glutamate metabolism, glyoxylate and dicarboxylate metabolism, histidine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis.

Metabolic transformation of CIN GC tumors vs adjacent healthy tissues

The CIN tumors had 31 genetic alterations (alteration frequency > 56%). *JAK2*, *PSIP1*, and *PTPRD* changed the gene copy-number in all patients with CIN GC. *CDH20*, *CDKN2A*, *DCC*, and *MALT1* demonstrated gene mutations and were changed in copy-number in 78% of patients. *TP53*, *AFF1*, *CDH1*, *CDH11*, *CDH2*, *CDH5*, *CDKN2B*, *MBD1*, *MMP2*, and *SMAD2* were altered in 67% of the patients. *ITGB2*, *ZNF521*, *ATM*, *BCL2*, *CYLD*, *ERG*, *FANCA*, *IL2*, *MAF*, *MTOR*, *PBRM1*, *RUNX1*, *TCF12*, and *TLR4* were additionally altered in 56% of

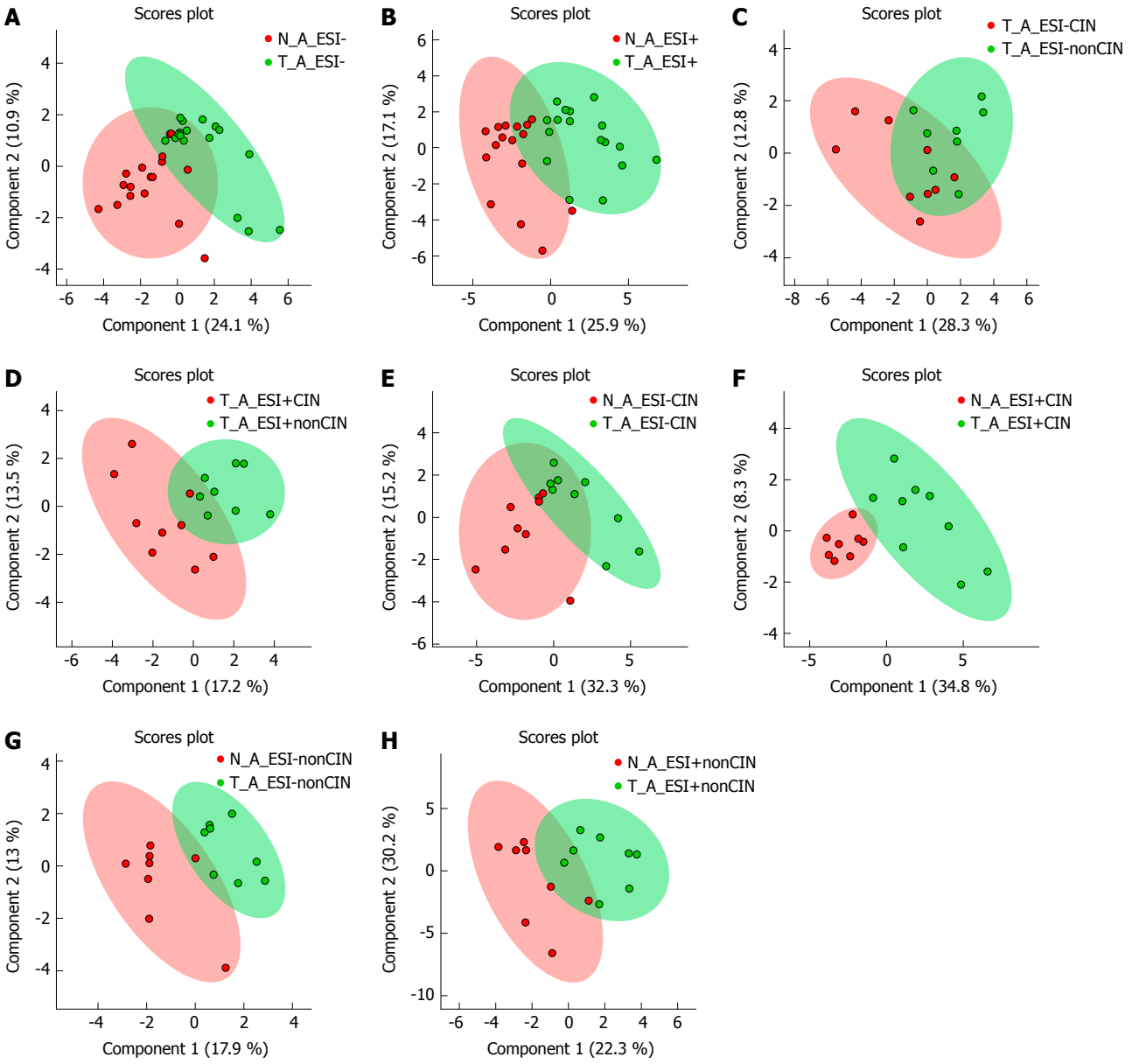


Figure 1 Metabolite-concentration distribution of gastric cancer tumor and its surrounding healthy tissue using the partial least squares discriminate analysis statistical method. A and B: Tumor (green, *n* = 19) *vs* healthy tissue (red, *n* = 19); C: and D: Tumor between chromosomal instability (CIN) type (red, *n* = 9) and non-CIN type (green, *n* = 10); E and F: Tumor (green, *n* = 9) and healthy tissue (red, *n* = 9) in the CIN type; G and H: Tumor (green, *n* = 10) and healthy tissue (red, *n* = 10) in the non-CIN type. A/C/E/G and B/D/F/H defined metabolites from ESI- and ESI+ LC-MS. Arrows indicate the variation in the two groups of metabolite distribution. The straight line indicates the difference of metabolite distribution between two patient types. Within the ellipse was the 95% confidence region. CIN: Chromosomal instability; ESI: Electrospray ionization; LC-MS: Liquid chromatography-mass spectrometry.

the patients (Table 3).

The metabolite distributions in tumor and healthy tissue could be clearly distinguished in CIN GC (Figure 1E and F). In the GC tumors, the levels of aspartic acid, citicoline, glutamic acid, oxidized glutathione, succinyladenosine, and uridine diphosphate-N-acetylglucosamine significantly increased, whereas those of butyrylcarnitine, glutathione hydroxyhexanoycarnitine, inosinic acid, isovalerylcarnitine, and threonine significantly decreased ($P < 0.05$) compared with the adjacent healthy tissues (Table 5).

These metabolic alterations indicated alterations in eight pathways (aminoacyl-tRNA biosynthesis, glutamine and glutamate metabolism, alanine, aspartate, and glutamate metabolism, glutathione metabolism, arginine and proline metabolism, histidine metabolism, glyoxylate and dicarboxylate metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis; $P < 0.05$).

Metabolic transformation of non-CIN GC tumors vs adjacent healthy tissues

The non-CIN tumors demonstrated most alterations in *TP53* and *SYNE1* (40%), followed by *ARID1A* (30%). *ITGB2*, *KMT2C*, *LRP1B*, *AKAP9*, *SAMD9*, *SMARCA4*, *TNK2*, *RNF213*, *EPHB6*, and *ERBB3* were altered in

Table 2 Altered metabolites with statistical significance between gastric cancer tumor tissue and its adjacent healthy tissue

Metabolites		VIP score (VIP > 1.2)	Fold change (tumor/healthy > 1.2 or < 0.8)	*P* value (*P* < 0.05)
Increased	Butyrylcarnitine	1.204	1.498	0.002
	Hydroxyhexanoycarnitine	2.021	2.342	0.002
	Inosinic acid	1.218	1.720	0.042
	Isovalerylcarnitine	1.424	2.265	0.006
	Threonine	1.316	1.911	0.019
Decreased	Aspartic acid	1.534	0.544	0.004
	Citicoline	1.973	0.371	0.005
	Glutamic acid	1.356	0.536	0.001
	Glutamine	1.221	0.518	0.005
	Isoleucine	1.323	0.593	0.015
	Oxidized glutathione	1.591	0.54	0.008
	Proline	1.231	0.574	0.001
	Succinyladenosine	1.855	0.293	0.001
	Xanthine	1.966	0.443	0.002

VIP: Variable importance in the projection.

Table 3 Altered genes in patients with gastric cancer

	Patients (%)	Alteration gene
All	53	*TP53*
	47	*JAK2, PSIP1, PTPRO*
	37	*ARID1A, CDH20, CDKN2A, DCC,ITGB2, MALT1, SYNE1, KMT2C*
	32	*AFF1, CDH1, CDH11, CDH2, CDH5, CDKN2B, LRP1B, MBD1, MMP2, SMAD2, SMAD4, ZNF521*
	26	*AKAP9, APC, ATM, BCL2, CYLD, EP400, ERG, FANCA, FN1, IL2, LTF, MAF, MTOR, PBRM1, RUNX1, SAMD9, SMARCA4, TAF1L, TCF12, TLR4, TNK2*
	< 21	364 genes
CIN tumor	100	*JAK2, PSIP1, PTPRD*
	78	*CDH20, CDKN2A, DCC, MALT1*
	67	*TP53, AFF1, CDH1, CDH11, CDH2, CDH5, CDKN2B, MBD1, MMP2, SMAD2*
	56	*ITGB2, ZNF521, ATM, BCL2, CYLD, ERG, FANCA, IL2, MAF, MTOR, PBRM1, RUNX1, TCF12, TLR4*
	44	43 genes
	33	70 genes
	< 22	265 genes
Non-CIN tumor	> 50	None
	40	*TP53, SYNE1*
	30	*ARID1A*
	20	*ITGB2, KMT2C, LRP1B, AKAP9, SAMD9, SMARCA4, TNK2, RNF213, EPHB6, ERBB3*
	10	50 genes
	0	346 genes

GC: Gastric cancer; CIN: Chromosomal instability.

Table 4 Altered metabolites with statistical significance between tumor and non-cancerous tissue

	Metabolites	VIP score (VIP > 1.2)	Fold change (CIN/non-CIN > 1.2 or < 0.8)	*P* value (*P* < 0.05)
Tumor tissue	Beta-citryl-L-glutamic acid	1.859	0.567	0.040
	Phosphocholine	1.998	2.093	0.017
	Uridine 5'-monophosphate	1.677	1.632	0.032
Non-cancerous tissue	NAD	1.649	0.607	0.027

VIP: Variable importance in the projection; CIN: Chromosomal instability; NAD: Nicotinamide adenine dinucleotide.

20% of the non-CIN tumors. The other 346 genes demonstrated no alterations (Table 3). The metabolite distributions in tumor and healthy tissue in the non-CIN GC could be clearly distinguished (Figure 1G and H). In the non-CIN tumors, the level of glutamic acid, oxidized glutathione, proline, succinyladenosine, and xanthine significantly increased ($P < 0.05$), whereas those of nicotinamide adenine dinucleotide (NAD) significantly decreased ($P = 0.047$), compared with the adjacent healthy tissues. The six metabolites and 13 genes were associated with four pathways in the non-CIN GC tumors: glutathione metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, and glutamine and glutamate metabolism.

Table 5 Altered metabolites with statistical significance between chromosomal instability and non-chromosomal instability types

		Metabolites	VIP score (VIP > 1.2)	Fold change (tumor/healthy > 1.2 or < 0.8)	*P* value (*P* < 0.05)
CIN tumor	Increased	Aspartic acid	1.309	2.029	0.043
		Citicoline	1.751	2.886	0.033
		Glutamic acid	1.219	2.101	0.011
		Oxidized glutathione	1.642	2.249	0.018
		Succinyladenosine	1.671	3.434	0.011
		Uridine diphosphate-N-acetylglucosamine	1.264	2.006	0.036
	Decreased	Butyrylcarnitine	1.218	0.590	0.014
		Glutathione	1.214	0.582	0.042
		Hydroxyhexanoycarnitine	1.883	0.397	0.013
		Inosinic acid	1.572	0.506	0.008
		Isovalerylcarnitine	1.657	0.346	0.004
		Threonine	1.714	0.394	0.006
non-CIN tumor	Increased	Glutamic acid	1.424	1.688	0.031
		Oxidized glutathione	1.678	1.676	0.019
		Proline	1.542	1.569	0.003
		Succinyladenosine	1.929	3.207	0.031
		Xanthine	2.360	2.085	0.007
	Decreased	NAD	1.584	0.599	0.047

VIP: Variable importance in the projection; CIN: Chromosomal instability; NAD: Nicotinamide adenine dinucleotide.

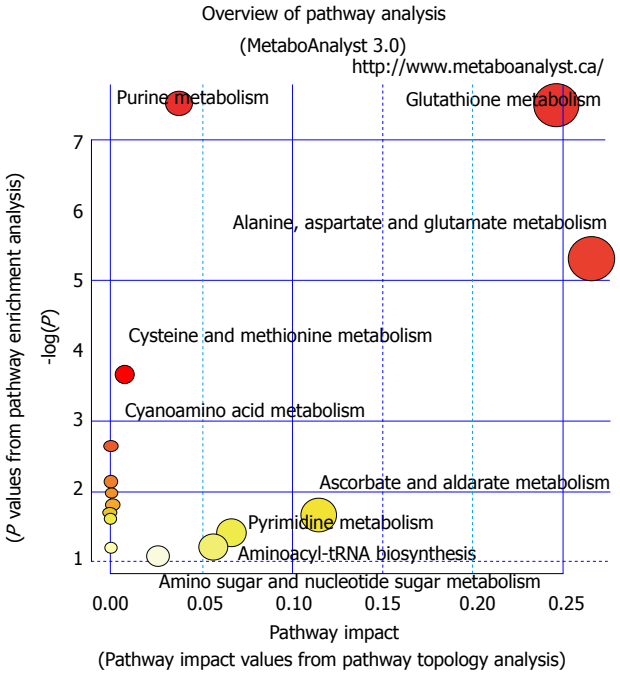


Figure 2 Pathways in chromosomal instability (CIN) and non-chromosomal instability gastric cancer. Pathways involving glutathione metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, and glutamine and glutamate metabolism were found in CIN and non-CIN types. Pathways involving alanine, aspartate, and glutamate metabolism, glyoxylate and dicarboxylate metabolism, histidine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis were observed in the CIN type but not the non-CIN type. CIN: Chromosomal instability.

Metabolomic profiling of healthy tissues: CIN vs non-CIN type

The metabolite distributions in healthy tissue between the CIN and non-CIN type tumors could be clearly distinguished, and the decreased NAD level was significantly different ($P = 0.027$). The concentration of NAD in the healthy tissue was 0.607 times that in the non-CIN type.

Nonspecific metabolic patterns based on the Lauren classifications

In the study, 87.5% of the Lauren intestinal-type tumors belonged to the CIN GC group, whereas all of the Lauren diffuse-type tumors belonged to the non-CIN GC group. The Lauren mixed-type tumors belonged to both CIN (50%) and non-CIN type (50%) (Supplementary Table 4). The intestinal-type tumors demonstrated a high alteration rate of 92.2% (377 genes), particularly those with copy-number changes, whereas the diffuse-type tumors indicated a low alteration rate of 8.56% (35 genes). Therefore, the metabolite distribution of tumors could not be distinguished based on the Lauren classification system.

DISCUSSION

In the present study, we used the TCGA molecular classification method rather than using the traditional Lauren histological classification. In line with our study, CIN tumors were 80% intestinal type, 12% diffuse type, and 7% mixed type in a TCGA report[21]. The CIN phenotype can be induced by dysfunctions of different cellular processes, which can be categorized into (1) inaccurate chromosome segregation during mitosis, (2) cell-cycle checkpoint defects, (3) oncogene-induced mitotic stress, and (4) replication stress[12]. Accelerated loss of heterozygosity in tumor suppressor genes or accelerated gain of oncogene copies due to chromosomal duplication results from CIN, which leads to cancer[22]. To our knowledge, the present study is the first report combining the CIN status of gastric cancer with the metabolomics data. According to our data,

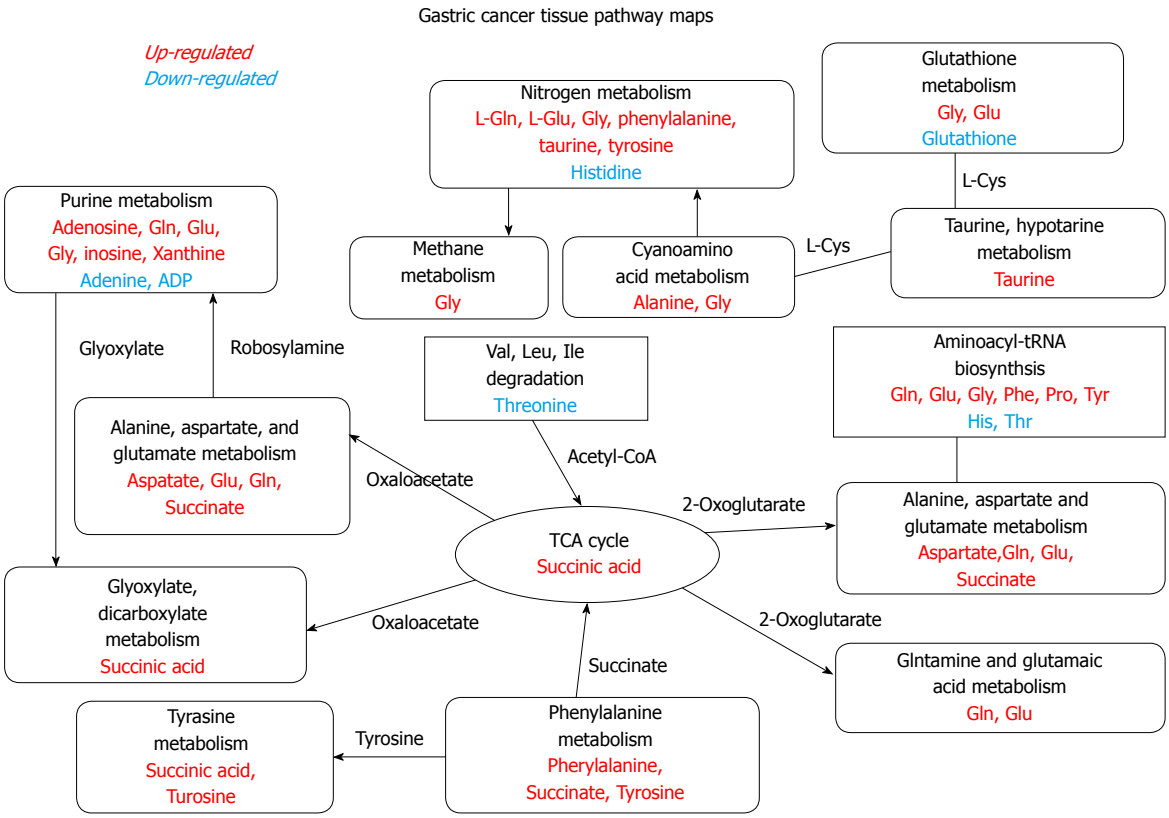


Figure 3 Metabolites in the chromosomal instability type and the non-chromosomal instability type. Upregulated (red) or down-regulated (blue) metabolites of pathways (black) involved in CIN and non-CIN types. CIN: Chromosomal instability.

we revealed the metabolomic profiling of GC directly correlated with the genetic CIN status, but not with the traditional Lauren's classification. We demonstrated that the CIN was associated with downstream biochemical alterations.

Genetic alterations continue to accumulate over time, resulting in serious disruptive changes in cell biochemical metabolism and finally leading to GC tumor formation. Through mRNA expression analysis, Carvalho *et al*[14] discovered that upregulated gene sets associated with CIN were also associated with GC compared with benign adenomas. Disruption of specific biological processes, such as CIN maintenance and altered metabolism, is the key factor in the progression from adenoma to carcinoma. In the present study, CIN GC tumors contained significantly higher levels of phosphocholine and uridine 5′-monophosphate and significantly lower levels of beta-citryl-L-glutamic acid compared with the non-CIN GC tumors. Eight metabolic pathways were involved in GC tumor formation. Pathways involving glutathione metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, as well as glutamine and glutamate metabolism, were found in both CIN and non-CIN types (Figure 2), and those involving alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, histidine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis were observed in the CIN type alone. NAD could be a predictor for GC tissue development in both CIN and non-CIN GC. Through gas chromatography-MS analysis, Song *et al*[15] revealed that the levels of several intermediate products of aerobic glycolytic pathways, such as fumaric and alpha-ketoglutaric acid, significantly increased in cancer tissues compared with healthy mucosa, suggesting that altered glucose metabolism is a vital parameter in distinguishing GC cells from healthy cells. Similarly, abnormal glucose metabolism has been observed by other researchers in GC tissue[16,23-25]. Signaling pathways are involved in glycolysis pathways with changes in hypoxia-inducible factor, insulin signaling pathway, and PI3K-Akt-mTOR levels[26].

A critical clinical implication of our GC metabolomics study is biomarker discovery. Sohn *et al*[27] demonstrated that patients with CIN GC had better overall survival than those with non-CIN GC. Metabolomics might offer an alternative way for stratification that could help to select more appropriate adjuvant chemotherapy. Shaukat *et al*[28] also demonstrated that CIN induction sensitizes GC to metabolic stress. Mild metabolic disruption that does not affect healthy cells can lead to high levels of oxidative stress and subsequent cell death in CIN GC cells because they are already managing elevated stress levels. Our data illustrated novel therapeutic possibilities regarding GC, as untargeted metabolomics screening identified key regulators that can exploit these changes that cause cell death, which may provide cancer-specific potential drug targets,

particularly for advanced cancers exhibiting CIN. The interaction between CIN status and metabolic alteration is linked by aberrant DNA methylation, which could lead to CIN and transcriptional gene silencing of tumor suppressors or overexpression of oncogenes. Vitamin B has essential roles in carbon metabolism providing critical metabolites for DNA methylation, DNA repair, and nucleic acid synthesis[29], although they were not significantly changed in our study. Adjustment of vitamin B levels and genetic polymorphisms of related key enzymes in one carbon metabolism pathway might govern the bioavailability of metabolites and therefore cause changes in CIN phenotypes. Another aspect is the balance between the oxidized and reduced forms of NAD, which is a vital component of a redox state in a cell, and reflects both the metabolic activities and the health of cells[30]. NAD acts as a coenzyme in redox reactions, as a donor of ADP-ribose moieties in ADP-ribosylation reactions, as a precursor of the second messenger molecule cyclic ADP-ribose, and as a substrate for DNA ligases (Figure 3)[31,32].

One of the study limitations was a small number of participants undergoing an operation who were willing to contribute their tissue samples in each category, and therefore we did not correct for multiple comparisons. Metabolomics studies on GC have provided metabolite biomarkers that can differentiate GC patients from healthy controls[33]. We found that the metabolomic profiling of GC was directly correlated with the genetic CIN status but not the traditional Lauren's classification system. The transomics approach in this study to analyze the genomics and metabolomics data inadvertently limited the cohort size of this study. Nevertheless, in this discovery phase, we demonstrated that CIN was associated with downstream biochemical alterations. Therefore, larger studies are required for validating this biomarker's utility followed by its translation into a clinical setting. In future works, we would like to collect more patient survival data to understand what metabolites can affect cancer progression or patient prognosis.

In conclusion, we combined the classification method of gene molecules with a metabolomics method to discover metabolic information to accurately classify tumors. These findings on metabolomics profiling based on CIN status have translational potential for biomarker discovery and novel therapeutic development.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most common malignancies. GC can be histologically classified using the Lauren classification system, which divides GC into diffuse and intestinal subtypes. The Cancer Genome Atlas Research Group (TCGA) has developed molecular classification systems based on gene expression profiling. Metabolomics may offer practical solutions to the traditional methods for GC detection and treatment. We hypothesize that metabolic alternations reflect the chromosomal instability (CIN) or genomic stability status of GC and aim to study the comprehensive metabolomic profiles of GC and correlate them with its CIN status.

Research motivation

The numerous biomarkers discovered from metabolomic studies may play a noteworthy role in GC with regard to early-stage detection, diagnosis, prognosis, drug development, and chemosensitivity predictions, but the evidence of metabolomics' association with CIN status remains lacking.

Research objectives

The aim of our study was to explore the correlation of metabolomics profiles of GC and its CIN status.

Research methods

Based on 409 oncogenes and tumor suppressor genes sequenced, 19 GC patients were classified as CIN and non-CIN type by TCGA. The aqueous metabolites of the GC tumor and its surrounding adjacent healthy tissues were identified through liquid chromatography-mass spectrometry.

Research results

GC tumors demonstrated significantly higher aspartic acid, citicoline, glutamic acid, oxidized glutathione, succinyladenosine, and uridine diphosphate-N-acetylglucosamine levels, but significantly lower butyrylcarnitine, glutathione hydroxyhexanoycarnitine, inosinic acid, isovalerylcarnitine, and threonine levels compared to the adjacent healthy tissues. CIN tumors contained significantly higher phosphocholine and uridine 5'-monophosphate levels but significantly lower beta-citryl-L-glutamic acid level than did non-CIN tumors. CIN GC tumors demonstrated additional altered pathways involving alanine, aspartate, and glutamate metabolism, glyoxylate and dicarboxylate metabolism, histidine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis.

Research conclusions

Metabolomic profiles of GC tumors and the adjacent healthy tissue are distinct, and the CIN status is associated with downstream metabolic alterations in GC.

Research perspectives

The combination of classification method of gene molecules and a metabolomics method may reveal that metabolic information can be used to accurately classify tumors. These findings on metabolomics profiling based on CIN status have translational potential for biomarker discovery and novel therapeutic development.

ACKNOWLEDGMENTS

The authors express their thanks to the Metabolomics Core Laboratory, Healthy Aging Research Center, Chang Gung University and Clinical Metabolomics Core Laboratory, Chang Gung Memorial Hospital for performing the metabolomics analysis using liquid chromatography-mass spectrometry and/or NMR spectroscopy.

REFERENCES

1 **Emadi-Baygi M**, Nikpour P, Emadi-Andani E. SIX1 overexpression in diffuse-type and grade Ⅲ gastric tumors: Features that are associated with poor prognosis. *Adv Biomed Res* 2015; **4**: 139 [PMID: 26322287 DOI: 10.4103/2277-9175.161540]

2 **Quéro L**, Guillerm S, Hennequin C. Neoadjuvant or adjuvant therapy for gastric cancer. *World J Gastrointest Oncol* 2015; **7**: 102-110 [PMID: 26306142 DOI: 10.4251/wjgo.v7.i8.102]

3 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

4 **Compare D**, Rocco A, Nardone G. Risk factors in gastric cancer. *Eur Rev Med Pharmacol Sci* 2010; **14**: 302-308 [PMID: 20496539]

5 **Lauren P**. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675 DOI: 10.1111/apm.1965.64.1.31]

6 **Dicken BJ**, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM. Gastric adenocarcinoma: review and considerations for future directions. *Ann Surg* 2005; **241**: 27-39 [PMID: 15621988]

7 **Grabsch HI**, Tan P. Gastric cancer pathology and underlying molecular mechanisms. *Dig Surg* 2013; **30**: 150-158 [PMID: 23867592 DOI: 10.1159/000350876]

8 **Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

9 **Rochfort S**. Metabolomics reviewed: a new "omics" platform technology for systems biology and implications for natural products research. *J Nat Prod* 2005; **68**: 1813-1820 [PMID: 16378385 DOI: 10.1021/np050255w]

10 **Lim B**, Kim JH, Kim M, Kim SY. Genomic and epigenomic heterogeneity in molecular subtypes of gastric cancer. *World J Gastroenterol* 2016; **22**: 1190-1201 [PMID: 26811657 DOI: 10.3748/wjg.v22.i3.1190]

11 **Geigl JB**, Obenauf AC, Schwarzbraun T, Speicher MR. Defining 'chromosomal instability'. *Trends Genet* 2008; **24**: 64-69 [PMID: 18192061 DOI: 10.1016/j.tig.2007.11.006]

12 **Maleki SS**, Röcken C. Chromosomal Instability in Gastric Cancer Biology. *Neoplasia* 2017; **19**: 412-420 [PMID: 28431273 DOI: 10.1016/j.neo.2017.02.012]

13 **Jayavelu ND**, Bar NS. Metabolomic studies of human gastric cancer: review. *World J Gastroenterol* 2014; **20**: 8092-8101 [PMID: 25009381 DOI: 10.3748/wjg.v20.i25.8092]

14 **Carvalho B**, Sillars-Hardebol AH, Postma C, Mongera S, Terhaar Sive Droste J, Obulkasim A, van de Wiel M, van Criekinge W, Ylstra B, Fijneman RJ, Meijer GA. Colorectal adenoma to carcinoma progression is accompanied by changes in gene expression associated with ageing, chromosomal instability, and fatty acid metabolism. *Cell Oncol* (Dordr) 2012; **35**: 53-63 [PMID: 22278361 DOI: 10.1007/s13402-011-0065-1]

15 **Song H**, Wang L, Liu HL, Wu XB, Wang HS, Liu ZH, Li Y, Diao DC, Chen HL, Peng JS. Tissue metabolomic fingerprinting reveals metabolic disorders associated with human gastric cancer morbidity. *Oncol Rep* 2011; **26**: 431-438 [PMID: 21567103 DOI: 10.3892/or.2011.1302]

16 **Hur H**, Paik MJ, Xuan Y, Nguyen DT, Ham IH, Yun J, Cho YK, Lee G, Han SU. Quantitative measurement of organic acids in tissues from gastric cancer patients indicates increased glucose metabolism in gastric cancer. *PLoS One* 2014; **9**: e98581 [PMID: 24911788 DOI: 10.1371/journal.pone.0098581]

17 **Chan AW**, Gill RS, Schiller D, Sawyer MB. Potential role of metabolomics in diagnosis and surveillance of gastric cancer. *World J Gastroenterol* 2014; **20**: 12874-12882 [PMID: 25278684 DOI: 10.3748/wjg.v20.i36.12874]

18 **Abbassi-Ghadi N**, Kumar S, Huang J, Goldin R, Takats Z, Hanna GB. Metabolomic profiling of oesophago-gastric cancer: a systematic review. *Eur J Cancer* 2013; **49**: 3625-3637 [PMID: 23896378 DOI: 10.1016/j.ejca.2013.07.004]

19 **FOLCH J**, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957; **226**: 497-509 [PMID: 13428781]

20 **Want EJ**, Wilson ID, Gika H, Theodoridis G, Plumb RS, Shockcor J, Holmes E, Nicholson JK. Global metabolic profiling procedures for urine using UPLC-MS. *Nat Protoc* 2010; **5**: 1005-1018 [PMID: 20448546 DOI: 10.1038/nprot.2010.50]

21 **Fontana E**, Smyth EC. Novel targets in the treatment of advanced gastric cancer: a perspective review. *Ther Adv Med Oncol* 2016; **8**: 113-125 [PMID: 26929787 DOI: 10.1177/1758834015616935]

22 **Rajagopalan H**, Lengauer C. Aneuploidy and cancer. *Nature* 2004; **432**: 338-341 [PMID: 15549096 DOI: 10.1038/nature03099]

23 **Wu H**, Xue R, Tang Z, Deng C, Liu T, Zeng H, Sun Y, Shen X. Metabolomic investigation of gastric cancer tissue using gas chromatography/mass spectrometry. *Anal Bioanal Chem* 2010; **396**: 1385-1395 [PMID: 20012946 DOI: 10.1007/s00216-009-3317-4]

24 **Hirayama A**, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, Kinoshita T, Saito N, Ochiai A, Tomita M, Esumi H, Soga T. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer Res* 2009; **69**: 4918-4925 [PMID: 19458066 DOI: 10.1158/0008-5472.CAN-08-4806]

25 **Cai Z**, Zhao JS, Li JJ, Peng DN, Wang XY, Chen TL, Qiu YP, Chen PP, Li WJ, Xu LY, Li EM, Tam JP, Qi RZ, Jia W, Xie D. A combined proteomics and metabolomics profiling of gastric cardia cancer reveals characteristic dysregulations in glucose metabolism. *Mol Cell Proteomics* 2010; **9**: 2617-2628 [PMID: 20699381 DOI: 10.1074/mcp.M110.000661]

26 **Yuan LW**, Yamashita H, Seto Y. Glucose metabolism in gastric cancer: The cutting-edge. *World J Gastroenterol* 2016; **22**: 2046-2059 [PMID: 26877609 DOI: 10.3748/wjg.v22.i6.2046]

27 **Sohn BH**, Hwang JE, Jang HJ, Lee HS, Oh SC, Shim JJ, Lee KW, Kim EH, Yim SY, Lee SH, Cheong JH, Jeong W, Cho JY, Kim J, Chae J, Lee J, Kang WK, Kim S, Noh SH, Ajani JA, Lee JS. Clinical Significance of Four Molecular Subtypes of Gastric Cancer Identified by The Cancer Genome Atlas Project. *Clin Cancer Res* 2017 [PMID: 28747339 DOI: 10.1158/1078-0432.CCR-16-2211]

28 **Shaukat Z**, Liu D, Choo A, Hussain R, O'Keefe L, Richards R, Saint R, Gregory SL. Chromosomal instability causes sensitivity to metabolic stress. *Oncogene* 2015; **34**: 4044-4055 [PMID: 25347746 DOI: 10.1038/onc.2014.344]

29 **Donkena KV**, Yuan H, Young CY. Vitamin Bs, one carbon metabolism and prostate cancer. *Mini Rev Med Chem* 2010; **10**: 1385-1392 [PMID: 20937030 DOI: 10.2174/138955710793564106]

30 **Schafer FQ**, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001; **30**: 1191-1212 [PMID: 11368918 DOI: 10.1016/S0891-5849(01)00480-4]

31 **Smyth LM**, Bobalova J, Mendoza MG, Lew C, Mutafova-Yambolieva VN. Release of beta-nicotinamide adenine dinucleotide upon stimulation of postganglionic nerve terminals in blood vessels and urinary bladder. *J Biol Chem* 2004; **279**: 48893-48903 [PMID: 15364945 DOI: 10.1074/jbc.M407266200]

32 **Billington RA**, Bruzzone S, De Flora A, Genazzani AA, Koch-Nolte F, Ziegler M, Zocchi E. Emerging functions of extracellular pyridine nucleotides. *Mol Med* 2006; **12**: 324-327 [PMID: 17380199 DOI: 10.2119/2006-00075.Billington]

33 **Ni Y**, Xie G, Jia W. Metabonomics of human colorectal cancer: new approaches for early diagnosis and biomarker discovery. *J Proteome Res* 2014; **13**: 3857-3870 [PMID: 25105552 DOI: 10.1021/pr500443c]

P- Reviewer: Chen Z, De Re V, Kimura A **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3770-3775

DOI: 10.3748/wjg.v24.i33.3770

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Beta-blockers and physical frailty in patients with end-stage liver disease

Selena Z Kuo, Blanca Lizaola, Hilary Hayssen, Jennifer C Lai

Selena Z Kuo, Blanca Lizaola, Hilary Hayssen, Jennifer C Lai, Division of Gastroenterology/Hepatology, Department of Medicine, University of California, San Francisco, San Francisco, CA 94143, United States

ORCID number: Selena Z Kuo (0000-0002-8532-0489); Blanca Lizaola (0000-0003-0130-0091); Hilary Hayssen (0000-0002-8833-2880); Jennifer C Lai (0000-0003-2092-6380).

Author contributions: Kuo SZ and Lai JC designed research, and analyzed data; Kuo SZ performed research; Kuo SZ, Lizaola B, Hayssen H and Lai JC wrote the paper.

Supported by the Paul B. Beeson Career Development Award in Aging Research, No. K23AG048337.

Institutional review board statement: This study was reviewed and approved by the International Review Board of the University of California, San Francisco

Conflict-of-interest statement: We have no conflict of interests to disclose.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement – checklist of items, and the manuscript was prepared and revised according to the STROBE Statement – Checklist of items.

Manuscript source: Unsolicited manuscript

Correspondence to: Jennifer C Lai, MD, MBA, Assistant Professor, Gastroenterology, University of California, San Francisco, 513 Parnassus Ave, San Francisco, CA 94143, United States. jennifer.lai@ucsf.edu
Telephone: +415-476-2777
Fax: +415-476-0659

Received: May 19, 2018
Peer-review started: May 19, 2018
First decision: June 5, 2018
Revised: July 18, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: September 7, 2018

Abstract

AIM

To investigate beta-blocker (BB) use in patients with cirrhosis and determine their effects on physical frailty and overall survival.

METHODS

Adult outpatients with cirrhosis listed for liver transplantation underwent testing of physical frailty using the performance-based Liver Frailty Index, comprised of chair stands, grip strength, and balance testing, as well as self-reported assessments of exhaustion and physical activity. BB use was assessed from medical chart review. Univariable and multivariable logistic regression were performed to determine BB use and their association with measures of physical frailty. Competing risk analyses were performed to determine the effect of BB use on wait-list mortality, as defined by death or delisting for being too sick for transplant.

RESULTS

Of 344 patients, 35% were female, median age was 60, median model for end stage liver disease was 15, and 53% were prescribed a BB. Compared to those not on

BB, patients on BB were similar except for percentage female (25% *vs* 46%; $P < 0.001$) and BMI (29 *vs* 28; $P = 0.008$). With respect to tests of physical frailty, BB use was not associated with increased odds of frailty (by the Liver Frailty Index), exhaustion, or low physical activity. BB use was, however, significantly associated with a decreased adjusted risk of mortality (SHR 0.55; $P = 0.005$).

CONCLUSION

In patients with cirrhosis awaiting liver transplantation, BB use is not associated with physical frailty. We confirmed the known survival benefits with BB use, and concerns about adverse effects should not deter their utilization when indicated.

Key words: Beta-blockers; Cirrhosis; End-stage liver disease; Frailty

Core tip: In patients with cirrhosis, beta-blockers are the main medical treatment for prevention of variceal bleeds, but fatigue and weakness are commonly reported side effects. This study demonstrates that use of beta-blockers is not associated with physical frailty and improves survival in patients with cirrhosis.

Kuo SZ, Lizaola B, Hayssen H, Lai JC. Beta-blockers and physical frailty in patients with end-stage liver disease. *World J Gastroenterol* 2018; 24(33): 3770-3775 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3770.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3770

INTRODUCTION

Physical frailty is reported to be prevalent in patients with cirrhosis and has emerged as a critical determinant of outcomes in this population[1-3]. Resulting from the loss of homeostatic balance of multiple physiologic systems including (but not limited to) musculoskeletal, inflammatory, endocrine, and neurocognitive, physical frailty has been operationalized using standardized instruments that measure multiple domains such as fatigue, weakness, slowness, weight loss, and low activity[4]. Two of these physical frailty components, fatigue and weakness, overlap with the frequent side effects of non-selective beta-blockers (BBs).

This overlap is of particular importance for patients with cirrhosis. Beta-blockers are the main pharmacologic therapy for both primary and secondary prevention of variceal hemorrhage in patients with cirrhosis[5]. Despite the proven clinical effectiveness and known mortality benefits of beta-blockers, there is mounting evidence that patients are not receiving these treatments due to physicians under prescribing, suboptimal adherence, and side effects. More recently, investigators have hypothesized that a "therapeutic window" exists during which a patient with cirrhosis might benefit from beta-blocker therapy for variceal prevention, with a patient losing benefit once they develop refractory ascites[6,7]. One study showed that as few as 6%-22% of patients with known medium or large varices received primary prophylaxis with beta-blockers[8]. Furthermore, concern regarding side effects led to discontinuation of beta-blockers in about 15% of patients with cirrhosis[9].

Reluctance to prescribe beta-blocker therapy may arise from concerns about their commonly reported symptoms of weakness and fatigue[10,11]. Most of the studies of beta-blocker side effects are performed within the cardiac population, but little is known of their effects in patients with cirrhosis. The adverse effects of weakness and fatigue, factors that are integral to the frail phenotype[4], are of particular importance in this population given the recent studies indicating physical frailty as a predictor of mortality in patients awaiting liver transplant[2,3]. Therefore, in this study, we aimed to evaluate the association between beta-blocker use and physical frailty in patients with cirrhosis.

MATERIALS AND METHODS

Study population

The Functional Assessment in Liver Transplantation (FrAILT) study is an ongoing prospective study of adult patients (≥ 18 years) with cirrhosis actively listed for liver transplantation and seen as outpatients at the University of California, San Francisco (UCSF). This retrospective study included data from patients enrolled in the FrAILT study from July 2012 until January 2014. Included were patients with model for end stage liver disease (MELD) score ≥ 12 and excluded were patients with severe hepatic encephalopathy, defined by the time to complete the Numbers Connection Test of > 120 s. All participants gave informed consent and the UCSF Institutional Review Board approved this study.

Study procedures and data collection

At enrollment and subsequent clinic visits, all patients underwent tests of physical frailty in the outpatient clinic setting: (1) the Liver Frailty Index (LFI), consisting of chair stands, grip strength, and balance (calculator available at https://liverfrailtyindex.ucsf.edu)[1]; (2) exhaustion, determined by self-report using two questions from the Center for Epidemiological Studies-Depression scale that have been included in the Fried Frailty Phenotype[4]; and (3) physical activity, determined by self-report using the Minnesota Leisure Time Physical Activity Scale (MNLTPA) that has also been included in the Fried Frailty Phenotype[4].

Patient demographics, medical co-morbidities, degree of ascites, vital signs, beta-blocker use and beta-blocker indications were collected from the clinic visit note from the same date of their physical frailty

assessment. Hepatic encephalopathy (HE) was defined as moderate if the patient's Numbers Connection Test score was > 60 s and mild/none if < 60 s. Laboratory studies within 3 mo of the study visit were collected from the patient's electronic health record.

Statistical analysis

Patients were classified as "frail" if they had an LFI score of ≥ 4.5, as these cutoffs have been associated with worse outcomes in patients awaiting liver transplant[1]. Patients were classified as "robust" if they had an LFI score of < 3.2[1]. Frailty by the MNLTPA activity level was defined as < 383 Kcals/wk for males and < 270 Kcals/wk for females[4].

Differences in baseline characteristics between beta-blocker users *vs* non-users were compared using chi-square or Wilcoxon ranksum tests for categorical and continuous variables, respectively. Differences in heart rate, performance measures of physical frailty, and self-reported physical frailty were also compared by chi-square and Wilcoxon ranksum tests. Univariable logistic regression was performed to determine associations between beta-blocker use and physical frailty. Multivariable models initially included heart rate, age, MELD, creatinine, albumin, sodium, presence of hepatocellular carcinoma (HCC), degree of ascites, and presence of hepatic encephalopathy. All variables associated with a *P*-value < 0.2 in the univariable analysis were included in the multivariable model and a backwards stepwise selection was used to only include variables with a $P < 0.05$.

Competing risk analysis evaluated the effect of beta-blocker use on wait-list mortality, as defined by death or delisting for being too sick for transplant, with liver transplant as a competing risk. Patients were censored at the time of waitlist removal if removed for "other" reasons (*e.g.*, violation of substance abuse contract, inadequate social support). All variables associated with a *P*-value < 0.2 in the univariate analysis were included in the multivariate model and a backward stepwise selection was used to eliminate covariates using a threshold *P*-value < 0.05 to determine the subdistribution hazard ratio (SHR).

All statistical analyses were performed using STATA® version 11 (College Station, TX, United States). The statistical methods of this study were reviewed by Lai JC, MD, from UCSF who is trained in advanced clinical research methodologies.

RESULTS

Baseline characteristics of the cohort

A total of 344 patients with end-stage liver disease were included in this study. Baseline characteristics are listed in Table 1, column A. The median (IQR) age was 60 years (54-64) and 35% were female. The etiology of underlying liver disease was hepatitis C in 50%, alcoholic liver disease in 15%, nonalcoholic steatohepatitis in 13%, autoimmune/cholestatic liver disease in 11%, hepatitis B in 4% and "other" in 6%. Median weight and body mass index (BMI) were 83.5 kg and 28.4 kg/m^2, respectively. With respect to medical co-morbidities, 44% had hypertension, 32% had diabetes, and 7% had coronary artery disease. Hepatic encephalopathy was present in 21% of patients. Ascites was absent in 67%, 30% had a mild to moderate degree, and 3% had severe ascites. The median (IQR) MELD score was 15 (12-18), and the proportion with Child Pugh Score A, B, and C was 19%, 60%, and 21%, respectively.

Characteristics associated with beta-blocker use

Out of the 344 patients, 181 (53%) were taking a beta-blocker at the time of assessment: 68% were taking propranolol, 15% nadolol, and 17% other beta-blockers (Supplemental Table 1).

Baseline characteristics of beta-blocker users *vs* non-users are shown in Table 1, columns B and C, respectively. The two groups were similar with respect to age, race/ethnicity, etiology of liver disease, degree of ascites, rates of hepatic encephalopathy, MELD scores and Child Pugh scores. Beta-blocker users *vs* non-users had a lower percentage of females (25% *vs* 46%; $P < 0.001$), higher body mass index (29.3 kg/m^2 *vs* 27.7 kg/m^2; $P = 0.008$), and higher rates of HCC (32% *vs* 22%; $P = 0.04$).

Median (IQR) heart rate (in beats per minute) differed significantly between beta-blocker users and non-users [67 (61-74) *vs* 76 (69-85); $P < 0.001$]. There was no significant difference in systolic or diastolic blood pressure between the two groups.

Associations between beta-blocker use and physical frailty

A comparison of characteristics by beta-blocker use is presented in Table 2. Median LFI was statistically, but not clinically, significantly worse in beta-blocker users compared to non-users (3.75 *vs* 3.64; $P = 0.04$). Rates of frailty were similar between the two groups (14% *vs* 14%), but there was a trend toward a lower rate of patients who were classified as robust among beta-blocker users *vs* non-users (16% *vs* 25%; $P = 0.06$). There was no difference in rates of self-reported exhaustion or physical activity between the two groups (Table 2).

In univariable logistic regression, beta-blocker use was not significantly associated with physical frailty as defined by LFI ≥ 4.5 [OR 0.99 (95%CI: 0.54-1.83)], exhaustion [OR 0.97 (95%CI: 0.63-1.48)], or low physical activity [OR 0.92 (95%CI: 0.60-1.41)]. The associations between beta-blocker use and physical frailty by LFI ≥ 4.5 [OR 0.97 (95%CI: 0.50-1.87)], exhaustion [OR 0.97 (95%CI: 0.63-1.50)], or low physical activity [OR 1.18 (95%CI: 0.74-1.89)] did not change after multivariable adjustment. There was no association between physical frailty and each unit increase in dosing of either propranolol [OR 1.00 (95%CI: 0.98-1.02)] or nadolol [OR 0.99 (95%CI: 0.92-1.08)].

Table 1 Patient demographics

Characteristics	All *n* = 344	On beta blockers *n* = 181 (53%)	Not on beta blockers *n* = 163 (47%)	*P* value
Age (yr)	60 (54-64)	61 (54-65)	60 (54-63)	0.16
Female	35%	25%	46%	< 0.001
Race/ethnicity				0.26
Non-Hispanic White	57%	56%	58%	
Black	4%	3%	6%	
Hispanic White	27%	30%	23%	
Asian	7%	5%	8%	
Other	6%	7%	5%	
Etiology of liver disease				0.39
Hepatitis C	50%	51%	49%	
Alcohol	15%	15%	15%	
Nonalcoholic steatohepatitis	13%	15%	11%	
Autoimmune/cholestatic	11%	9%	14%	
Hepatitis B	4%	2%	6%	
Other	6%	7%	6%	
HCC	27%	32%	22%	0.04
BMI (kg/m^2)	28.4 (24.9-33.0)	29.3 (25.8-33.7)	27.7 (24.2-31.8)	0.0081
Medical co-morbidities				
Hypertension	44%	48%	40%	0.12
Diabetes	32%	36%	27%	0.06
Coronary artery disease	7%	10%	4%	0.04
Lab tests				
Lab MELD	15 (12-18)	15 (13-18)	15 (12-18)	0.55
Total bilirubin (mg/dL)	2.3 (1.6-3.4)	2.3 (1.5-3.2)	2.4 (1.7-3.6)	0.41
INR	1.4 (1.2-1.6)	1.4 (1.3-1.6)	1.4 (1.2-1.6)	0.54
Creatinine (mg/dL)	0.9 (0.8-1.2)	1 (0.8-1.2)	0.9 (0.7-1.2)	0.0034
Sodium (mEq/L)	137 (134-139)	137 (135-139)	137 (134-139)	0.75
Ascites				
Mild-moderate	30%	30%	30%	0.40
Refractory	3%	4%	2%	
Hepatic encephalopathy[1]	21%	22%	21%	0.86
Child Pugh Score				0.89
A	19%	18%	20%	
B	60%	60%	59%	
C	21%	22%	21%	

[1]Defined as a Numbers Connection test > 60 s. HCC: Hepatocellular carcinoma; BMI: Body mass index; MELD: Model for End Stage Liver Disease.

Table 2 Comparison of metrics of physical frailty by beta-blocker use

Outcome	On beta blockers *n* = 181 (53%)	Not on beta blockers *n* = 163 (47%)	*P* value
Liver frailty index[1]	3.75 (3.37-4.15)	3.64 (3.23-4.04)	0.04
Chair stands (s)	12.5 (10-16.2)	10.9 (8.3-13.2)	0.003
Grip strength (kg)	33.3 (24.3-40)	29 (22-37)	0.03
Balance (s)	30 (30-30)	30 (26.6-30)	0.20
LFI Frail	14%	14%	0.98
LFI Robust	16%	25%	0.06
Exhaustion	51%	52%	0.89
MNLTPA frailty	58%	60%	0.69

[1]Five missing data points. LFI: Liver frailty index; MNLTPA: Minnesota leisure time physical activity scale.

Beta-blocker use and wait-list mortality

Median (IQR) follow-up time was 12 (4-22) mo. By the end of follow-up, 92 (27%) patients died or were delisted for being too sick, 167 (48%) underwent liver transplant, 47 (14%) were delisted for other reasons, and 37 (11%) remained on the waitlist. Patients who were not beta-blocker users *vs* beta-blocker users had a higher proportion who died/delisted for being too sick (33% *vs* 22%; *P* = 0.02).

In univariable competing risks regression, beta-blocker use was significantly associated with decreased mortality, whereas creatinine, HE and LFI score were significantly associated with increased waitlist mortality (Table 3). After adjustment for all independent predictors, beta-blocker use remained associated with decreased hazard of waitlist mortality [SHR 0.55 (95%CI 0.36-0.83)

Table 3 Competing risks survival analysis

Variable	Univariable SHR (95%CI) *P* value	Multivariable SHR (95%CI) *P* value
Being on a Beta Blocker	0.57 (0.38-0.85) 0.006	0.55 (0.36-0.83) 0.005
Liver Frailty Index	1.52 (1.13-2.03) 0.005	1.35 (1.02-1.80) 0.04
MELD score	1.03 (0.99-1.07) 0.19	
Creatinine	1.17 (1.07-1.28) 0.001	1.10 (1.00-1.22) 0.006
Albumin	0.79 (0.56-1.12) 0.18	
Sodium	0.99 (0.95-1.04) 0.76	
HCC	0.80 (0.50-1.29) 0.37	
Ascites	0.98 (0.63-1.51) 0.92	
Age	1.02 (0.99-1.04) 0.15	
Gender (female)	1.09 (0.72-1.65) 0.68	
Hepatic encephalopathy (> 60 s)	1.99 (1.30-3.05) 0.001	1.64 (1.05-2.57) 0.03

HCC: Hepatocellular carcinoma; MELD: Model for End Stage Liver Disease; SHR: subdistribution hazard ratio.

P = 0.005].

DISCUSSION

Non-selective beta-blockers have become a cornerstone of medical management for patients with cirrhosis to reduce the risk of variceal hemorrhage and have been associated with mortality benefit[9,12,13]. However, beta-blockers also have a number of well-known side effects, including fatigue and weakness, which may be particularly challenging for patients with cirrhosis who already experience a high burden of fatigue and weakness from their cirrhosis, in addition to polypharmacy[14]. These common side effects of non-selective beta-blockers could, in turn, theoretically accelerate physical frailty, a potent determinant of mortality in this population[1-3].

In this study, we demonstrated that the addition of a beta-blocker is not associated with clinically-significantly increased rates of physical frailty or its associated symptoms of exhaustion or low physical activity. While patients on non-selective beta-blockers had *statistically* worse LFI scores than patients not on non-selective beta-blockers, the difference in the median values for each group was 0.11, which does not meet the 0.2 threshold for the minimum clinically important difference in LFI[15]. This is supported by the fact that rates of physical frailty, using the LFI cut-off of ≥ 4.5[1], were similar between the two groups. Furthermore, our analyses confirmed the known overall mortality benefits of beta-blocker therapy[9,12,13]. The fact that the vast majority of our cohort did not have refractory ascites supports the concept of a "therapeutic window" for benefit of beta-blockers on mortality in patients with cirrhosis[7]. Importantly, our data add to the existing body of literature by adjusting for the differences in frailty, which is now established to be an important determinant of mortality in patients with end-stage liver disease[1].

We acknowledge several limitations to our study. Since this was a cross-sectional study, we were only able to ascertain beta-blocker use at a single visit, but recognize that beta-blocker prescription could have changed during the course of the patient's time on the waitlist. Adherence to beta-blocker therapy could not be verified, but the median heart rate was significantly lower in the beta-blocker users. Inclusion of only outpatients with MELD scores ≥ 12 limits the study's generalizability to the liver transplant population as a whole, but we expect those with MELD scores < 12 to be less frail and therefore, even less affected by beta-blocker use. Lastly, given the observational nature of this study, we could not conclude the absence of causality between non-selective beta-blockers and physical frailty.

Despite these limitations, our data provide important data for clinicians who manage patients with cirrhosis and portal hypertension. This study is, to our knowledge, the first to investigate the association between commonly reported beta-blocker side effects of fatigue and weakness on physical frailty, of which fatigue and weakness are major components. The lack of any clinically meaningful difference in rates of physical frailty in addition to the strong association with mortality benefit of non-selective beta-blockers (in this study and in others[9,12,13]) provide reassuring evidence in support of non-selective beta-blocker use when indicated.

ARTICLE HIGHLIGHTS

Research background

Patients with cirrhosis are vulnerable to developing physical frailty, and it is becoming increasingly apparent that frailty predicts poor waitlist mortality. Frequently reported side effects of beta-blockers include weakness and fatigue, which overlap with aspects of frailty.

Research motivation

There are an increasing number of studies that indicate physical frailty as a predictor of mortality in patients awaiting liver transplant. Given that beta-blockers have commonly reported side effects of fatigue and weakness, it is possible that they could accelerate physical frailty.

Research objectives

The objective of this study was to determine the association between beta-blocker use with physical frailty, exhaustion, physical activity and mortality in patients with cirrhosis.

Research methods

Three-hundred-forty-four patients with cirrhosis underwent physical frailty testing using the Liver Frailty Index, which includes chair stands, grip strength and balance testing. Data was also collected on self-reported assessments of exhaustion and amount of physical activity. Data on beta-blocker usage was obtained from chart review. Both univariable and multivariable logistic regression were performed to determine if there was an association with

physical frailty and beta-blocker use.

Research results

Fifty three percent of the patients were prescribed a beta-blocker. In both univariable and multivariable models, beta-blocker users did not have increased odds of physical frailty (as defined by LFI ≥ 4.5), higher rates of exhaustion, or lower physical activity levels. Patients on beta-blockers had a 45% reduction in odds of waitlist mortality compared to patients not on beta-blockers.

Research conclusions

Our study demonstrates that in patients with cirrhosis, beta-blocker use is not associated with physical frailty, exhaustion, or lower physical activity. Furthermore, our study confirms the survival benefits of beta-blocker use.

Research perspectives

Taken together, our findings suggest that there is no association with beta-blocker use and physical frailty, and that concerns about side effects should not prevent their use when indicated. Since this is an observational study, future studies will be needed to conclude the absence of causality.

REFERENCES

1 **Lai JC**, Covinsky KE, Dodge JL, Boscardin WJ, Segev DL, Roberts JP, Feng S. Development of a novel frailty index to predict mortality in patients with end-stage liver disease. *Hepatology* 2017; **66**: 564-574 [PMID: 28422306 DOI: 10.1002/hep.29219]

2 **Lai JC**, Dodge JL, Sen S, Covinsky K, Feng S. Functional decline in patients with cirrhosis awaiting liver transplantation: Results from the functional assessment in liver transplantation (FrAILT) study. *Hepatology*2016; **63**: 574-580 [PMID: 26517301 DOI: 10.1002/hep.28316]

3 **Lai JC**, Feng S, Terrault NA, Lizaola B, Hayssen H, Covinsky K. Frailty predicts waitlist mortality in liver transplant candidates. *Am J Transplant* 2014; **14**: 1870-1879 [PMID: 24935609 DOI: 10.1111/ajt.12762]

4 **Fried LP**, Tangen CM, Walston J, Newman AB, Hirsch C, Gottdiener J, Seeman T, Tracy R, Kop WJ, Burke G, McBurnie MA; Cardiovascular Health Study Collaborative Research Group. Frailty in older adults: evidence for a phenotype. *J Gerontol A Biol Sci Med Sci* 2001; **56**: M146-M156 [PMID: 11253156]

5 **Ge PS**, Runyon BA. The changing role of beta-blocker therapy in patients with cirrhosis. *J Hepatol* 2014; **60**: 643-653 [PMID: 24076364 DOI: 10.1016/j.jhep.2013.09.016]

6 **Kok B**, Tandon P. Frailty in Patients With Cirrhosis. *Curr Treat Options Gastroenterol* 2018; **16**: 215-225 [PMID: 29589278 DOI: 10.1007/s11938-018-0179-x]

7 **Krag A**, Wiest R, Albillos A, Gluud LL. The window hypothesis: haemodynamic and non-haemodynamic effects of β-blockers improve survival of patients with cirrhosis during a window in the disease. *Gut* 2012; **61**: 967-969 [PMID: 22234982 DOI: 10.1136/gutjnl-2011-301348]

8 **Wilbur K**, Sidhu K. Beta blocker prophylaxis for patients with variceal hemorrhage. *J Clin Gastroenterol* 2005; **39**: 435-440 [PMID: 15815213]

9 **Garcia-Tsao G**, Abraldes JG, Berzigotti A, Bosch J. Portal hypertensive bleeding in cirrhosis: Risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the study of liver diseases. *Hepatology* 2017; **65**: 310-335 [PMID: 27786365 DOI: 10.1002/hep.28906]

10 **Ko DT**, Hebert PR, Coffey CS, Sedrakyan A, Curtis JP, Krumholz HM. Beta-blocker therapy and symptoms of depression, fatigue, and sexual dysfunction. *JAMA* 2002; **288**: 351-357 [PMID: 12117400]

11 **Koch-Weser J**, Frishman WH. beta-Adrenoceptor antagonists: new drugs and new indications. *N Engl J Med* 1981; **305**: 500-506 [PMID: 6114433 DOI: 10.1056/NEJM198108273050907]

12 **Bernard B**, Lebrec D, Mathurin P, Opolon P, Poynard T. Beta-adrenergic antagonists in the prevention of gastrointestinal rebleeding in patients with cirrhosis: a meta-analysis. *Hepatology* 1997; **25**: 63-70 [PMID: 8985266 DOI: 10.1053/jhep.1997.v25.pm0008985266]

13 **Poynard T**, Calès P, Pasta L, Ideo G, Pascal JP, Pagliaro L, Lebrec D. Beta-adrenergic-antagonist drugs in the prevention of gastrointestinal bleeding in patients with cirrhosis and esophageal varices. An analysis of data and prognostic factors in 589 patients from four randomized clinical trials. Franco-Italian Multicenter Study Group. *N Engl J Med* 1991; **324**: 1532-1538 [PMID: 1674104 DOI: 10.1056/NEJM199105303242202]

14 **Kuo SZ**, Haftek M, Lai JC. Factors Associated with Medication Non-adherence in Patients with End-Stage Liver Disease. *Dig Dis Sci* 2017; **62**: 543-549 [PMID: 27933471 DOI: 10.1007/s10620-016-4391-z]

15 **Lai JC**, Segev DL, McCulloch CE, Covinsky KE, Dodge JL, Feng S. Physical frailty after liver transplantation. *Am J Transplant* 2018; **18**: 1986-1994 [PMID: 29380529 DOI: 10.1111/ajt.14675]

P- Reviewer: Kim DJ, Panza F **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3776-3785

DOI: 10.3748/wjg.v24.i33.3776

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

ORIGINAL ARTICLE

Retrospective Study

Secondary endoscopic submucosal dissection for locally recurrent or incompletely resected gastric neoplasms

Da Hyun Jung, Young Hoon Youn, Jie-Hyun Kim, Jae Jun Park, Hyojin Park

Da Hyun Jung, Young Hoon Youn, Jie-Hyun Kim, Jae Jun Park, Hyojin Park, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul 06273, South Korea

ORCID number: Da Hyun Jung (0000-0001-6668-3113); Young Hoon Youn (0000-0002-0071-229X); Jie-Hyun Kim (0000-0002-9198-3326); Jae Jun Park (0000-0001-5297-5414); Hyojin Park (0000-0002-5759-5135).

Author contributions: All authors performed the research; Jung DH contributed to manuscript writing, performing procedures, and data analysis; Youn YH contributed to manuscript writing, conception and design, performing procedures, and data analysis; Kim JH, Park JJ, and Park H contributed to writing the manuscript and performing procedures.

Supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Science and ICT(NRF-2015R1C1A1A01054352).

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Gangnam Severance Hospital.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Young Hoon Youn, MD, PhD, Associate Professor, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, 211 Eonjuro, Gangnam-gu, Seoul 06273, South Korea. dryoun@yuhs.ac
Telephone: +82-2-20193453
Fax: +82-2-34633882

Received: June 11, 2018
Peer-review started: June 11, 2018
First decision: July 6, 2018
Revised: July 31, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 7, 2018

Abstract

AIM

To investigate the feasibility and safety of secondary endoscopic submucosal dissection (ESD) for residual or locally recurrent gastric tumors.

METHODS

Between 2010 and 2017, 1623 consecutive patients underwent ESD for gastric neoplasms at a single tertiary referral center. Among these, 28 patients underwent secondary ESD for a residual or locally recurrent tumor. Our analysis compared clinicopathologic factors between primary ESD and secondary ESD groups.

RESULTS

The en bloc resection and curative rate of resection of secondary ESD were 92.9% and 89.3%, respectively. The average procedure time of secondary ESD was significantly longer than primary ESD (78.2 min *vs* 55.1 min, $P = 0.004$), and the adverse events rate was not significantly different but trended slightly higher in the secondary ESD group compared to the primary ESD group (10.7% *vs* 3.8%, $P = 0.095$). Patients who received secondary ESD had favorable outcomes without severe adverse events. During a mean follow-

up period, no local recurrence occurred in patients who received secondary ESD.

CONCLUSION
Secondary ESD of residual or locally recurrent gastric tumors appears to be a feasible and curative treatment though it requires greater technical efficiency and longer procedure time.

Key words: Secondary endoscopic submucosal dissection; Endoscopic submucosal dissection; Gastric neoplasms; Residual tumors; Recurrent tumors

Core tip: Although secondary endoscopic submucosal dissection (ESD) is technically demanding, it can be applied to residual or recurrent tumors. We categorized secondary ESD into three groups according to the surgical strategy and analyzed them. There is no consensus on the timing of salvage ESD. This is the first study to report the feasibility and safety of secondary ESD according to the timing of ESD. Although secondary ESD requires greater technical efficiency and a longer procedure time, secondary ESD of residual or locally recurrent gastric tumors appears to be a feasible and curative treatment.

Jung DH, Youn YH, Kim JH, Park JJ, Park H. Secondary endoscopic submucosal dissection for locally recurrent or incompletely resected gastric neoplasms. *World J Gastroenterol* 2018; 24(33): 3776-3785 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3776.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3776

INTRODUCTION

Endoscopic submucosal dissection (ESD) technique has evolved, and various lesions can now be treated with ESD, regardless of their size and site, including ulcer scars with severe fibrosis[1,2]. To achieve curative resection, it is very important to accurately predict the margin of neoplasm before ESD. Therefore, chromoendoscopy, magnifying endoscopy, and magnifying image-enhanced endoscopy are widely used to determine the appropriate lesion margin[3,4]. However, despite these efforts, the positive lateral margins of a neoplasm can still occur after ESD. According to the Japanese gastric cancer treatment guidelines, surgical resection should be performed after non-curative resection of early gastric cancer[5]. However, for patients with differentiated carcinoma in which the positive lateral margin is the only non-curative factor, secondary ESD can be performed given the low risk of lymph node metastasis in such cases.

Primary ESD is a relatively established technique that dissects along the loose submucosal layer. However, secondary ESD is difficult because the submucosal layer is eliminated by the previous endoscopic resection and is replaced with fibrosis. In patients who have positive lateral margin after primary ESD, early secondary ESD is performed within a few days. This can be technically difficult because the orientation of the residual lesion might be confusing and development of submucosal fibrosis may have begun[6]. Late secondary ESD is performed a few months after the primary ESD and is also technically demanding because of severe submucosal fibrosis. Until recently, there have only been a few reports of secondary ESD for residual or locally recurrent tumors[6-8]. Therefore, this study aimed to investigate the feasibility and safety of secondary ESD procedures for residual or locally recurrent gastric neoplasms.

MATERIALS AND METHODS

Patients

Between January 2010 and February 2017, 1623 consecutive patients underwent gastric ESD for gastric neoplasms at Gangnam Severance Hospital in Seoul, Korea. Among these, 28 patients received secondary ESD for residual or locally recurrent tumors. We compared clinicopathologic factors between secondary ESD (28 patients) and primary ESD (1595 patients). The prospectively collected database of ESD and medical records were then reviewed and analyzed retrospectively to determine the feasibility and safety of ESD for residual or locally recurrent tumors. The Institutional Review Board (IRB) of Gangnam Severance Hospitals approved this study.

ESD procedure

Procedures were primarily performed using one or two ESD knives, including a DualKnife (Olympus Co., Tokyo, Japan), FlexKnife (Olympus Co., Tokyo, Japan), HookKnife (Olympus Co., Tokyo, Japan), and/or an insulated-tip knife (Olympus Co., Tokyo, Japan). Following circumferential marking (*via* argon plasma coagulation), a mixture of indigo carmine, epinephrine, and 10% glycerol (Cerol; JW Pharmaceutical Co, Seoul, South Korea) was used to inject the submucosa. The mucosa surrounding each lesion was then incised and dissection of the submucosal layer ensued. Hemostasis during ESD and ablation of visible vessels at the post-ESD ulcer site were achieved using hemostatic forceps (Coagrasper; Olympus Co, Tokyo, Japan). For the electrosurgical unit, the VIO300D (ERBE, Tuebingen, Germany) was used. Mucosal incision was performed using the Endocut I current (effect 2, cut duration 4, interval 3), and submucosal dissection was carried out with the swift coagulation current (effect 3, 40 W). The visible vessels were ablated using the soft coagulation current (effect 3, 60 W).

Secondary ESD procedure

In this study, secondary ESD refers to an additional ESD procedure for residual or locally recurrent tumors.

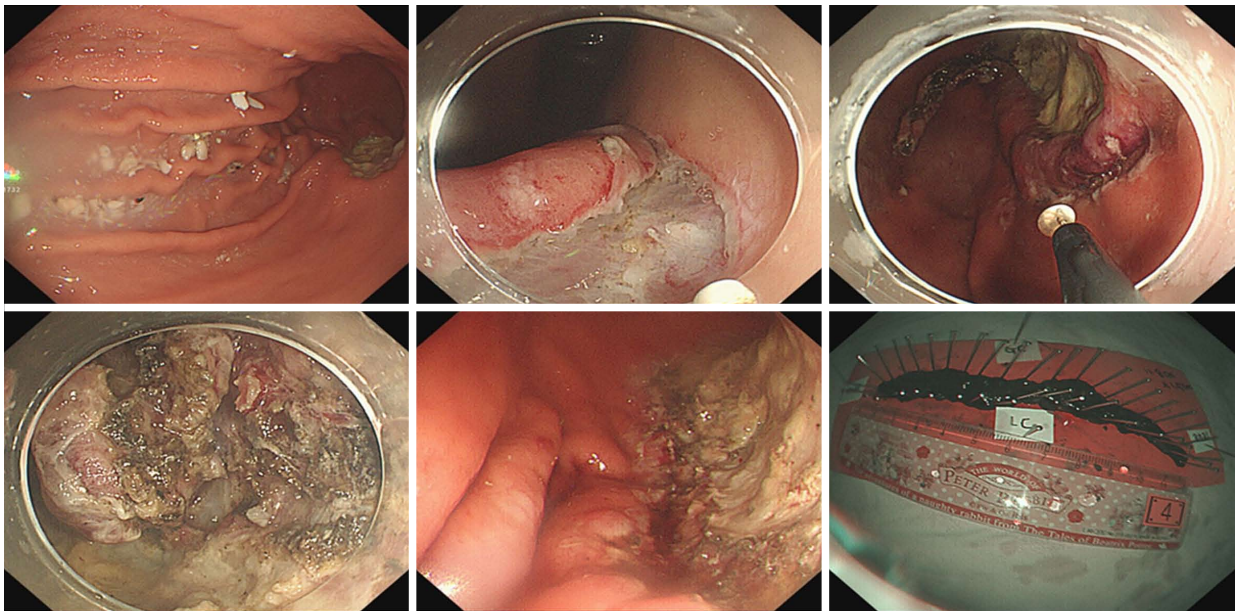


Figure 1 Case of early salvage endoscopic submucosal dissection. A 52 year old female patient was diagnosed with a 6 cm early gastric cancer on the posterior wall of the lower body. The primary ESD procedure was performed, and en bloc resection was achieved. The pathology report indicated the positive lateral margin. Early salvage ESD was performed after histological confirmation of positive lateral margins of the initial ESD specimen. ESD: Endoscopic submucosal dissection.

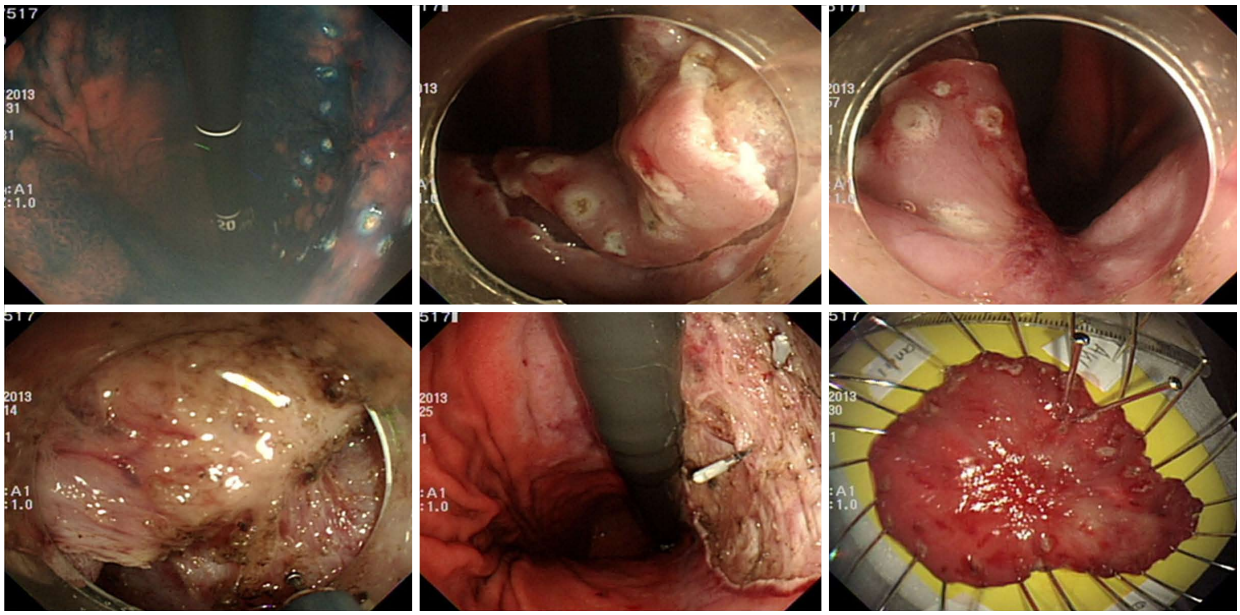


Figure 2 Case of late salvage endoscopic submucosal dissection. A 52 year old male patient was diagnosed with a 3 cm early gastric cancer on the posterior wall of the lower body. The primary ESD procedure was performed, and piecemeal resection was done incompletely due to severe bleeding and adhesion. Late salvage ESD was performed after complete healing of the artificial ulcer caused by the primary ESD. ESD: Endoscopic submucosal dissection.

Early salvage ESD was performed immediately after histological confirmation of positive lateral margins of the initial ESD specimen (Figure 1). Late salvage ESD was prescribed when the positive lateral margin was histologically confirmed after the initial ESD, and was performed after complete healing of the artificial ulcer caused by the initial ESD (Figure 2). Late secondary ESD was performed when local recurrence was proven histologically during the follow-up period after initially curative primary ESD (Figure 3). The general sequence of early or late salvage and late secondary ESD were similar to that of the primary ESD. However, sodium hyaluronate (LG Life Science Co., Seoul, South Korea) was frequently used as a submucosal injection material to overcome non-lifting signs induced by ulceration and fibrosis of the submucosal layer caused by the primary ESD[9]. In general, a non-insulated knife was used for dissecting fibrotic areas that were not lifted due to fibrosis. Additionally, to achieve an appropriate angle for a dissecting view of the submucosa in fibrotic area,

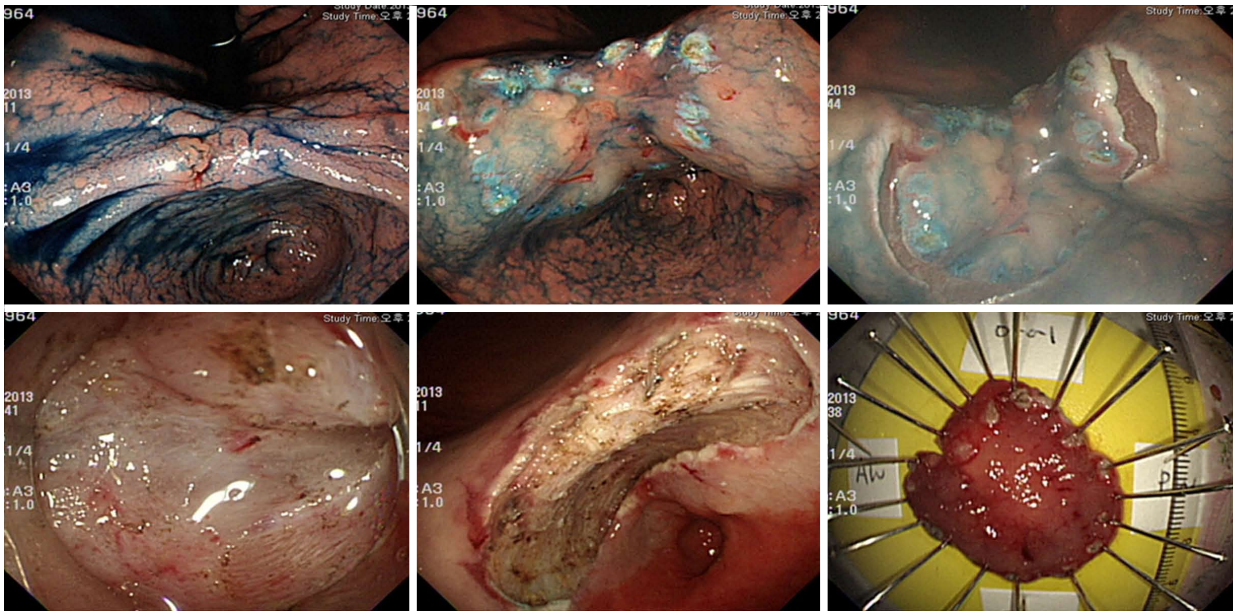


Figure 3 Case of late secondary endoscopic submucosal dissection. A 61 year old male patient was diagnosed with a 1.7 cm high grade dysplasia on the lesser curvature of the angle. The primary ESD procedure was performed, and en bloc resection was achieved. The local recurrence was shown five years after initial curative primary ESD. Late secondary ESD was performed. ESD: Endoscopic submucosal dissection.

the submucosal dissection was initiated in a non-fibrotic area of sufficient distance from the fibrotic area.

Histopathological evaluation of tumors

En bloc resection was defined as a tumor that was removed whole as a single piece. Curative resection was defined as expanded indications according to the Japanese gastric cancer treatment guidelines[5]. According to the Japanese gastric cancer treatment guidelines, the expanded indications for curative endoscopic resection (ER) were en bloc resection, negative lateral and vertical margins, no lymphovascular invasion (LVI) and one of the following: (1) Tumor size > 2 cm, differentiated type, mucosa, and ulcer (-); (2) tumor size ≤ 3 cm, differentiated type, mucosa, and ulcer (+); (3) tumor size ≤ 2 cm, undifferentiated type, mucosa, and ulcer (-); or (4) tumor size ≤ 3 cm, differentiated type, and submucosa1 (SM1, < 500 μm from the muscularis mucosa).

Statistical analysis

Comparison of clinicopathologic factors between primary ESD and secondary ESD was performed using the chi-square test and Fisher's exact test. The Student's *t*-test was used for intergroup comparison of non-categorical variables. The accepted significance level was a *P* value < 0.05. All statistical analyses were performed using the software SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Demographic and clinicopathologic characteristics

Among 1623 patients, 1595 patients received primary ESD and 28 patients underwent secondary ESD. Six of these patients (21.4%) and eight of these patients (28.6%) underwent early and late salvage ESD for residual lesions, respectively, while fourteen of these patients (50.0%) underwent late secondary ESD for locally recurrent lesions. Baseline characteristics of the gastric lesions treated by ESD are shown in Table 1. There were significantly more depressed macroscopic lesions in the secondary ESD group and there were no significant differences in lesion location or size between the two groups. We investigated the initial lesion corresponding to secondary ESD. The initial lesions corresponding to secondary ESD were dysplasia in 13 patients, and carcinoma in 15 patients. A total of 15 patients with carcinoma had mucosal cancer without lymphovascular invasion.

Procedure outcomes

When we compared the procedure outcomes of the 1595 patients in the primary ESD group and the 28 patients in the secondary ESD group, the resected specimen size in the secondary ESD group was significantly larger than that of the primary ESD group, though the lesion size of two groups was not different. Next, significant differences in procedure outcomes were identified by comparing the primary ESD group and the secondary ESD group (Table 2). Patients in the secondary ESD group had a longer mean ESD procedure time than patients in the primary ESD group (78.2 min *vs* 55.1 min, $P = 0.004$). Additionally, the dissection times for the secondary ESD group were significantly longer than that of the primary ESD group (63.4 min *vs* 36.7 min, $P < 0.001$). Furthermore, the dissection speed was significantly lower in the secondary ESD group than in the primary ESD

Table 1 Baseline characteristics

Characteristics	ESD (n = 1595, %)	Secondary ESD (n = 28, %)	*P* value
Gender			0. 836
Male	1117 (70.0)	19 (67.9)	
Female	478 (30.0)	9 (32.1)	
Age [yr, mean (SD)]	64.8 (10.4)	63.5 (9.2)	0.528
Lesion location			0.217
Upper	127 (8.0)	3 (11.1)	
Middle	633 (39.7)	15 (53.6)	
Lower	835 (52.4)	10 (35.7)	
Multiplicity	250 (15.7)	2 (7.1)	0.296
Lesion size [mm, mean (SD)]	17.4 (12.8)	16.4 (13.2)	0.664
Macroscopic type			< 0.001
Elevated	674 (42.3)	7 (25.0)	
Flat	685 (42.9)	9 (32.1)	
Depressed	236 (14.8)	12 (42.9)	
WHO classification			< 0.001
Low grade dysplasia	610 (38.2)	8 (28.6)	
High grade dysplasia	221 (13.9)	4 (14.3)	
Well differentiated	356 (22.3)	4 (14.3)	
Moderately differentiated	268 (16.8)	7 (25.0)	
Poorly differentiated	76 (4.8)	3 (10.7)	
Signet ring cell carcinoma	64 (4.0)	0 (0.0)	
No residual lesion	0 (0.0)	2 (7.1)	
Depth of invasion			< 0.001
Dysplasia	831 (52.1)	12 (42.9)	
Mucosal cancer	609 (38.2)	14 (50.0)	
Submucosal cancer	155 (9.7)	0 (0.0)	
No residual lesion	0 (0.0)	2 (7.1)	
LVI			1.000
No	1546 (96.9)	28 (100.0)	
Yes	49 (3.1)	0 (0.0)	
Lateral margin			0.381
Negative	1521 (95.4)	26 (92.9)	
Positive	74 (4.6)	2 (7.1)	
Vertical margin			0.615
Negative	1542 (96.7)	27 (96.4)	
Positive	53 (3.3)	1 (3.6)	

ESD: Endoscopic submucosal dissection; LVI: Lymphovascular invasion.

Table 2 Comparison of procedural outcomes and oncologic outcomes between the primary endoscopic submucosal dissection group and secondary endoscopic submucosal dissection group

	ESD (n = 1595, %)	Secondary ESD (n = 28, %)	*P* value
Lesion size [mm, mean (SD)]	17.4 (12.8)	16.4 (13.2)	0.664
Specimen size [mm, mean (SD)]	38.1 (14.6)	47.8 (19.6)	0.001
Whole procedure time [min, mean (SD)]	55.1 (41.5)	78.2 (38.0)	0.004
Dissection time [min, mean (SD)]	36.7 (34.7)	63.4 (38.0)	< 0.001
Dissection speed [mm^2/min, mean (SD)]	38.7 (32.8)	22.1 (12.7)	< 0.001
Complication	61 (3.8)	3 (10.7)	0.095
Perforation	37 (2.3)	0 (0.0)	
Bleeding	15 (0.9)	1 (3.6)	
Aspiration pneumonia	13 (0.8)	2 (7.1)	
Hospital stay [d, mean (SD)]	3.6 (3.2)	3.9 (2.5)	0.639
En bloc resection	1574 (98.7)	26 (92.9)	0.058
Curative resection	1383 (86.7)	25 (89.3)	1.000
Additive salvage treatment	104 (6.5)	3 (10.7)	0.425
Additive surgery	85 (5.3)	2 (7.1)	
Redo ESD	16 (1.0)	1 (3.6)	
Argon plasma coagulation	3 (0.2)	0 (0.0)	

ESD: Endoscopic submucosal dissection.

Table 3 Clinicopathologic characteristics according to surgical strategy

Characteristics	Early salvage ESD (*n* = 6, %)	Late salvage ESD (*n* = 8, %)	Late secondary ESD (*n* = 14, %)	*P* value
Gender				0. 909
Male	4 (66.7)	5 (62.5)	10 (71.4)	
Female	2 (33.3)	3 (37.5)	4 (28.6)	
Age [yr, mean (SD)]	65.4 (10.8)	61.6 (7.3)	63.8 (9.9)	0.749
Lesion location				0.255
Upper	2 (33.3)	0 (0.0)	1 (7.1)	
Middle	2 (33.3)	4 (50.0)	9 (64.3)	
Lower	2 (33.3)	4 (50.0)	4 (28.6)	
Multiplicity	0 (0.0)	1 (12.5)	1 (7.1)	0.668
Lesion size [mm, mean (SD)]	19.8 (22.4)	13.6 (10.9)	16.4 (9.8)	0.701
Initial method				0.341
EMR	0 (0.0)	0 (0.0)	2 (14.3)	
ESD	6 (100.0)	8 (100.0)	12 (85.7)	
Macroscopic type				0.014
Elevated	0 (0.0)	0 (0.0)	7 (50.0)	
Flat	4 (66.7)	2 (25.0)	3 (21.4)	
Depressed	2 (33.3)	6 (75.0)	4 (28.6)	
WHO classification				0.081
Low grade dysplasia	2 (33.3)	3 (37.5)	3 (21.4)	
High grade dysplasia	0 (0.0)	1 (12.5)	3 (21.4)	
Well differentiated	1 (16.7)	0 (0.0)	3 (21.4)	
Moderately differentiated	1 (16.7)	4 (50.0)	2 (14.3)	
Poorly differentiated	0 (0.0)	0 (0.0)	3 (21.4)	
Signet ring cell carcinoma	0 (0.0)	0 (0.0)	0 (0.0)	
No residual lesion	2 (33.3)	0 (0.0)	0 (0.0)	
Depth of invasion				0.090
Dysplasia	2 (33.3)	4 (50.0)	6 (42.9)	
Mucosal cancer	2 (33.3)	4 (50.0)	8 (57.1)	
Submucosal cancer	0 (0.0)	0 (0.0)	0 (0.0)	
No residual lesion	2 (33.3)	0 (0.0)	0 (0.0)	
LVI				1.000
No	6 (100.0)	8 (100.0)	14 (100.0)	
Yes	0 (0.0)	0 (0.0)	0 (0.0)	
Lateral margin				0.341
Negative	6 (100.0)	8 (100.0)	12 (85.7)	
Positive	0 (0.0)	0 (0.0)	2 (14.3)	
Vertical margin				0.617
Negative	6 (100.0)	8 (100.0)	13 (92.9)	
Positive	0 (0.0)	0 (0.0)	1 (7.1)	

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; LVI: Lymphovascular invasion.

group (22.1 mm^2/min *vs* 38.7 mm^2/min, $P < 0.001$). The adverse events rate in the secondary ESD group was not significantly different but trended slightly higher than that in the primary ESD group (10.7% *vs* 3.8%, $P = 0.095$). There were no perforations in the secondary ESD group.

Oncologic outcomes

The en bloc resection and curative rates of resection of secondary ESD were 92.9% and 89.3%, respectively. These rates were comparable with those of primary ESD, which had an en bloc resection rate of 98.7% and a curative resection rate of 86.7% (Table 2). Three patients in the secondary ESD group had additive salvage treatments due to a positive lateral margin ($n = 2$) and other synchronous lesions that had vertical margin involvement after secondary ESD ($n = 1$). One patient in the secondary ESD group showed a positive vertical margin with a very focal extension of dysplasia. This patient was 75 years old and decided to be observed and was followed up closely. During follow-up, one patient who received secondary ESD presented with metachronous recurrence 25 months after secondary ESD. We evaluated the outcomes according to an institutional learning curve based upon the initial phase and the late phase of the study period. The en bloc resection rate was not significantly different (initial phase *vs* late phase, 98.4% *vs* 98.8%, $P = 0.533$). Curative resection rate was significantly lower in initial phase *vs* late phase (84.6% *vs* 88.8%, $P = 0.013$). When we evaluated the outcomes in the secondary ESD group, the en bloc and curative resection rates were not statistically different (initial phase *vs* late phase, 100% *vs* 88.2%, $P = 0.505$ and 90.9 % *vs* 88.2%, $P = 1.000$, respectively).

Subgroup analysis according to surgical strategy

We categorized secondary ESD into three groups according to the surgical strategy (early salvage, late salvage, or late secondary ESD) (Table 3). The median interval times from the primary ESD to the secondary

Table 4 Comparison of procedural outcomes and oncologic outcomes according to surgical strategy

	Early salvage ESD (n = 6, %)	Late salvage ESD (n = 8, %)	Late secondary ESD (n = 14, %)	P value
Lesion size [mm, mean (SD)]	19.8 (22.4)	13.6 (10.9)	16.4 (9.8)	0.701
Specimen size [mm, mean (SD)]	63.0 (33.0)	46.4 (11.6)	42.1 (12.8)	0.084
Whole procedure time [min, mean (SD)]	81.7 (48.5)	85.9 (24.3)	72.3 (41.1)	0.714
Dissection time [min, mean (SD)]	73.2 (46.8)	73.0 (23.4)	53.8 (40.7)	0.421
Dissection speed [mm^2/min, mean (SD)]	19.4 (6.8)	19.0 (7.5)	24.9 (12.7)	0.512
Method				0.341
ESD only	6 (100.0)	8 (100.0)	12 (85.7)	
ESD plus snaring	0 (0.0)	0 (0.0)	2 (14.3)	
Complication	0 (0.0)	1 (12.5)	2 (14.3)	0.627
Perforation	0 (0.0)	0 (0.0)	0 (0.0)	
Bleeding	0 (0.0)	0 (0.0)	1 (7.1)	
Aspiration pneumonia	0 (0.0)	1 (12.5)	1 (7.1)	
Hospital stay [d, mean (SD)]	7.2 (2.9)	3.0 (1.8)	3.0 (1.2)	< 0.001
Median interval [d, mean (SD)]	6.5 (3.1)	129.0 (133.6)	712.8 (546.5)	0.001
En bloc resection	6 (100.0)	8 (100.0)	12 (85.7)	0.341
Curative resection	6 (100.0)	8 (100.0)	11 (78.6)	0.186
Additive treatment	0 (0.0)	0 (0.0)	3 (21.4)	0.186
Additive surgery	0 (0.0)	0 (0.0)	2 (14.3)	
Redo ESD	0 (0.0)	0 (0.0)	1 (7.1)	

ESD: Endoscopic submucosal dissection.

ESD were 6.5, 129.0, and 712.8 d in patients who received early salvage, late salvage, or late secondary ESD, respectively. Hospital stay was significantly longer in early salvage ESD group compared with late salvage and late secondary ESD groups (7.2 d *vs* 3.0 d and 3.0 d, $P \leq 0.001$). There was no other significant difference in procedure outcomes among the three groups (Table 4). The en bloc and curative resection rates of early and late salvage groups were both 100%. However, the en bloc and curative resection rates of the late secondary ESD group tended to be lower than that of the other groups (Table 4).

DISCUSSION

ESD is now widely performed for gastric neoplasms and has favorable outcomes. However, the incomplete resection rate of ESD is 2.2% to 26.3%[10-21] and the local recurrence rate after ESD is 0% to 3%[15,16,22-24]. Therefore, the management of residual or locally recurrent gastric neoplasms after ESD continues to be problematic. Secondary ESD for a locally recurrent tumor is difficult to perform due to severe fibrosis and ulcer scar formation, and there are few published reports about secondary ESD for residual or locally recurrent gastric neoplasms. Bae *et al*[6] reported a total of 16 early secondary ESD cases in which the curative resection rate was 93.8% (15/16). Therefore, they asserted that early secondary ESD was a feasible and useful treatment in patients with a positive lateral margin. Additionally, secondary ESD was also determined to be an effective treatment for locally recurrent lesions after endoscopic mucosal resection (EMR)[7]. Oka *et al*[7] reported that the complete resection rate of ESD for a residual or locally recurrent lesion is 93.3% (14/15). Therefore, they suggested that secondary ESD was an effective and minimally invasive procedure for patients with residual or local recurrent tumors after EMR.

Secondary ESD is technically difficult due to the severe fibrosis and scar formation that results from primary ESD[25]. In this study, the mean procedure time and dissection time of secondary ESD was longer than that for primary ESD. In addition, the dissection speed was lower in the secondary ESD group than in the primary ESD group, while the adverse events rate of secondary ESD was slightly higher than that of primary ESD. That is, secondary ESD is associated with a greater technical difficulty, longer procedure time, and increased incidence of adverse events. However, in this study, bleeding after secondary ESD was managed endoscopically, without the need for further emergent operation; aspiration pneumonia was treated with antibiotics and conservative care. The aspiration pneumonia occurred in 15 (0.9%) patients. The procedure time was longer in patients with aspiration pneumonia than others (83.3 min *vs* 55.1 min, $P = 0.125$). However, the difference was not statistically significant. Although many endoscopists worry that submucosal fibrosis might cause perforation, in this study, there were no perforations reported in the secondary ESD group. We suspected that deep excavation by benign peptic ulcers would not preserve the muscle layer. However, the artificial ulcer caused by ESD was contracted, preserving the muscle layer, which was thicker than before ESD. Therefore, this observation may explain the rarity of perforation. Primary ESD procedures in this study were performed by one of five ESD endoscopists. Two of them were fully skilled experts (YYH, far more than a thousand cases of ESD experience; KJH, more than five hundreds cases of ESD experience) and the other three were less-

skilled ESD endoscopists whose ESD experience were less than a hundred cases. However, secondary ESD in this study was exclusively performed only by the two fully skilled ESD experts (YYH and KJH). That might be a reason why the outcomes of the secondary ESD group were relatively good despite the technical difficulty.

ESD has multiple advantages compared to other treatment options for residual or locally recurrent tumors, such as gastrectomy and additional endoscopic treatment. Compared to gastrectomy, ESD offers improved quality of life, decreased morbidity, longer length of recovery, and lower healthcare costs. It also has advantages over other endoscopic treatments, such as argon plasma coagulation, including a high chance of en bloc resection, which allows for complete histologic assessment. Therefore, the application of secondary ESD for residual or locally recurrent tumors is an important treatment consideration.

When we categorized secondary ESD according to the time interval from primary ESD, the main difference between early secondary ESD and late secondary ESD was scar formation. Early secondary ESD is performed while the ESD ulcer is still open, therefore, there is minimal fibrosis. However, by the time late secondary ESD is performed, the ulcer has healed with severe fibrosis[26]. In this study, we compared three groups: early salvage ESD, late salvage ESD, and late secondary ESD, and hospital stay of the early salvage ESD group was the longest. This difference was due to many patients in the early salvage ESD group that underwent primary and secondary ESD successively during a single hospitalization. There was no other significant difference in procedure outcomes regarding the whole procedure time, dissection time, and dissection speed among the three groups. Although the late salvage ESD and late secondary ESD groups had severe fibrosis and scar formations, no perforations occurred.

According to this study, secondary ESD for residual or locally recurrent tumors is a safe, minimally invasive, and effective treatment. However, secondary ESD should be performed by an experienced endoscopist with precautions, including the appropriate electrosurgical knife, the appropriate dissection depth, and the careful gradual dissection of the severe fibrotic tissue along the plane of the deep submucosa or superficial proper muscle. Furthermore, the surrounding non-fibrotic tissue should first be dissected sufficiently to make a flap of the specimen and directly visualize the plane of the submucosa from the non-lifting fibrotic area to the fibrotic area[27,28]. Therefore, in this study, we found that the specimen size of the secondary ESD group was significantly larger than the primary ESD group. In addition, one recommendation for dissecting the fibrotic area is to dissect deeply along the surface of the muscle fiber because the ESD ulcer scar has an intact muscle layer.

Correct diagnosis of the depth of invasion of residual or locally recurrent tumors is often difficult due to scar formation after ESD. Therefore, residual or locally recurrent tumors with submucosal invasion are sometimes misdiagnosed as mucosal cancer[29]. In this study, the depth of invasion of all secondary ESD was within the mucosa.

In previous reports, the curative resection rate for residual gastric neoplasms was 92% to 100%[6,26,30] and curative resection rate for locally recurrent gastric neoplasms was 76% (35/46) to 87% (13/15)[26,28]. In this study, the en bloc and curative resection rates of secondary ESD were 92.9% and 89.3%, respectively, which were comparable with those reported previously for secondary ESD. Two patients in the early salvage ESD group had no residual tumor. Although, they showed histological confirmation of positive lateral margins of the initial ESD specimen, no residual lesion was detected due to burning effects on the tissue by electrosurgical unit.

Early and late salvage ESD, which were both performed after histological confirmation of positive lateral margins of the primary ESD specimen, did not involve en bloc resection of the primary lesion. Therefore, the risks of local recurrence and lymph node metastasis resulting from piecemeal resection were always taken into consideration. Hence, careful surveillance after early and late salvage ESD is also very important. In this study, there was no local recurrence during the mean follow-up period in any patient who received secondary ESD.

There is no consensus on the timing of salvage ESD. Until now, there were few reports about the feasibility and effectiveness of early salvage ESD[6,26,31]. In this study, we compared the clinicopathologic factors between early salvage ESD and late salvage ESD and found no significant differences in procedure or oncologic outcomes. However, further study about the timing of salvage ESD will be needed.

Although this study was retrospective with a small number of patients, this is the first study to report the feasibility and safety of secondary ESD according to the timing of ESD.

In conclusion, we found that secondary ESD appears to be a feasible and curative treatment. When we consider the morbidity of gastrectomy, secondary ESD of residual or locally recurrent gastric neoplasms is a good therapeutic option worth pursuing, although the procedure time is longer and associated with higher technical difficulty.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) is an accepted curative treatment option for gastric tumors with very low local recurrence. However, residual or locally recurrent tumors occur rarely after ESD. Although secondary ESD is technically demanding, it can be applied to residual or recurrent tumors with scar and dense fibrotic submucosa. We investigated the feasibility and safety of secondary ESD for gastric tumors. We also categorized secondary ESD into three groups according to the surgical strategy (early salvage, late salvage, or

late secondary ESD) and analyzed them.

Research motivation
There is no consensus on the timing of salvage ESD. Until now, there were few reports about the feasibility and effectiveness of early salvage ESD.

Research objectives
To investigate the feasibility and safety of secondary ESD for residual or locally recurrent gastric tumors.

Research methods
Between 2010 and 2017, 1623 consecutive patients underwent ESD for gastric neoplasms at a single tertiary referral center. Among these, 28 patients underwent secondary ESD for a residual or locally recurrent tumor. Our analysis compared clinicopathologic factors between primary ESD and secondary ESD groups.

Research results
The en bloc resection and curative rate of resection of secondary ESD were 92.9% and 89.3%. The average procedure time of secondary ESD was significantly longer than primary ESD, and the adverse events rate was not statistically different but trended slightly higher in the secondary ESD group compared to the primary ESD group. Patients who received secondary ESD had favorable outcomes without severe adverse events. During a mean follow-up period, no local recurrence occurred in patients who received secondary ESD.

Research conclusions
Although it requires greater technical efficiency and longer procedure time, secondary ESD of residual or locally recurrent gastric tumors appears to be feasible and curative treatment.

Research perspectives
Secondary ESD of residual or locally recurrent gastric tumors is a feasible and curative treatment.

REFERENCES

1 **Hoteya S**, Iizuka T, Kikuchi D, Yahagi N. Benefits of endoscopic submucosal dissection according to size and location of gastric neoplasm, compared with conventional mucosal resection. *J Gastroenterol Hepatol* 2009; **24**: 1102-1106 [PMID: 19383079 DOI: 10.1111/j.1440-1746.2009.05811.x]

2 **Tanaka M**, Ono H, Hasuike N, Takizawa K. Endoscopic submucosal dissection of early gastric cancer. *Digestion* 2008; **77** Suppl 1: 23-28 [PMID: 18204258 DOI: 10.1159/000111484]

3 **Otsuka Y**, Niwa Y, Ohmiya N, Ando N, Ohashi A, Hirooka Y, Goto H. Usefulness of magnifying endoscopy in the diagnosis of early gastric cancer. *Endoscopy* 2004; **36**: 165-169 [PMID: 14765314 DOI: 10.1055/s-2004-814184]

4 **Zhao Z**, Yin Z, Wang S, Wang J, Bai B, Qiu Z, Zhao Q. Meta-analysis: The diagnostic efficacy of chromoendoscopy for early gastric cancer and premalignant gastric lesions. *J Gastroenterol Hepatol* 2016; **31**: 1539-1545 [PMID: 26860924 DOI: 10.1111/jgh.13313]

5 **Japanese Gastric Cancer Association**. Japanese gastric cancer treatment guidelines 2014 (ver. 4). *Gastric Cancer* 2017; **20**: 1-19 [PMID: 27342689 DOI: 10.1007/s10120-016-0622-4]

6 **Bae SY**, Jang TH, Min BH, Lee JH, Rhee PL, Rhee JC, Kim JJ. Early additional endoscopic submucosal dissection in patients with positive lateral resection margins after initial endoscopic submucosal dissection for early gastric cancer. *Gastrointest Endosc* 2012; **75**: 432-436 [PMID: 22248614 DOI: 10.1016/j.gie.2011.09.044]

7 **Oka S**, Tanaka S, Kaneko I, Mouri R, Hirata M, Kanao H, Kawamura T, Yoshida S, Yoshihara M, Chayama K. Endoscopic submucosal dissection for residual/local recurrence of early gastric cancer after endoscopic mucosal resection. *Endoscopy* 2006; **38**: 996-1000 [PMID: 17058164 DOI: 10.1055/s-2006-944780]

8 **Sekiguchi M**, Suzuki H, Oda I, Abe S, Nonaka S, Yoshinaga S, Taniguchi H, Sekine S, Kushima R, Saito Y. Favorable long-term outcomes of endoscopic submucosal dissection for locally recurrent early gastric cancer after endoscopic resection. *Endoscopy* 2013; **45**: 708-713 [PMID: 23918620 DOI: 10.1055/s-0033-1344332]

9 **Yamamoto H**, Kawata H, Sunada K, Sasaki A, Nakazawa K, Miyata T, Sekine Y, Yano T, Satoh K, Ido K, Sugano K. Successful en-bloc resection of large superficial tumors in the stomach and colon using sodium hyaluronate and small-caliber-tip transparent hood. *Endoscopy* 2003; **35**: 690-694 [PMID: 12929067 DOI: 10.1055/s-2003-41516]

10 **Chung IK**, Lee JH, Lee SH, Kim SJ, Cho JY, Cho WY, Hwangbo Y, Keum BR, Park JJ, Chun HJ, Kim HJ, Kim JJ, Ji SR, Seol SY. Therapeutic outcomes in 1000 cases of endoscopic submucosal dissection for early gastric neoplasms: Korean ESD Study Group multicenter study. *Gastrointest Endosc* 2009; **69**: 1228-1235 [PMID: 19249769 DOI: 10.1016/j.gie.2008.09.027]

11 **Akasaka T**, Nishida T, Tsutsui S, Michida T, Yamada T, Ogiyama H, Kitamura S, Ichiba M, Komori M, Nishiyama O, Nakanishi F, Zushi S, Nishihara A, Iijima H, Tsujii M, Hayashi N. Short-term outcomes of endoscopic submucosal dissection (ESD) for early gastric neoplasm: multicenter survey by osaka university ESD study group. *Dig Endosc* 2011; **23**: 73-77 [PMID: 21198921 DOI: 10.1111/j.1443-1661.2010.01062.x]

12 **Ahn JY**, Jung HY, Choi KD, Choi JY, Kim MY, Lee JH, Choi KS, Kim DH, Song HJ, Lee GH, Kim JH, Park YS. Endoscopic and oncologic outcomes after endoscopic resection for early gastric cancer: 1370 cases of absolute and extended indications. *Gastrointest Endosc* 2011; **74**: 485-493 [PMID: 21741645 DOI: 10.1016/j.gie.2011.04.038]

13 **Shin KY**, Jeon SW, Cho KB, Park KS, Kim ES, Park CK, Chung YJ, Kwon JG, Jung JT, Kim EY, Kim KO, Jang BI, Lee SH, Park JB, Yang CH. Clinical outcomes of the endoscopic submucosal dissection of early gastric cancer are comparable between absolute and new expanded criteria. *Gut Liver* 2015; **9**: 181-187 [PMID: 25167797 DOI: 10.5009/gnl13417]

14 **Nakamura K**, Honda K, Akahoshi K, Ihara E, Matsuzaka H, Sumida Y, Yoshimura D, Akiho H, Motomura Y, Iwasa T, Komori K, Chijiiwa Y, Harada N, Ochiai T, Oya M, Oda Y, Takayanagi R. Suitability of the expanded indication criteria for the treatment of early gastric cancer by endoscopic submucosal dissection: Japanese multicenter large-scale retrospective analysis of short- and long-term outcomes. *Scand J Gastroenterol* 2015; **50**: 413-422 [PMID: 25635364 DOI: 10.3109/00365521.2014.940377]

15 **Takenaka R**, Kawahara Y, Okada H, Hori K, Inoue M, Kawano S, Tanioka D, Tsuzuki T, Yagi S, Kato J, Uemura M, Ohara N, Yoshino T, Imagawa A, Fujiki S, Takata R, Yamamoto K. Risk factors associated with local recurrence of early gastric cancers after endoscopic submucosal dissection. *Gastrointest Endosc* 2008; **68**: 887-894 [PMID: 18565523 DOI: 10.1016/j.gie.2008.03.1089]

16 **Oka S**, Tanaka S, Kaneko I, Mouri R, Hirata M, Kawamura T, Yoshihara M, Chayama K. Advantage of endoscopic submucosal dissection compared with EMR for early gastric cancer. *Gastrointest Endosc* 2006; **64**: 877-883 [PMID: 17140890 DOI: 10.1016/j.gie.2006.03.932]

17 **Oda I**, Saito D, Tada M, Iishi H, Tanabe S, Oyama T, Doi T, Otani Y, Fujisaki J, Ajioka Y, Hamada T, Inoue H, Gotoda T, Yoshida S. A multicenter retrospective study of endoscopic resection for early gastric cancer. *Gastric Cancer* 2006; **9**: 262-270 [PMID: 17235627 DOI: 10.1007/s10120-006-0389-0]

18 **Isomoto H**, Shikuwa S, Yamaguchi N, Fukuda E, Ikeda K, Nishiyama H, Ohnita K, Mizuta Y, Shiozawa J, Kohno S. Endoscopic submucosal dissection for early gastric cancer: a large-scale feasibility study. *Gut* 2009; **58**: 331-336 [PMID: 19001058 DOI: 10.1136/gut.2008.165381]

19 **Goto O**, Fujishiro M, Kodashima S, Ono S, Omata M. Outcomes of endoscopic submucosal dissection for early gastric cancer

with special reference to validation for curability criteria. *Endoscopy* 2009; **41**: 118-122 [PMID: 19214889 DOI: 10.1055/s-0028-1119452]
20 **Kang HY**, Kim SG, Kim JS, Jung HC, Song IS. Clinical outcomes of endoscopic submucosal dissection for undifferentiated early gastric cancer. *Surg Endosc* 2010; **24**: 509-516 [PMID: 19585066 DOI: 10.1007/s00464-009-0614-0]
21 **Nakamoto S**, Sakai Y, Kasanuki J, Kondo F, Ooka Y, Kato K, Arai M, Suzuki T, Matsumura T, Bekku D, Ito K, Tanaka T, Yokosuka O. Indications for the use of endoscopic mucosal resection for early gastric cancer in Japan: a comparative study with endoscopic submucosal dissection. *Endoscopy* 2009; **41**: 746-750 [PMID: 19681023 DOI: 10.1055/s-0029-1215010]
22 **Tanabe S**, Ishido K, Higuchi K, Sasaki T, Katada C, Azuma M, Naruke A, Kim M, Koizumi W. Long-term outcomes of endoscopic submucosal dissection for early gastric cancer: a retrospective comparison with conventional endoscopic resection in a single center. *Gastric Cancer* 2014; **17**: 130-136 [PMID: 23576197 DOI: 10.1007/s10120-013-0241-2]
23 **Park JC**, Lee SK, Seo JH, Kim YJ, Chung H, Shin SK, Lee YC. Predictive factors for local recurrence after endoscopic resection for early gastric cancer: long-term clinical outcome in a single-center experience. *Surg Endosc* 2010; **24**: 2842-2849 [PMID: 20428894 DOI: 10.1007/s00464-010-1060-8]
24 **Onozato Y**, Ishihara H, Iizuka H, Sohara N, Kakizaki S, Okamura S, Mori M. Endoscopic submucosal dissection for early gastric cancers and large flat adenomas. *Endoscopy* 2006; **38**: 980-986 [PMID: 17058161 DOI: 10.1055/s-2006-944809]
25 **Kakushima N**, Fujishiro M, Kodashima S, Kobayashi K, Tateishi A, Iguchi M, Imagawa A, Motoi T, Yahagi N, Omata M. Histopathologic characteristics of gastric ulcers created by endoscopic submucosal dissection. *Endoscopy* 2006; **38**: 412-415 [PMID: 16680644 DOI: 10.1055/s-2006-925166]
26 **Hoteya S**, Iizuka T, Kikuchi D, Mitani T, Matsui A, Ogawa O, Furuhata T, Yamashta S, Yamada A, Kaise M, Yahagi N. Secondary endoscopic submucosal dissection for residual or recurrent tumors after gastric endoscopic submucosal dissection. *Gastric Cancer* 2014; **17**: 697-702 [PMID: 24310295 DOI: 10.1007/s10120-013-0325-z]
27 **Shimamura Y**, Ishii N, Nakano K, Ikeya T, Nakamura K, Takagi K, Fukuda K, Suzuki K, Fujita Y. Repeat endoscopic submucosal dissection for recurrent gastric cancers after endoscopic submucosal dissection. *World J Gastrointest Endosc* 2013; **5**: 600-604 [PMID: 24368936 DOI: 10.4253/wjge.v5.i12.600]
28 **Yokoi C**, Gotoda T, Hamanaka H, Oda I. Endoscopic submucosal dissection allows curative resection of locally recurrent early gastric cancer after prior endoscopic mucosal resection. *Gastrointest Endosc* 2006; **64**: 212-218 [PMID: 16860071 DOI: 10.1016/j.gie.2005.10.038]
29 **Okada K**, Fujisaki J, Kasuga A, Omae M, Yoshimoto K, Hirasawa T, Ishiyama A, Yamamoto Y, Tsuchida T, Hoshino E, Igarashi M, Takahashi H. Endoscopic ultrasonography is valuable for identifying early gastric cancers meeting expanded-indication criteria for endoscopic submucosal dissection. *Surg Endosc* 2011; **25**: 841-848 [PMID: 20734082 DOI: 10.1007/s00464-010-1279-4]
30 **Higashimaya M**, Oka S, Tanaka S, Numata N, Sanomura Y, Yoshida S, Arihiro K, Chayama K. Endoscopic submucosal dissection for residual early gastric cancer after endoscopic submucosal dissection. *Gastrointest Endosc* 2013; **77**: 298-302 [PMID: 23206812 DOI: 10.1016/j.gie.2012.09.003]
31 **Kikuchi D**, Iizuka T, Hoteya S, Yamada A, Furuhata T, Yamashita S, Domon K, Nakamura M, Matsui A, Mitani T, Ogawa O, Watanabe S, Yahagi N, Kaise M. Safety and efficacy of secondary endoscopic submucosal dissection for residual gastric carcinoma after primary endoscopic submucosal dissection. *Digestion* 2012; **86**: 288-293 [PMID: 23051712 DOI: 10.1159/000342114]

P- Reviewer: Akiho H, Nishida T **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3786-3798

DOI: 10.3748/wjg.v24.i33.3786

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

ORIGINAL ARTICLE

Retrospective Study

Differentiation of intrahepatic cholangiocarcinoma from hepatocellular carcinoma in high-risk patients: A predictive model using contrast-enhanced ultrasound

Li-Da Chen, Si-Min Ruan, Jin-Yu Liang, Zheng Yang, Shun-Li Shen, Yang Huang, Wei Li, Zhu Wang, Xiao-Yan Xie, Ming-De Lu, Ming Kuang, Wei Wang

Li-Da Chen, Si-Min Ruan, Jin-Yu Liang, Yang Huang, Wei Li, Zhu Wang, Xiao-Yan Xie, Ming-De Lu, Ming Kuang, Wei Wang, Department of Medical Ultrasonics, Institute of Diagnostic and Interventional Ultrasound, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Zheng Yang, Department of Pathology, The Seventh Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Shun-Li Shen, Ming-De Lu, Ming Kuang, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

ORCID number: Li-Da Chen (0000-0001-9904-2195); Si-Min Ruan (0000-0002-1121-3664); Jin-Yu Liang (0000-0002-1121-3664); Zheng Yang (0000-0002-1121-3664); Shun-Li Shen (0000-0002-1121-3664); Yang Huang (0000-0002-1121-3664); Wei Li (0000-0002-1121-3664); Zhu Wang (0000-0002-4272-3501); Xiao-Yan Xie (0000-0002-4272-3501); Ming-De Lu (0000-0002-4272-3501); Ming Kuang (0000-0002-7397-5779); Wei Wang (0000-0002-9485-583X).

Author contributions: Chen LD, Ruan SM, Wang W, Xie XY, Lu MD, Kuang M designed the research; Chen LD, Ruan SM, Wang W, Liang JY, Huang Y performed the research; Chen LD and Ruan SM contributed equally to the design and preparation of this study and should be considered co-first authors; Li W, Wang Z contributed new reagents or analytical tools; Yang Z, Shen SL analyzed data; Chen LD, Ruan SM wrote the paper.

Supported by the National Nature Science Foundation of China, No. 81701719; the Guangdong Science and Technology Foundation, No. 2017A020215195; and the Guangdong Medical Scientific Research Foundation, No. A2016254.

Institutional review board statement: This study was approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-Sen University.

Informed consent statement: Informed consent was obtained from each patient.

Conflict-of-interest statement: The authors have declared no conflicts of interest.

Manuscript source: Unsolicited manuscript

Correspondence to: Wei Wang, MD, PhD, Associate Professor, Department of Medical Ultrasonics, Institute of Diagnostic and Interventional Ultrasound, The First Affiliated Hospital of Sun Yat-Sen University, 58 Zhongshan Road 2, Guangzhou 510080, Guangdong Province, China. wangw73@mail.sysu.edu.cn
Telephone: +86-20-87765183
Fax: +86-20-87765183

Received: May 7, 2018
Peer-review started: May 7, 2018
First decision: May 16, 2018
Revised: June 30, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 7, 2018

Abstract

AIM

To develop a contrast-enhanced ultrasound (CEUS)

predictive model for distinguishing intrahepatic cholangiocarcinoma (ICC) from hepatocellular carcinoma (HCC) in high-risk patients.

METHODS

This retrospective study consisted of 88 consecutive high-risk patients with ICC and 88 high-risk patients with HCC selected by propensity score matching between May 2004 and July 2016. Patients were assigned to two groups, namely, a training set and validation set, at a 1:1 ratio. A CEUS score for diagnosing ICC was generated based on significant CEUS features. Then, a nomogram based on the CEUS score was developed, integrating the clinical data. The performance of the nomogram was then validated and compared with that of the LR-M of the CEUS Liver Imaging Reporting and Data System (LI-RADS).

RESULTS

The most useful CEUS features for ICC were as follows: rim enhancement (64.5%), early washout (91.9%), intratumoral vein (58.1%), obscure boundary of intratumoral non-enhanced area (64.5%), and marked washout (61.3%, all $P < 0.05$). In the validation set, the area under the curve (AUC) of the CEUS score (AUC = 0.953) for differentiation between ICC and HCC was improved compared to the LI-RADS (AUC = 0.742) ($P < 0.001$). When clinical data were added, the CEUS score nomogram was superior to the LI-RADS nomogram (AUC: 0.973 *vs* 0.916, $P = 0.036$, Net Reclassification Improvement: 0.077, Integrated Discrimination Index: 0.152). Subgroup analysis demonstrated that the CEUS score model was notably improved compared to the LI-RADS in tumors smaller than 5.0 cm ($P < 0.05$) but not improved in tumors smaller than 3.0 cm ($P > 0.05$).

CONCLUSION

The CEUS predictive model for differentiation between ICC and HCC in high-risk patients had improved discrimination and clinical usefulness compared to the CEUS LI-RADS.

Key words: Ultrasonography; Hepatocellular carcinoma; Intrahepatic cholangiocarcinoma; Hepatitis

Core tip: A contrast-enhanced ultrasound (CEUS) score for predicting intrahepatic cholangiocarcinoma (ICC) consisting of more detailed CEUS features was constructed. The diagnostic performance of the CEUS score for differentiation between ICC and hepatocellular carcinoma were improved compared to the LR-M of the Liver Imaging Reporting and Data System (LI-RADS). A CEUS score nomogram, which added the clinical risk factors, was superior to the LI-RADS nomogram.

Chen LD, Ruan SM, Liang JY, Yang Z, Shen SL, Huang Y, Li W, Wang Z, Xie XY, Lu MD, Kuang M, Wang W. Differentiation of intrahepatic cholangiocarcinoma from hepatocellular carcinoma in high-risk patients: A predictive model using contrast-enhanced ultrasound. *World J Gastroenterol* 2018; 24(33): 3786-3798 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3786.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3786

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant epithelial cancer originating from bile ducts with cholangiocyte differentiation[1]. In recent years, chronic cirrhosis and viral hepatitis have been recognized as important risk factors for ICC development[2]. ICC has been increasingly found in patients with cirrhosis[3], and distinguishing between ICC and hepatocellular carcinoma (HCC) is a major clinical issue because the management and prognosis of these conditions differ significantly[4].

In recent years, the value of contrast-enhanced ultrasound (CEUS) for distinguishing ICC from HCC has been controversial. Vilana *et al*[5] has pointed out that ICC in cirrhosis shares a similar enhancement pattern to that of HCC on CEUS (47.6%, 10/21), which may lead to a false-positive diagnosis of HCC. Therefore, CEUS has been eliminated from the HCC diagnostic flowchart in the updated American Association for the Study of Liver Diseases (AASLD) 2011 guidelines[6]. This removal has caused controversy and has not been widely accepted in Europe or Asia[7-10] because the study by Vilana R was only based on a rather small sample size without differential diagnostic analysis of ICC versus HCC.

In 2016, the American College of Radiology (ACR) released the CEUS Liver Imaging Reporting and Data System® (LI-RADS®). The CEUS LI-RADS® standardizes the CEUS diagnostic system for patients at risk for developing HCC. In this system, the diagnosis of HCC should be distinguished from that of not only benign lesions (LR-1 or LR-2) but also other malignancies of the liver, namely, LR-M. LR-M represents a category for lesions that are definitely or probably malignant, and their features are defined as rim enhancement and early and/or marked washout. These features most closely refer to the appearance of ICC. The CEUS LI-RADS® sets the specific category of LR-M for distinguishing ICC from HCC, but the diagnostic dilemma remains unresolved. Additionally, no study has validated the performance of LR-M as the differential diagnostic criterion for ICC and HCC.

Since our first study in 2010, several studies have assessed the usefulness of CEUS in the differentiation of ICC and HCC[11-17]. Because ICCs are rare in cirrhotic livers[17], only two of these studies were able to test the performance of differential diagnosis from HCC in high-risk patients[15,16]. Several reports indicated that the typical rim-like hyperenhancement of ICC,

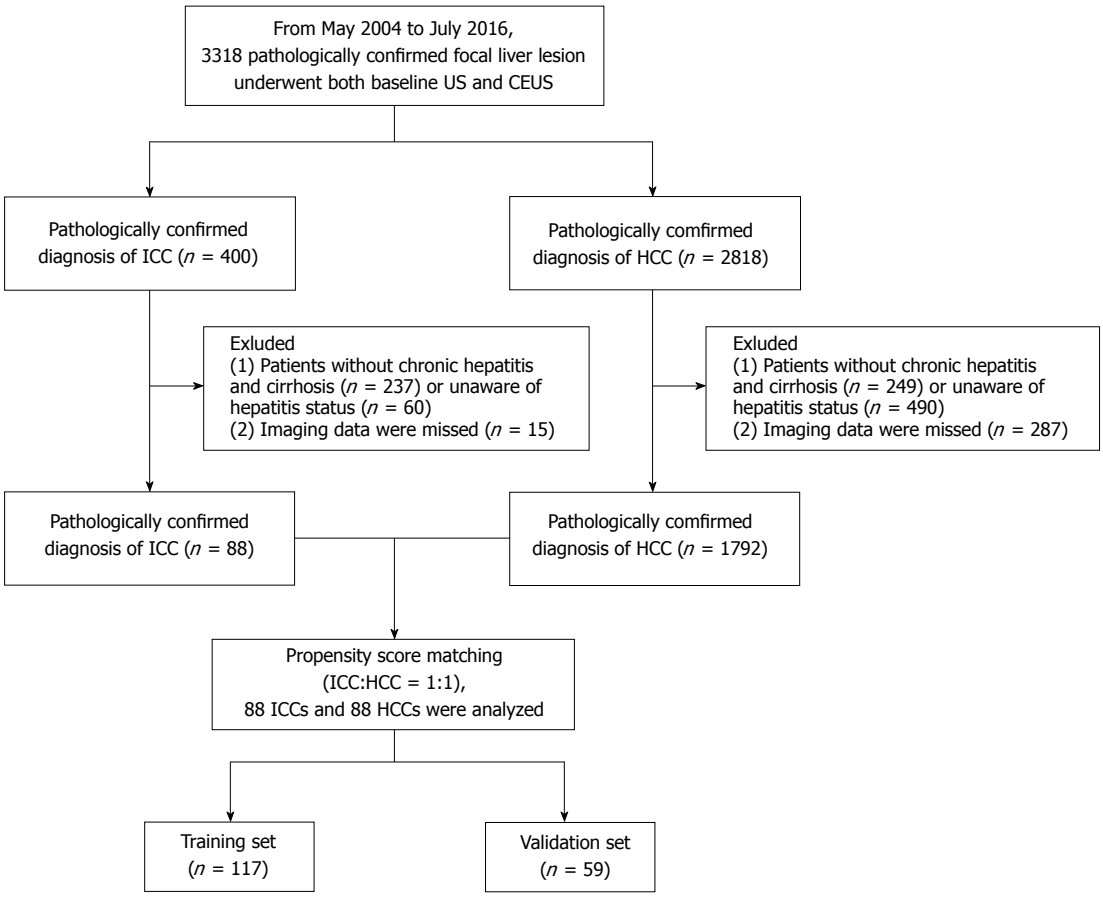


Figure 1 Flowchart of the intrahepatic cholangiocarcinoma and hepatocellular carcinoma patient selection process. CEUS: Contrast-enhanced ultrasound; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma.

recommended by the EFSUMB guideline, was observed in 8.0% to 69.2% of high-risk patients[16,18-20]. The time point for early washout was also defined within 40 s, 43 s or 60 s after contrast agent injection in three different cohorts[14-16]. Unfortunately, the differentiation criteria based on the above features varied greatly, with the sensitivity ranging from 64.1% to 87.9% and the specificity ranging from 17.9% to 97.4%[15,16]. The inconsistency indicated that ICCs in high-risk patients required further investigation from real-time CEUS, especially in comparative studies with HCC using a larger sample size.

In light of the abovementioned issues, we sought to identify important imaging predictors of ICC on CEUS, to develop a novel diagnostic nomogram incorporating clinical, CEUS and laboratory characteristics that could be used to accurately predict the risk of ICC in high-risk patients and to compare the nomogram with modified CEUS LI-RADS.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board, and informed consent was obtained from each patient. From May 2004 to July 2016, we enrolled 400 consecutive patients with ICC and 2818 consecutive patients with HCC who underwent both baseline ultrasound (US) and CEUS.

The inclusion criteria were (1) a pathologically confirmed diagnosis of ICC or HCC and (2) high-risk patients comprising patients with chronic hepatitis B and/or hepatitis C infection confirmed *via* laboratory tests[21] and cirrhosis of any cause confirmed by pathological examination via liver biopsy or surgery.

The exclusion criteria included (1) mixed hepatocellular cholangiocarcinoma (n = 45) or (2) missing imaging data (n = 302). Finally, 88 patients with ICC and 1792 patients with HCC were included at baseline (to match for the propensity score).

Propensity score matching was used to reduce the effect of selection bias in retrospective observational studies[22]. The variables for matching were tumor size and number of nodules. ICC and HCC patients were then matched 1:1 using a three-digit matching algorithm with the nearest modality.

The selection flow diagram for the study population is presented in Figure 1.

Basic clinical data, including age and sex, were recorded. Laboratory tests included hepatitis status, alpha-fetoprotein (AFP) levels, and CA 19-9 levels.

Histopathological standard

Histopathological examination was the reference stan-

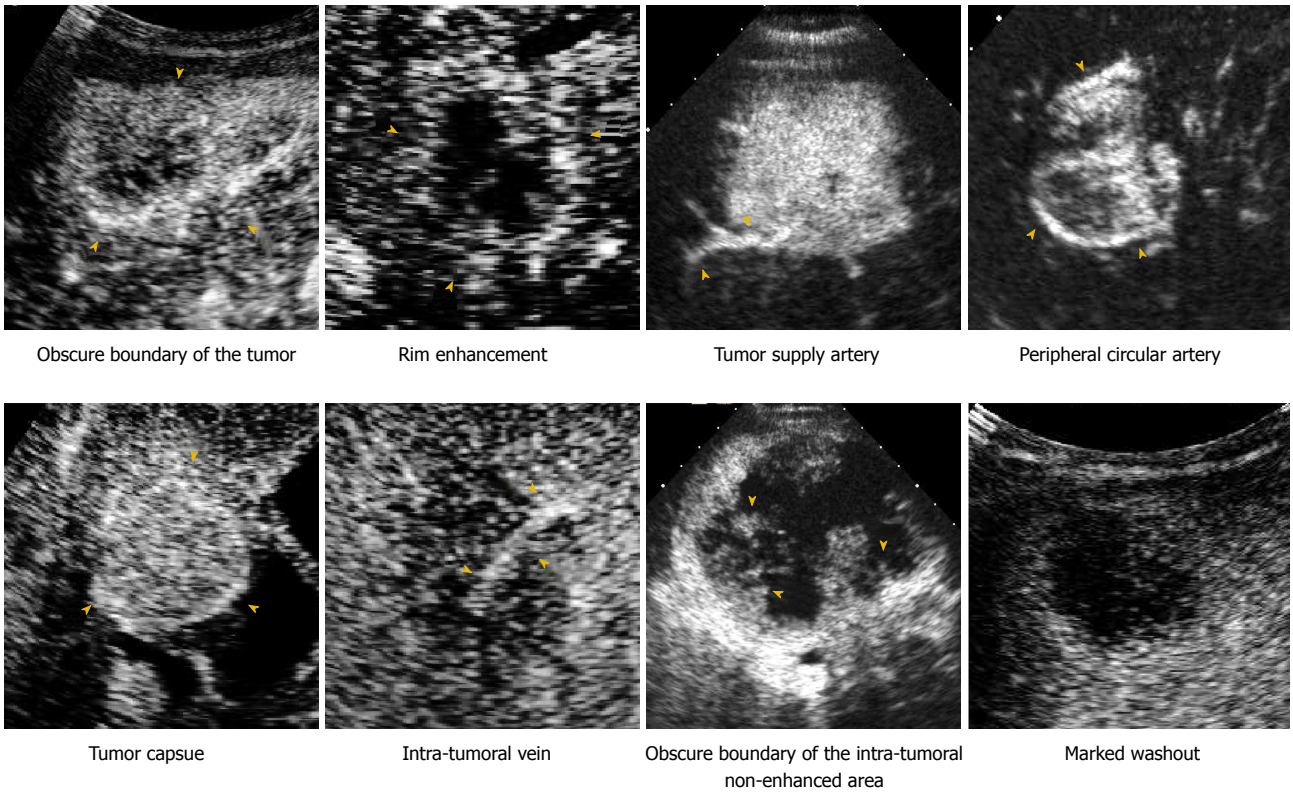


Figure 2 Contrast-enhanced ultrasound images demonstrate the enhancement features of intrahepatic cholangiocarcinoma and hepatocellular carcinoma.

dard of this study. Biopsy or surgical specimens of the hepatic lesion were fixed with 10% formalin and embedded in paraffin. The tissue slices were stained with hematoxylin-eosin. The hematoxylin-eosin slices were evaluated by a senior pathologist (Yang Z) who had more than ten years of experience with liver cancer and who was unaware of the results of any imaging or clinical examination.

Imaging techniques: The US equipment was as follows: (1) Acuson Sequoia 512 (Siemens Medical Solutions, Mountain View, CA, United States) with a 4V1 vector transducer (frequency range, 1.0-4.0 MHz) and a contrast-specific mode of contrast pulse sequencing; (2) Aplio SSA-770 or Aplio 500 (Toshiba Medical Systems, Tokyo, Japan) with a 375BT convex transducer (frequency range, 1.9-6.0 MHz) and a Contrast Harmonic Imaging mode; and (3) Aixplorer Ultrasound system (SuperSonic Imagine, Aix-en-Provence, France) equipped with the SC6-1 convex probe (frequency range, 1.0-6.0 MHz). All examinations were separately performed by three skilled radiologists (Wang W, Liang JY, Xie YX) who each had at least 15 years of experience in liver CEUS. First, the entire liver was scanned with baseline ultrasound (BUS). Then, the imaging mode was changed to CEUS, and a volume of 2.4 mL of SonoVue (Bracco, Milan, Italy) was administered. For patients with multiple nodules, only the largest lesion was selected. The target lesion was observed continuously for at least 5 min, and all imaging data were recorded. The CEUS process was classified into arterial (6-30 s after contrast agent injection), portal venous (31-120 s) and late phases (121-300 s).

Image analysis: All BUS and CEUS images were anonymized, randomized, and independently reviewed in two separate review sessions by two radiologists (Chen LD and Liang JY), who both had at least ten years of experience in liver CEUS. Neither the patient details nor the clinical or pathological results were available to them. In cases of discordance, a third investigator (Lu MD, with at least 15 years of experience in liver CEUS) reviewed the images to make the final decision. The readers were simply asked to review the enhancement appearances of the lesion instead of making a diagnosis. Therefore, the readers were informed of the fact that all patients had either ICC or HCC, but they were blinded to the final diagnosis of the target lesion.

The features on CEUS were recorded and characterized as follows (Figure 2): (1) The number of lesions; (2) maximum diameter of the target lesion; (3) shape of the target lesion; (4) boundary of the lesion; (5) enhancement level in the arterial/portal/late phase (hyper/iso-/hypo-); (6) enhancement patterns of the lesion in the arterial phase (rim/homogeneous/in homogeneous/others); (7) time to enhanced commencement; (8) washout time (within 60 s or not)[23]; (9) duration of enhancement (washout time- time to enhanced commencement); (10) tumor supply artery (defined as an artery extending from the surrounding liver parenchyma into the tumor)[24]; (11) peripheral circular artery (defined as an annular

strip artery around the tumor in the arterial phase)[24]; (12) tumor capsule (defined as an enhancement line that surrounds the tumor during the portal venous phase)[25]; (13) intratumoral vein (defined as straight vessel branches extending through the mass during the portal venous and late phase)[12,26]; (14) boundary of the intratumoral non-enhanced area (if it was present); and (15) marked washout (defined as the lesion appearing as a uniform black defect within the enhanced liver parenchyma)[23].

CEUS scoring through least absolute shrinkage and selection operator in the training set

Because of the multicollinearity of the CEUS features, we used a method of least absolute shrinkage and selection operator (LASSO)[27] regularized regression to select the most useful independent features for predicting ICC in the training set. A CEUS score was calculated for each patient via a linear combination of selected features that were weighted by their respective coefficients.

Diagnostic validation of CEUS score and comparison with CEUS LI-RADS

The constructed CEUS score was first assessed in the training set and then validated in the independent validation set and subgroups with different lesion sizes. The receiver operating characteristic (ROC) for differential diagnosis between ICC and HCC was analyzed by calculating the area under the curve (AUC) and compared with the CEUS LI-RADS. According to the CEUS LI-RADS, we classified the lesions as ICC using the definition of LR-M: rim enhancement in the arterial phase and/or early onset washout (< 60 s) and/or a marked (punched out) appearance; other lesions were defined as HCC.

Development of an individualized prediction model using the CEUS score and CEUS LI-RADS

A nomogram was developed that integrated the CEUS score and clinical information. Multivariable logistic regression analysis began with the following clinical candidate predictors: age, sex, AFP level, and CA 19-9 level. Backward stepwise selection was applied by using the likelihood ratio test with Akaike's information criterion. To provide the clinician with a quantitative tool to predict the individual probability of ICC, we built the CEUS score nomogram on the basis of multivariable logistic analysis in the training set.

With the same method, we also developed the LI-RADS nomogram after combining the CEUS LI-RADS and independent clinical predictors.

Validation of the CEUS score nomogram

The constructed predictive model was first assessed in the training set and then validated in the independent validation set. The performance of the CEUS score nomogram for predicting ICC was evaluated with respect to discrimination, calibration, and clinical usefulness and compared with the LI-RADS nomogram. The improvement in the predictive accuracy of the nomogram was evaluated by calculating the integrated discrimination improvement (IDI) and the net reclassification improvement (NRI). Subgroup analysis was also performed based on the lesion size.

Statistical analysis

Statistical analysis was performed using R software (R Foundation for Statistical Computing, version 3.2.5, http://www.r-project.org/, Austria) and Medcalc (version 11.2, Mariakerke, Belgium). Significance was set at a two-tailed $P < 0.05$. Computer-generated random numbers were used to assign 2/3 of the patients to the training dataset and 1/3 of the patients to the validation dataset. The comparison of the clinical characteristics was performed using the chi-square test and independent *t* test for continuous variables. To test the intra-observer variability of the enhancement patterns, the intraclass correlation coefficient (ICC) was calculated. The ICC was graded as follows: poor (< 0.20), moderate (0.20 to < 0.40), fair (0.40 to < 0.60), good (0.60 to < 0.80) or very good (0.80 to 1.00).

The ROC curves were plotted to demonstrate the performance of the CEUS score, LI-RADS, and nomograms in discriminating ICC from HCC in the training cohort, validation cohort and subgroups with different lesion sizes. Discrimination was quantified with the AUC. Calibration curves (*i.e.*, agreement between observed outcome frequencies and predicted probabilities) were plotted to explore the predictive accuracy of the nomograms[28]. Decision curve analysis (DCA) was conducted to determine the clinical usefulness of the nomograms by quantifying the net benefits at different threshold probabilities in the validation cohort[29].

The "*glmnet*" package of R was used for LASSO regression. The "*glm*" function was used for univariate and multivariate logistic regression analysis. The "*Hmisc*" package was used to plot the nomogram. The "*pROC*" package was used to plot the ROC curves and measure the AUC. The "*CalibrationCurves*" package was used for calibration curves. The "*DecisionCurve*" package was used to perform DCA.

RESULTS

Patients

Eighty-eight ICC and 88 HCC nodules were observed, and the study group comprised 176 nodules in 176 patients (136 men and 40 women; mean age ± SD, 55 years ± 11; range, 22-82 years) (Table 1). Hepatitis B was confirmed in 84 (95.5%) ICC patients, as well as in 86 (97.7%) HCC patients. Hepatitis B + C was confirmed in 4 (4.5%) ICC patients, as well as in 2 (2.3%) HCC patients. Alpha-fetoprotein (AFP) was elevated (> 20 μg/L) in 14 (15.9%) ICC patients and 40 (45.5%) HCC patients ($P < 0.0001$). CA19-9 was

Table 1 Demography of patients with intrahepatic cholangiocarcinoma or hepatocellular carcinoma *n* (%)

Characteristic	Training set (*n* = 117)		Validation set (*n* = 59)		*P* value
	ICC	HCC	ICC	HCC	
Number of patients	56 (47.9)	61 (52.1)	32 (27.4)	27 (23.1)	0.425
Gender					0.148
Male	35 (29.9)	56 (47.9)	24 (20.5)	21 (17.9)	
Female	21 (17.9)	5 (4.3)	8 (6.8)	6 (5.1)	
Age (yr)[1]	55 ± 11 (32-84)	55 ± 11 (32-84)	53 ± 10 (18-76)	57 ± 11 (33-82)	0.646
Hepatitis status					0.627
Hepatitis B	53 (45.3)	59 (50.4)	31 (26.5)	27 (23.1)	
Hepatitis B + C	3 (2.6)	2 (1.7)	1 (0.9)	0 (0)	
AFP > 20 (μg/L)	11 (9.4)	29 (24.8)	3 (2.6)	11 (9.4)	0.655
CA 19-9 > 35 (U/mL)	22 (18.8)	6 (5.1)	15 (12.8)	3 (2.6)	0.691
Nodule size					0.782
⩽ 3.0 cm	5 (4.3)	9 (7.7)	2 (1.7)	3 (2.6)	
3.1-5.0 cm	9 (7.7)	17 (14.5)	8 (6.8)	6 (5.1)	
> 5.0 cm	42 (35.9)	35 (29.9)	22 (18.8)	18 (15.4)	
Number of nodules					0.156
One	39 (33.3)	42 (35.9)	25 (21.4)	24 (20.5)	
Multiple	17 (14.5)	19 (16.2)	7 (6.0)	3 (2.6)	

[1]Data are means ± SD, with ranges in parentheses. Unless otherwise indicated, data are number of nodules, with percentages in parentheses. ICC: Intrahepatic cholangiocarcinoma; HCC: Hepatocellular carcinoma.

Table 2 Comparison and univariate analysis of contrast-enhanced ultrasound features between intrahepatic cholangiocarcinoma and hepatocellular carcinoma *n* (%)

CEUS features	ICC[1] (*n* = 62)	HCC[1] (*n* = 55)	*P* value	OR	(95%CI)
Irregular shape	31 (50.0)	9 (16.4)	0.000	5.037	(2.002, 13.786)
Hyper-enhanced in arterial phase	55 (88.7)	54 (98.2)	0.065	0.147	(0.003, 1.210)
Hypo/iso-enhanced in arterial phase	7 (11.3)	1 (1.8)	0.065	6.783	(0.827, 314.886)
Hypo-enhanced in portal phase	61 (98.4)	40 (72.7)	0.000	22.391	(3.206, 973.549)
Hypo-enhanced in late phase	61 (98.4)	51 (92.7)	0.186	4.728	(0.449, 239.097)
Rim-enhancement	40 (64.5)	1 (1.8)	0.000	94.271	(14.202, 3946.676)
Early washout (< 60 s)	57 (91.9)	17 (30.9)	0.000	24.563	(8.022, 92.533)
Duration of enhancement (< 30 s)	49 (79.0)	11 (20)	0.000	14.614	(5.653, 41.160)
Tumor supply artery	12 (19.4)	29 (52.7)	0.000	4.581	(1.904, 11.618)
Peripheral circular artery or tumor capsule	2 (3.2)	14 (25.5)	0.000	10.060	(2.137, 95.937)
Intra-tumoral vein	36 (58.1)	2 (3.6)	0.000	35.556	(8.118, 327.503)
Obscure boundary of tumor	43 (69.4)	12 (21.8)	0.000	7.942	(3.268, 20.550)
Obscure boundary of intra-tumoral non-enhanced area	40 (64.5)	1 (1.8)	0.000	94.271	(14.202, 3946.676)
Marked washout	38 (61.3)	1 (1.8)	0.000	82.367	(12.448, 3454.264)

[1]Data are number of cases, with percentages in parentheses. CEUS: Contrast-enhanced ultrasound; ICC: Intrahepatic cholangiocarcinoma; HCC: Hepatocellular carcinoma; OR: Odds ratio.

elevated (> 35 U/mL) in 37 (42.0%) ICC patients and 9 (10.2%) HCC patients ($P < 0.001$). No difference in any clinical characteristic was found between the training dataset and the validation dataset (all $P > 0.05$).

CEUS features distinguishing ICC from HCC

The interobserver reproducibility of the CEUS features assessment was high (Supplementary Table 1). Therefore, all results were based on the records of the first radiologist (Chen LD).

In the training set, the following features were observed more frequently in ICC than in HCC: irregular shape (31/62, 50.0%), hypo-enhancement in the portal phase (61/62, 98.4%), rim enhancement (40/62, 64.5%), early washout (< 60 s, 57/62, 91.9%), short duration of enhancement (< 30 s, 49/62, 79.0%), intratumoral vein (36/62, 58.1%), obscure boundary of tumor (43/62, 69.4%), obscure boundary of intratumoral non-enhanced area (40/62, 64.5%), and marked washout (38/62, 61.3%) (all $P < 0.05$) (Table 2).

CEUS scoring

The most useful CEUS independent variables selected by LASSO regression in the training set were as follows: rim enhancement in the arterial phase, rapid washout within 60 s, intratumoral vein, boundary of the intratumoral non-enhanced area and marked washout. Then, a CEUS score for diagnosing ICC was constructed based on the independent features as follows:

CEUS score = -1.3499017 + 1.2090675 × rim enhancement + 0.4303147 × washout within 60 s + 0.2192697 × intratumoral vein + 0.9281196 × unclear

Table 3 Comparison of the diagnostic performance of the contrast-enhanced ultrasound score *vs* contrast-enhanced ultrasound liver imaging reporting and data system in distinguishing intrahepatic cholangiocarcinoma from hepatocellular carcinoma

	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	(95%CI)	*P* value
Training set (n = 117)								
CEUS LI-RADS	0.936	0.691	0.773	0.905	0.821	0.813	(0.744, 0.882)	0.000
CEUS score	0.871	0.946	0.947	0.867	0.906	0.958	(0.924, 0.993)	
Validation set (n = 59)								
CEUS LI-RADS	1.000	0.485	0.605	1.000	0.712	0.742	(0.656, 0.829)	0.000
CEUS score	0.885	0.909	0.885	0.909	0.898	0.953	(0.907, 0.999)	
≤ 5.0 cm subgroup (n = 59)								
CEUS LI-RADS	0.917	0.600	0.611	0.913	0.729	0.758	(0.658, 0.858)	0.000
CEUS score	0.750	0.886	0.818	0.838	0.831	0.902	(0.824, 0.980)	
≤ 3.0 cm subgroup (n = 19)								
CEUS LI-RADS	0.857	0.750	0.667	0.900	0.790	0.804	(0.614, 0.993)	0.512
CEUS score	0.571	0.917	0.800	0.786	0.790	0.833	(0.636, 1.000)	

Numbers are raw data. *P* values were CEUS Score *vs* CEUS LI-RADS. CEUS: Contrast-enhanced ultrasound; LI-RADS: Liver imaging reporting and data system; ICC: Intrahepatic cholangiocarcinoma; HCC: Hepatocellular carcinoma; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the ROC curve.

Table 4 Univariate and multivariate logistic regression of independent variables in the prediction of intrahepatic cholangiocarcinoma

Factors	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Gender (female)	0.149 (0.046, 0.403)	< 0.001	0.190 (0.034,0.908)	0.044
Age (yr)				
> 40	0.786 (0.220, 2.618)	0.696	NA	NA
> 50	1.630 (0.778, 3.450)	0.197	NA	NA
AFP (mg/L) > 20	0.331 (0.148, 0.717)	0.006	0.508 (0.107, 2.212)	0.370
CA199 (U/mL) > 35	10.577 (4.152, 31.070)	< 0.001	5.352 (1.108, 30.336)	0.043
CEUS score	12.188 (5.475, 37.787)	< 0.001	14.078 (5.608, 52.831)	< 0.001

Numbers in parentheses are raw data, with 95%CI in parentheses. ICC: Intrahepatic cholangiocarcinoma; OR: Odds ratio; NA: Not available; AFP: Alpha-fetoprotein; CEUS: Contrast-enhanced ultrasound.

boundary of the intratumoral non-enhanced area + 1.1565281 × marked washout.

Diagnostic performance of the CEUS score

In the training and validation sets, the ROC analysis demonstrated that the AUCs of the CEUS score for differentiation between ICC and HCC were 0.958 and 0.953, respectively. The CEUS score showed significantly higher discriminative performance than CEUS LI-RADS (AUC = 0.742-0.813, $P < 0.001$) (Table 3).

Subgroup analysis in the validation set was also performed based on the tumor size. For tumors ≤ 5.0 cm, the AUC of the CEUS score (0.902) was much higher than that of the LI-RADS (0.758, $P < 0.001$). However, in tumors smaller than 3.0 cm, the AUC of the CEUS score was not improved compared with that of the LI-RADS ($P > 0.05$) (Table 3).

The CEUS score nomogram with clinical risk factors added

Among the clinical data, the selected independent variables for the prediction of ICC by multivariate regression analysis were sex (OR: 0.190, 95%CI: 0.034 - 0.908, $P = 0.044$) and CA 19-9 (OR: 5.352, 95%CI: 1.108-30.336, $P = 0.043$) (Table 4). Sex, CA 19-9, and the CEUS score were integrated to develop a CEUS score nomogram. A CEUS LI-RADS nomogram was also constructed integrating sex, CA 19-9, and the CEUS LI-RADS classification (Figure 3).

Discrimination of the CEUS score nomogram: The AUCs of the CEUS score nomogram in the training and validation sets were 0.971 and 0.973, respectively, which were statistically improved compared with the AUCs of the LI-RADS nomogram (AUC = 0.891-0.916, $P < 0.05$). Relative to the LI-RADS nomogram, the use of the CEUS score nomogram resulted in an NRI of 0.077 ($P = 0.488$) and an IDI of 0.152 ($P = 0.006$) in distinguishing ICC from HCC in the validation cohort (Table 5).

The results of the subanalysis in the validation set based on the tumor size demonstrated that the diagnostic performance of the CEUS score nomogram (AUC = 0.929) was far superior to that of the LI-RADS nomogram (AUC = 0.835) in differentiating a ≤ 5.0 cm ICC from HCC ($P = 0.008$). The use of the CEUS score nomogram resulted in an NRI of 0.382 ($P = 0.017$) and an IDI of 0.177 ($P = 0.002$) compared to the LI-RADS nomogram. However, in distinguishing tumors under 3 cm, the AUC, NRI, and IDI of the CEUS score

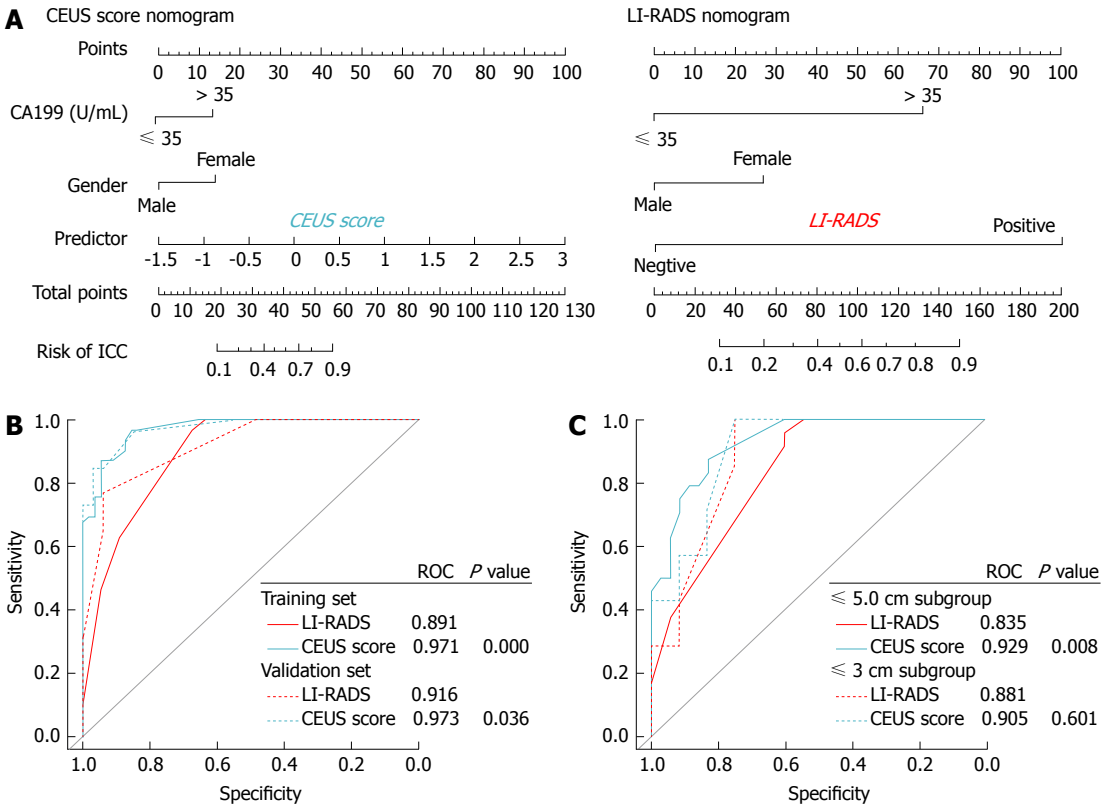


Figure 3 Contrast-enhanced ultrasound score nomogram and liver imaging reporting and data system nomogram for intrahepatic cholangiocarcinoma prediction. A: Constructed contrast-enhanced ultrasound score nomogram and liver imaging reporting and data system nomogram; B: ROC curves for the two nomograms in the training and validation set; C: ROC curves for the two nomograms in ⩽ 5.0 cm and ⩽ 3.0 cm subgroup analysis. CEUS: Contrast-enhanced ultrasound; LI-RADS: Liver imaging reporting and data system; ICC: Intrahepatic cholangiocarcinoma; HCC: Hepatocellular carcinoma.

nomogram did not show statistical improvements over the LI-RADS nomogram (all $P < 0.05$, Table 5).

Calibration for the CEUS score nomogram: The calibration plots of the CEUS score nomogram and LI-RADS nomogram applied in the validation cohort are shown in Figure 4. The CEUS score nomogram showed good agreement on the presence of ICC between the prediction and histopathological confirmation on surgical specimens. We found that the CEUS score nomogram had minimum errors (E max = 0.160) compared to the LI-RADS nomogram (E max = 0.282) in the validation set, which was consistent in tumors under ⩽ 5.0 cm (E max: 0.075 *vs* 0.119). This indicated the smallest difference in the predicted and calibrated probabilities when using the CEUS score nomogram to predict the probability of ICC. However, in distinguishing tumors under 3.0 cm, the E max of the LI-RADS nomogram (E max = 0.098) was lower than that of the CEUS score nomogram (E max = 0.121).

Clinical usefulness of the CEUS score nomogram: In both the training and validation sets, the DCA showed that the CEUS score nomogram had the highest overall net benefit compared with the LI-RADS nomogram at different threshold probabilities across the majority of the range between 0% and 95%, and the CEUS score nomogram was more beneficial than the treat-all-patients strategy or the treat-none strategy (Figure 5).

DISCUSSION

This study extends the analysis of individual specific CEUS features to a predictive model-based differential diagnosis approach. A multifeature-based CEUS diagnostic tool was identified to be an independent factor for ICC in patients at high risk of HCC in this study, with incremental value to LR-M in the LI-RADS classification. The predictive model performed better than the LR-M of the LI-RADS system in identifying ICC, which thoroughly demonstrated the incremental value of the predictive model for individualized ICC predictions in high-risk patients.

The differentiation between ICC and HCC in high-risk patients has been a challenging issue for the identification of HCC in focal liver lesions. Since the study by Vilana *et al*[5], which directed extensive attention to the diagnosis of ICC in high-risk patients, several studies have found some useful features for identifying ICC other than rim enhancement in the arterial phase. Galassi *et al*[20] found that the degree of washout intensity in the late phase was marked in 24% of ICCs ($n = 6$). Li *et al*[15,18] found that 26 out of 33 ICCs (78.8%) demonstrated both early washout (< 60 s) and marked washout in the late part of the portal phase, whereas only 6 of 50 HCCs (12.0%) showed

Table 5 Comparison of the AUC, NRI and IDI of the contrast-enhanced ultrasound score nomogram *vs* contrast-enhanced ultrasound liver imaging reporting and data system nomogram in distinguishing intrahepatic cholangiocarcinoma from hepatocellular carcinoma

	AUC	(95%CI)	*P* value	NRI	(95%CI)	*P* value	IDI	(95%CI)	*P* value
Training set (n = 117)									
CEUS LI-RADS nomogram	0.891	(0.834, 0.948)	< 0.001	0.446	(0.263, 0.629)	< 0.001	0.210	(0.140, 0.280)	< 0.001
CEUS score nomogram	0.971	(0.948, 0.995)							
Validation set (n = 59)									
CEUS LI-RADS nomogram	0.916	(0.854, 0.978)	0.036	0.077	(-0.141, 0.295)	0.488	0.152	(0.044, 0.260)	0.006
CEUS score nomogram	0.973	(0.941, 1.000)							
⩽ 5.0 cm subgroup (n = 59)									
CEUS LI-RADS nomogram	0.835	(0.744, 0.926)	0.008	0.382	(0.069, 0.695)	0.017	0.177	(0.065, 0.289)	0.002
CEUS score nomogram	0.929	(0.870, 0.988)							
⩽ 3.0 cm subgroup (n = 19)									
CEUS LI-RADS nomogram	0.881	(0.732, 1.000)	0.601	-0.202	(-0.572, 0.167)	0.283	-0.117	(-0.284, 0.050)	0.171
CEUS score nomogram	0.905	(0.772, 1.000)							

Numbers are raw data. *P* values were CEUS Score nomogram *vs* CEUS LI-RADS nomogram. AUC: Area under the ROC curve; NRI: Net reclassification improvement; IDI: Integrated discriminatory improvement; CEUS: Contrast-enhanced ultrasound; LI-RADS: Liver imaging reporting and data system; ICC: Intrahepatic cholangiocarcinoma; HCC: Hepatocellular carcinoma.

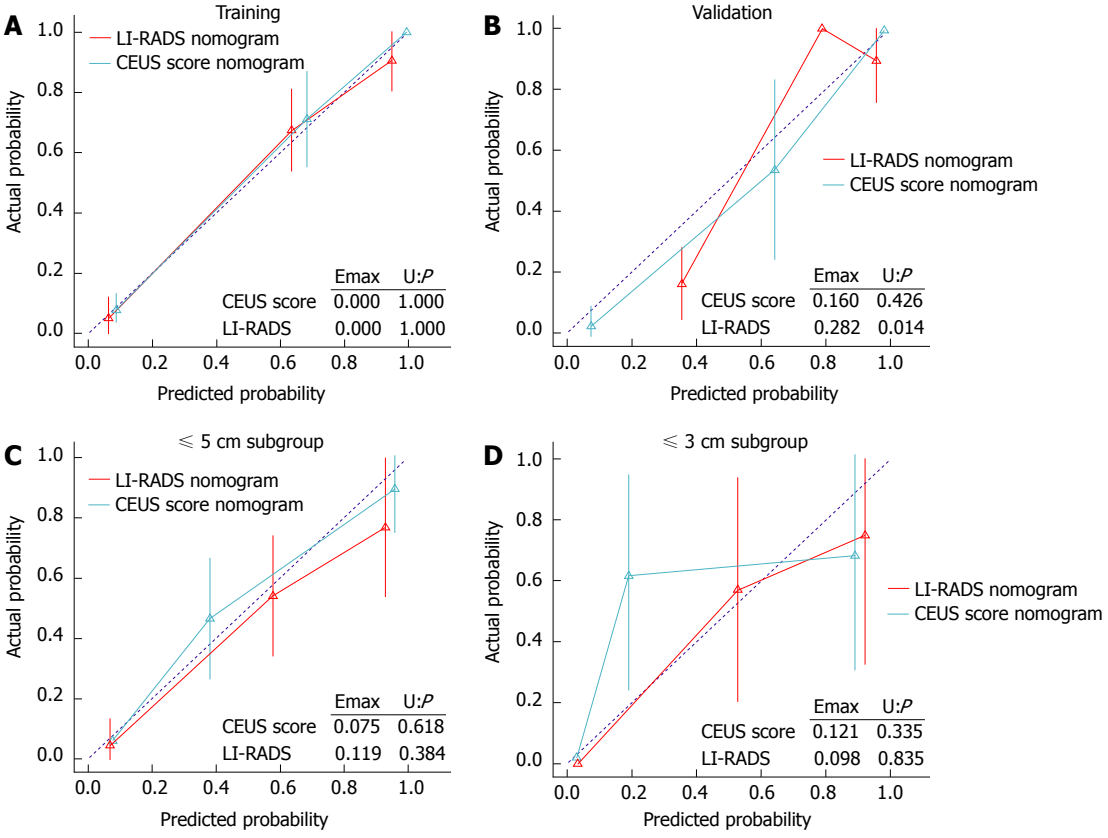


Figure 4 Calibration plots of the contrast-enhanced ultrasound score nomogram and liver imaging reporting and data system nomogram applied in the training (A), validation cohort (B), as well as ⩽ 5.0 cm (C) and ⩽ 3.0 cm subgroup (D). CEUS: Contrast-enhanced ultrasound; LI-RADS: Liver imaging reporting and data system.

these temporal enhancement features (P = 0.000). Kong *et al*[14] found that 70% of ICCs (7/10) showed more rapid washout than HCCs (P < 0.05). However, these studies depicted diverse incidence rates or sensitivities of a single feature due to small numbers of cases. The diagnostic performance and weight of these features are unknown.

To harmonize the interpretation of CEUS with that of CT and MR, the CEUS LI-RADS® system was officially released by the ACR in 2016[23]. Although the category of LR-M in the CEUS LI-RADS® represents various non-HCC malignant liver cancers, the most common malignancy aside from HCC in patients at risk for HCC is ICC[30]. As shown in our study, the sensitivity of LR-M achieved 100.0% when LR-M was used as the diagnostic criterion for ICC, which was in accordance

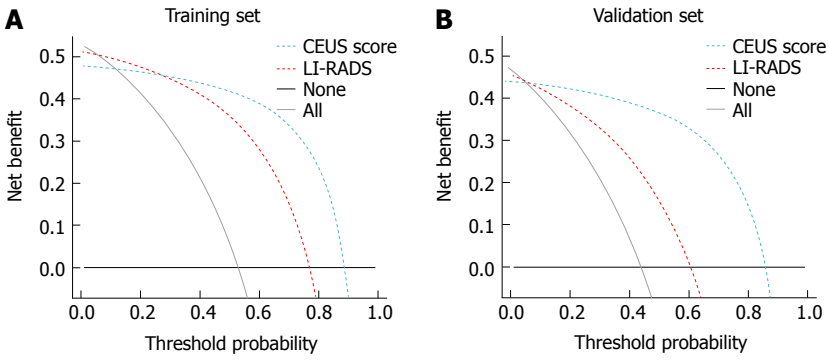


Figure 5 Decision curve analysis of the contrast-enhanced ultrasound score nomogram and liver imaging reporting and data system nomogram in the training (A) and validation cohort (B). CEUS: Contrast-enhanced ultrasound; LI-RADS: Liver imaging reporting and data system.

with the purpose of this category to preserve a high sensitivity for the detection of malignancy. However, the specificity was low (48.5%-69.1%), and that alone was insufficient for a reliable differentiation between ICC and HCC. Differentiating between ICC and HCC remains challenging. A more detailed definition of ICC might help resolve this dilemma.

In our study, we selected five specific features of ICC by LASSO regression, which is useful for addressing the collinearity between parameters. In addition to the tumor enhancement patterns of arterial phase rim enhancement, rapid washout, and marked washout proposed by the CEUS LI-RADS, we categorized two other features as tumor growth patterns and vascular invasion patterns. The boundary of the non-enhanced area in the tumor is different between ICC and HCC if the boundary is present. The non-enhanced area in ICC represents abundant fibrous tissue in the center of the tumor[31]. The border between fibrous tissue and peripheral tumor cells is obscured due to the infiltrative tumor growth of ICC, which arises from cholangiocytes[32]. However, the non-enhancing area in HCC represents necrosis, and thus, the border between necrosis and the enhanced tumor area is sharp[33]. For vascular invasion patterns, an intratumoral vessel is a characteristic of ICC. This feature is relatively frequent in CT scans[34]. Nishibori *et al*[35] have postulated that the tumor does not directly invade the portal vein but that the vein is extrinsically compressed by the tumor. On the other hand, portal vein tumor thrombosis is specific for HCC[36,37].

Another advantage of LASSO regression analysis in this study was that we could assign each feature a weight score to construct a predictive model for predicting ICC, as individualized work-up and management is often essential[38]. Since the specificity of LI-RADS was low, we integrated the above specific features to optimize the diagnostic criterion, which fully considered both sensitivity and specificity. The diagnostic performance of the CEUS score was improved compared with that of the LR-M of CEUS LI-RADS. With much higher specificity, the predictive model may have incremental value to the CEUS LI-RADS for individualized precise predictions of ICC in high-risk patients. Furthermore, we incorporated clinical risk factors into the CEUS score to construct a predictive model, which may be more applicable in clinical practice. The discriminative performance of the CEUS score nomogram was also better than that of the LI-RADS nomogram.

We performed subgroup analysis according to the tumor size and found that the CEUS score significantly improved the discriminative performance in the $\leqslant$ 5.0 cm subgroup compared to the LI-RADS. However, the discriminative performance did not improve in the $\leqslant$ 3.0 cm subgroup. This may be because small ICCs lack specific enhancement characteristics selected by LASSO regression. Since the NRI and IDI were decreased in the $\leqslant$ 3.0 cm lesions, the LI-RADS nomogram was superior to the CEUS score nomogram for small lesions, while the CEUS score nomogram was recommended for lesions larger than 3.0 cm.

Our study has several limitations. First, we did not enroll all focal liver lesions to validate the diagnostic accuracy of the CEUS LI-RADS. The main purpose of this study was to refine the CEUS diagnostic algorithm for ICC in high-risk patients, so we only selected patients with ICC or HCC. The readers did not know the purpose of the study and were only asked to record CEUS features. Therefore, the design and process of this study are reasonable. Second, this is a retrospective study. Prospective studies are mandatory in our future work. Third, the study focused on high-risk patients with chronic hepatitis B or hepatitis C or cirrhosis. As a result, only 88 high-risk patients with ICC were evaluable for the purpose of the study, and this decreased the strength of the study. Fourth, specimens from liver biopsy or surgery and selection of HCC group could affect the present result. Fifth, we analyzed the records on radiologist. This will increase the subjectivity of the study. Finally, we did not compare the diagnostic performance of CEUS with that of MRI/CT. Because of the large number of patients in our hospital, the delay phase scanning of MRI/CT was not performed routinely for each patient with a liver lesion. As a result,

the comparison was not achieved due to the missing information of the delay phase, which was crucial for the differentiation of ICC and HCC by MRI/CT. This is a relevant limitation of the study given that MRI/CT scans must be performed in clinical practice according to international guidelines.

In conclusion, we developed a CEUS predictive model for predicting ICC in high-risk patients with improved discriminative accuracy compared to the LR-M of the CEUS LI-RADS in tumors larger than 3.0 cm. This tool may serve as a useful supplement to the CEUS LI-RADS and further improve the diagnostic efficiency of CEUS. For small tumors, the CEUS LI-RADS is recommended. A large prospective trial is necessary to confirm the conclusion.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant epithelial cancer originating from bile ducts with cholangiocyte differentiation. In recent years, chronic cirrhosis and viral hepatitis have been recognized as important risk factors for ICC development. ICC has been increasingly found in patients with cirrhosis, and distinguishing between ICC and hepatocellular carcinoma (HCC) is a major clinical issue because the management and prognosis of these conditions differ significantly.

Research motivation

The contrast-enhanced ultrasound (CEUS) Liver Imaging Reporting and Data System (LI-RADS®) sets the specific category of LR-M for distinguishing ICC from HCC, but the diagnostic dilemma remains unresolved. Additionally, no study has validated the performance of LR-M as the differential diagnostic criterion for ICC and HCC.

Research objectives

To identify important imaging predictors of ICC on CEUS, to develop a novel diagnostic nomogram incorporating clinical, CEUS and laboratory characteristics that could be used to accurately predict the risk of ICC in high-risk patients and to compare the nomogram with modified CEUS LI-RADS.

Research methods

This retrospective study consisted of 88 consecutive high-risk patients with ICC and 88 high-risk patients with HCC selected by propensity score matching between May 2004 and July 2016. Patients were assigned to two groups, namely, a training set and validation set, at a 1:1 ratio. A CEUS score for diagnosing ICC was generated based on significant CEUS features. Then, a nomogram based on the CEUS score was developed, integrating the clinical data. The performance of the nomogram was then validated and compared with that of the LR-M of the CEUS LI-RADS.

Research results

The most useful CEUS features for ICC were as follows: rim enhancement (64.5%), early washout (91.9%), intratumoral vein (58.1%), obscure boundary of intratumoral non-enhanced area (64.5%), and marked washout (61.3%, all $P < 0.05$). In the validation set, the area under the curve (AUC) of the CEUS score (AUC = 0.953) for differentiation between ICC and HCC were improved compared to the LI-RADS (AUC = 0.742) ($P < 0.001$). When clinical data were added, the CEUS score nomogram was superior to the LI-RADS nomogram (AUC: 0.973 *vs* 0.916, $P = 0.036$, NRI: 0.077, IDI: 0.152). Subgroup analysis demonstrated that the CEUS score model was notably improved compared to the LI-RADS in under 5.0 cm tumors ($P < 0.05$) but not improved in tumors under 3.0 cm ($P > 0.05$).

Research conclusions

A CEUS score for predicting ICC consisted of more detailed CEUS features (rim enhancement, early washout, intratumoral vein, obscure boundary of intratumoral non-enhanced area, and marked washout) was constructed.

The diagnostic performance of the CEUS score (AUC = 0.953) for differentiation between ICC and HCC was improved compared to the LR-M of LI-RADS (AUC = 0.742) ($P < 0.001$). A CEUS score nomogram that added the clinical risk factors was superior to the LI-RADS nomogram (AUC: 0.973 *vs* 0.916, $P = 0.036$, NRI: 0.077, IDI: 0.152). The CEUS score predictive model was notably improved compared to the LI-RADS in ≤ 5.0 cm tumors ($P < 0.05$) but not improved in tumors ≤ 3.0 cm ($P > 0.05$).

Research perspectives

The CEUS predictive model for differentiation between ICC and HCC in high-risk patients had improved discrimination and clinical usefulness compared to the CEUS LI-RADS.

REFERENCES

1 **Lim JH.** Cholangiocarcinoma: morphologic classification according to growth pattern and imaging findings. *AJR Am J Roentgenol* 2003; **181**: 819-827 [PMID: 12933488 DOI: 10.2214/ajr.181.3.1810819]

2 **Khan SA**, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 2005; **366**: 1303-1314 [PMID: 16214602 DOI: 10.1016/S0140-6736(05)67530-7]

3 **Blechacz B**, Gores GJ. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. *Hepatology* 2008; **48**: 308-321 [PMID: 18536057 DOI: 10.1002/hep.22310]

4 **Kim SJ**, Lee JM, Han JK, Kim KH, Lee JY, Choi BI. Peripheral mass-forming cholangiocarcinoma in cirrhotic liver. *AJR Am J Roentgenol* 2007; **189**: 1428-1434 [PMID: 18029881 DOI: 10.2214/AJR.07.2484]

5 **Vilana R**, Forner A, Bianchi L, García-Criado A, Rimola J, de Lope CR, Reig M, Ayuso C, Brú C, Bruix J. Intrahepatic peripheral cholangiocarcinoma in cirrhosis patients may display a vascular pattern similar to hepatocellular carcinoma on contrast-enhanced ultrasound. *Hepatology* 2010; **51**: 2020-2029 [PMID: 20512990 DOI: 10.1002/hep.23600]

6 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

7 **Omata M**, Lesmana LA, Tateishi R, Chen PJ, Lin SM, Yoshida H, Kudo M, Lee JM, Choi BI, Poon RT, Shiina S, Cheng AL, Jia JD, Obi S, Han KH, Jafri W, Chow P, Lim SG, Chawla YK, Budihusodo U, Gani RA, Lesmana CR, Putranto TA, Liaw YF, Sarin SK. Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. *Hepatol Int* 2010; **4**: 439-474 [PMID: 20827404 DOI: 10.1007/s12072-010-9165-7]

8 **Dietrich CF**, Cui XW, Boozari B, Hocke M, Ignee A. Contrast-enhanced ultrasound (CEUS) in the diagnostic algorithm of hepatocellular and cholangiocellular carcinoma, comments on the AASLD guidelines. *Ultraschall Med* 2012; **33** Suppl 1: S57-S66 [PMID: 22723030 DOI: 10.1055/s-0032-1312903]

9 **Barreiros AP**, Piscaglia F, Dietrich CF. Contrast enhanced ultrasound for the diagnosis of hepatocellular carcinoma (HCC): comments on AASLD guidelines. *J Hepatol* 2012; **57**: 930-932 [PMID: 22739095 DOI: 10.1016/j.jhep.2012.04.018]

10 **Quaia E**, Lorusso A, Grisi G, Stacul F, Cova MA. The role of CEUS in the characterization of hepatocellular nodules detected during the US surveillance program--current practices in Europe. *Ultraschall Med* 2012; **33** Suppl 1: S48-S56 [PMID: 22723029 DOI: 10.1055/s-0032-1312899]

11 **Bohle W**, Clemens PU, Heubach T, Zoller WG. Contrast-enhanced ultrasound (CEUS) for differentiating between hepatocellular

and cholangiocellular carcinoma. *Ultraschall Med* 2012; **33**: E191-E195 [PMID: 22194045 DOI: 10.1055/s-0031-1282029]
12 **Chen LD**, Xu HX, Xie XY, Xie XH, Xu ZF, Liu GJ, Wang Z, Lin MX, Lu MD. Intrahepatic cholangiocarcinoma and hepatocellular carcinoma: differential diagnosis with contrast-enhanced ultrasound. *Eur Radiol* 2010; **20**: 743-753 [PMID: 19760416 DOI: 10.1007/s00330-009-1599-8]
13 **Han J**, Liu Y, Han F, Li Q, Yan C, Zheng W, Wang J, Guo Z, Wang J, Li A, Zhou J. The Degree of Contrast Washout on Contrast-Enhanced Ultrasound in Distinguishing Intrahepatic Cholangiocarcinoma from Hepatocellular Carcinoma. *Ultrasound Med Biol* 2015; **41**: 3088-3095 [PMID: 26386477 DOI: 10.1016/j.ultrasmedbio.2015.08.001]
14 **Kong WT**, Wang WP, Huang BJ, Ding H, Mao F. Value of wash-in and wash-out time in the diagnosis between hepatocellular carcinoma and other hepatic nodules with similar vascular pattern on contrast-enhanced ultrasound. *J Gastroenterol Hepatol* 2014; **29**: 576-580 [PMID: 24118042 DOI: 10.1111/jgh.12394]
15 **Li R**, Yuan MX, Ma KS, Li XW, Tang CL, Zhang XH, Guo DY, Yan XC. Detailed analysis of temporal features on contrast enhanced ultrasound may help differentiate intrahepatic cholangiocarcinoma from hepatocellular carcinoma in cirrhosis. *PLoS One* 2014; **9**: e98612 [PMID: 24874413 DOI: 10.1371/journal.pone.0098612]
16 **Liu GJ**, Wang W, Lu MD, Xie XY, Xu HX, Xu ZF, Chen LD, Wang Z, Liang JY, Huang Y, Li W, Liu JY. Contrast-Enhanced Ultrasound for the Characterization of Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *Liver Cancer* 2015; **4**: 241-252 [PMID: 26779444 DOI: 10.1159/000367738]
17 **Wildner D**, Bernatik T, Greis C, Seitz K, Neurath MF, Strobel D. CEUS in hepatocellular carcinoma and intrahepatic cholangiocellular carcinoma in 320 patients - early or late washout matters: a subanalysis of the DEGUM multicenter trial. *Ultraschall Med* 2015; **36**: 132-139 [PMID: 25812115 DOI: 10.1055/s-0034-1399147]
18 **Li R**, Zhang X, Ma KS, Li XW, Xia F, Zhong H, Tang CL, Guo Y, Yan XC. Dynamic enhancing vascular pattern of intrahepatic peripheral cholangiocarcinoma on contrast-enhanced ultrasound: the influence of chronic hepatitis and cirrhosis. *Abdom Imaging*2013; **38**: 112-119 [PMID: 22323003 DOI: 10.1007/s00261-012-9854-x]
19 **Lu Q**, Xue LY, Wang WP, Huang BJ, Li CX. Dynamic enhancement pattern of intrahepatic cholangiocarcinoma on contrast-enhanced ultrasound: the correlation with cirrhosis and tumor size. *Abdom Imaging* 2015; **40**: 1558-1566 [PMID: 25725793 DOI: 10.1007/s00261-015-0379-y]
20 **Galassi M**, Iavarone M, Rossi S, Bota S, Vavassori S, Rosa L, Leoni S, Venerandi L, Marinelli S, Sangiovanni A, Veronese L, Fraquelli M, Granito A, Golfieri R, Colombo M, Bolondi L, Piscaglia F. Patterns of appearance and risk of misdiagnosis of intrahepatic cholangiocarcinoma in cirrhosis at contrast enhanced ultrasound. *Liver Int* 2013; **33**: 771-779 [PMID: 23445369 DOI: 10.1111/liv.12124]
21 **Stroffolini T**, Gaeta GB, Mele A. AASLD Practice Guidelines on chronic hepatitis B and HBV infection in Italy. *Hepatology* 2007; **46**: 608-609; author reply 609 [PMID: 17661422 DOI: 10.1002/hep.21841]
22 **Baek S**, Park SH, Won E, Park YR, Kim HJ. Propensity score matching: a conceptual review for radiology researchers. *Korean J Radiol* 2015; **16**: 286-296 [PMID: 25741190 DOI: 10.3348/kjr.2015.16.2.286]
23 **Piscaglia F**, Wilson SR, Lyshchik A, Cosgrove D, Dietrich CF, Jang HJ, Kim TK, Salvatore V, Willmann JK, Sirlin CB, Kono Y. American College of Radiology Contrast Enhanced Ultrasound Liver Imaging Reporting and Data System (CEUS LI-RADS) for the diagnosis of Hepatocellular Carcinoma: a pictorial essay. *Ultraschall Med* 2017; **38**: 320-324 [PMID: 28329875 DOI: 10.1055/s-0042-124661]
24 **Catalano O**, Nunziata A, Lobianco R, Siani A. Real-time harmonic contrast material-specific US of focal liver lesions. *Radiographics* 2005; **25**: 333-349 [PMID: 15798053 DOI: 10.1148/rg.252045066]
25 **Schellhaas B**, Görtz RS, Pfeifer L, Kielisch C, Neurath MF, Strobel D. Diagnostic accuracy of contrast-enhanced ultrasound for the differential diagnosis of hepatocellular carcinoma: ESCULAP versus CEUS-LI-RADS. *Eur J Gastroenterol Hepatol* 2017; **29**: 1036-1044 [PMID: 28562394 DOI: 10.1097/MEG.0000000000000916]
26 **Xu HX**, Lu MD, Liu GJ, Xie XY, Xu ZF, Zheng YL, Liang JY. Imaging of peripheral cholangiocarcinoma with low-mechanical index contrast-enhanced sonography and SonoVue: initial experience. *J Ultrasound Med* 2006; **25**: 23-33 [PMID: 16371552 DOI: 10.7863/jum.2006.25.1.23]
27 **Zhang JX**, Song W, Chen ZH, Wei JH, Liao YJ, Lei J, Hu M, Chen GZ, Liao B, Lu J, Zhao HW, Chen W, He YL, Wang HY, Xie D, Luo JH. Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis. *Lancet Oncol* 2013; **14**: 1295-1306 [PMID: 24239208 DOI: 10.1016/S1470-2045(13)70491-1]
28 **Coutant C**, Olivier C, Lambaudie E, Fondrinier E, Marchal F, Guillemin F, Seince N, Thomas V, Levêque J, Barranger E, Darai E, Uzan S, Houvenaeghel G, Rouzier R. Comparison of models to predict nonsentinel lymph node status in breast cancer patients with metastatic sentinel lymph nodes: a prospective multicenter study. *J Clin Oncol* 2009; **27**: 2800-2808 [PMID: 19349546 DOI: 10.1200/JCO.2008.19.7418]
29 **Vickers AJ**, Cronin AM, Elkin EB, Gonen M. Extensions to decision curve analysis, a novel method for evaluating diagnostic tests, prediction models and molecular markers. *BMC Med Inform Decis Mak* 2008; **8**: 53 [PMID: 19036144 DOI: 10.1186/1472-6947-8-53]
30 **Joo I**, Lee JM, Lee SM, Lee JS, Park JY, Han JK. Diagnostic accuracy of liver imaging reporting and data system (LI-RADS) v2014 for intrahepatic mass-forming cholangiocarcinomas in patients with chronic liver disease on gadoxetic acid-enhanced MRI. *J Magn Reson Imaging* 2016; **44**: 1330-1338 [PMID: 27087012 DOI: 10.1002/jmri.25287]
31 **Xu HX**, Chen LD, Liu LN, Zhang YF, Guo LH, Liu C. Contrast-enhanced ultrasound of intrahepatic cholangiocarcinoma: correlation with pathological examination. *Br J Radiol* 2012; **85**: 1029-1037 [PMID: 22374276 DOI: 10.1259/bjr/21653786]
32 **Bridgewater J**, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol* 2014; **60**: 1268-1289 [PMID: 24681130 DOI: 10.1016/j.jhep.2014.01.021]
33 **Ogawa S**, Kumada T, Toyoda H, Ichikawa H, Kawachi T, Otobe K, Hibi T, Takeshima K, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Yamaguchi A, Isogai M, Kaneoka Y, Washizu J. Evaluation of pathological features of hepatocellular carcinoma by contrast-enhanced ultrasonography: comparison with pathology on resected specimen. *Eur J Radiol* 2006; **59**: 74-81 [PMID: 16545532 DOI: 10.1016/j.ejrad.2006.02.003]
34 **Han JK**, Choi BI, Kim AY, An SK, Lee JW, Kim TK, Kim SW. Cholangiocarcinoma: pictorial essay of CT and cholangiographic findings. *Radiographics* 2002; **22**: 173-187 [PMID: 11796906 DOI: 10.1148/radiographics.22.1.g02ja15173]
35 **Nishibori H**, Kanematsu M, Hoshi H, Kondo H, Yamawaki Y, Kawaguchi Y, Kato M, Yamada T. Small peripheral cholangiocarcinoma with undisturbed transiting portal vein: radiologic-pathologic correlation. *AJR Am J Roentgenol* 1999; **173**: 1243-1245 [PMID: 10541096 DOI: 10.2214/ajr.173.5.10541096]
36 **Tarantino L**, Francica G, Sordelli I, Esposito F, Giorgio A, Sorrentino P, de Stefano G, Di Sarno A, Ferraioli G, Sperlongano P. Diagnosis of benign and malignant portal vein thrombosis in cirrhotic patients with hepatocellular carcinoma: color Doppler US, contrast-enhanced US, and fine-needle biopsy. *Abdom Imaging* 2006; **31**: 537-544 [PMID: 16865315 DOI: 10.1007/s00261-005-0150-x]
37 **Sparchez Z**, Radu P, Zaharia T, Kacso G, Diaconu B, Grigorescu

I, Badea R. B-mode and contrast enhanced ultrasound guided biopsy of portal vein thrombosis. Value in the diagnosis of occult hepatocellular carcinoma in liver cirrhosis. *Med Ultrason* 2010; **12**: 286-294 [PMID: 21210013]

38 **An C**, Kim DW, Park YN, Chung YE, Rhee H, Kim MJ. Single Hepatocellular Carcinoma: Preoperative MR Imaging to Predict Early Recurrence after Curative Resection. *Radiology* 2015; **276**: 433-443 [PMID: 25751229 DOI: 10.1148/radiol.15142394]

P- Reviewer: Kao JT, Rimassa L, Xu LB **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3799-3805

DOI: 10.3748/wjg.v24.i33.3799

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

ORIGINAL ARTICLE

Retrospective Study

Percutaneous transhepatic extraction and balloon dilation for simultaneous gallbladder stones and common bile duct stones: A novel technique

Bin Liu, De-Shun Wu, Pi-Kun Cao, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Hai-Yang Chang, Dong Li, Xiao Li, Yancu Hertzanu, Yu-Liang Li

Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Hai-Yang Chang, Yancu Hertzanu, Yu-Liang Li, Department of Interventional Medicine, The Second Hospital of Shandong University, Jinan 250033, Shandong Province, China

Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Hai-Yang Chang, Yu-Liang Li, Interventional Oncology Institute of Shandong University, Jinan 250033, Shandong Province, China

De-Shun Wu, Department of General Surgery, Jiyang County People's Hospital, Jinan 251400, Shandong Province, China

Pi-Kun Cao, Dong Li, Xiao Li, School of Medicine, Shandong University, Jinan 250014, Shandong Province, China

Yancu Hertzanu, Department of Radiology, Ben-Gurion University, Negev 88874, Israel

ORCID number: Bin Liu (0000-0003-1686-1553); De-Shun Wu (0000-0002-3799-4681); Pi-Kun Cao (0000-0003-2973-7527); Yong-Zheng Wang (0000-0001-5889-2118); Wu-Jie Wang (0000-0001-9431-7153); Wei Wang (0000-0002-2124-5328); Hai-Yang Chang (0000-0001-8684-4877); Dong Li (0000-0002-3944-3680); Xiao Li (0000-0002-6459-0885); Yancu Hertzanu (0000-0001-9915-4074); Yu-Liang Li (0000-0001-8117-4317).

Author contributions: Liu B designed and performed the research, analyzed and interpreted data, wrote the paper, and obtained funding; Wu DS designed the research, analyzed, and interpreted data; Cao PK collected, analyzed, and interpreted data; Wang YZ, Wang WJ, Wang W, and Chang HY technically supported the research; Li D and Li X collected data; Hertzanu Y critically revised the manuscript for important intellectual content; Li YL designed the research, supervised the report, and obtained funding.

Supported by the National Natural Science Foundation of China, No. 61671276; and the Natural Science Foundation of Shandong Province, No. 2014ZRE27479 and No. ZR2018PH032.

Institutional review board statement: This study was approved by the ethics committee of the Second Hospital of Shandong University [KYLL-2018(LW)007].

Informed consent statement: All participants provided written informed consent.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Yu-Liang Li, MD, Chief Doctor, Professor, Department of Interventional Medicine, The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, Shandong Province, China. lyl.pro@sdu.edu.cn
Telephone: +86-531-85875462
Fax: +86-531-85875462

Received: May 30, 2018
Peer-review started: May 30, 2018
First decision: July 6, 2018
Revised: July 9, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: September 7, 2018

Abstract

AIM

To evaluate the clinical efficacy and safety of an innovative percutaneous transhepatic extraction and balloon dilation (PTEBD) technique for clearance of gallbladder stones in patients with concomitant stones in the common bile duct (CBD).

METHODS

The data from 17 consecutive patients who underwent PTEBD for clearance of gallbladder stones were retrospectively analyzed. After removal of the CBD stones by percutaneous transhepatic balloon dilation (PTBD), the gallbladder stones were extracted to the CBD and pushed into the duodenum with a balloon after dilation of the sphincter of Oddi. Large stones were fragmented using a metallic basket. The patients were monitored for immediate adverse events including hemorrhage, perforation, pancreatitis, and cholangitis. During the two-year follow-up, they were monitored for stone recurrence, reflux cholangitis, and other long-term adverse events.

RESULTS

Gallbladder stones were successfully removed in 16 (94.1%) patients. PTEBD was repeated in one patient. The mean hospitalization duration was 15.9 ± 2.2 d. Biliary duct infection and hemorrhage occurred in one (5.9%) patient. No severe adverse events, including pancreatitis or perforation of the gastrointestinal or biliary tract occurred. Neither gallbladder stone recurrence nor refluxing cholangitis had occurred two years after the procedure.

CONCLUSION

Sequential PTBD and PTEBD are safe and effective for patients with simultaneous gallbladder and CBD stones. These techniques provide a new therapeutic approach for certain subgroups of patients in whom endoscopic retrograde cholangiopancreatography/endoscopic sphincterotomy or surgery is not appropriate.

Key words: Common bile duct; Gallstones; Removing; Percutaneous; Balloon

Core tip: Simultaneous gallbladder and common bile duct stones present a challenge in certain subgroups of patients with pulmonary or cardiac comorbidities who cannot tolerate the risk of general anesthesia with tracheal intubation, endoscopic retrograde cholangiopancreatography/endoscopic sphincterotomy, or surgery. For these patients, sequential percutaneous transhepatic balloon dilation and percutaneous transhepatic extraction and balloon dilation, providing a path with compliance and only requiring intravenous anesthesia, could be a safe and effective procedure.

Liu B, Wu DS, Cao PK, Wang YZ, Wang WJ, Wang W, Chang HY, Li D, Li X, Hertzanu Y, Li YL. Percutaneous transhepatic extraction and balloon dilation for simultaneous gallbladder stones and common bile duct stones: A novel technique. *World J Gastroenterol* 2018; 24(33): 3799-3805 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3799.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3799

INTRODUCTION

Gallstones constitute a significant health issue, affecting 10% to 15% of the adult population in developed societies[1,2] and approximately 13% of the Chinese population[3]. Gallstones cause pain and discomfort in the right upper abdomen and are associated with other symptoms such as nausea, vomiting, and postprandial fullness, which can seriously affect a patient's quality of life. In the United States, 20 to 25 million patients are newly diagnosed with gallstones each year, and the medical expenses for the prevention and treatment of gallstone disease reach almost $62 billion annually[1]. The goal of treatment is to resolve ongoing infections, thereby preventing recurrent cholecystitis, subsequent cholangitis, hepatic fibrosis, and progression to cholangiocarcinoma[4].

Approximately 15% of patients with gallbladder stones have concomitant common bile duct (CBD) stones[5]. Open exploration of the CBD was historically the therapeutic option for patients with CBD stones. During recent decades, endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy (EST) has gained wide acceptance as an effective and minimally invasive alternative. After ERCP/EST, gallbladder stones must be removed through open cholecystectomy, laparoscopic cholecystectomy, or percutaneous cholecystolithotomy. Whether removal of CBD stones should be followed by cholecystectomy to prevent recurrent symptoms has long been debated. Recent prospective randomized trials have indicated benefits of subsequent cholecystectomy[6].

However, some older patients have pulmonary or cardiac comorbidities and cannot tolerate the risk of general anesthesia with tracheal intubation, ERCP/EST, or surgery. Therefore, for treatment of this subgroup, we aimed to evaluate the clinical efficacy of an innovative percutaneous transhepatic extraction and balloon dilation (PTEBD) following percutaneous transhepatic balloon dilation (PTBD).

MATERIALS AND METHODS

From December 2013 to June 2014, 17 consecutive patients with 35 simultaneous gallbladder and CBD stones (demonstrated by ultrasonography, computed tomography, or magnetic resonance cholangiopancreatography) underwent PTEBD after percutaneous

Table 1 Patient and treatment characteristics

Characteristic	*n* (%)
No. of patients	17
Gender	
Female	7 (41.2)
Male	10 (58.8)
Comorbidity	
Emphysema	3 (17.6)
Pulmonary insufficiency	5 (29.4)
Coronary artery disease	3 (17.6)
Cardiac insufficiency	5 (29.4)
Hypoproteinemia	1 (6.0)
Number of PTEBD procedures	
One	16 (94.1)
Two	1 (5.9)
Diameter of stones	
< 10 mm	10 (28.6)
10-20 mm	21 (60.0)
≥ 20 mm	4 (11.4)
Types of stones	
Cholesterol stone	16 (45.7)
Mixed stone	15 (42.9)
Bilirubin stone	4 (11.4)

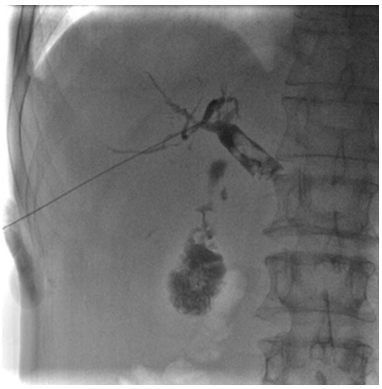


Figure 1 Puncture of the bile duct, and cholangiography showing the number, size, and location of simultaneous gallbladder and common bile duct stones.

CBD stone removal in our hospital (Table 1). The hospital ethics committee approved this prospective study, and all patients provided written informed consent.

The study included ten men and seven women aged 51 to 79 years with a mean age of 65.8 ± 8.9 years old. All patients suffered from pulmonary or cardiac comorbidities such as emphysema, pulmonary insufficiency, coronary artery disease, cardiac insufficiency, or other conditions that lowered their tolerance for general anesthesia with tracheal intubation, EST, or surgery. Gallbladder and CBD stone diameters ranged from 0.6-2.2 cm. Ten (28.6%) stones were < 10 mm, twenty-one (60%) ranged from 10-20 mm, and four (11.4%) were > 20 mm. Six patients (35.3%) were admitted with acute cholecystitis, nine (52.9%) with acute cholangitis, and two (11.8%) with pancreatitis. PTEBD was repeated in one patient because of overlooked stones. Laboratory values, including WBC count, aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), albumin (ALB) and serum amylase were obtained using routine laboratory tests.

Inclusion criteria were: (1) Concomitant gallbladder and CBD stones with symptoms of acute cholangitis, pancreatitis, or cholecystitis; (2) inability to tolerate or refusal to undergo general anesthesia with tracheal intubation, ERCP/EST, or surgery because of cardiac or lung insufficiency; (3) ERCP/EST not possible due to prior Billroth II surgery; (4) leukocyte count ≥ 4.0 × 10^9 /L, platelet count ≥ 60 × 10^9 /L, and hemoglobin concentration ≥ 100 g/L; (5) predicted life span of ≥ 6 mo; and (6) Karnofsky score > 70.

Exclusion criteria were: (1) Concomitant intrahepatic bile duct stones; (2) severe cardiac insufficiency (New York Heart Association class III-IV) or advanced lung disease (determined by consultation with respiratory disease specialists), liver disease (Child-Pugh class C), or kidney disease (grade 3 chronic kidney disease); or (3) severe coagulopathy (prothrombin time > 17 s or platelet count ≤ 60 × 10^9 /L).

Percutaneous CBD stone removal: PTBD

The intrahepatic bile duct was punctured (Figure 1) using conventional percutaneous transhepatic cholangiodrainage with aseptic technique under ultrasonographic and fluoroscopic guidance and intravenous general anesthesia. Cholangiography revealed the number, size, and location of the CBD stones. Balloons (Cristal Balloon; Balt Extrusion, Montmorency, France) sized appropriately for the diameter of the CBD and stones were then introduced crossing the stones using a 0.035-inch-diameter guide wire (Radifocus Guidewire M; Terumo Medical Corporation, Tokyo, Japan), and the sphincter of Oddi was gradually and intermittently dilated (Figure 2)[7]. The sphincter could be dilated to a maximum of 22 mm. The empty balloon was then withdrawn above the stones, inflated, and used to push the stones into the duodenum through the sphincter of Oddi (Figure 3)[8,9]. An 8.5-Fr catheter (COOK Medical, Bloomington, IN 47404, United States) was placed in the CBD for drainage and cholangiography in case of residual stones.

Percutaneous clearance of gallbladder stones: PTEBD

One week after PTBD, contrast was injected through the CBD catheter to perform cholangiography, identifying the anatomic characteristics of the bile duct tree and if there was any residual CBD stones. If yes, PTBD was performed again. If no, a guide wire and a 4-Fr single-angle catheter (Terumo Medical Corporation, Tokyo, Japan) were introduced through the previous transhepatic tract into the gallbladder through the cystic duct in sequence. Cholangiography revealed the number, size, and location of the gallbladder stones (Figure 4A). Large stones were extracted to the CBD with a basket (Olympus, Japan) and then pushed into the duodenum using PTBD (Figure 4B). Sandy stones could be aspirated through a guiding catheter (Launcher, Medtronic, Minneapolis, MN, United States) (Figure 4C). An 8.5-Fr

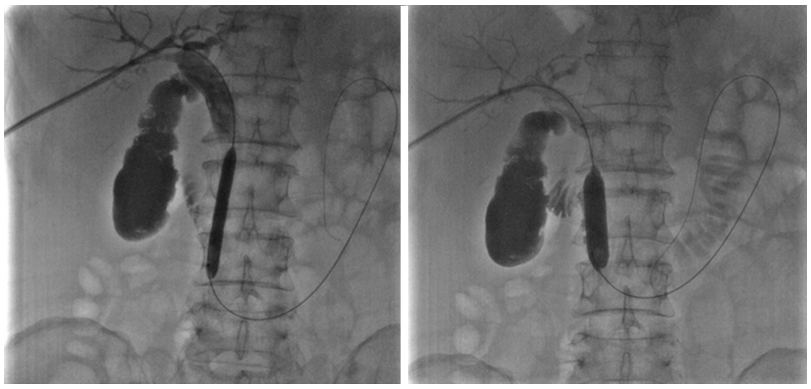


Figure 2 Sequential dilation of the sphincter of Oddi with an 8 mm × 60 mm and a 14 mm × 40 mm balloon.

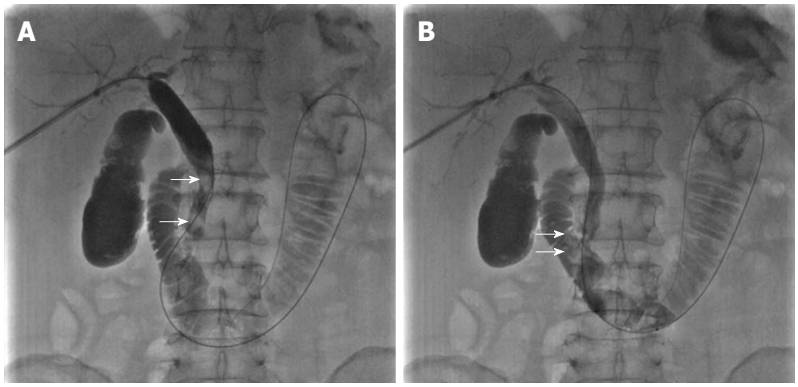


Figure 3 Empty balloon was then withdrawn above the stones, inflated, and used to push the stones into the duodenum through the sphincter of Oddi. A: The empty balloon was withdrawn above the common bile duct stones (white arrow) and then re-inflated. B: The common bile duct stones (white arrow) were pushed into the duodenum through the dilated sphincter of Oddi.

drainage catheter was placed in the gallbladder and removed after two weeks without residual stones.

Outcome measures

Outcomes recorded included postoperative hospital stay, success rate, causes of failure, and procedure-related complications. The AST, TBIL, DBIL, ALB, serum amylase concentrations, and WBC count were recorded before the procedure and at one week and one month after the procedure. Short-term adverse events, such as biliary duct infection, hemorrhage, pancreatitis, and gastrointestinal and biliary duct perforation were assessed before discharging. Ultrasonography, computed tomography, or magnetic resonance cholangiopancreatography were performed at 1, 3, 6, 9, 12, 18 and 24 mo after the procedure. Refluxing cholangitis, and recurrence of gallbladder or CBD stones, considered as long-term complications, were monitored for two years.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 24.0. Categorical variables were presented as number and percentage. Continuous data were presented as mean ± standard deviation. We used paired *t*-tests for the same indexes before and after the procedure in the same patient. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

A total of 35 gallbladder stones were successfully removed by PTEBD in 16 (94.1%) of the 17 patients. PTEBD was repeated in one patient. The mean hospitalization duration was 15.9 ± 2.2 d.

The diameter of ten stones (28.6%) was smaller than 10 mm, twenty-one stones (60.0%) ranged from 10-20 mm, and four stones (11.4%) were larger than 20 mm. Sixteen stones (45.7%) were cholesterol type, four (11.4%) were bilirubin type, and fifteen (42.9%) were mixed type.

The concentrations of AST, TBIL, DBIL and WBC count declined markedly after PTBD and PTEBD. The differences in these indexes before PTBD, one week after PTBD, and one week after PTEBD were all significant ($P < 0.01$). In contrast, ALB concentration significantly increased after PTBD and PTEBD (Table 2).

One (5.9%) of the seventeen patients developed a high fever (39.5 ℃) and shivering. *Escherichia coli* was found in the bile, and a biliary duct infection was confirmed. Another patient developed bile duct hemorrhage and recovered after treatment with 1000 IU of reptilase and drainage clamping. No severe adverse

Table 2 Relevant variables before and one week after percutaneous transhepatic balloon dilation and one week after percutaneous transhepatic extraction and balloon dilation

Item	Before PTBD	One week after PTBD	One week after PTEBD
AST (U/L)	128.1 ± 47.7	42.7 ± 23.1[a]	23.5 ± 10.1[ab]
TBIL (μmol/L)	169.0 ± 56.6	62.8 ± 21.5[a]	16.1 ± 8.8[ab]
DBIL (μmol/L)	110.6 ± 40.3	36.5 ± 12.1[a]	11.8 ± 8.0[ab]
WBC ($\times 10^9$/L)	16.5 ± 2.6	11.2 ± 2.1[a]	7.3 ± 1.6[ab]
ALB (g/L)	21.4 ± 4.9	32.5 ± 3.2[a]	37.6 ± 3.1[ab]

[a]$P < 0.01$ compared with parameters before PTBD; [b]$P < 0.01$ compared with parameters one week after PTBD. AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; WBC: White blood cell; ALB: Albumin; PTBD: Percutaneous transhepatic balloon dilation: PTEBD: Percutaneous transhepatic extraction and balloon dilation.

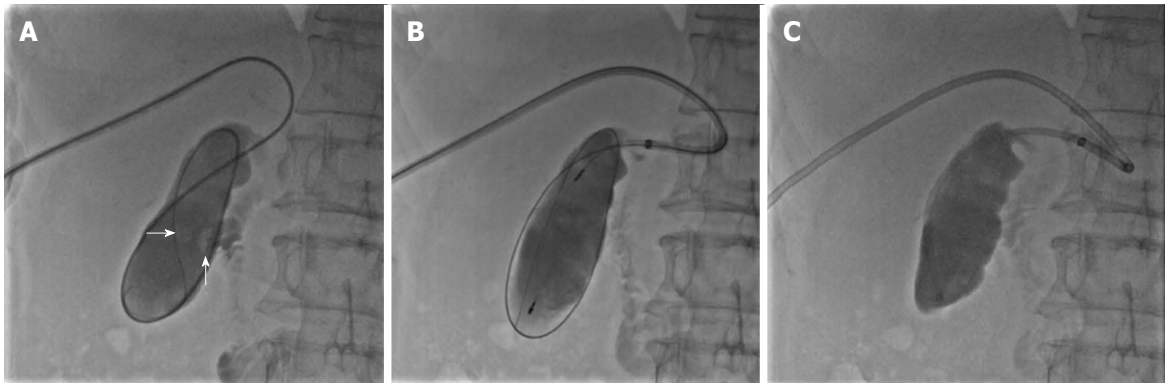


Figure 4 Percutaneous clearance of gallbladder stones. A: Sequential introduction of a guidewire and 4-Fr single-angle catheter into the gallbladder through the cystic duct, and cholangiography showing the number, size, and location of the gallbladder stones (white arrow); B: Capture of stones in a metallic basket; C: Aspiration of sandy stones out of the body through a guide catheter.

events occurred during the perioperative period, including pancreatitis or perforation of the gastrointestinal or biliary duct. Neither recurrence of gallbladder or CBD stones nor refluxing cholangitis had occurred two years after the procedure.

DISCUSSION

Several different methods for management of simultaneous gallbladder and CBD stones have been proposed and are currently in clinical use[4-6]. Furthermore, since the introduction of laparoscopic cholecystectomy, the frequency of preoperative removal of CBD stones by ERCP/EST has increased[10]. However, certain subgroups of patients cannot tolerate or refuse to undergo general anesthesia with tracheal intubation, ERCP/EST, or surgery due to insufficient pulmonary or cardiac function. In other patients, prior Billroth II surgery causes overwhelming obstacles for endoscopy, preventing subsequent ERCP/EST.

Percutaneous transhepatic papillary dilation was reported to be a safe and effective procedure for CBD stone removal[11-13]. The present study indicates that PTBD and PTEBD are safe and effective and have a low incidence of biliary duct infection and hemorrhage without causing pancreatitis or perforation of the gastrointestinal or biliary duct. Furthermore, because the sphincter of Oddi is preserved, the incidence of late adverse events such as refluxing cholangitis and recurrence of gallbladder and CBD stones is lower than that after EST[14-17].

Theoretically, gallstones can be treated either surgically or nonsurgically after PTBD. In fact, only cholesterol gallstones can be treated without surgery. With a functioning gallbladder, cholesterol gallstones will dissolve slowly when ingestion of ursodiol or chenodiol induces the secretion of unsaturated bile[18]. Stone dissolution could be enhanced by increasing the surface area of the stone *via* extracorporeal shock wave lithotripsy, which fragments stones rapidly and safely, accelerating their dissolution rate. Some organic solvents such as methyl tert-butyl ether can be instilled into the gallbladder through a catheter *via* either a percutaneous transhepatic or endoscopic approach, which also dissolves the stones rapidly[19]. However, gallstones will eventually recur in about 50% of patients who undergo these nonsurgical treatments because the gallbladder is left in place and the fundamental pathogenic abnormalities are not corrected[20]. Compared with surgery (open or laparoscopic cholecystectomy) or nonsurgical medication, PTEBD has the advantages of being less invasive, being well tolerated by patients, and having a lower recurrence rate.

Several key points of PTBD and PTEBD should be addressed: (1) usually puncture of the right front hepatic duct is recommended to obtain a more compliant

operating tract; (2) a stiff guide wire is necessary along the puncture tract, bile duct, duodenum, and jejunum for improved balloon support; (3) the sphincter of Oddi should be gradually and intermittently dilated to a maximum diameter of 22 mm to avoid tearing; (4) baskets should be applied for fragmentation of large stones (> 10 mm); (5) aspiration through a guide catheter is usually effective for sandy stones in the gallbladder; and (6) postoperative drainage decompresses the bile duct and decreases the incidence of pancreatitis.

Our study has two main limitations. First, as a pilot study, the number of patients was small. Second, this treatment method is devised for a specific subset of patients in which it has been well tested.

In conclusion, our data indicate that sequential PTBD and PTEBD is a safe, feasible, and effective treatment option for simultaneous gallbladder and CBD stones. It is an innovative alternative procedure for a subgroup of patients who cannot tolerate the risk of general anesthesia. Larger studies and generalizability of the results to more widespread populations will be investigated in the future.

ARTICLE HIGHLIGHTS

Research background

Gallstones constitute a significant health issue, and 15% of these cases have concomitant common bile duct (CBD) stones. Open exploration of CBD, endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy (EST) followed by open or laparoscopic cholecystectomy may solve the problem.

Research motivation

However, a certain subgroup of patients with pulmonary or cardiac comorbidities cannot tolerate the risk of general anesthesia with tracheal intubation, ERCP/EST, or surgery.

Research objectives

We aimed to evaluate the clinical efficacy of an innovative percutaneous transhepatic extraction and balloon dilation (PTEBD) following percutaneous transhepatic balloon dilation (PTBD).

Research methods

From December 2013 to June 2014, 17 consecutive patients with 35 simultaneous gallbladder and CBD stones underwent PTEBD after percutaneous CBD stone removal in our hospital. Laboratory values, including WBC count, aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), albumin (ALB) and serum amylase were obtained using routine laboratory tests. Gallbladder and CBD stone diameters ranged from 0.6-2.2 cm. Ten (28.6%) stones were < 10 mm, twenty-one stones ranged from 10-20 mm, and four were > 20 mm. Six patients were admitted with acute cholecystitis, nine with acute cholangitis, and two with pancreatitis. All statistical analyses were performed using IBM SPSS Statistics 24.0. Categorical variables were presented as number and percentage. Continuous data were presented as mean ± standard deviation. We used paired *t*-tests for the same indexes before and after the procedure in the same patient. $P < 0.05$ was considered statistically significant.

Research results

Thirty-five gallbladder stones were successfully removed by PTEBD in 16 of the 17 patients. PTEBD was repeated in one patient. The mean hospitalization duration was 15.9 ± 2.2 d. The concentrations of AST, TBIL, DBIL and WBC count declined markedly after PTBD and PTEBD. The differences in these indexes before PTBD, one week after PTBD, and one week after PTEBD were all significant. In contrast, ALB concentration significantly increased after PTBD and PTEBD. No severe adverse events, including pancreatitis or perforation of the gastrointestinal or biliary duct occurred during the perioperative period. Neither recurrence of gallbladder or CBD stones nor refluxing cholangitis had occurred two years after the procedure.

Research conclusions

As our data indicate, sequential PTBD and PTEBD is a safe, feasible, and effective treatment option for simultaneous gallbladder and CBD stones. It is an innovative alternative procedure for a subgroup of patients who cannot tolerate the risk of general anesthesia.

Research perspectives

In the future, larger studies and generalizability of the results to more widespread populations will be investigated.

REFERENCES

1 **Stinton LM**, Shaffer EA. Epidemiology of gallbladder disease: cholelithiasis and cancer. *Gut Liver* 2012; **6**: 172-187 [PMID: 22570746 DOI: 10.5009/gnl.2012.6.2.172]

2 **Lammert F**, Miquel JF. Gallstone disease: from genes to evidence-based therapy. *J Hepatol* 2008; **48** Suppl 1: S124-S135 [PMID: 18308417 DOI: 10.1016/j.jhep.2008.01.012]

3 **Zhu L**, Aili A, Zhang C, Saiding A, Abudureyimu K. Prevalence of and risk factors for gallstones in Uighur and Han Chinese. *World J Gastroenterol* 2014; **20**: 14942-14949 [PMID: 25356055 DOI: 10.3748/wjg.v20.i40.14942]

4 **Patrone L**, Parthipun A, Diamantopoulos A, Pinna F, Valdata A, Ahmed I, Filauro M, Sabharwal T, de Caro G. Innovative percutaneous antegrade clearance of intrahepatic and extrahepatic biliary stones with the use of a hysterosalpingography catheter. *Eur J Gastroenterol Hepatol* 2015; **27**: 419-424 [PMID: 25874515 DOI: 10.1097/MEG.0000000000000291]

5 **Sarli L**, Iusco DR, Roncoroni L. Preoperative endoscopic sphincterotomy and laparoscopic cholecystectomy for the management of cholecystocholedocholithiasis: 10-year experience. *World J Surg* 2003; **27**: 180-186 [PMID: 12616433 DOI: 10.1007/s00268-002-6456-8]

6 **Almadi MA**, Barkun JS, Barkun AN. Management of suspected stones in the common bile duct. *CMAJ* 2012; **184**: 884-892 [PMID: 22508980 DOI: 10.1503/cmaj.110896]

7 **Li YL**, Geng JL, Jia YM, Lu HL, Wang W, Liu B, Wang WJ, Chang HY, Wang YZ, Li Z. [Clinical study of percutaneous transhepatic balloon dilation:a novel procedure for common bile duct stone]. *Zhonghua Yi Xue Za Zhi* 2013; **93**: 3586-3589 [PMID: 24534307]

8 **Wang W**, Wang C, Qi H, Wang Y, Li Y. Percutaneous transcystic balloon dilation for common bile duct stone removal in high-surgical-risk patients with acute cholecystitis and co-existing choledocholithiasis. *HPB* (Oxford) 2018; **20**: 327-331 [PMID: 29146469 DOI: 10.1016/j.hpb.2017.10.002]

9 **Li D**, Li YL, Wang WJ, Liu B, Chang HY, Wang W, Wang YZ, Li Z. Percutaneous transhepatic papilla balloon dilatation combined with a percutaneous transcystic approach for removing concurrent gallbladder stone and common bile duct stone in a patient with billroth II gastrectomy and acute cholecystitis: A case report. *Medicine* (Baltimore) 2017; **96**: e7964 [PMID: 28858128 DOI: 10.1097/MD.0000000000007964]

10 **Berci G**, Morgenstern L. Laparoscopic management of common bile duct stones. A multi-institutional SAGES study. Society of American Gastrointestinal Endoscopic Surgeons. *Surg Endosc* 1994; **8**: 1168-1174; discussion 1174-1175 [PMID: 7809799]

11 **Shirai N**, Hanai H, Kajimura M, Kataoka H, Yoshida K, Nakagawara M, Nemoto M, Nagasawa M, Kaneko E. Successful treatment of percutaneous transhepatic papillary dilation in patients with obstructive jaundice due to common bile duct stones after

Billroth II gastrectomy: report of two emergent cases. *J Clin Gastroenterol* 2000; **30**: 91-93 [PMID: 10636221]
12 **Gil S**, de la Iglesia P, Verdú JF, de España F, Arenas J, Irurzun J. Effectiveness and safety of balloon dilation of the papilla and the use of an occlusion balloon for clearance of bile duct calculi. *AJR Am J Roentgenol* 2000; **174**: 1455-1460 [PMID: 10789811 DOI: 10.2214/ajr.174.5.1741455]
13 **Park YS**, Kim JH, Choi YW, Lee TH, Hwang CM, Cho YJ, Kim KW. Percutaneous treatment of extrahepatic bile duct stones assisted by balloon sphincteroplasty and occlusion balloon. *Korean J Radiol* 2005; **6**: 235-240 [PMID: 16374081 DOI: 10.3348/kjr.2005.6.4.235]
14 **Cuschieri A**. Ductal stones: pathology, clinical manifestations, laparoscopic extraction techniques, and complications. *Semin Laparosc Surg* 2000; **7**: 246-261 [PMID: 11323762]
15 **Fletcher DR**. Changes in the practice of biliary surgery and ERCP during the introduction of laparoscopic cholecystectomy to Australia: their possible significance. *Aust NZJ Surg* 1994; **64**: 75-80 [PMID: 8291982]
16 **Itoi T**, Wang HP. Endoscopic management of bile duct stones. *Dig Endosc* 2010; **22** Suppl 1: S69-S75 [PMID: 20590776 DOI: 10.1111/j.1443-1661.2010.00953.x]
17 **Prat F**, Malak NA, Pelletier G, Buffet C, Fritsch J, Choury AD, Altman C, Liguory C, Etienne JP. Biliary symptoms and complications more than 8 years after endoscopic sphincterotomy for choledocholithiasis. *Gastroenterology* 1996; **110**: 894-899 [PMID: 8608900]
18 **Podda M**, Zuin M, Battezzati PM, Ghezzi C, de Fazio C, Dioguardi ML. Efficacy and safety of a combination of chenodeoxycholic acid and ursodeoxycholic acid for gallstone dissolution: a comparison with ursodeoxycholic acid alone. *Gastroenterology* 1989; **96**: 222-229 [PMID: 2642440]
19 **Zakko SF**, Hofmann AF. Microprocessor-assisted solvent transfer system for effective contact dissolution of gallbladder stones. *IEEE Trans Biomed Eng* 1990; **37**: 410-416 [PMID: 2338354 DOI: 10.1109/10.52348]
20 **O'Donnell LD**, Heaton KW. Recurrence and re-recurrence of gall stones after medical dissolution: a longterm follow up. *Gut* 1988; **29**: 655-658 [PMID: 3396952]

P- Reviewer: Amin S, Apisarnthanarax S, Currie IS, Marion R
S- Editor: Wang XJ **L- Editor**: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 7; 24(33): 3806-3812

DOI: 10.3748/wjg.v24.i33.3806

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

CASE REPORT

Neurofibromatosis type 1-associated multiple rectal neuroendocrine tumors: A case report and review of the literature

Rui Xie, Kuang-I Fu, Shao-Min Chen, Bi-Guang Tuo, Hui-Chao Wu

Rui Xie, Kuang-I Fu, Shao-Min Chen, Bi-Guang Tuo, Hui-Chao Wu, Department of Gastroenterology, Affiliated Hospital to Zunyi Medical College, Zunyi 563003, Guizhou Province, China

Kuang-I Fu, Department of Endoscopy, Kanma Memorial Hospital, Tokyo 3250046, Japan.

ORCID number: Rui Xie (0000-0001-7970-5916); Kuang-I Fu (0000-0003-3655-6759); Shao-Min Chen (0000-0001-7569-093X); Bi-Guang Tuo (0000-0003-3147-3487); Hui-Chao Wu (0000-0003-0740-5273).

Author contributions: Tuo BG and Wu HC are the co-corresponding authors; Xie R and Chen SM managed patients and collected data; Xie R was in charge of pathological examination; Fu KI, Tuo BG and Wu HC were responsible for case design and writing.

Informed consent statement: Written informed consent was obtained.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Manuscript source: Unsolicited manuscript

Correspondence to: Hui-Chao Wu, PhD, Full Professor, Department of Gastroenterology, Affiliated Hospital to Zunyi Medical College, Zunyi 563003, Guizhou Province, China. wuhui-chao_gzzy@aliyun.com
Telephone: +86-851-28609206
Fax: +86-851-28609205

Received: May 18, 2018
Peer-review started: May 18, 2018
First decision: June 6, 2018
Revised: June 11, 2018
Accepted: July 21, 2018
Article in press: July 21, 2018
Published online: September 7, 2018

Abstract

Neurofibromatosis type 1 (NF-1) is commonly associated with benign or malignant tumors in both the central and peripheral nervous systems. However, rare cases of NF-1-associated multiple rectal neuroendocrine tumors have been reported. This report describes a case of a 39 year old female with NF-1 and intermittent hematochezia as a primary symptom. Physical examination showed multiple subcutaneous nodules and café au lait spots with obvious scoliosis of the back. Imaging examinations and colonoscopy found malformation of the left external iliac vein and multiple gray-yellow nodules with varying sizes and shapes in the rectal submucosal layer. Histological and immunohistochemical results suggested multiple rectal neuroendocrine tumors, a rare disease with few appreciable symptoms and a particularly poor prognosis. The patient with NF-1 presented here had not only multiple rectal neuroendocrine neoplasms but also vascular malformations, scoliosis and other multiple system lesions. This case therefore contributes to improving clinical understanding, diagnosis and treatment of related complications for patients with NF-1 who present with associated medical conditions.

Key words: Neurofibromatosis type 1; Multiple rectal neuroendocrine tumors; Vascular malformations; Scoliosis

Core tip: Neurofibromatosis type 1 (NF-1) is commonly complicated with either benign or malignant tumors in both the central and peripheral nervous systems. However, there are rare reported cases of NF-1 associated with multiple rectal neuroendocrine tumors. This study reports a case of a 39 year old female NF-1 patient with not only multiple rectal neuroendocrine neoplasms but also vascular malformations and scoliosis.

Xie R, Fu KI, Chen SM, Tuo BG, Wu HC. Neurofibromatosis type 1-associated multiple rectal neuroendocrine tumors: A case report and review of the literature. *World J Gastroenterol* 2018; 24(33): 3806-3812 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i33/3806.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i33.3806

INTRODUCTION

Neurofibromatosis (NF) is an autosomal dominant genetic disorder that includes three subtypes: type 1 NF (NF-1), type 2 NF, and schwannoma[1,2]. Approximately 50% of patients have a family history. The incidence rate of NF-1 is approximately 1/3000-1/4000, and it is associated with a pathogenic mutation on chromosome 17q11.2[3]. Physiologically, the disease is characterized by abnormal skin pigmentation (*i.e.*, milk coffee spots), Lisch nodules, and multiple skin nodules. NF could affect many tissues and organs, including the peripheral and central nervous systems, bones, and internal organs[4,5]. Previous studies have reported that NF-1 can lead to Ras pathway abnormalities, which may also result in peripheral neurilemmomas, central nervous system tumors, stromal tumors, neuroendocrine tumors, and other benign and malignant tumors[6-8]. However, the incidence of NF-1 in combination with gastrointestinal neuroendocrine tumor is less than two percent. Neuroendocrine tumors are commonly found in the duodenum and pancreas[9-12] and usually present as a single malignant lesion. Multiple rectal neuroendocrine tumors are particularly rare, with diverse and non-specific clinical symptoms. Common symptoms include changes in bowel habits, hematochezia, and abdominal pain, which are similar to those of more common rectal diseases such as hemorrhoids, rectal polyps, and colorectal cancers, thus complicating the accuracy of disease diagnosis[13]. Our department has received and treated a patient diagnosed with NF-1 that was combined with multiple rectal neuroendocrine tumors, vascular malformations, and scoliosis.

CASE REPORT

A 39 year old woman was admitted to our department because of intermittent bloody stools without vomiting, abdominal pain, diarrhea, skin flushes, *etc*. The patient had suffered from a slightly curved spinal column since childhood, with the abnormal curvature becoming noticeable 13 years prior. Systemic skin pimples then occurred gradually without pain or itching. Physical examination showed multiple hemispherical subcutaneous nodules with varying sizes and with soft and clear boundaries on the chest and abdomen. There were coffee pigment spots with varied sizes and colors between these nodules, with a maximal size of 3 cm × 2 cm (Figure 1A). The patient's father also had definitive NF-1. Blood examination showed the hemoglobin level of the patient was 101 g/L, with no other abnormalities. Computed tomography and magnetic resonance imaging (MRI) of the chest revealed enlarged mediastinal lymph nodes, dermatologic nodules with long T1 and T2 values, uniform densities, clear boundaries, diameters of < 10 mm (Figure 1B), thoracolumbar scoliosis and thoracic deformities (Figure 1C-D). A pelvic MRI detected segmental thickening of the right external iliac vein, with a thickness of 27.4 mm and a sausage-like appearance (Figure 2). The middle and lower rectal mucosae were irregularly thickened, with 26.5 mm at the widest point and an irregular signal with long T1 and slightly longer T2 values. Obvious uneven enhancement was noted in the post-contrast arterial phase, while separation and necrosis were visible in parts. These presentations suggested a diagnosis of multiple rectal lesions (Figure 3A and B). No obvious abnormalities were noted in the computed tomography of the head. Colonoscopy revealed multiple yellow-white nodular uplifts under the rectal mucosa at approximately 1-10 cm from the anal verge. These uplifts varied between 0.3-2.5 cm in diameter and presented with a patchy distribution. The lesion involved the entire rectal lumen (Figure 3C and D). The uplifted surface was smooth but congestive, and blood vessels were apparent on the surface of the nodules under the narrow-band imaging (Figure 3E). Endoscopic ultrasound revealed multiple hypoechoic lesions in the mucosa and submucosa, with enlarged lymph nodes in the outer membrane (Figure 3F). Pathohistological and immunohistochemical examinations (n = 10) at many sites from rectal samples showed that tumor cells were present in the lesions and mutually linked to form cord, nest, or gland-like structures. The tumor cells were round, oval or columnar, of varying sizes, with round nuclei, and without obvious mitosis. Cells were CD117 (-), CD56 (+), CK (+), CgA (+), Syn (+), and TTF-1 (-), with a Ki-67 index of < 2%, thus supporting the diagnosis of a grade 1 rectal neuroendocrine tumor (Figure 4A-B). Specimens from many nodules were taken throughout the body and were examined by pathohistology and immunohistochemistry. The subdermal nerve fibers were in a disordered arrangement, and the cells were elongated, spindle-shaped and oddly distributed in the light-stained collagen matrix. Immune staining revealed CD34 (+) and S-100 (+) expression, deep and S-shaped

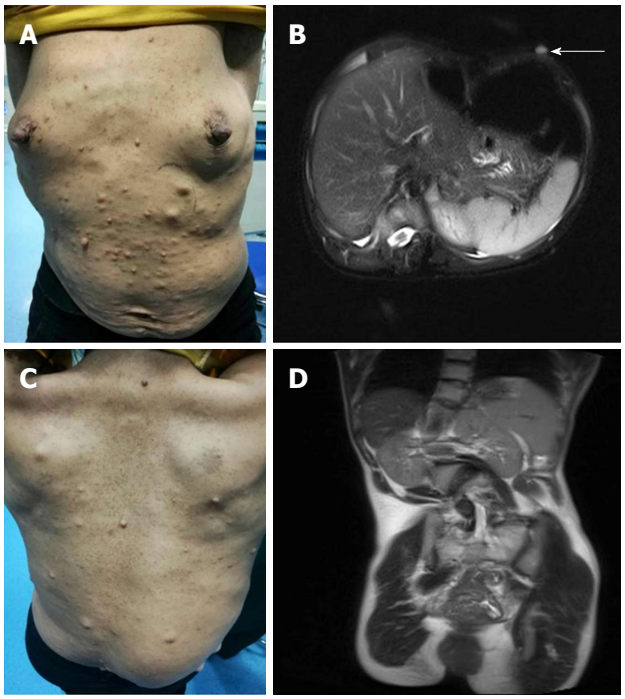


Figure 1 Patient skin changes and spinal deformities. A: Patient presented with multiple subcutaneous nodules and café au lait spots; B: MRI detected multiple nodules with uniform densities and clear boundaries that were visible on the chest and abdomen (white arrow); C: Scoliosis was noted at the spinal column; D: MRI identified scoliosis and thoracic deformity. MRI: Magnetic resonance imaging.

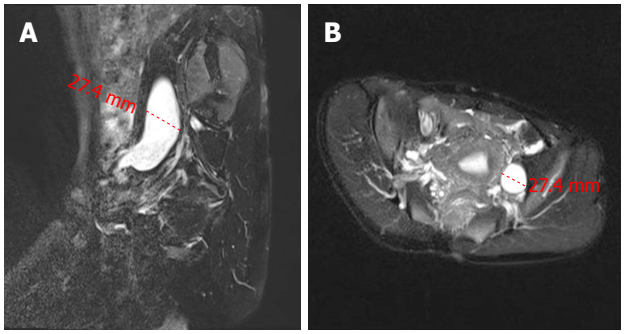


Figure 2 Imaging of left external iliac vein malformation in the patient. A: MRI sagittal image of the left iliac vein; B: MRI coronal image of the left iliac vein (red dotted line marking the widest point of the vein, measuring approximately 27.4 mm). MRI: Magnetic resonance imaging.

nuclei, and scattered mast cells. These pathological features corresponded to a diagnosis of type I neurofibromatosis (Figure 4C-E). This patient had multiple rectal neuroendocrine tumors with a diameter > 20 mm. The probability of lymph node metastasis and distant metastasis was considered to be very high. Surgical intervention was advised, however the patient rejected surgery and favored surveillance by regular follow-ups every 3-6 mo.

DISCUSSION

NF-1 is an autosomal dominant genetic disorder caused by abnormal ectodermal development that results in peripheral and central nervous system impairment. This disease has a 68.6% and 31.4% likelihood of maternal and paternal heritability, respectively. The etiology of NF-1 is not fully understood. NF-1 is currently considered to be related to gene mutations, hormones, telomerase, angiogenic factors, tumor microenvironment, electrophysiological changes and other factors related to tumor promotion[7]. Neurofilament, encoded by the *NF-1* gene, is a negative regulator of the Ras pathway, and the GAP-related domains encoded by exons 21-27 are homologous to the GTPase-activating protein family. This protein may convert the active form of Ras-GTP into the inactive form of Ras-GDP, thereby inhibiting the activation of Ras and its downstream signaling pathways including Raf-MEK-ERK and Paf-MAPK-PI3-K/Akt[6,7]. Therefore, patients with an NF-1 mutation could present with complications such as spinal malformations, vascular malformations, and benign and malignant tumors in both the central and peripheral nervous systems due to excessive Ras pathway activation. The clinical symptoms are diverse, complex and difficult to treat. Neuroendocrine tumors refer to a group of heterogeneous tumors that originate from neuroendocrine cells. They grow slowly with malignant potential and can occur in multiple systems throughout the body, although they are most commonly found in the gastrointestinal tract[14]. Clinical data confirmed that approximately two percent of patients diagnosed with NF-1 also have neuroendocrine tumors, which may be related to Ras-PI3K over-activation that leads to an imbalance of rapamycin (mTOR) expression[15]. The case presented in this report contradicts previous studies claiming that complicated neuroendocrine tumors are commonly located in the region around the ampulla of the duodenum and pancreas[9-12]. There are very few cases of NF-1 that are associated with multiple rectal neuroendocrine tumors. Rectal neuroendocrine neoplasms (NENs) are often derived from peptidergic neurons and neuroendocrine cells of the rectal mucosal epithelium, and are often divided into functional or non-functional types[16]. The clinical symptoms of functional NENs are most often related to peptides and hormones secreted from the primary site, while non-functional NENs have no specific clinical symptoms. Imaging, endoscopic ultrasound and biopsy are used as the main diagnostic methods for non-functional NENs.

Clinically, the rectal neuroendocrine tumors are mostly non-functional. In addition, rectal neuroendocrine tumors are usually single-onset, with only two to four percent being multiple-onset. Previous research suggests that the MEN1 (neuroendocrine tumor) gene, PI3-K/AKT, Raf/MEK/ERK, Notch, GSK-3β and other signaling pathways may be involved in the occurrence and metastasis of multiple rectal tumors[17]. We have summarized the relevant literature in the past 20 years and found that only one case, combined with NF-1 in 14 cases, reports of multiple rectal neuroendocrine tumors

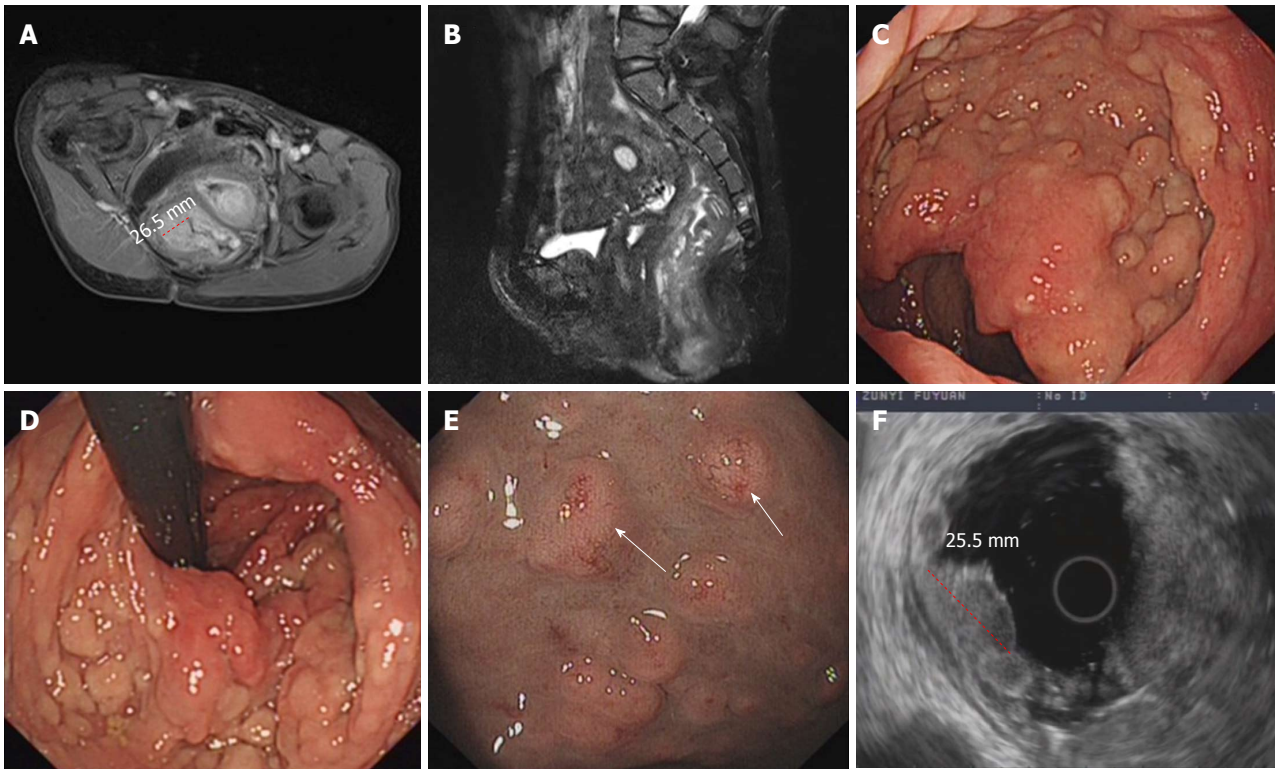


Figure 3 Imaging, endoscopy and endoscopy ultrasonographic findings of multiple rectal neuroendocrine tumors in the patient. A and B: Magnetic resonance imaging (red dotted line marking the widest point of the tumors, measuring approximately 26.5 mm); C and D: Endoscopic manifestations; E: Blood vessels were apparent on the surface of the nodules under the NBI (neuroendocrine tumors marked by the white arrow); F: Endoscopic ultrasonography of the multiple rectal neuroendocrine tumors (red dotted line marking the widest point of the tumors, measuring approximately 25.5 mm). NBI: Narrow-band imaging.

(Table 1)[18-26]. Moreover, compared with Ghassemi[26]'s case report in 2010, the number of rectal endocrine tumors in our patient significant increased (n > 30), and the lesions were more widely spread over the entire lumen of the rectum. The tumor size was also unprecedented. According to the data in Table 1, most rectal endocrine tumor diameters were between 4-10 mm, while the largest tumor diameter in this case measured up to 25 mm, with the deepest lesion invading into the submucosa. Clinical evidence revealed that the diameters of rectal endocrine tumors are greater than 20 mm, the rate of lymph node metastasis may be as high as 60%-80%, and the rate of distant metastasis can reach up to 40%[27,28]. Surgical resection is considered to be the most appropriate treatment. Common surgical methods include endoscopic submucosal dissection, endoscopic mucosal resection, transanal endoscopic microsurgery, anterior resection and abdominoperineal resection. The choice of surgical methods depends on the size of the tumor, the depth of invasion, the regional lymph node, the distant metastasis and the malignancy grade of the tumor. The 2010 guide for the diagnosis and treatment of rectal neuroendocrine cancer suggested that endoscopic resection or endoscopic dissection was the first choice for rectal carcinoids that had lower levels of malignancy with sizes of no more than 2 cm in the mucosal or submucosal layers. However, cases with tumors > 2 cm in diameter that show intravascular myometrium infiltration and vascular metastasis require surgical treatment. In addition, in light of the malignant tendency and metastasis of most gastrointestinal NENs, in addition to surgery, rectal neuroendocrine tumors require a combination of multidisciplinary and multiple interventions. For example, somatostatin analogs and molecular targeting drugs like sunitinib and everolimus inhibit tumor growth, are anti-angiogenic, and have been successfully applied in clinical applications. Additionally, the chemotherapeutic drug streptozocin, as well as similar types of temozolomides, have certain effects on patients who have failed with standardized treatments of neuroendocrine carcinomas. In recent years, peptide receptor-mediated radio receptor therapy has proven to have a definite effect on alleviating symptoms and shrinking tumors, however its severe side effects restrict its use and promotion. Although the long-term effects of the aforementioned adjuvant therapies are still not fully confirmed, multidisciplinary and multi-system combination therapy is an inevitable trend in the treatment of neuroendocrine tumors. However, the patient rejected surgery and so the pathological data are therefore not available in this case. The risk of malignancy and metastasis in this patient is very high, and she should receive regular follow-ups every 3-6 mo.

In addition to rectal neuroendocrine tumors, the patient also presented with malformations of the external iliac veins and the spinal column. A pelvic MRI revealed segmental thickening of the right external iliac vein, which was nearly double the normal diameter

Table 1 Summary of multiple rectal carcinoid case reports

Case	Sex	Age	Number	Size (mm)	The depth of invasion	Lymph node metastasis	Histological stage	Treatment	Complicated with NF-1
Kato *et al*[18]	M	61	52	1-6	SM	NA	NA	NA	No
Maruyama *et al*[19]	M	52	5	4-10	M3	No	NA	AR	No
Okamoto *et al*[20]	M	54	4	< 6	SM	NA	NA	ESMR-L	No
Haraguchi *et al*[21]	M	69	30	< 10	SM	Yes	NA	APR	No
Sasou *et al*[22]	M	51	7	< 8	SM	Yes	G1	APR	No
	M	58	3	< 7	M3	Yes	G2	AR	No
Zhou *et al*[23]	M	47	3	5-8	SM	No	G1	TEM	No
Park *et al*[24]	M	52	2	4	SM	No	G1	ESMR-L	No
	M	32	3	5-7	SM	No	G1	ESMR-L	No
	F	65	3	5-7	SM	No	NA	EMR	No
	M	62	2	5	SM	No	G1	ESMR-L	No
	F	48	2	NA	SM	No	G1	ESMR-L	No
Hua *et al*[25]	F	61	12	3-10	SM	No	G1	TEM	No
Ghassemi *et al*[26]	F	53	6	2-3	SM	No	G1	NA	Yes

APR: Abdominoperineal resection; AR: Anterior resection; ESMR-L: Endoscopic submucosal resection with a ligation device; EMR: Endoscopic mucosal resection; TEM: Transmission electron microscope; SM: Submucosa; M3: Mina muscularismiucosae.

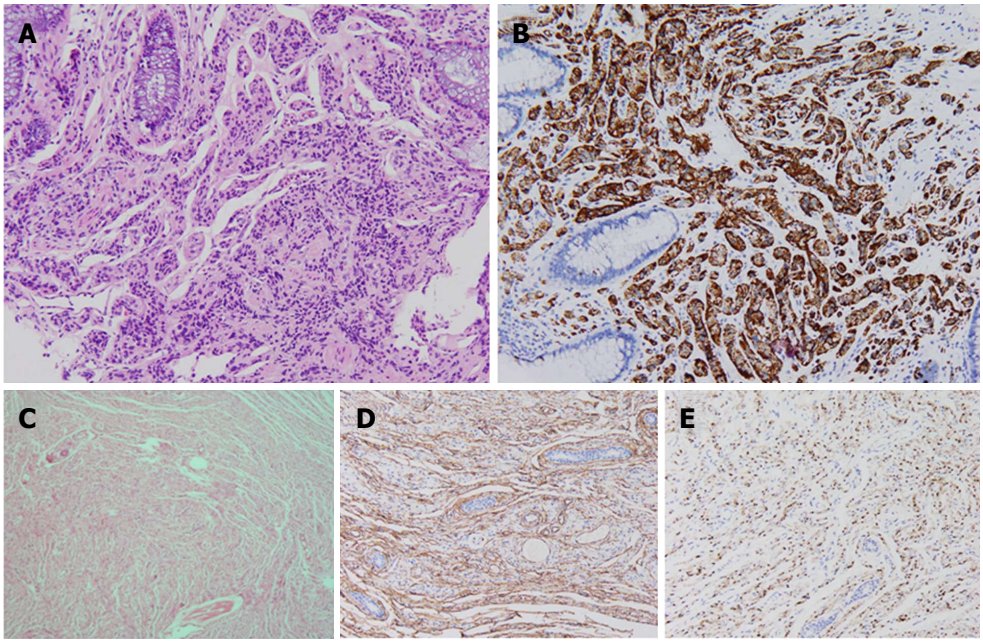


Figure 4 Immunohistochemical results of skin neurofibromatosis and multiple rectal neuroendocrine tumors. A: HE staining of multiple rectal neuroendocrine tumors (× 200); B: CgA staining pattern of multiple rectal neuroendocrine tumors (× 200); C: HE staining of the skin neurofibromatosis (× 200); D and E: S-100 and CD34 staining patterns of skin neurofibromatosis (× 200). HE: Hematoxylin and eosin.

and showed a sausage-like appearance. However, there was no obvious stenosis below the venous expansion, suggesting a congenital deformity instead of a compensatory increase caused by stenosis. Neurofibromatosis is associated with a one to three percent risk of vascular lesions. The lesions can affect varying sizes of blood vessels and present as stenosis, occlusions, hemorrhages, aneurysms, arteriovenous malformations and arteriovenous fistulas. The renal artery is the most vulnerable blood vessel, followed by the superior mesenteric artery, intracranial artery, cardiovascular, *etc*[29]. NF-1 patients often present with hypertension or arteriovenous malformations and bleeding prior to definitive diagnosis. To our knowledge, this is the first case of NF-1 that is complicated with abdominal iliac vein malformation. Previous studies have suggested that vascular dysplasia may be associated with mutations in the *NF-1* gene, which may lead to dysregulated vascular development in the mesoderm. Concentric growth, rupture of elastic fibers, and nodule hyperplasia occur in the intima of blood vessels. The reduction of smooth muscle, decrease in elastic components in the media, and increase in brittleness of the vessel wall are all observed, ultimately leading to thinning of the blood vessel wall, poor elasticity, and formation of a large number of sinus cavities in the diseased tissue, which can cause bleeding[29,30]. Currently, the preferred treatment for vascular malfor-

mations includes symptomatic treatment, surgical resection or other surgical interventions. However, no clinical symptoms can be observed in this patient, and follow-up observations can be continued. Conversely, both the patient and her daughter were diagnosed with scoliosis during childhood, which supported the possibility of heredity. Scoliosis is a common clinical manifestation of NF-1, with 10%-33% of children simultaneously diagnosed with NF-1 and scoliosis [31]. The incidence of scoliosis in adult patients with NF-1 has been reported to be between 10%-77%[32]. The pathogenesis of NF-1-associated spinal deformity is not yet clear and may encompass several factors, including the direct erosion of neurofibroma, dural dilatation of the spinal canal, osteoporosis, precocious puberty, and mesoderm dysplasia[31,32]. Patients with scoliosis may also develop lung damage as time progresses. Surgical correction of NF-1-related spinal deformities may improve the clinical curative effect.

In summary, this case suggests that an NF-1 diagnosis may be complicated by multiple system diseases. The clinical symptoms are complex, non-specific, and not easily identified. We thus need to develop individualized treatment based on the different symptoms of NF-1 patients. Although surgical and symptomatic treatments are currently preferred for multiple rectal neuroendocrine tumors, patients often require multi-system and multi-disciplinary comprehensive treatment. It is necessary to formulate the most appropriate intervention based on individual complications, with the comprehensive application of various technologies and inspection methods, in order to reduce the psychological burden on patients and improve overall quality of life.

ARTICLE HIGHLIGHTS

Case characteristics

A 39 year old woman was admitted to our department because of intermittent bloody stools. The diagnosis was confirmed to be neurofibromatosis type Ⅰ (NF-1) with multiple rectal neuroendocrine neoplasms, vascular malformations and scoliosis.

Clinical diagnosis

A female woman had a primary symptom of intermittent hematochezia without vomiting, abdominal pain, diarrhea, skin flushes, *etc*.

Differential diagnosis

There were three different diagnoses considered: hemorrhoids, rectal polyps and colorectal cancer.

Laboratory diagnosis

Blood examination showed the hemoglobin levels of the patient was 101 g/L, without other abnormalities such as liver and renal function, tumor markers, *etc*.

Imaging diagnosis

Computed tomography and magnetic resonance imaging of the chest revealed enlarged mediastinal lymph nodes, dermatologic nodules with long T1 and T2 values, uniform density, clear boundaries and diameters of < 10 mm. A pelvic MRI detected segmental thickening of the right external iliac vein. The middle and lower rectal mucosae were irregularly thickened, with 26.5 mm at the widest point and an irregular signal with long T1 and slightly longer T2 values.

Pathological diagnosis

Pathohistological and immunohistochemical examinations showed that neuroendocrine tumor cells were present in the lesions and mutually linked to form cord, nest, or gland-like structures. The tumor cells were round, oval or columnar, of varying sizes, with round nuclei, and without obvious mitosis. Cells were CD117 (-), CD56 (+), CK (+), CgA (+), Syn (+), and TTF-1 (-), with a Ki-67 index of < 2%. The subdermal nerve fibers were in a disordered arrangement, and the cells were elongated, spindle-shaped and oddly distributed in the light-stained collagen matrix. Immune staining revealed CD34 (+) and S-100 (+) expression, deep and S-shaped nuclei, and scattered mast cells.

Treatment

Surgical intervention was advised, however the patient rejected surgery and favored surveillance by regular follow-ups every 3-6 mo.

Related reports

Neuroendocrine tumors are commonly found in the duodenum and pancreas, and rare cases of NF-1-associated multiple rectal neuroendocrine tumors have been reported. We have summarized the relevant literature in the past 20 years and found that only one case, combined with NF-1 in 14 cases, reports of rectal multiple neuroendocrine tumors. In addition, this is the first case where NF-1 is complicated by abdominal iliac vein malformation.

Term explanation

Rectal neuroendocrine neoplasms (NENs) are often derived from peptidergic neurons and neuroendocrine cells of the rectal mucosal epithelium, and are often divided into functional and non-functional types. Non-functional NENs have no specific clinical symptoms. Imaging, endoscopic ultrasound and biopsy are used as the main diagnostic methods for non-functional NENs.

Experiences and lessons

NF-1 diagnosis may be complicated by multiple system diseases. The clinical symptoms are complex, non-specific, and not easily identified. We need to develop individualized treatment based on the different symptoms of NF-1 patients. Although surgical and symptomatic treatments are currently preferred for multiple rectal neuroendocrine tumors, patients often require multi-system and multi-disciplinary comprehensive treatment.

REFERENCES

1 **Huffman JL**, Gahtan V, Bowers VD, Mills JL. Neurofibromatosis and arterial aneurysms. *Am Surg* 1996; **62**:311-314 [PMID: 8600855]

2 **Ilgit ET**, Vural M, Oguz A, Ozdogan ME. Peripheral arterial involvement in neurofibromatosis type 1--a case report. *Angiology* 1999; **50**: 955-958 [PMID: 10580361 DOI: 10.1177/000331979905001111]

3 **O'Connell P**, Leach RJ, Ledbetter DH, Cawthon RM, Culver M, Eldridge JR, Frej AK, Holm TR, Wolff E, Thayer MJ. Fine structure DNA mapping studies of the chromosomal region harboring the genetic defect in neurofibromatosis type I. *Am J Hum Genet* 1989; **44**: 51-57 [PMID: 2562822]

4 **Williams VC**, Lucas J, Babcock MA, Gutmann DH, Korf B, Maria BL. Neurofibromatosis type 1 revisited. *Pediatrics* 2009; **123**: 124-133 [PMID: 19117870 DOI: 10.1542/peds.2007-3204]

5 **Boyd KP**, Korf BR, Theos A. Neurofibromatosis type 1. *J Am Acad Dermatol* 2009; **61**: 1-14; quiz 15-16 [PMID: 19539839 DOI: 10.1016/j.jaad.2008.12.051]

6 **Harrisingh MC**, Perez-Nadales E, Parkinson DB, Malcolm DS, Mudge AW, Lloyd AC. The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation. *EMBO J* 2004; **23**: 3061-3071 [PMID: 15241478 DOI: 10.1038/sj.emboj.7600309]

7 **Gottfried ON**, Viskochil DH, Fults DW, Couldwell WT. Molecular, genetic, and cellular pathogenesis of neurofibromas and surgical implications. *Neurosurgery* 2006; **58**: 1-16; discussion 1-16

[PMID: 16385324 DOI: 10.1227/01.NEU.0000190651.45384.8B]
8 **Pasmant E**, Vidaud M, Vidaud D, Wolkenstein P. Neurofibromatosis type 1: from genotype to phenotype. *J Med Genet* 2012; **49**: 483-489 [PMID: 22889851 DOI: 10.1136/jmedgenet-2012-100978]
9 **Mao C**, Shah A, Hanson DJ, Howard JM. Von Recklinghausen's disease associated with duodenal somatostatinoma: contrast of duodenal versus pancreatic somatostatinomas. *J Surg Oncol* 1995; **59**: 67-73 [PMID: 7745981 DOI: 10.1002/jso.2930590116]
10 **Clements WM**, Martin SP, Stemmerman G, Lowy AM. Ampullary carcinoid tumors: rationale for an aggressive surgical approach. *J Gastrointest Surg* 2003; **7**: 773-776 [PMID: 13129555 DOI: 10.1016/S1091-255X(03)00114-8]
11 **Klein A**, Clemens J, Cameron J. Periampullary neoplasms in von Recklinghausen's disease. *Surgery* 1989; **106**: 815-819 [PMID: 2510333]
12 **Relles D**, Baek J, Witkiewicz A, Yeo CJ. Periampullary and duodenal neoplasms in neurofibromatosis type 1: two cases and an updated 20-year review of the literature yielding 76 cases. *J Gastrointest Surg* 2010; **14**: 1052-1061 [PMID: 20300877 DOI: 10.1007/s11605-009-1123-0]
13 **Modlin IM**, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A. Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008; **9**: 61-72 [PMID: 18177818 DOI: 10.1016/S1470-2045(07)70410-2]
14 **Mandair D**, Caplin ME. Colonic and rectal NET's. *Best Pract Res Clin Gastroenterol* 2012; **26**: 775-789 [PMID: 23582918 DOI: 10.1016/j.bpg.2013.01.007]
15 **Rosner M**, Hanneder M, Siegel N, Valli A, Fuchs C, Hengstschläger M. The mTOR pathway and its role in human genetic diseases. *Mutat Res* 2008; **659**: 284-292 [PMID: 18598780 DOI: 10.1016/j.mrrev.2008.06.001]
16 **Blonski WC**, Reddy KR, Shaked A, Siegelman E, Metz DC. Liver transplantation for metastatic neuroendocrine tumor: a case report and review of the literature. *World J Gastroenterol* 2005; **11**: 7676-7683 [PMID: 16437698 DOI: 10.3748/wjg.v11.i48.7676]
17 **Ning L**, Chen H, Kunnimalaiyaan M. Focal adhesion kinase, a downstream mediator of Raf-1 signaling, suppresses cellular adhesion, migration, and neuroendocrine markers in BON carcinoid cells. *Mol Cancer Res*2010; **8**: 775-782 [PMID: 20407018 DOI: 10.1158/1541-7786.MCR-09-0525]
18 **Kato M**, Yonemura Y, Sugiyama K, Hashimoto T, Shima Y, Miyazaki I, Sugiura H, Kurumaya H, Hoso M, Yao T. [Multiple rectal carcinoids--with special reference to the histogenesis of these lesions]. *Gan No Rinsho*1986; **32**: 1894-1900 [PMID: 3795507]
19 **Maruyama M**, Fukayama M, Koike M. A case of multiple carcinoid tumors of the rectum with extraglandular endocrine cell proliferation. *Cancer* 1988; **61**: 131-136 [PMID: 2446733 DOI: 10.1002/1097-0142(19880101)61:1<131::AID-CNCR2820610123>3.0.CO;2-G]
20 **Okamoto Y**, Fujii M, Tateiwa S, Sakai T, Ochi F, Sugano M, Oshiro K, Masai K, Okabayashi Y. Treatment of multiple rectal carcinoids by endoscopic mucosal resection using a device for esophageal variceal ligation. *Endoscopy* 2004; **36**: 469-470 [PMID: 15100972 DOI: 10.1055/s-2004-814386]
21 **Haraguchi M**, Kinoshita H, Koori M, Tsuneoka N, Kosaka T, Ito Y, Furui J, Kanematsu T. Multiple rectal carcinoids with diffuse ganglioneuromatosis. *World J Surg Oncol* 2007; **5**: 19 [PMID: 17306015 DOI: 10.1186/1477-7819-5-19]
22 **Sasou S**, Suto T, Satoh T, Tamura G, Kudara N. Multiple carcinoid tumors of the rectum: report of two cases suggesting the origin of carcinoid tumors. *Pathol Int* 2012; **62**: 699-703 [PMID: 23005597 DOI: 10.1111/j.1440-1827.2012.02852.x]
23 **Zhou JL**, Lin GL, Zhao DC, Zhong GX, Qiu HZ. Resection of multiple rectal carcinoids with transanal endoscopic microsurgery: case report. *World J Gastroenterol* 2015; **21**: 2220-2224 [PMID: 25717261 DOI: 10.3748/wjg.v21.i7.2220]
24 **Park CS**, Lee SH, Kim SB, Kim KO, Jang BI. Multiple rectal neuroendocrine tumors: report of five cases. *Korean J Gastroenterol* 2014; **64**: 103-109 [PMID: 25168053 DOI: 10.4166/kjg.2014.64.2.103]
25 **Bai H,** Meng F, Yang X. One case of multiple rectal carcinoids and literature review. *Zhongguo Yiyao Daokan* 2016;**18:** 1282-1283
26 **Ghassemi KA**, Ou H, Roth BE. Multiple rectal carcinoids in a patient with neurofibromatosis. *Gastrointest Endosc* 2010; **71**: 216-218 [PMID: 19836738 DOI: 10.1016/j.gie.2009.06.026]
27 **Caplin M**, Sundin A, Nillson O, Baum RP, Klose KJ, Kelestimur F, Plöckinger U, Papotti M, Salazar R, Pascher A; Barcelona Consensus Conference participants. ENETS Consensus Guidelines for the management of patients with digestive neuroendocrine neoplasms: colorectal neuroendocrine neoplasms. *Neuroendocrinology* 2012; **95**: 88-97 [PMID: 22261972 DOI: 10.1159/000335594]
28 **Konishi T**, Watanabe T, Kishimoto J, Kotake K, Muto T, Nagawa H; Japanese Society for Cancer of the Colon and Rectum. Prognosis and risk factors of metastasis in colorectal carcinoids: results of a nationwide registry over 15 years. *Gut* 2007; **56**: 863-868 [PMID: 17213340 DOI: 10.1136/gut.2006.109157]
29 **Serranito-García R**, Gelabert M, García-Allut A. [Aneurysm of anterior communicating artery associated with type 1 neurofibromatosis]. *Neurologia* 2007; **22**: 547-550 [PMID: 17641990]
30 **Deans WR**, Bloch S, Leibrock L, Berman BM, Skultety FM. Arteriovenous fistula in patients with neurofibromatosis. *Radiology* 1982; **144**: 103-107 [PMID: 6806851 DOI: 10.1148/radiology.144.1.6806851]
31 **Abdel-Wanis ME**, Kawahara N. Aetiology of spinal deformities in neurofibromatosis 1: new hypotheses. *Med Hypotheses* 2001; **56**: 400-404 [PMID: 11359370 DOI: 10.1054/mehy.2000.1232]
32 **Tsirikos AI**, Saifuddin A, Noordeen MH. Spinal deformity in neurofibromatosis type-1: diagnosis and treatment. *Eur Spine J* 2005; **14**: 427-439 [PMID: 15712001 DOI: 10.1007/s00586-004-0829-7]

P- Reviewer: Serban ED **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Huang Y

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 September 14; 24(34): 3813-3964

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 34 September 14, 2018

META-ANALYSIS

CASE REPORT

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Mitsushige Sugimoto, MD, PhD, Associate Professor, Division of Digestive Endoscopy, Shiga University of Medical Science Hospital, Otsu 520-2192, Japan

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
September 14, 2018

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3813-3820

DOI: 10.3748/wjg.v24.i34.3813

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

EDITORIAL

Clinical impact of microbiome in patients with decompensated cirrhosis

Theodora Oikonomou, George V Papatheodoridis, Michael Samarkos, Ioannis Goulis, Evangelos Cholongitas

Theodora Oikonomou, Ioannis Goulis, Fourth Department of Internal Medicine, Hippokration General Hospital, Medical School of Aristotle University of Thessaloniki, Thessaloniki 54642, Greece

Michael Samarkos, Evangelos Cholongitas, First Department of Internal Medicine, Laiko General Hospital, Medical School of National and Kapodistrian University of Athens, Athens 11527, Greece

George V Papatheodoridis, Academic Department of Gastroenterology, Laiko General Hospital, Medical School of National and Kapodistrian University of Athens, Athens 11527, Greece

ORCID number: Theodora Oikonomou (0000-0002-9077-4960); George Papatheodoridis (0000-0002-3518-4060); Ioannis Goulis (0000-0002-2765-4157); Michael Samarkos (0000-0001-9630-9712); Evangelos Cholongitas (0000-0002-3645-582X).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Manuscript source: Invited manuscript

Correspondence to: Evangelos Cholongitas, MD, PhD, Associate Professor, First Department of Internal Medicine, Medical School of National & Kapodistrian University of Athens, Laiko General Hospital, Agiou Thoma 17, Athens 11527, Greece. cholongitas@yahoo.gr

Telephone: +30-6936-378903
Fax: +30-2310-992940

Received: May 28, 2018
Peer-review started: May 28, 2018
First decision: July 4, 2018
Revised: July 11, 2018
Accepted: July 21, 2018
Article in press: July 21, 2018
Published online: September 14, 2018

Abstract

Cirrhosis is an increasing cause of morbidity and mortality. Recent studies are trying to clarify the role of microbiome in clinical exacerbation of patients with decompensated cirrhosis. Nowadays, it is accepted that patients with cirrhosis have altered salivary and enteric microbiome, characterized by the presence of dysbiosis. This altered microbiome along with small bowel bacterial overgrowth, through translocation across the gut, is associated with the development of decompensating complications. Studies have analyzed the correlation of certain bacterial families with the development of hepatic encephalopathy in cirrhotics. In general, stool and saliva dysbiosis with reduction of autochthonous bacteria in patients with cirrhosis incites changes in bacterial defenses and higher risk for bacterial infections, such as spontaneous bacterial peritonitis, and sepsis. Gut microbiome has even been associated with oncogenic pathways and under circumstances might promote the development of hepatocarcinogenesis. Lately, the existence of the oral-gut-liver axis has been related with the development of decompensating events. This link between the liver and the oral cavity could be *via* the gut through impaired intestinal permeability that allows direct translocation of bacteria from the oral cavity to the systemic circulation. Overall, the contribution of the microbiome to pathogenesis becomes more pronounced with progressive disease and therefore may represent an important therapeutic

target in the management of cirrhosis.

Key words: Microbiome; Dysbiosis; Oral-gut-liver axis; Hepatic encephalopathy; Decompensated cirrhosis; Liver carcinoma

Core tip: Human microbiome of the oral-gut-liver axis is implicated in the progression of hepatic diseases and the development of decompensated events. Its significance over diagnostic, prognostic and therapeutic possibilities drives a new era in the management of patients with cirrhosis.

Oikonomou T, Papatheodoridis GV, Samarkos M, Goulis I, Cholongitas E. Clinical impact of microbiome in patients with decompensated cirrhosis. *World J Gastroenterol* 2018; 24(34): 3813-3820 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3813.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3813

INTRODUCTION

Cirrhosis is an increasing cause of morbidity and mortality in more developed countries, being the 14th most common cause of death worldwide[1]. Decompensated disease has an annual mortality rate of 57%, and acute decompensating events present mortality of 30%[1]. Traditionally, clinicians use models to triage patients with advanced liver diseases; Model for End stage Liver Disease (MELD) and Child-Pugh (CTP) scores have been validated and provide significant prognostic information[2,3].

In the era of advanced molecular techniques, human microbiome is being studied for the pathogenesis and superior prognostication of decompensated patients[4]. The microbial imbalance or dysbiosis that occurs in the gut in patients with cirrhosis has recently been linked with complications of cirrhosis, including hepatic encephalopathy (HE), spontaneous bacterial peritonitis (SBP), and sepsis[5].

At least for the last decade, it is known that the gut flora contributes in the pathogenesis of cirrhosis' complications, regarding the development of infections or the hyperdynamic circulatory state of cirrhosis[6]. Here, we reviewed data regarding the role of microbiome in clinical exacerbation of patients with decompensated cirrhosis.

HUMAN MICROBIOME

Human microbiome stands the community of bacteria, archaea, fungi, and viruses which are found and interact within a body habitat, such as oral cavity or gut[7]. It is characterized by its diversity and microbial abundance and performs specific metabolic and functional pathways[8]. Using culture-independent techniques, which analyze the DNA extracted directly from a sample, allow us to investigate several aspects of microbial communities, their causative or modulatory roles[7]. The challenge in microbiome analysis concerns the relation between differences in community composition to differences in function, therefore identify the human microbiome as a biomarker for specific clinical conditions[9].

ALTERED MICROBIOME IN PATIENTS WITH CIRRHOSIS

A few studies tried to map the human microbiome of patients with advanced liver diseases. Data regarding decompensated cirrhotic patients are far more scarce. A first comprehensive view into the intestinal microbiome of patients with cirrhosis showed that the fecal microbial composition of patients with cirrhosis is distinct from healthy controls. Patients presented with prevalence of potentially pathogenic bacteria, such as *Enterobacteriaceae Veillonellaceae* and *Streptococcaceae*, which had a positive correlation with CTP score. Proteobacteria and Fusobacteria were highly enriched along with the reduction of beneficial populations such as *Lachnospiraceae* which correlated negatively with CTP score[10]. A next analysis of stool microbiome conducted in cirrhotics showed that the composition differed significantly[11].

Bajaj *et al*[12] studied 54 decompensated cirrhotic patients and proposed the cirrhosis dysbiosis ratio (CDR), ratio of autochthonous to non-autochthonous taxa, as a tool to estimate dysbiosis in cirrhotics. Microbiota and CDR were relatively stable over time within patients whose disease remained unchanged and altered when the underlying disease worsened. CDR for controls was significantly higher compared to all cirrhotic patients.

In 2014 Qin *et al*[13] analyzed data regarding the gut microbiome of patients with cirrhosis and reported two principal findings; patients with cirrhosis had altered gut microbiome profile compared to healthy controls, and most (54%) of the patient-enriched species were of buccal origin, suggesting a massive invasion of the gut by oral bacterial species from the mouth, responsible for this change of the gut microbiota seen in cirrhosis. These findings established new perspectives over the role of oral-gut-liver axis in patients with cirrhosis.

Accordingly, a further evaluation of the salivary and stool microbiome in decompensated cirrhotic patients showed dysbiosis represented by reduction in autochthonous bacteria, both in saliva and stool samples[14]. This was related to impaired salivary defenses and worse salivary and systemic inflammation, more prominent in patients with HE. Patients with cirrhosis had a significantly lower relative abundance of autochthonous taxa (*Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales* XIV) and significantly lower stool cirrhosis dysbiosis ratio

(*Lachnospiraceae* + *Ruminococcaceae* + *Clostridiales Incertae Sedis* XIV + *Veillonelllaceae*/*Enterobacteriaceae* + *Bacteroidaceae*) and salivary microbiota dysbiosis ratio (*Lachnospiraceae* + *Ruminococcaceae* + *Clostridiales Incertae Sedis* XIV/*Streptococcaceae*)[14]. Chen *et al*[15], showed that even the duodenal mucosa microbiota in cirrhotic patients is dramatically different from healthy controls, possibly in accordance with alterations of oral microbiota and changes in duodenal micro-environment. *Veillonella*, *Prevotella*, *Neisseria*, and *Haemophilus*, found to be the most discriminative taxa between cirrhosis and controls.

Dysbiosis of the oral microbiota was present in patients with chronic liver disease; *i.e.*, chronic hepatitis B and hepatitis B related cirrhosis. One correspondent study supported that the higher proportion of *Firmicutes* than of *Bacteroidetes* organisms is responsible for the weak oral defenses that contributes to the breakdown of oral defenses and invasion of the gut. So, dysbiosis was introduced as inversion of the *Firmicutes*/*Bacteroidetes* ratio[16]. Lately, oral microbiome was characterized by significant dysbiosis in cirrhotic patients with hepatocellular carcinoma (HCC), suggesting that certain key bacterial species may characterize patients' microbiota[17]. Overall, these findings suggest new potential prognostic and therapeutic targets.

PATHOPHYSIOLOGY: THE ROLE OF MICROBIOME OVER THE ORAL-GUT-LIVER AXIS

Published research findings reveal implications of the altered microbiome in the progress of liver diseases. The microbiome determines likelihood and rate of progression of liver injury, complications of cirrhosis and ultimately outcome[18]. The principal theory highlights the role of the gut-liver axis in the development and progression of cirrhosis and portal hypertension; bacterial translocation (BT) from the intestine reaches the liver and increases portal pressure, while, on the other hand, portal hypertension leads to intestinal edema, disruption of epithelial integrity and more translocation[19].

The microbiome has been considered the "core facility" for the production of a myriade of bacterial metabolites and products to which the gut-vascular barrier and each member of the gut-liver-axis are exposed[20]. Cirrhotic patients are exposed to a higher risk of dysbiosis because of a variety of pathological interactions between the liver and the gastrointestinal tract. Alteration in intestinal motility, higher gastric pH and reduced bile acid concentration in the colon, may lead to a failure in the control of bacterial intestinal growth[21]. During progression of cirrhosis, the microbiome, through their metabolism, cell wall components (LPS) and translocation, leads to inflammation. Inflammation suppresses synthesis of bile acids in liver supporting a positive-feedback mechanism. Decrease in bile acids entering the intestines appears to favor overgrowth of pathogenic and pro-inflammatory members of the microbiome[22]. Moreover, dysbiosis seems to co-exist with small intestinal bacterial overgrowth (SIBO) related to delayed intestinal transit and the development of cirrhotic complications[23]. Overall, intestinal dysbiosis is established in decompensated liver disease. This was found to represent a condition of reduced relative abundance of taxa considered benign and autochthonous, including *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridialies Incertae Sedis* XIV and a relatively higher abundance of others, particularly *Enterobacteriaceae* and *Bacteroidaceae*[12,14,16].

Portal hypertension, alterations in the intestinal microbiota, inflammation and oxidative stress can affect intestine barrier function, which becomes more permeable[24]. Eventually, pathologically increased BT from the gut to mesenteric lymph nodes arise in cirrhosis as an interplay of microbiome, deficiencies in secretory and mechanical intestinal barrier functions along with immune tolerant and deficient gut-associated lymphatic tissue. Small intestine has been suggested as the predominant site of BT in cirrhosis and SIBO has the greatest potential for promoting BT[25].

More recent data raise implications on buccal origin of the gut microbiome supporting the emerging role of oral-liver-gut axis in decompensated cirrhosis[26]. Generally, oral dysbiosis has been correlated with local and distal infections, postulating that a baseline for the healthy core oral microbiota provides an opportunity to examine shifts during the onset and recurrence of disease[27]. Research findings imply a link between dental infections and accelerated progression of liver diseases trying to understand the clinical significance of oral-derived endotoxemia/bacteremia in the course of liver disease[28].

A first study on omeprazole in compensated cirrhotics showed that gastric acid suppression allows intestinal overgrowth of bacteria normally present in the oral cavity and implicated a link between the gut microbiota changes and complications of cirrhosis[29]. Later, Bajaj *et al*[14] found that dysbiosis, represented by reduction in autochthonous bacteria, is present in both saliva and stool in patients with cirrhosis. The major change of the gut microbiota was considered to stem from a massive invasion of the gut by oral bacterial species[13]. This link between the liver and the oral cavity could be *via* the gut through impaired intestinal permeability that in turn could allow direct translocation of bacteria and/or their products and inflammatory mediators from the oral cavity to the systemic circulation[26]. Patients with cirrhosis have salivary and enteric dysbiosis along with small bowel bacterial overgrowth, and translocation across the leaky gut. The latter is exacerbated by underlying portal hypertension and endothelial dysfunction and is associated with the development of decompensating complications[4] (Figure 1).

CLINICAL IMPACT OF THE ALTERED MICROBIOME

Considering its key role in bacterial translocation, gut

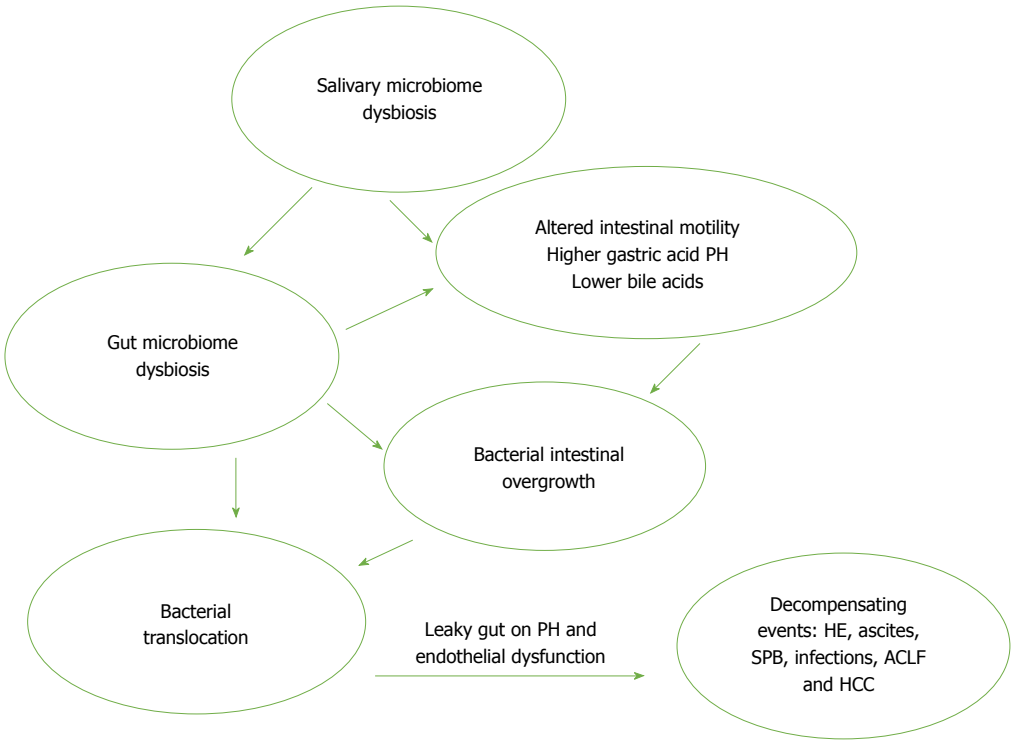


Figure 1 Pathophysiological mechanism showing the role of microbiome over the oral-gut-liver axis. (Arrows imply the successive steps over the pathophysiology of complications.) PH: Portal hypertension; HE: Hepatic encephalopathy; SBP: Spontaneous bacterial peritonitis; ACLF: Acute-on-chronic liver failure; HCC: Hepatocellular carcinoma.

microbiome has been implicated in the pathogenesis of complications on the course of decompensated cirrhosis[21]. This culminates in systemic inflammation and endotoxemia, which induces innate immune dysfunction predisposing to infection, and development of acute conditions, such as hemorrhage, sepsis, and hepatic encephalopathy[4].

Hepatic encephalopathy

Dysbiosis, or altered microbiota represented by reduction in autochthonous bacteria, is present in both saliva and stool in patients with cirrhosis[14]. After HE development, there is significant change in microbial relative abundance[12]. Interestingly, two consecutive studies analyzed the correlation of certain bacterial families with cognition in cirrhotics. Stool microbiome analyses along with magnetic resonance imaging (MRI) brain assessment, revealed that specific bacterial taxa are associated with astrocytic changes and neuronal changes in humans with cirrhosis and HE in accordance with the clinically impaired cognition[30,31]. Indeed, patients with HE presented with altered flora (higher *Veillonellaceae*), poor cognition, endotoxemia, and inflammation (IL-6, TNF-α, IL-2, and IL-13) compared with cirrhotics without HE[30]. Healthy controls had a significantly higher proportion of autochthonous bacterial families than cirrhotics. Similarly cirrhotic patients with HE had a higher relative abundance of autochthonous families and a higher abundance pattern of *Staphylococcaceae*, *Enterococcaceae*, *Porphyromonadaceae* and *Lactobacillaceae* compared to controls and cirrhotics without HE[31].

Salivary and gut microbiome dysbiosis along with small bowel bacterial overgrowth and translocation of bacteria and their products across the leaky gut epithelial barrier underpin the endotoxemia and systemic inflammatory response that predispose to the manifestation of covert and overt HE[32]. Moreover, microbiome variations have associations with systemic inflammation and ammonia levels[31] and contribute to the development of HE by means of *ammoniagenesis* and generation of endotoxin-driven inflammatory response[18]. These microbial functions enriched in the microbiota in patients with cirrhosis supports the concept that the microbiome contributes to HE[33]. The enrichment of the modules for ammonia production gut bacteria might contribute to increased levels of ammonia in blood. Manganese-related transport system modules enriched in patients possibly contribute to the changes in concentrations of manganese, which accumulated within the basal ganglia in patients with end-stage liver disease and may have a role in hepatic encephalopathy. Modules for GABA biosynthesis enriched in patients are involved in the pathogenesis of hepatic encephalopathy too[13].

Ascites-infections-SBP-acute on chronic liver failure

One recently published study shed light in the role of microbiome in patients with ascites. Santiago *et al*[34], found that specific serum microbiome is linked to the presence of ascites and proposed that these patients might have a greater deterioration of the intestinal barrier

integrity, also a higher degree of microbial translocation thus leading to a higher microbial diversity in serum.

In general, stool and saliva dysbiosis with reduction of autochthonous bacteria in patients with cirrhosis incites a systemic proinflammatory milieu due to changes in bacterial defenses[14]. Buccal proinflammatory environment in accordance with impaired local innate defenses of the oral cavity, represents a global mucosal-immune interface change in cirrhosis affecting the gut microbiota[4]. Moreover, pathologic bacterial translocation is associated with higher risk of developing infections; bacterial overgrowth, increased intestinal permeability, and integrity of immune surveillance mechanisms that allow bacteria (or parts of bacteria) to translocate to mesenteric lymph nodes and the systemic circulation, are important in driving SBP, in particular[18,21].

Progressive severe changes in the gut microbiome present in cirrhotic patients on acute on chronic liver failure[12,35]. Changes in salivary and stool microbiota have been independently associated with the prediction of hospitalizations in cirrhotic patients[14,36]. Besides, the great interest regarding microbiota has been shifting from pathogenesis toward the prediction of clinically relevant outcomes in cirrhosis[5].

HCC

In 2016, Lu *et al*[17] identified oral microbiota dysbiosis in cirrhotic patients with liver carcinoma recognizing specific discriminatory bacteria that could provide novel and non-invasive diagnostic biomarkers of the presence of liver carcinoma. The suggested pathogenic pathway introduced the role of endotoxemia produced by gut microbiota; associated with oncogenic pathways when increased promotes the development of hepatocarcinogenesis[37]. Experimental animal models support this promoting effect of the gut microbiota-driven inflammation in hepatocarcinogenesis[38], though data on human are scarce; Zhang *et al*[39] demonstrated that the induction of dysbiosis, with increased growth rate of *E. coli* and *Atopobium* cluster and significantly decreased percentages of benign bacteria (*Lactobacillus* group, *Bifidobacterium* group, and *Enterococcus* group), is sufficient to promote hepatocarcinogenesis by enhanced portal LPS levels. So, there is profound impact of intestinal microbiota and gut homeostasis on HCC development[40] (Table 1).

THERAPEUTIC ASPECT OF MICROBIOME

Overall, the contribution of the microbiota to pathogenesis becomes more pronounced with progressive disease and therefore remains an important therapeutic target in the management of cirrhosis[18]. The mutual interdependence between the pathogenesis of the cirrhotic process, intestinal bacterial translocation and portal pressure makes the gut–liver axis an attractive target for specific therapeutic interventions[19]. The gut microbiome can be modulated in different ways, using prebiotics, probiotics, synbiotics, antibiotics, and even fecal microbiota transplantation[41].

Lactulose is probably the best-studied prebiotic in liver disease and is commonly used for the treatment of HE[41]. It acts by acidifying and modifying the colonic flora. This could lead to a displacement of urease-producing bacteria with non-urease-producing Lactobacillus, and, therefore, to a reduction in the formation of potentially toxic short-chain fatty acids[19]. Probiotics are living microorganisms that confer a health benefit on their host through antimicrobial effects, enhancement of mucosal barrier integrity, and immunomodulation[41]. Probiotics and synbiotics (a combination of probiotics and prebiotics in a form of synergism) have been used broadly in trials addressing HE, showing improvements in endotoxemia, endothelial dysfunction, and dysbiosis, along with modifications on bile acid pool composition; though larger clinical trials with clinically significant outcomes are needed[19].

The traditional and most logical first approach to diminish translocation of microbial components and products is to reduce the enteric burden of the bacteria that contribute the most to this, with antibiotics[20]. Rifaximin, a minimally-absorbed oral antimicrobial agent, has been intensively studied on advanced liver cirrhosis. It is supported that rifaximin diminishes the risk of HE recurrence and HE-related hospitalizations but also improves endotoxemia, systemic hemodynamics and renal function[23]. Finally, new perspectives came from fecal microbiota transplant, which seems to influence the microbiota through limiting the colonization of pathogens and affecting microbial metabolic function[41]. In a latest mouse study the gut microbiota was reprogrammed by transplanting bacteria with minimal urease gene content, thus reduction in fecal urease activity. This led to reduced fecal ammonia levels, and neurobehavioral deficits and decreased the morbidity and mortality associated with liver damage[42].

CONCLUSION

Microbiome has been rather implicated in the pathogenesis of various clinical conditions ranging from chronic liver diseases to decompensated complications[5,43-45]. Several studies presented different aspects of the altered microbiome seen in patients with cirrhosis. There is no consensus among the researchers. Specifically, microbiome dysbiosis has been introduced either as a reduced ratio of autochthonous to non-autochthonous taxa (CDR) or as inversion of the *Firmicutes*/*Bacteroidetes* ratio[12,14,16]. Whatever the expression, salivary and stool dysbiosis has been described as an interplay in the development of cirrhosis-related complications. Besides, modulation of the microbiome with the existing therapeutic strategies remains the cornerstone of the management of cirrhosis. Thus, estimation of microbiome in patients with cirrhosis seems to facilitate new prognostic and therapeutic strategies.

Table 1 Published studies regarding microbiome over cirrhotic patients

Study	Analysis	Conclusion
Chen *et al*[10], 2011	36 patients with liver cirrhosis and 24 healthy controls; analysis of fecal microbial community	Fecal microbial communities are distinct in patients with cirrhosis compared with healthy individuals. The prevalence of potentially pathogenic bacteria, such as *Enterobacteriaceae* and *Streptococcaceae*, along with the reduction of beneficial populations such as *Lachnospiraceae* in patients with cirrhosis may affect prognosis
Bajaj *et al*[11], 2012	60 patients with cirrhosis (24 patients without HE/36 with HE) and 17 age-matched healthy controls; stool and colonic mucosal microbiome analysis, linkage of them with changes in peripheral inflammation and cognition	There was higher abundance of autochthonous genera and a lower abundance of potentially pathogenic ones in controls compared with cirrhotic patients' mucosa. Significant change was recorded in the microbiome of the mucosa compared with stool. In general HE patients had less "healthy" microbiome
Bajaj *et al*[30], 2012	25 patients (17 HE and 8 without HE) and 10 controls; fecal microbiota analysis	Cirrhosis, especially when complicated with HE, is associated with significant alterations in the stool microbiome compared with healthy individuals. Specific bacterial families (*Alcaligeneceae*, *Porphyromonadaceae*, *Enterobacteriaceae*) are strongly associated with cognition and inflammation in HE.
Bajaj *et al*[29], 2014	15 patients with compensated cirrhosis and 15 age-matched healthy controls; stool microbiota profiling before (pre) and after PPI	Significant microbiota change was seen in both controls and cirrhotics after omeprazole. Omeprazole is associated with a microbiota shift and functional change in the distal gut in patients with compensated cirrhosis that could set the stage for bacterial overgrowth
Bajaj *et al*[12], 2014	219 cirrhotics (121 compensated outpatients, 54 decompensated outpatients, 44 inpatients) and 25 age-matched controls); stool analysis and introduction of the cirrhosis dysbiosis ratio (CDR)	Relative stability of the microbiota and CDR over time within cirrhotics whose disease remained unchanged. Microbiota changed when the underlying disease worsened in HE and infections reflected by CDR reduction. Associations of increased dysbiosis, with lower CDR and higher gram-negative taxa relative abundance. CDR for controls was significantly higher compared to all cirrhotic patients
Kakiyama *et al*[45], 2014	19 healthy, 6 drinkers without liver disease, and 78 cirrhotic (compensated and decompensated) patients; fecal and serum bile acids (BA), serum endotoxin, and stool microbiota analysis	Bile acids affect the composition of the intestinal microbiota Higher total BA pool in alcoholic cirrhotics could lead to a higher substrate for microbiota. Inflammation and gut barrier injury in alcoholic liver disease
Qin *et al*[13], 2014	98 cirrhotic patients and 83 healthy controls; gene catalogue of gut microbes analysis	Patients with liver cirrhosis have a less "healthy" gut microbiome, enriched with *Veillonella*, *Streptococcus*, *Clostridium*. Healthy individuals' microbiome was enriched with autochthonous species (*Lachnospiraceae* and *Ruminococcaceae*). Proof that the major change of the gut microbiota in patients with liver cirrhosis, is mainly because of a massive invasion of the gut by oral bacterial species
Bajaj *et al*[14], 2015	102 patients with cirrhosis (with/without HE) and 32 age-matched healthy controls; stool and saliva microbiome analysis along with evaluation of systemic and salivary inflammatory response	Dysbiosis, represented by reduction in autochthonous bacteria (*Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales* XIV), is present in both saliva and stool in patients with cirrhosis, compared to controls. Stool cirrhosis dysbiosis ratio (*Lachnospiraceae* + *Ruminococcaceae* + *Clostridiales Incertae Sedis* XIV + *Veillonelllaceae*/*Enterobacteriaceae* + *Bacteroidaceae*) was significantly lower in patients with cirrhosis. Salivary microbiota dysbiosis ratio (*Lachnospiraceae* + *Ruminococcaceae* + *Clostridiales Incertae Sedis* XIV/*Streptococcaceae*), was lower in patients with cirrhosis, compared to controls
Bajaj *et al*[36], 2015	278 cirrhotics (39% with HE, 31% with DM); stool microbial analysis and 72 underwent mucosal analyses	Cirrhotic subjects who required non-elective 90 d hospitalization had a different microbial profile DM in the presence of cirrhosis alters the mucosal and stool microbiota compared to cirrhotics without DM, it does not add to the 90 d hospitalization risk
Chen *et al*[35], 2015	79 ACLF patients and 50 controls; fecal microbiota analysis	ACLF patients had lower abundance of *Bacteroidaceae*, *Ruminococcaceae*, and *Lanchnospiraceae*, but higher abundance of *Pasteurellaceae*, *Streptococcaceae*, and *Enterecoccaceae*. Abundance of *Lachnospiraceae* was decreased in ACLF patients with HE. Gut dysbiosis in ACLF has predictive value for mortality and could represent diagnostic biomarker
Ling *et al*[16], 2015	10 CHB patients, 10 patients with HBV-associated compensated liver cirrhosis (LC), and 10 healthy controls (HC)	Differences in the compositions of the oral microbiota revealed the dysbiosis involved in the development of HBV-CLD
Lu *et al*[17], 2016	35 early liver carcinoma (LC) patients with cirrhosis and 25 matched healthy subjects; study of microbiome of the tongue coat	Certain key bacterial species may characterize LCT microbiota; *Oribacterium* and *Fusobacterium* could distinguish LC patients from healthy subjects. Microbiota dysbiosis of tongue coat in LC patients, may provide novel and non-invasive potential diagnostic biomarker of LC.

Ahluwalia *et al*[31], 2016	40 healthy controls and 147 cirrhotics (85 cirrhotic patients had HE); stool samples and brain MRI assessment	Effort to understand the role of impaired gut-liver-brain axis in cirrhosis. Gut microbial changes are linked with systemic inflammation, ammonia and ultimately with neuronal and astrocytic dysfunction in cirrhotic patients, especially those with HE
Chen *et al*[15], 2016	30 cirrhotic patients and 28 healthy subjects; study of the duodenal microbiome	Duodenal mucosa microbiota in cirrhotic patients is dramatically different from healthy controls. The duodenum dysbiosis might be related to alterations of oral microbiota and changes in duodenal micro-environment. Possible associations between small intestinal microbiota of oral origins and hepatic encephalopathy.
Santiago *et al*[34], 2016	27 patients (13 with ascites and 14 without ascites), 17 healthy controls; stool (n = 17) and serum (n = 7) microbiome analysis	Patients with ascites have a greater deterioration of the intestinal barrier integrity, also a higher degree of microbial translocation than those without ascites, thus leading to a higher microbial diversity and higher concentration of lipopolysaccharide binding protein (LBP) in serum Specific serum microbiome is linked to the presence of ascites Alteration of the serum and fecal microbiome composition be considered indicators of cirrhosis progression

HE: Hepatic encephalopathy; PPI: Proton-pump inhibitor; CDR: Cirrhosis dysbiosis ratio; ACLF: Acute-on-chronic liver failure; HBV: Hepatitis B virus; CLD: Chronic liver disease; DM: Diabetes mellitus.

REFERENCES

1 **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

2 **Said A**, Williams J, Holden J, Remington P, Gangnon R, Musat A, Lucey MR. Model for end stage liver disease score predicts mortality across a broad spectrum of liver disease. *J Hepatol* 2004; **40**: 897-903 [PMID: 15158328 DOI: 10.1016/j.jhep.2004.02.010]

3 **Kamath PS**, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464-470 [PMID: 11172350 DOI: 10.1053/jhep.2001.22172]

4 **Patel VC**, Shawcross DL. Salivary microbiota-immune profiling in cirrhosis: could this be the noninvasive strategy that will revolutionize prognostication in hepatology? *Hepatology* 2015; **62**: 1001-1003 [PMID: 25998053 DOI: 10.1002/hep.27870]

5 **Lachar J**, Bajaj JS. Changes in the Microbiome in Cirrhosis and Relationship to Complications: Hepatic Encephalopathy, Spontaneous Bacterial Peritonitis, and Sepsis. *Semin Liver Dis* 2016; **36**: 327-330 [PMID: 27997972 DOI: 10.1055/s-0036-1593881]

6 **Garcia-Tsao G**, Wiest R. Gut microflora in the pathogenesis of the complications of cirrhosis. *Best Pract Res Clin Gastroenterol* 2004; **18**: 353-372 [PMID: 15123075 DOI: 10.1016/j.bpg.2003.10.005]

7 **Morgan XC**, Huttenhower C. Chapter 12: Human microbiome analysis. *PLoS Comput Biol* 2012; **8**: e1002808 [PMID: 23300406 DOI: 10.1371/journal.pcbi.1002808]

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

9 **Hamady M**, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Res* 2009; **19**: 1141-1152 [PMID: 19383763 DOI: 10.1101/gr.085464.108]

10 **Chen Y**, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 2011; **54**: 562-572 [PMID: 21574172 DOI: 10.1002/hep.24423]

11 **Bajaj JS**, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, Monteith P, Noble NA, Sikaroodi M, Gillevet PM. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G675-G685 [PMID: 22821944 DOI: 10.1152/ajpgi.00152.2012]

12 **Bajaj JS**, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M, Gillevet PM. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol* 2014; **60**: 940-947 [PMID: 24374295 DOI: 10.1016/j.jhep.2013.12.019]

13 **Qin N**, Yang F, Li A, Prifti E, Chen Y, Shao L, Guo J, Le Chatelier E, Yao J, Wu L, Zhou J, Ni S, Liu L, Pons N, Batto JM, Kennedy SP, Leonard P, Yuan C, Ding W, Chen Y, Hu X, Zheng B, Qian G, Xu W, Ehrlich SD, Zheng S, Li L. Alterations of the human gut microbiome in liver cirrhosis. *Nature* 2014; **513**: 59-64 [PMID: 25079328 DOI: 10.1038/nature13568]

14 **Bajaj JS**, Betrapally NS, Hylemon PB, Heuman DM, Daita K, White MB, Unser A, Thacker LR, Sanyal AJ, Kang DJ, Sikaroodi M, Gillevet PM. Salivary microbiota reflects changes in gut microbiota in cirrhosis with hepatic encephalopathy. *Hepatology* 2015; **62**: 1260-1271 [PMID: 25820757 DOI: 10.1002/hep.27819]

15 **Chen Y**, Ji F, Guo J, Shi D, Fang D, Li L. Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. *Sci Rep* 2016; **6**: 34055 [PMID: 27687977 DOI: 10.1038/srep34055]

16 **Ling Z**, Liu X, Cheng Y, Jiang X, Jiang H, Wang Y, Li L. Decreased Diversity of the Oral Microbiota of Patients with Hepatitis B Virus-Induced Chronic Liver Disease: A Pilot Project. *Sci Rep* 2015; **5**: 17098 [PMID: 26606973 DOI: 10.1038/srep17098]

17 **Lu H**, Ren Z, Li A, Zhang H, Jiang J, Xu S, Luo Q, Zhou K, Sun X, Zheng S, Li L. Deep sequencing reveals microbiota dysbiosis of tongue coat in patients with liver carcinoma. *Sci Rep* 2016; **6**: 33142 [PMID: 27605161 DOI: 10.1038/srep33142]

18 **Macnaughtan J**, Jalan R. Clinical and pathophysiological consequences of alterations in the microbiome in cirrhosis. *Am J Gastroenterol* 2015; **110**: 1399-1410; quiz 1411 [PMID: 26416191 DOI: 10.1038/ajg.2015.313]

19 **Arab JP**, Martin-Mateos RM, Shah VH. Gut-liver axis, cirrhosis and portal hypertension: the chicken and the egg. *Hepatol Int* 2018; **12**: 24-33 [PMID: 28550391 DOI: 10.1007/s12072-017-9798-x]

20 **Wiest R**, Albillos A, Trauner M, Bajaj JS, Jalan R. Targeting the gut-liver axis in liver disease. *J Hepatol* 2017; **67**: 1084-1103 [PMID: 28526488 DOI: 10.1016/j.jhep.2017.05.007]

21 **Giannelli V**, Di Gregorio V, Iebba V, Giusto M, Schippa S, Merli M, Thalheimer U. Microbiota and the gut-liver axis: bacterial translocation, inflammation and infection in cirrhosis. *World J Gastroenterol* 2014; **20**: 16795-16810 [PMID: 25492994 DOI: 10.3748/wjg.v20.i45.16795]

22 **Ridlon JM**, Alves JM, Hylemon PB, Bajaj JS. Cirrhosis, bile acids and gut microbiota: unraveling a complex relationship. *Gut Microbes* 2013; **4**: 382-387 [PMID: 23851335 DOI: 10.4161/gmic.25723]

23 **Fukui H**. Gut Microbiome-based Therapeutics in Liver Cirrhosis: Basic Consideration for the Next Step. *J Clin Transl Hepatol* 2017; **5**: 249-260 [PMID: 28936406 DOI: 10.14218/JCTH.2017.00008]

24 **Pijls KE**, Jonkers DM, Elamin EE, Masclee AA, Koek GH. Intestinal epithelial barrier function in liver cirrhosis: an extensive review of the literature. *Liver Int* 2013; **33**: 1457-1469 [PMID: 23879434 DOI: 10.1111/liv.12271]

25 **Wiest R**, Lawson M, Geuking M. Pathological bacterial translocation in liver cirrhosis. *J Hepatol* 2014; **60**: 197-209 [PMID: 23993913

DOI: 10.1016/j.jhep.2013.07.044]
26 **Acharya C**, Sahingur SE, Bajaj JS. Microbiota, cirrhosis, and the emerging oral-gut-liver axis. *JCI Insight* 2017; **2**: [PMID: 28978799 DOI: 10.1172/jci.insight.94416]
27 **Hall MW**, Singh N, Ng KF, Lam DK, Goldberg MB, Tenenbaum HC, Neufeld JD, G Beiko R, Senadheera DB. Inter-personal diversity and temporal dynamics of dental, tongue, and salivary microbiota in the healthy oral cavity. *NPJ Biofilms Microbiomes* 2017; **3**: 2 [PMID: 28649403 DOI: 10.1038/s41522-016-0011-0]
28 **Aberg F**, Helenius-Hietala J, Meurman J, Isoniemi H. Association between dental infections and the clinical course of chronic liver disease. *Hepatol Res* 2014; **44**: 349-353 [PMID: 23607641 DOI: 10.1111/hepr.12126]
29 **Bajaj JS**, Cox IJ, Betrapally NS, Heuman DM, Schubert ML, Ratneswaran M, Hylemon PB, White MB, Daita K, Noble NA, Sikaroodi M, Williams R, Crossey MM, Taylor-Robinson SD, Gillevet PM. Systems biology analysis of omeprazole therapy in cirrhosis demonstrates significant shifts in gut microbiota composition and function. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G951-G957 [PMID: 25258407 DOI: 10.1152/ajpgi.00268.2014]
30 **Bajaj JS**, Ridlon JM, Hylemon PB, Thacker LR, Heuman DM, Smith S, Sikaroodi M, Gillevet PM. Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G168-G175 [PMID: 21940902 DOI: 10.1152/ajpgi.00190.2011]
31 **Ahluwalia V**, Betrapally NS, Hylemon PB, White MB, Gillevet PM, Unser AB, Fagan A, Daita K, Heuman DM, Zhou H, Sikaroodi M, Bajaj JS. Impaired Gut-Liver-Brain Axis in Patients with Cirrhosis. *Sci Rep* 2016; **6**: 26800 [PMID: 27225869 DOI: 10.1038/srep26800]
32 **Patel VC**, White H, Støy S, Bajaj JS, Shawcross DL. Clinical science workshop: targeting the gut-liver-brain axis. *Metab Brain Dis* 2016; **31**: 1327-1337 [PMID: 26446022 DOI: 10.1007/s11011-015-9743-4]
33 **Gorham J**, Gleeson M. Cirrhosis and dysbiosis: New insights from next-generation sequencing. *Hepatology* 2016; **63**: 336-338 [PMID: 26313376 DOI: 10.1002/hep.28133]
34 **Santiago A**, Pozuelo M, Poca M, Gely C, Nieto JC, Torras X, Román E, Campos D, Sarrabayrouse G, Vidal S, Alvarado-Tapias E, Guarner F, Soriano G, Manichanh C, Guarner C. Alteration of the serum microbiome composition in cirrhotic patients with ascites. *Sci Rep* 2016; **6**: 25001 [PMID: 27112233 DOI: 10.1038/srep25001]
35 **Chen Y**, Guo J, Qian G, Fang D, Shi D, Guo L, Li L. Gut dysbiosis in acute-on-chronic liver failure and its predictive value for mortality. *J Gastroenterol Hepatol* 2015; **30**: 1429-1437 [PMID: 25711972 DOI: 10.1111/jgh.12932]
36 **Bajaj JS**, Betrapally NS, Hylemon PB, Thacker LR, Daita K, Kang DJ, White MB, Unser AB, Fagan A, Gavis EA, Sikaroodi M, Dalmet S, Heuman DM, Gillevet PM. Gut Microbiota Alterations can predict Hospitalizations in Cirrhosis Independent of Diabetes Mellitus. *Sci Rep* 2015; **5**: 18559 [PMID: 26692421 DOI: 10.1038/srep18559]
37 **Tao X**, Wang N, Qin W. Gut Microbiota and Hepatocellular Carcinoma. *Gastrointest Tumors* 2015; **2**: 33-40 [PMID: 26673641 DOI: 10.1159/000380895]
38 **Sanduzzi Zamparelli M**, Rocco A, Compare D, Nardone G. The gut microbiota: A new potential driving force in liver cirrhosis and hepatocellular carcinoma. *United European Gastroenterol J* 2017; **5**: 944-953 [PMID: 29163959 DOI: 10.1177/2050640617705576]
39 **Zhang HL**, Yu LX, Yang W, Tang L, Lin Y, Wu H, Zhai B, Tan YX, Shan L, Liu Q, Chen HY, Dai RY, Qiu BJ, He YQ, Wang C, Zheng LY, Li YQ, Wu FQ, Li Z, Yan HX, Wang HY. Profound impact of gut homeostasis on chemically-induced pro-tumorigenic inflammation and hepatocarcinogenesis in rats. *J Hepatol* 2012; **57**: 803-812 [PMID: 22727732 DOI: 10.1016/j.jhep.2012.06.011]
40 **Roderburg C**, Luedde T. The role of the gut microbiome in the development and progression of liver cirrhosis and hepatocellular carcinoma. *Gut Microbes* 2014; **5**: 441-445 [PMID: 25006881 DOI: 10.4161/gmic.29599]
41 **Anand G**, Zarrinpar A, Loomba R. Targeting Dysbiosis for the Treatment of Liver Disease. *Semin Liver Dis* 2016; **36**: 37-47 [PMID: 26870931 DOI: 10.1055/s-0035-1571276]
42 **Shen TC**, Albenberg L, Bittinger K, Chehoud C, Chen YY, Judge CA, Chau L, Ni J, Sheng M, Lin A, Wilkins BJ, Buza EL, Lewis JD, Daikhin Y, Nissim I, Yudkoff M, Bushman FD, Wu GD. Engineering the gut microbiota to treat hyperammonemia. *J Clin Invest* 2015; **125**: 2841-2850 [PMID: 26098218 DOI: 10.1172/JCI79214]
43 **Wang J**, Wang Y, Zhang X, Liu J, Zhang Q, Zhao Y, Peng J, Feng Q, Dai J, Sun S, Zhao Y, Zhao L, Zhang Y, Hu Y, Zhang M. Gut Microbial Dysbiosis Is Associated with Altered Hepatic Functions and Serum Metabolites in Chronic Hepatitis B Patients. *Front Microbiol* 2017; **8**: 2222 [PMID: 29180991 DOI: 10.3389/fmicb.2017.02222]
44 **Preveden T**, Scarpellini E, Milić N, Luzza F, Abenavoli L. Gut microbiota changes and chronic hepatitis C virus infection. *Expert Rev Gastroenterol Hepatol* 2017; **11**: 813-819 [PMID: 28621554 DOI: 10.1080/17474124.2017.1343663]
45 **Kakiyama G**, Hylemon PB, Zhou H, Pandak WM, Heuman DM, Kang DJ, Takei H, Nittono H, Ridlon JM, Fuchs M, Gurley EC, Wang Y, Liu R, Sanyal AJ, Gillevet PM, Bajaj JS. Colonic inflammation and secondary bile acids in alcoholic cirrhosis. *Am J Physiol Gastrointest Liver Physiol* 2014; **306**: G929-G937 [PMID: 24699327 DOI: 10.1152/ajpgi.00315.2013]

P- Reviewer: Huang LY, Kadayifci A, Kreisel W **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3821-3833

DOI: 10.3748/wjg.v24.i34.3821

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

REVIEW

Implication of neurohormonal-coupled mechanisms of gastric emptying and pancreatic secretory function in diabetic gastroparesis

Bashair M Mussa, Sanjay Sood, Anthony JM Verberne

Bashair M Mussa, Sanjay Sood, Department of Basic Medical Science, College of Medicine, University of Sharjah, Sharjah 27272, United Arab Emirates

Anthony JM Verberne, Department of Medicine, Austin Health, University of Melbourne, Melbourne 3084, Australia

ORCID number: Bashair M Mussa (0000-0002-1554-6319); Sanjay Sood (0000-0003-3104-9379); Anthony JM Verberne (0000-0002-2049-1439).

Author contributions: Mussa BM developed, designed and wrote the review; Sood S designed the figures and revised the manuscript; Verberne AJ edited and revised the manuscript; all authors approved the final version.

Conflict-of-interest statement: The authors have no personal, financial or non-financial conflicts of interest.

Manuscript source: Unsolicited manuscript

Correspondence to: Bashair M Mussa, PhD, Assistant Professor, Department of Basic Medical Science, College of Medicine, University of Sharjah, Sharjah 27272, United Arab Emirates. bmussa@sharjah.ac.ae
Telephone: +971-6-5057220
Fax: +971-6-558579

Received: May 21, 2018
Peer-review started: May 21, 2018
First decision: June 13, 2018
Revised: June 22, 2018
Accepted: June 27, 2018

Article in press: June 27, 2018
Published online: September 14, 2018

Abstract

Recently, diabetic gastroparesis (DGP) has received much attention as its prevalence is increasing in a dramatic fashion and management of patients with DGP represents a challenge in the clinical practice due to the limited therapeutic options. DGP highlights an interrelationship between the gastric emptying and pancreatic secretory function that regulate a wide range of digestive and metabolic functions, respectively. It well documented that both gastric emptying and pancreatic secretion are under delicate control by multiple neurohormonal mechanisms including extrinsic parasympathetic pathways and gastrointestinal (GI) hormones. Interestingly, the latter released in response to various determinants that related to the rate and quality of gastric emptying. Others and we have provided strong evidence that the central autonomic nuclei send a dual output (excitatory and inhibitory) to the stomach and the pancreas in response to a variety of hormonal signals from the abdominal viscera. Most of these hormones released upon gastric emptying to provide feedback, and control this process and simultaneously regulate pancreatic secretion and postprandial glycemia. These findings emphasize an important link between gastric emptying and pancreatic secretion and its role in maintaining homeostatic processes within the GI tract. The present review deals with the neurohormonal-coupled mechanisms of gastric emptying and pancreatic secretory function that implicated in DGP and this provides new insights in our understanding of the pathophysiology of DGP. This also enhances the process of identifying potential therapeutic targets to treat DGP and limit the complications of current management practices.

Key words: Gastroparesis; Gastric emptying; Pancreatic secretion; Postprandial glycemia; Neurohormonal control

Core tip: Prevalence of diabetic gastroparesis (DGP) is increasing in a dramatic fashion, however there are still gaps in our understanding of the pathophysiology of DGP. It well documented that gastric emptying and subsequent pancreatic secretion are interrelated and regulated by several neurohormonal mechanisms. Dysfunction of these mechanisms affects gastric emptying, pancreatic secretion and postprandial glycemia. Therefore, the present article reviews the neurohormonal-coupled mechanisms that control gastric emptying and pancreatic secretion and their plausible involvement in DGP. This will help in identification of novel therapeutic targets to treat DGP with minimal adverse effects on postprandial glycemia.

Mussa BM, Sood S, Verberne AJ. Implication of neurohormonal-coupled mechanisms of gastric emptying and pancreatic secretory function in diabetic gastroparesis. *World J Gastroenterol* 2018; 24(34): 3821-3833 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3821.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3821

INTRODUCTION

Gastroparesis (or stomach paralysis) is a chronic and symptomatic disorder characterized by a complex pathogenesis which mainly includes delayed gastric emptying in the absence of mechanical obstruction[1,2]. It may also involve reduced antral contraction, impaired gastric accommodation, slow wave dysrhythmia and partial loss of the interstitial cells of Cajal (ICCs)[3-5]. Therefore, comprehensive criteria have been recommended to evaluate and diagnose gastoparesis. Documented delay in gastric emptying is one of the main requirements to confirm the diagnosis of gastoparesis and this can be achieved by measuring gastric retention of solids by scintigraphy[6].

Given the fact that more than 30% of gastroparesis cases are related to diabetes mellitus (DM), several studies have investigated the pathophysiological nature of diabetic gastroparesis (DGP)[7]. Understanding of the relationship between DM and gastoparesis has evolved during the last decade as a result of several research studies and initiatives such as the Gastoparesis Clinical Research Consortium[8].

High prevalence of DGP has been reported in Type 1 DM (approximately 40%) and Type 2 DM (approximately 30%) and it was found that in a cohort of unselected patients with DM, DGP was present in 28% of cases[9]. The prevalence of DGP seems to be significantly dependent on the duration of DM and gender. Compared to newly diagnosed patients with DM, patients with long-standing DM are more likely to experience DGP[10,11]. Similarly, the prevalence of DGP is higher among females compared to males and, although the reason for this gender-difference is unknown, the fact that gastric emptying is slower in females may explain this observation[12,13].

High rate of mortality is not directly related to DGP however, quality of life seems to be impaired independently of several factors including age and type of DM[14]. In addition, poor glycemic control is one of the main challenges that the patients with DGP face during the course of the disease[15]. It has been found that delayed gastric emptying leads to time mismatch between blood glucose and insulin secretion jeopardizing the regulation of postprandial glycemia[16]. Several studies have demonstrated that patients with DGP experience a blunted postprandial glucose response and hypoglycemia which further complicates the management of DM in this group of patients. These findings highlight an important aspect about the interrelationship between gastric emptying and pancreatic secretory function.

Although the prevalence of gastroparesis dramatically increased among DM patients with consequent adverse effects on glycemic control, the exact pathophysiology of DGP is yet to be determined. Multiple gastrointestinal (GI) hormonal mechanisms, autonomic neuropathy with loss of the ICCs as well as myopathy, have been proposed[17]. The role of the ICCs and myopathy is beyond the scope of the present review and other investigators including Bashashati's group have comprehensively reviewed the involvement of Cajal-opathy in gastroparesis[18].

We believe that identification of the exact pathophysiological processes that are involved in DGP is a crucial step towards development of potential targets for management of DGP. The hormonal coupled mechanisms of gastric emptying and pancreatic secretory function may be an important element that requires further characterization.

The focus of this review is to discuss the extrinsic neural pathways and the neurohormonal mechanisms that regulate both gastric emptying and pancreatic secretory function and the interrelationship between these two elements. In addition, the review sheds light on how the dysfunction of these processes may contribute to development of DGP.

PHASES OF THE DIGESTIVE PROCESS AND PANCREATIC SECRETION

Digestion is an essential homeostatic process that is involved in maintenance of homeostasis and general health. It is a complex phenomenon, consists of multiple phases that eventually lead to absorption, assimilation and uptake of nutrients. Digestion starts with the

smell or the taste of food and this sensory information is conveyed to the central nervous system (CNS) *via* trigeminal, facial, glossopharyngeal and vagal afferents which innervate different parts of the digestive tract including the tongue, pharynx, esophagus, stomach, intestine and pancreas[19]. The majority of vagal afferents terminate in the nucleus of the solitary tract (NTS) for sensory signals integration. Subsequently, this information is conveyed to motor neurons such as those in the dorsal motor nucleus of the vagus (DMV) which then transforms the information into motor output. The vagal efferent fibres which originate in the DMV, in turn, control subsequent phases of digestion including the cephalic, gastric and intestinal components of pancreatic secretion (PS)[19,20]. It is noteworthy that the phases of PS strongly correlated with the phases of digestion highlighting the importance of the former in the digestive process. During the cephalic phase of digestion, the pancreatic exocrine acinar cells are stimulated by a vagal mechanism to secrete digestive enzymes. However, the latter remain inactive due to the low pH environment and inadequate levels of bicarbonate. This phase followed by the gastric phases that include an increase in the number of digestive zymogens that release the active digestive enzymes when pH rises after bicarbonate secretion. Gastric emptying of stomach contents into the small intestine is described as the intestinal phase and represents the final phase of PS and is controlled mainly by vagovagal pathways and GI hormones such as cholecystokinin (CCK) and secretin[19,21]. It is noteworthy that gastric emptying is strongly coupled to the neurohormonal mechanisms that control PS. Interestingly, most of the GI hormones and agents that control PS are also involved in regulation of gastric emptying.

As early as 1642, the pancreatic ducts were identified by Virsung and in the same century the first collection of PS *via* a pancreatic fistula was made by Regner de Graaf[22]. However, it took more than two centuries to appreciate the significance of PS in digestion[22]. Later, Pavlov highlighted the role of the CNS in control of PS[23]. Subsequent discovery of various GI hormones and peptides such as secretin, modified Pavlov's theory[24,25]. However, it was not until the late 1970s that there was a renewed focus on the relationship between the CNS and PS[26-28]. The results of these investigations showed for the first time the importance of vagovagal reflexes and PS as common factors in controlling different GI functions including gastric emptying. Since then several lines of evidence have implicated various interacting factors including hormones, paracrine mediators and vagovagal reflexes in regulation of gastric emptying[29]. The latter represents one of the significant determinants of postprandial glycemia in health and in glycemic disorders including DM. Therefore, delayed gastric emptying (gastroparesis) that is associated with DM affects several aspects of glycemic control in patients with DM.

GASTRIC EMPTYING AND PANCREATIC SECRETION: MULTIFACTORIAL PHYSIOLOGICAL PROCESSES

Gastric emptying defined as the process of ejecting the stomach's content (chyme) into the duodenum. The rate of gastric emptying is dependent on several physiological factors including fundal relaxation, pyloric control of flow into the duodenum and antro-duodenal coupling. In addition, the physical nature and the composition of the chyme are important determinants of the rate of gastric emptying[30].

This process is precisely tuned to react to various intrinsic and extrinsic signals and therefore it is not surprising that highly complex systems are involved in the regulation of gastric emptying. This includes (1) intrinsic neural plexuses (2) extrinsic autonomic factors and (3) neurohormonal mechanisms[30]. Although, it seems that intrinsic neural pathways have some degree of independence in regulating GI functions, the extrinsic control of the parasympathetic and sympathetic pathways are still the predominant players that modulate various gastric processes along with the output of the intrinsic plexuses[31]. In particular, regulation of gastric motility is largely dependent on excitatory (cholinergic) inputs and inhibitory (nitrergic) inputs[17]. In addition, ICCs are also involved to some extent in electrical control of gastric motility. Given the PS has two main types: (1) Exocrine secretion and (2) endocrine secretion, it is considered to be one of the main factors that regulates both digestive and metabolic processes.

Hormonal regulation of PS was demonstrated as early as 1902 when the first hormone, secretin, was discovered by Bayliss and Starling[24]. Strong evidence has shown that the shortest circulation times for maximal doses of GI hormones are significantly longer than the observed latency of pancreatic responses to nutrient stimuli[27]. Moreover, this latency increased 10-fold when neuronal influences were excluded, supporting the theory of neurohormonal regulation of PS (for review see Niebergall-Roth[29]). This theory proposes that hormonal and neural factors, which were previously thought to act separately, act together to regulate PS. Although both divisions of the autonomic nervous system; the parasympathetic nervous system (PNS) and sympathetic nervous system (SNS), are known to innervate pancreatic exocrine and endocrine tissues, the parasympathetic (vagal) pathways have the greatest influence on PS[32]. While not wishing to diminish the important role of the SNS, which is mainly concerned with GI smooth muscle function, blood flow, and mucosal secretion, the PNS is the principal regulator of gastric emptying and secretion. Therefore, dysfunction of the latter is always associated with disruption of the parasympathetic pathways[33].

To understand the potential pathophysiological mechanisms that underpin the delayed gastric emptying

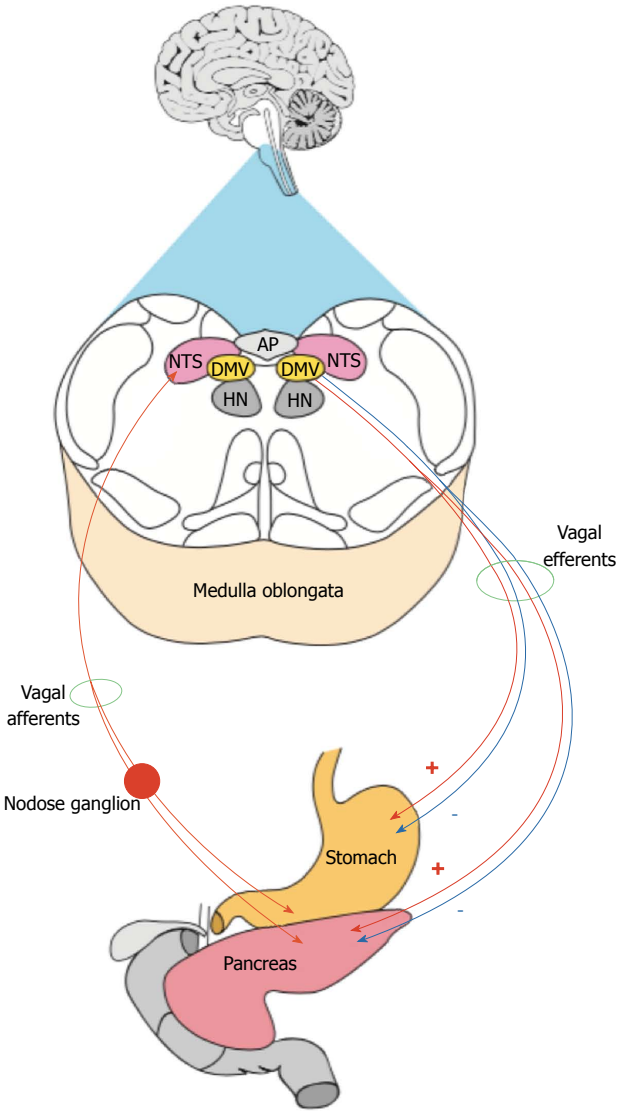


Figure 1 Vasovagal model and the dual, excitatory and inhibitory, pathways from the dorsal motor nucleus of the vagus to the pancreas and stomach. AP: Area postrema; DMV: Dorsal motor nucleus of the vagus; NTS: Nucleus of solitary tract.

noted in DGP, it is very important to identify and characterize these mechanisms. The following sections discuss these systems in detail and highlight the relationship between gastric emptying and pancreatic secretory function in each process.

Extrinsic control of gastric emptying and pancreatic secretion

The involvement of the vago-vagal pathways and reflexes in control of gastric emptying and pancreatic secretory function is well-documented[33-36]. It facilitates the complex processes that are associated with gastric emptying and also explains the interrelationship between gastric emptying and pancreatic secretory function.

The vago-vagal model consists of three major components (1) vagal afferents, (2) central neurons in the NTS and the DMV and (3) vagal efferents (Figure 1). These reflexes seem mediated by GI hormones such as secretin, serotonin (5-hydroxytryptamine, 5-HT), glucagon-like peptide-1 (GLP-1) and CCK[37-40].

The first component: Vagal afferent pathways

The vagal afferents respond to different components of the chyme including nutrients, osmotic pressure and chemicals[41]. Therefore, the vagal innervation plays a major role in monitoring and regulating the gastric emptying process. On the other hand, the vagal innervation controls PS by responding to various pancreatic secretagogues, such as CCK and 5-HT that are secreted from the intestinal enteroendocrine and enterochromaffin cells, respectively. It is well-documented that these agents provoke excitatory influences on PS *via* vagal mechanisms[42]. Thus, it is reasonable to postulate that GI hormones mediate their actions on gastric emptying and PS *via* activation of gastric and pancreatic vagal afferents, respectively[38,40,43,44]. It is noteworthy that a wide range of GI hormones are engaged in the dual control of PS and gastric emptying however, CCK and 5-HT represent an important classic neurohormonal examples that have been studied extensively.

In addition, it is evident that CCK and 5-HT mediate their physiological effects on gastric and pancreatic vagal afferents *via* activation of CCK and 5-HT receptors, respectively. These findings are in good agreement with previous reports which have shown that under physiological conditions CCK_1 and $5\text{-}HT_3$ receptors on the vagus nerve are the main players in the regulation of PS and gastric emptying[39,45].

The second component: Central nuclei-NTS and DMV

All GI vagal afferents terminate in and activate NTS neurons mainly *via* glutamatergic transmission[46-49]. NTS neurons, in turn, integrate and assimilate this sensory information and eventually influence DMV neurons mainly *via* GABAergic transmission although blockade of $GABA_A$ receptors indirectly enhances glutamatergic transmission[34,50]. A large body of evidence supports a role for GABAergic inputs from the NTS to the DMV in the regulation of the vagal efferent output from the DMV to the GI tract. DMV neurons are the main source of vagal motor output to various GI organs including the pancreas and the stomach[49-53].

The hypothesis that GABA receptors in the DMV are involved in modulation of PS was tested. Mussa *et al*[42]. have shown that blockade of $GABA_A$ receptors using bilateral microinjection of Bicuculline methionine ($GABA_A$ receptor blocker) into the DMV produced pronounced excitatory effects on both pancreatic exocrine secretion and glucose-induced insulin secretion[53,54]. Interestingly, the excitatory effects of chemical activation of the DMV were sensitive to muscarinic acetylcholine receptor blockade, confirming the involvement of a peripheral cholinergic pathway. These findings support the hypothesis that pancreatic secretagogues activate pancrea-

tic vagal afferent input into the NTS, which in turn, stimulates cholinergic efferent output from the DMV possibly *via* inhibition of GABAergic transmission. The excitatory effects of $GABA_A$ receptor blockade in the DMV on glucose-induced insulin secretion are enhanced in the presence of the nitric oxide (NO) synthase inhibitor L-NAME. This suggests that a nitrergic inhibitory pathway is involved in pancreatic insulin secretion[54,55].

Similarly, a considerable number of studies have shown that blockade of GABA receptors within the DMV has a profound effect on gastric emptying. Stimulation of the DMV by $GABA_A$ receptor blockade led to a significant increase in gastric motility suggesting that GABAergic transmission in the DMV is involved in control of gastric emptying. Therefore, gastric emptying is very sensitive to any sort of disruption of GABAergic transmission to the GI tract[56,57].

The third component: Vagal efferent pathways

Functional studies *in vivo* have emphasized the relationship between the DMV and the pancreas by showing that electrical and chemical stimulation of dorsal vagal motor neurons activates both pancreatic endocrine and exocrine secretion[53,58,59]. In addition, several *in vitro* studies have documented that all vagal efferents that project to the pancreas originate from the DMV[52,60,61]. However, major questions regarding the exact details of these pathways remain to be elucidated. Nevertheless, the electrophysiological and morphological characteristics of DMV pancreatic preganglionic neurons (PPNs) using whole cell patch clamp recording techniques have been described[62]. There were identifiable differences between the gastric and other preganglionic neurons and heterogeneity of the PPNs confirmed by this and other studies. This supports the finding that PS regulated by heterogeneous vagal efferent output from the DMV.

As mentioned previously, CCK and 5-HT are powerful stimulatory agents of the pancreatic secretion therefore their effects on DMV PPNs have been investigated[63]. The results of this investigation has shown that all DMV preganglionic neurons, the origin of the pancreatic vagal efferent, were activated in response to stimulation of the pancreatic branch of the vagus nerve and had axonal conduction velocities in the C-fibre range. This is not surprising since most of the subdiaphragmatic vagal efferents are of C-fibre type[64]. However, stimulation of peripheral CCK_1 and $5\text{-}HT_3$ receptors produced differential effects on the firing rates of these neurons. The majority of the preganglionic neurons within the intermediate DMV inhibited whereas the preganglionic neurons within the caudal and rostral DMV were activated or insensitive, respectively. This lends strong support to previous findings that emphasized heterogeneity of DMV PPNs. However, these results cast doubt on the hypothesis that pancreatic secretagogues, which known for their excitatory effects on pancreatic vagal afferents, would also activate the majority of DMV PPNs. Another possibility is that an inhibitory pathway is involved in modulation of the motor output from the DMV to the pancreas. This suggestion is supported further by the observation that nitrergic inhibitory inputs were actively involved in the regulation of pancreatic secretory function (Figure 1).

Interestingly, previous reports have shown that some gastric functions including gastric emptying are also under the control of both excitatory and inhibitory motor inputs from the DMV[65]. It has been found that, reflex-induced fundus relaxation is mainly controlled by the inhibitory pathways that originate in the DMV[66]. Studies in cats and rats have demonstrated that different regions within the DMV are involved in regulating gastric emptying.

The inhibitory pathway consists of cholinergic and nitrergic preganglionic neurons, and noncholinergic and nonadrenergic postganglionic neurons[65]. Given that postganglionic nitrergic nerves are involved in innervation of the stomach and the pancreas, it is possible that these nerves somehow inhibit the release of acetylcholine (Ach). This does not exclude the possibility that nitrergic nerves are tonically involved in control of gastric and pancreatic functions[67].

Neurohormonal control of gastric emptying and pancreatic secretory function

One of the most significant responses of the duodenum to gastric emptying is the release of several GI hormones and interestingly, this response depends on the composition of the chyme. For instance, CCK is released from the duodenum in response to the presence of nutrients, particularly fat and proteins. The role of neurohormonal mechanisms in regulation of gastric emptying and pancreatic secretion is well documented. Therefore, it has been hypothesized that dysfunction of these mechanisms is closely related to abnormally delayed gastric emptying. The holistic contribution of the GI hormones in regulation of the gastric emptying been emphasized by various findings. Importantly, it has been demonstrated that hypersensitivity to, and hypersecretion of, GI hormones were common features of the delayed gastric emptying that is associated with different metabolic disorders[68,69].

It is important to note that almost all the GI hormones that are released from the intestine in response to gastric emptying activate a feedback loop to control gastric emptying and simultaneously influence pancreatic secretory function. These findings highlight a critical interrelationship between gastric emptying and pancreatic secretory function that mainly controlled by neurohormonal processes. There are many GI hormones that are involved in regulation of gastric motility and pancreatic secretion including motilin, somatostatin, xenin, orexin A and B, ghrelin, gastrin, CCK, leptin, enterostatin, peptide YY (PYY), apolipoprotein A-Ⅳ, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glucose-dependent

insulinotropic polypeptide (GIP), pancreatic polypeptide, oxyntomodulin and amylin[69]. Taking into account all the studies that have discussed the neurohormonal involvement of CCK, 5-HT and GLP-1 in the GI activities, the present review focuses on the dual functions of CCK, 5-HT and GLP-1 in regulation of gastric emptying and pancreatic secretion.

CCK: A principal regulator of gastric emptying and pancreatic secretion

Under normal physiological conditions, CCK inhibits gastric emptying, stimulates the secretion of the digestive enzymes from the pancreas and bile from the gallbladder and regulates intestinal motility. These actions allow a slow delivery of food into the small intestine and provide enough time for the digestion and absorption of nutrients that have already been in the duodenum[70]. It has been known for more than 40 years that CCK inhibits gastric emptying *via* two main mechanisms; relaxation of the proximal stomach and contraction of the pyloric sphincter.

It is believed that CCK acts directly on pancreatic tissue to mediate PS in rodents. High and low affinity CCK receptors were detected in pancreatic acini and they possess high sensitivity to low levels of CCK[71]. In addition, the correlation between the increased CCK plasma levels after food ingestion and the elevation in PS well documented. *In vitro* studies support the hypothesis that CCK acts as a circulating hormone to stimulate PS by showing that activation of CCK_1 receptors on rat pancreatic cells by CCK elevates intracellular Ca^{2+} levels and subsequently PS. In addition, it has been shown that blockade of muscarinic receptors did not produce a significant change in pancreatic responses to CCK whereas CCK receptor antagonists were able to block the excitatory effects of CCK on PS[19]. The excitatory effects of CCK on pancreatic endocrine secretion were also reported in rats and dogs. Glucose-induced insulin secretion was enhanced in a dose-dependent manner after infusion of caerulein, a CCK analogue, in perfused rat pancreas[72]. In addition, it has been demonstrated that in perfused dog pancreas, pancreatic α-, β-, δ-cell secretion was stimulated in a dose-dependent fashion in response to CCK[73].

Previously, it was thought that CCK receptors in the human pancreas were undetectable or absent and thus the possibility of a direct action of CCK on the pancreas to mediate PS in human was excluded. However, Murphy and his group have demonstrated the presence of CCK receptors within the human exocrine pancreas[74].

Serotonin: A modulator of GI motility and secretory functions

5-HT is a potent activator of vagal afferent fibres that innervate the stomach and proximal intestine of different species and it has several types and subtypes of receptors. The 5-HT_{1A} receptor subtype has been detected in pancreatic neurons and 5-HT_3 receptor is abundant on sensory vagal afferents[75-77].

It is well documented that 5-HT is directly and indirectly involved in regulation of intestinal and gastric motility. It has been demonstrated that under normal physiological conditions, 5-HT reduces the rate of the gastric emptying and stimulates intestinal motility[78-80].

Fibres containing 5-HT were also found in different parts of the pancreas including the wall of the pancreatic blood vessels, ducts, acini and islets and thus 5-HT is one of the main factors that are involved in regulation of PS[81]. Studies in rats have shown that 5-HT_2 and 5-HT_3 receptor antagonists were able to inhibit approximately 94% of PS that was evoked by intragastric administration of rodent chow[39]. It has been found that luminal and mechanical factors stimulate PS *via* activation of 5-HT_2 and 5-HT_3 receptors which are present in intestinal vagal afferents. In addition, electrophysiological studies have shown that endogenously released and intraluminally perfused 5-HT activated vagal afferent neurons within the nodose ganglion. On the other hand, 5-HT is considered as one of the key factors that regulates food intake and mediates satiety due to its wide distribution within the GI tract[82].

Glucagon-like peptide-1: A unique pancreatic secretagogue and inhibitor of gastric emptying

GLP-1-(7-36) and GLP-1-(7-37) amides are signaling peptides that are produced in the enteroendocrine L-cells of the intestinal mucosa and released post-prandially in response to luminal nutrients including fat and carbohydrates[83,84]. It stimulates and inhibits insulin and glucagon, respectively, in a glucose-independent manner[83,85-87]. Interesting findings have demonstrated the involvement of TRPV2 ion channel in Lysophosphatidylinositol-induced GLP-1 secretion from enteroendocrine L cells[88].

Several studies have demonstrated the presence of GLP-1 receptors in various tissues including the pancreas, GIT and the brain[89,90]. The unique and powerful stimulatory effects of GLP-1 on insulin secretion in response to postprandial hyperglycemia have well documented using GLP-1 receptor agonists and antagonists. Interestingly, the application of the latter was sufficient to block insulin secretion in response to orally- and intraduodenally administered glucose[91,92]. In addition to the potent insulinotropic effects of GLP-1, a deceleration of gastric emptying was observed in response to GLP-1 administration. This observation was documented in healthy and Type 2 DM subjects supporting the fact that GLP-1 possesses an inhibitory influence in gastric emptying under physiological conditions[93,94]. Furthermore, additional experiments have shown that diversion of the duodenal delivery of nutrients affected the synergistic effects of GLP-1 on insulin secretion[95,96]. This finding not only emphasized

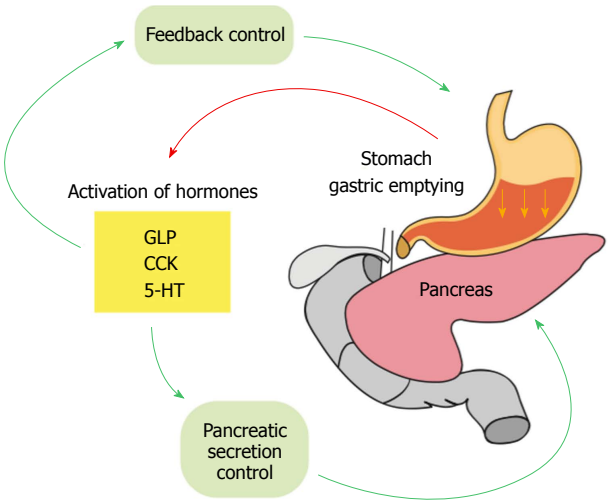


Figure 2 Postprandial events upon gastric emptying that are involved in secretion of gastrointestinal hormones and coupled control of pancreatic secretion and gastric emptying. CCK: Cholecystokinin; 5-HT: 5-hydroxytryptamine; GLP: Glucagon-like peptide.

the importance of the dual effects of GI hormones in gastric emptying and pancreatic secretion, but also sheds light on the involvement of neurohormonal factors in control of postprandial glycemia.

POSTPRANDIAL GLYCAEMIA: A CHECK POINT FOR THE INTEGRITY OF GASTRIC EMPTYING

The influence of gastric emptying on postprandial glycemia is evident and it is not surprising that the coupled mechanisms that are involved in regulation of gastric emptying also control postprandial glycemia[97-99]. The latter emphasizes the link between the digestive processes and pancreatic secretory function. The composition and rate of chyme emptying into the intestine precisely monitored and determined the feedback systems that control postprandial glycemia and pancreatic secretory functions. Interestingly, most of the neurohormonal processes that inhibit or decrease the rate of the gastric emptying simultaneously increase insulin secretion.

The initial involvement of gastric emptying in modulating postprandial glycemia started prior to the digestive process. This hypothesis is supported by the fact that the composition of each meal determines the rate of gastric emptying for that specific meal. High glucose content in a meal or infusion of glucose into the duodenum inhibits gastric emptying in a dose- dependent fashion[100]. The second important checkpoint for control of postprandial glycemia is the neurohormonal mechanisms and feedback that are triggered as the result of the interaction between the nutrients and the cells of the intestine (Figure 2). As mentioned previously, the hormones that are released from L and K cells of the intestine such as CCK and GLP-1, are able to feedback and control gastric emptying and at the same time modulate insulin secretion[101]. The integrity of these mechanisms are well maintained in healthy subjects and therefore any increase in digested glucose (hyperglycemia) stimulates insulin secretion and reduces glucagon levels. Similarly, feedback mechanisms are initiated to control the hormones that are involved directly in control of gastric emptying. A good example is ghrelin, which under normal physiological conditions, increases gastric emptying. However, during postprandial hyperglycemia, the secretion of this hormone is suppressed so that the gastric emptying is inhibited[102].

Taking into account the significance of the physiological conditions that control postprandial glycemia, it is not surprising to know that pronounced hyperglycemia in both Type 1 DM and Type 2 DM is associated with several abnormalities in gastric motility including DGP[103,104]. It proposed that DGP also occurs in response to the high level of insulin as a compensatory process. However, this proposal was challenged by the fact that patients with Type 1 DM also experienced delayed gastric emptying in response to hyperglycemia[105,106]. On the other hand, it was found that insulin-induced hypoglycemia was sufficient to provoke a counter-regulatory mechanism which involves acceleration of gastric emptying[107].

One of the key findings that emphasizes the central role of gastric emptying in the integrity of the response to postprandial glycemia, is that both healthy patients and patients with DM experienced an increase in almost all of the hormones that are insulin secretagogues in response to intraduodenal infusion of high loads of glucose[108].

NITRERGIC INHIBITORY PATHWAYS: MODULATION OF GASTRIC EMPTYING AND PANCREATIC SECRETION

A considerable number of studies have shown that NO produces inhibitory effects on insulin secretion and this has been demonstrated in several species[109-114]. The involvement of nitrergic pathways is strongly supported by the finding that peripheral inhibition of NO enhanced the excitatory effects of chemical stimulation of the DMV on insulin secretion[54]. In addition, a significant glucose uptake was reported as result of NO inhibition suggesting that NO is also involved in glucose metabolism.

A number of studies have documented the distribution of nitrergic postganglionic neurons within the pancreas. NO and nitric oxide (NOS) were localized within the pancreatic tissue or ganglia of a wide range of species including human, pig, monkey, dog, cat, rat, chick and kitten[109-114]. In particular, it is evident that NOS is localized within the endocrine islets and nerves as well as in the pancreatic β-cell line HIT-T15 from rat and mouse[115-120]. Interestingly, it has been found that NO evoked fast excitatory postsynaptic potentials in the

majority of neurons within the cat pancreatic ganglia supporting the hypothesis that postganglionic nitrergic neurons are modulators of pancreatic function[113].

Noncholinergic and nonadrenergic (NANC) neurons play a critical role in regulation of gastric motility, in particular gastric emptying, therefore any neural loss or dysfunction is always considered a major contributory factor to gastropathy[121]. Both cholinergic and nitrergic pathways are involved in regulation of the gastric fundic tone and imbalance between these two factors lead to dysfunction in the accommodation reflex and gastric emptying[122,123]. This hypothesis was strengthened by the finding that administration of NO inhibitors such as L-NAME in cats produced a significant increase in the fundic tone and these effects were reversible in the presence of L-arginine[124]. Several lines of evidence in different species including humans have shown that NO is a potent inhibitory neurotransmitter that mediates gastric relaxation and is considered a vital part of the accommodation reflex. In addition, recent reports have shown that mechanosensitive TRPV2 ion channel is co-expressed in nNOS-expressing inhibitory motor neurons in mouse stomach emphasizing the contribution of these inhibitory neurons gastric adaptive relaxation and gastric emptying in mice[125].

It is well documented that the effects of nitrergic inputs are mediated *via* a vagovagal reflex and NANC pathways[126]. This was strongly supported by several findings, which demonstrated that vagotomy led to significantly impaired accommodation and gastric emptying[127,128]. Vagotomy is used as a classic model to study the processes that are involved in delayed gastric emptying. However, studies in animals have shown that in vagotomized dogs, local gastric stimulation was able to improve gastric accommodation and emptying *via* a nitrergic pathway emphasizing the significance of local nitrergic inputs[129].

Interestingly, experiments in a diabetic gastroparesis model have provided evidence for loss of NOS neurons in this condition thus further emphasizing the key role of nitrergic neurons in regulating gastric emptying. Moreover, pharmacological studies have demonstrated that inhibition of NOS and knockout of NOS genes led to gastroparesis, gastric stasis and enlarged stomachs[130].

Taking this findings into account, we can propose that both pancreatic secretion and gastric emptying are under extrinsic and intrinsic inhibitory nitrergic neurotransmission.

DYSFUNCTION OF PANCREATIC SECRETION AND ITS ASSOCIATION WITH DIABETIC GASTROPARESIS

In DM, the exocrine pancreas loses its ability to secrete adequate amounts of pancreatic enzymes and to digest carbohydrates leading to Exocrine Pancreatic Insufficiency (EPI)[131,132]. It is important to emphasize that dysfunction of the neuronal pathways that innervate different organs within the GI tract produce negative impact on the interrelated functions of these organs. Normal gastric emptying is one of the critical determinants of the subsequent exocrine pancreatic secretion. It is not surprising to know that insufficiency of the exocrine pancreatic secretion is very prevalent in DM and strongly associated with DGP. Although it has been a long-standing debate that EPI is a cause or a sequel to DM, several studies have demonstrated that EPI is a complication of DM[132]. The exocrine pancreas is normally exposed to high concentrations of islet hormones since the blood flow from the endocrine pancreas pass through the exocrine pancreas in a very extensive manner and therefore, any changes in the levels of the endocrine hormones, including insulin, will affect the exocrine pancreatic secretory function[133]. It is well documented that insulin is a strong trophic factor for the exocrine pancreatic tissue and increases pancreatic enzyme output and this may partially explain the dysfunction of exocrine pancreas in DM[132,134]. However, given the fact that a considerable number of patients with Type 1 DM who experience a total loss of insulin still have normal exocrine pancreatic function, it remains unclear as to which factors are most important in development of EPI[132]. Autonomic neuropathy, on the other hand, may explain the etiology of diabetic EPI and its association with gastroparesis. Malfunction of the autonomic nervous system is one of the common complications, which can occur at any time during at the DM course. It affects several functions of the body including gastric emptying and pancreatic secretory function[133]. Since intact vagovagal reflexes and hormonal secretion play an important role in the regulation of these two digestive processes, interruption of this neurohormonal model interferes with the gastric emptying into the duodenum and, in turn, the feedback control of exocrine pancreatic secretion[135].

Chronic pancreatitis is another important progressive fibro-inflammatory disorder of the pancreas, which affects the digestive processes significantly and is associated with poor prognosis. The most common symptoms of this disorder include malabsorption, malnutrition, and abdominal pain[136]. Delayed gastric emptying is one of the hallmark features of pancreatitis and it has founded that the prevalence of gastroparesis in chronic pancreatitis is considerably high. Although the associative pathogenesis of the latter remains poorly understood, there are two main proposed etiological mechanisms[135]. The first mechanism involves increased levels of CCK that are a well-documented feature of chronic pancreatitis. Previous studies have shown that infusion of postprandial concentrations of CCK produced a marked delay in gastric emptying[137]. In addition, the involvement of CCK as contributory factor further supported by animal studies. It was found that the CCK failed to inhibit gastric emptying in CCK_A receptor gene knockout rat model suggesting that the action of CCK in gastric emptying is mediated *via* CCK_A receptors[138]. The second mechanism is autonomic neuropathy that

has received much attention due to the fact it explains the pathological background of severe abdominal pain. This type of pain is considered as one of the most problematic symptoms of chronic pancreatitis[139]. The concept of central sensitization which revolutionized the classic response to nociceptive stimuli has explained how the intensive nerve damage that is present in the chronic pancreatitis increases the efficiency of synaptic communication leading to severe pain sensation[140]. Interestingly, twenty years ago a study by Nakamura *et al*[141] has demonstrated that delayed gastric emptying that is associated with chronic pancreatitis is due to dysfunction of autonomic nerves.

Impaired awareness of hypoglycemia is another important aspect that highlights the interrelationship between the neurohormonal components and the postprandial glycemic control.

Previous research has shown that hypoglycemia is not only associated with transient impairment of cognition but also with high rates of functional mortality and morbidity[142]. It is well documented that type 1 DM patients experience at least two episodes of hypoglycemia per week and this represents a significant challenge in the clinical practice to achieve optimal therapeutic targets that involves insulin regimens[143].

A normal response to hypoglycemia includes an activation of a complex and sensitive counter-regulatory response leads eventually to a suppression of endogenous insulin and an increase in glucagon secretion. It has been found that in DM, the pancreatic α-cells, which are the main source of endogenous glucagon, loose their ability to secrete this hormone[143]. The notion that the pathophysiology of DM depends on the sole mechanism of insulin malfunction or resistance, has been revolutionized by the finding that loss of the glucagon response is a significant feature in DM[144].

Interestingly, recent studies have demonstrated that diabetic patients with DGP experience episodes hypoglycemia more frequent. This due to several factors including delayed gastric emptying and subsequent slow absorption of food. Insulin is an important therapeutic agent mainly for patients with type 1 DM however, type 2 DM patients also use insulin to improve their glycemic control. Dosing and administration of insulin in patients with DGP is almost impossible due to the delayed gastric emptying and slow absorption of food. This, in turn, leads to frequent episodes of postprandial hypoglycemia.

CONCLUSION

The dramatic increase in the prevalence of DGP has directed significant attention to the pathophysiology of DGP. In addition, the strong association between DGP and abnormal glycemic profile has highlighted the involvement of coupled mechanisms that control gastric emptying and endocrine and exocrine pancreatic secretion. Therefore, characterization of these mechanisms will enhance the understating of the etiology of DGP and in turn, this will facilitate the process of identifying novel therapeutic targets. The latter will further ease the burden of complex and challenging DGP management.

Under normal conditions, there is a delicate balance between the neurohormonal mechanisms that control gastric emptying and pancreatic secretion. Malfunction of any of these mechanisms affects the metabolic profile adversely and this has been strongly proven in DM where the delayed gastric emptying is associated with pancreatic secretory dysfunction.

This article has reviewed the neurohormonal-coupled mechanisms that control gastric emptying and pancreatic secretory function to identify the potential components and pathways that are involved in DGP and this will stimulate the development of novel therapeutic approaches hopefully for this disorder.

ACKNOWLEDGMENTS

The authors would like to thank Professor Joe Proietto for his valuable feedback and inputs regarding the clinical aspects of diabetic gastroparesis. The authors would also like to thank Ms. Judy Bastaty for her help in designing the Figures.

REFERENCES

1 **Waseem S**, Moshiree B, Draganov PV. Gastroparesis: current diagnostic challenges and management considerations. *World J Gastroenterol* 2009; **15**: 25-37 [PMID: 19115465 DOI: 10.3748/wjg.15.25]
2 **Falk GW**. Gastroparesis. *Gastroenterol Clin North Am* 2015; **44**: xiii [PMID: 25667033 DOI: 10.1016/j.gtc.2014.12.002]
3 **Kumar A**, Attaluri A, Hashmi S, Schulze KS, Rao SS. Visceral hypersensitivity and impaired accommodation in refractory diabetic gastroparesis. *Neurogastroenterol Motil* 2008; **20**: 635-642 [PMID: 18282170 DOI: 10.1111/j.1365-2982.2008.01081.x]
4 **Karamanolis G**, Caenepeel P, Arts J, Tack J. Determinants of symptom pattern in idiopathic severely delayed gastric emptying: gastric emptying rate or proximal stomach dysfunction? *Gut* 2007; **56**: 29-36 [PMID: 16840507 DOI: 10.1136/gut.2005.089508]
5 **Horváth VJ**, Vittal H, Lörincz A, Chen H, Almeida-Porada G, Redelman D, Ordög T. Reduced stem cell factor links smooth myopathy and loss of interstitial cells of cajal in murine diabetic gastroparesis. *Gastroenterology* 2006; **130**: 759-770 [PMID: 16530517 DOI: 10.1053/j.gastro.2005.12.027]
6 **Camilleri M**, Parkman HP, Shafi MA, Abell TL, Gerson L; American College of Gastroenterology. Clinical guideline: management of gastroparesis. *Am J Gastroenterol* 2013; **108**: 18-37; quiz 38 [PMID: 23147521 DOI: 10.1038/ajg.2012.373]
7 **Soykan I**, Sivri B, Sarosiek I, Kiernan B, McCallum RW. Demography, clinical characteristics, psychological and abuse profiles, treatment, and long-term follow-up of patients with gastroparesis. *Dig Dis Sci* 1998; **43**: 2398-2404 [PMID: 9824125 DOI: 10.1023/A:1026665728213]
8 **Marathe CS**, Rayner CK, Jones KL, Horowitz M. Novel insights into the effects of diabetes on gastric motility. *Expert Rev Gastroenterol Hepatol* 2016; **10**: 581-593 [PMID: 26647088 DOI: 10.1586/17474124.2016.1129898]
9 **Hasler WL**. Gastroparesis--current concepts and considerations. *Medscape J Med* 2008; **10**: 16 [PMID: 18324326]
10 **Parkman HP**, Fass R, Foxx-Orenstein AE. Treatment of patients with diabetic gastroparesis. *Gastroenterol Hepatol* (N Y) 2010; **6**: 1-16 [PMID: 20733935]

11 **Krishnan B**, Babu S, Walker J, Walker AB, Pappachan JM. Gastrointestinal complications of diabetes mellitus. *World J Diabetes* 2013; **4**: 51-63 [PMID: 23772273 DOI: 10.4239/wjd.v4.i3.51]
12 **Datz FL**, Christian PE, Moore J. Gender-related differences in gastric emptying. *J Nucl Med* 1987; **28**: 1204-1207 [PMID: 3598704]
13 **Jones KL**, Russo A, Stevens JE, Wishart JM, Berry MK, Horowitz M. Predictors of delayed gastric emptying in diabetes. *Diabetes Care* 2001; **24**: 1264-1269 [PMID: 11423513 DOI: 10.2337/diacare.24.7.1264]
14 **Kong MF**, Horowitz M, Jones KL, Wishart JM, Harding PE. Natural history of diabetic gastroparesis. *Diabetes Care* 1999; **22**: 503-507 [PMID: 10097936 DOI: 10.2337/diacare.22.3.503]
15 **Lyrenås EB**, Olsson EH, Arvidsson UC, Orn TJ, Spjuth JH. Prevalence and determinants of solid and liquid gastric emptying in unstable type I diabetes. Relationship to postprandial blood glucose concentrations. *Diabetes Care* 1997; **20**: 413-418 [PMID: 9051396 DOI: 10.2337/diacare.20.3.413]
16 **Mantides A**, Stefanides G, Kioulanis J, Tzovaras G, Epanomeritakis E, Xynos E. Cutaneous electrogastrography for the assessment of gastric myoelectrical activity in type I diabetes mellitus. *Am J Gastroenterol* 1997; **92**: 1190-1193 [PMID: 9219797]
17 **Sanders KM**, Koh SD, Ward SM. Interstitial cells of cajal as pacemakers in the gastrointestinal tract. *Annu Rev Physiol* 2006; **68**: 307-343 [PMID: 16460275 DOI: 10.1146/annurev.physiol.68.040504.094718]
18 **Bashashati M**, McCallum RW. Is Interstitial Cells of Cajal-opathy Present in Gastroparesis? *J Neurogastroenterol Motil* 2015; **21**: 486-493 [PMID: 26424037 DOI: 10.5056/jnm15075]
19 **Owyang C**, Logsdon CD. New insights into neurohormonal regulation of pancreatic secretion. *Gastroenterology* 2004; **127**: 957-969 [PMID: 15362050 DOI: 10.1053/j.gastro.2004.05.002]
20 **Nakabayashi H**, Nishizawa M, Nakagawa A, Takeda R, Niijima A. Vagal hepatopancreatic reflex effect evoked by intraportal appearance of tGLP-1. *Am J Physiol* 1996; **271**: E808-E813 [PMID: 8944665 DOI: 10.1152/ajpendo.1996.271.5.E808]
21 **Konturek SJ**, Pepera J, Zabielski K, Konturek PC, Pawlik T, Szlachcic A, Hahn EG. Brain-gut axis in pancreatic secretion and appetite control. *J Physiol Pharmacol* 2003; **54**: 293-317 [PMID: 14566070]
22 **Bernard C**, Memoir on the pancreas and on the role of pancreatic juice in digestive processes, particularly in the digestion of neutral fat. By Claude Bernard. 1856. Translated by John Henderson. *Monogr Physiol Soc* 1985; **42**: 1-131 [PMID: 3912660]
23 **Pavlov IP**. The work of the digestive glands. (Translated by Thompson WH) 1910; Charles Griffin and Company Ltd
24 **Bayliss WM**, Starling EH. The mechanism of pancreatic secretion. *J Physiol* 1902; **28**: 325-353 [PMID: 16992627 DOI: 10.1113/jphysiol.1902.sp000920]
25 **Chey WY**, Chang TM. Neural control of the release and action of secretin. *J Physiol Pharmacol* 2003; **54** Suppl 4: 105-112 [PMID: 15075453]
26 **Singer MV**, Solomon TE, Grossman MI. Effect of atropine on secretion from intact and transplanted pancreas in dog. *Am J Physiol* 1980; **238**: G18-G22 [PMID: 6986796 DOI: 10.1152/ajpgi.1980.238.1.G18]
27 **Singer MV**, Solomon TE, Wood J, Grossman MI. Latency of pancreatic enzyme response to intraduodenal stimulants. *Am J Physiol* 1980; **238**: G23-G29 [PMID: 6153511 DOI: 10.1152/ajpgi.1980.238.1.G23]
28 **Solomon TE**, Grossman MI. Effect of atropine and vagotomy on response of transplanted pancreas. *Am J Physiol* 1979; **236**: E186-E190 [PMID: 369390 DOI: 10.1152/ajpendo.1979.236.2.E186]
29 **Niebergall-Roth E**, Singer MV. Enteropancreatic reflexes mediating the pancreatic enzyme response to nutrients. *Auton Neurosci* 2006; **125**: 62-69 [PMID: 16490403 DOI: 10.1016/j.autneu.2006.01.003]
30 **Patrick A**, Epstein O. Review article: gastroparesis. *Aliment Pharmacol Ther* 2008; **27**: 724-740 [PMID: 18248660 DOI: 10.1111/j.1365-2036.2008.03637.x]
31 **McMenamin CA**, Travagli RA, Browning KN. Inhibitory neurotransmission regulates vagal efferent activity and gastric motility. *Exp Biol Med* (Maywood) 2016; **241**: 1343-1350 [PMID: 27302177 DOI: 10.1177/1535370216654228]
32 **Roze C**. Central regulation of pancreatic secretion. Brain-gut interactions 1991; Florida: CRC Press: 187-198.
33 **Browning KN**, Travagli RA. Central nervous system control of gastrointestinal motility and secretion and modulation of gastrointestinal functions. *Compr Physiol* 2014; **4**: 1339-1368 [PMID: 25428846 DOI: 10.1002/cphy.c130055]
34 **Travagli RA**, Browning KN. Central autonomic control of the pancreas. Central regulation of autonomic functions 2011; New York: Oxford University Press
35 **Hornby PJ**. Receptors and transmission in the brain-gut axis. Ⅱ. Excitatory amino acid receptors in the brain-gut axis. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1055-G1060 [PMID: 11352796 DOI: 10.1152/ajpgi.2001.280.6.G1055]
36 **Vanormelingen C**, Tack J, Andrews CN. Diabetic gastroparesis. *Br Med Bull* 2013; **105**: 213-230 [PMID: 23363458 DOI: 10.1093/bmb/ldt003]
37 **Li Y**, Owyang C. Pancreatic secretion evoked by cholecystokinin and non-cholecystokinin-dependent duodenal stimuli via vagal afferent fibres in the rat. *J Physiol* 1996; **494** (Pt 3): 773-782 [PMID: 8865073 DOI: 10.1113/jphysiol.1996.sp021531]
38 **Li Y**, Owyang C. Endogenous cholecystokinin stimulates pancreatic enzyme secretion via vagal afferent pathway in rats. *Gastroenterology* 1994; **107**: 525-531 [PMID: 8039628 DOI: 10.1016/0016-5085(94)90180-5]
39 **Li Y**, Hao Y, Zhu J, Owyang C. Serotonin released from intestinal enterochromaffin cells mediates luminal non-cholecystokinin-stimulated pancreatic secretion in rats. *Gastroenterology* 2000; **118**: 1197-1207 [PMID: 10833495 DOI: 10.1016/S0016-5085(00)70373-8]
40 **Li Y**, Wu XY, Zhu JX, Owyang C. Intestinal serotonin acts as paracrine substance to mediate pancreatic secretion stimulated by luminal factors. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G916-G923 [PMID: 11557511 DOI: 10.1152/ajpgi.2001.281.4.G916]
41 **Beyak MJ**, Bulmer DCE, Jiang W, Keating C, Rong W, Grundy D. Extrinsic sensory afferent nerves innervating the gastrointestinal tract. Physiology of the gastrointestinal tract 2006; 4th ed. Vol 1. Burlington, MA: Elsevier Academic Press: 685-725
42 **Mussa BM**, Sartor DM, Verberne AJ. Activation of cholecystokinin (CCK 1) and serotonin (5-HT 3) receptors increases the discharge of pancreatic vagal afferents. *Eur J Pharmacol* 2008; **601**: 198-206 [PMID: 19026634 DOI: 10.1016/j.ejphar.2008.11.007]
43 **Li Y**, Owyang C. Vagal afferent pathway mediates physiological action of cholecystokinin on pancreatic enzyme secretion. *J Clin Invest* 1993; **92**: 418-424 [PMID: 8100836 DOI: 10.1172/JCI116583]
44 **Powley TL**, Spaulding RA, Haglof SA. Vagal afferent innervation of the proximal gastrointestinal tract mucosa: chemoreceptor and mechanoreceptor architecture. *J Comp Neurol* 2011; **519**: 644-660 [PMID: 21246548 DOI: 10.1002/cne.22541]
45 **Li Y**, Hao Y, Owyang C. High-affinity CCK-A receptors on the vagus nerve mediate CCK-stimulated pancreatic secretion in rats. *Am J Physiol* 1997; **273**: G679-G685 [PMID: 9316472 DOI: 10.1152/ajpgi.1997.273.3.G679]
46 **McCann MJ**, Rogers RC. Functional and chemical neuroanatomy of a gastric vago-vagal reflex. Innervation of the Gut: Pathophysiological Implications, edited by Tache Y, Wingate DL and Burks TF 1994; Florida: CRC Press
47 **Niebergall-Roth E**, Singer MV. Central and peripheral neural control of pancreatic exocrine secretion. *J Physiol Pharmacol* 2001; **52**: 523-538 [PMID: 11787756]
48 **Smith BN**, Dou P, Barber WD, Dudek FE. Vagally evoked synaptic currents in the immature rat nucleus tractus solitarii in an intact in vitro preparation. *J Physiol* 1998; **512** (Pt 1): 149-162 [PMID: 9729625 DOI: 10.1111/j.1469-7793.1998.149bf.x]
49 **Streefland C**, Maes FW, Bohus B. Autonomic brainstem

projections to the pancreas: a retrograde transneuronal viral tracing study in the rat. *J Auton Nerv Syst* 1998; **74**: 71-81 [PMID: 9915620 DOI: 10.1016/S0165-1838(98)00047-2]
50 **Babic T**, Browning KN, Travagli RA. Differential organization of excitatory and inhibitory synapses within the rat dorsal vagal complex. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G21-G32 [PMID: 20947702 DOI: 10.1152/ajpgi.00363.2010]
51 **Berthoud HR**, Powley TL. Morphology and distribution of efferent vagal innervation of rat pancreas as revealed with anterograde transport of DiI. *Brain Res* 1991; **553**: 336-341 [PMID: 1718546 DOI: 10.1016/0006-8993(91)90846-N]
52 **Rinaman L**, Miselis RR. The organization of vagal innervation of rat pancreas using cholera toxin-horseradish peroxidase conjugate. *J Auton Nerv Syst* 1987; **21**: 109-125 [PMID: 3450690 DOI: 10.1016/0165-1838(87)90014-2]
53 **Mussa BM**, Verberne AJ. Activation of the dorsal vagal nucleus increases pancreatic exocrine secretion in the rat. *Neurosci Lett* 2008; **433**: 71-76 [PMID: 18241988 DOI: 10.1016/j.neulet.2007.12.048]
54 **Mussa BM**, Sartor DM, Rantzau C, Verberne AJ. Effects of nitric oxide synthase blockade on dorsal vagal stimulation-induced pancreatic insulin secretion. *Brain Res* 2011; **1394**: 62-70 [PMID: 21530944 DOI: 10.1016/j.brainres.2011.04.015]
55 **Alm P**, Ekström P, Henningsson R, Lundquist I. Morphological evidence for the existence of nitric oxide and carbon monoxide pathways in the rat islets of Langerhans: an immunocytochemical and confocal microscopical study. *Diabetologia* 1999; **42**: 978-986 [PMID: 10491758 DOI: 10.1007/s001250051256]
56 **Sivarao DV**, Krowicki ZK, Hornby PJ. Role of GABAA receptors in rat hindbrain nuclei controlling gastric motor function. *Neurogastroenterol Motil* 1998; **10**: 305-313 [PMID: 9697105 DOI: 10.1046/j.1365-2982.1998.00110.x]
57 **Travagli RA**, Hermann GE, Browning KN, Rogers RC. Brainstem circuits regulating gastric function. *Annu Rev Physiol* 2006; **68**: 279-305 [PMID: 16460274 DOI: 10.1146/annurev.physiol.68.040504.094635]
58 **Berthoud HR**, Powley TL. Identification of vagal preganglionics that mediate cephalic phase insulin response. *Am J Physiol* 1990; **258**: R523-R530 [PMID: 2178454 DOI: 10.1152/ajpregu.1990.258.2.R523]
59 **Ionescu E**, Rohner-Jeanrenaud F, Berthoud HR, Jeanrenaud B. Increases in plasma insulin levels in response to electrical stimulation of the dorsal motor nucleus of the vagus nerve. *Endocrinology* 1983; **112**: 904-910 [PMID: 6337050 DOI: 10.1210/endo-112-3-904]
60 **Fox EA**, Powley TL. Tracer diffusion has exaggerated CNS maps of direct preganglionic innervation of pancreas. *J Auton Nerv Syst* 1986; **15**: 55-69 [PMID: 3512681 DOI: 10.1016/0165-1838(86)90079-2]
61 **Loewy AD**, Haxhiu MA. CNS cell groups projecting to pancreatic parasympathetic preganglionic neurons. *Brain Res* 1993; **620**: 323-330 [PMID: 7690304 DOI: 10.1016/0006-8993(93)90174-L]
62 **Browning KN**, Coleman FH, Travagli RA. Characterization of pancreas-projecting rat dorsal motor nucleus of vagus neurons. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G950-G955 [PMID: 15637183 DOI: 10.1152/ajpgi.00549.2004]
63 **Mussa BM**, Sartor DM, Verberne AJ. Dorsal vagal preganglionic neurons: differential responses to CCK1 and 5-HT3 receptor stimulation. *Auton Neurosci* 2010; **156**: 36-43 [PMID: 20346737 DOI: 10.1016/j.autneu.2010.03.001]
64 **Berthoud HR**, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci* 2000; **85**: 1-17 [PMID: 11189015 DOI: 10.1016/S1566-0702(00)00215-0]
65 **Cruz MT**, Murphy EC, Sahibzada N, Verbalis JG, Gillis RA. A reevaluation of the effects of stimulation of the dorsal motor nucleus of the vagus on gastric motility in the rat. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**: R291-R307 [PMID: 16990483 DOI: 10.1152/ajpregu.00863.2005]
66 **Hermann GE**, Travagli RA, Rogers RC. Esophageal-gastric relaxation reflex in rat: dual control of peripheral nitrergic and cholinergic transmission. *Am J Physiol Regul Integr Comp Physiol* 2006; **290**: R1570-R1576 [PMID: 16439669 DOI: 10.1152/ajpregu.00717.2005]
67 **Toda N**, Herman AG. Gastrointestinal function regulation by nitrergic efferent nerves. *Pharmacol Rev* 2005; **57**: 315-338 [PMID: 16109838 DOI: 10.1124/pr.57.3.4]
68 **Sanger GJ**, Lee K. Hormones of the gut-brain axis as targets for the treatment of upper gastrointestinal disorders. *Nat Rev Drug Discov* 2008; **7**: 241-254 [PMID: 18309313 DOI: 10.1038/nrd2444]
69 **Khoo J**, Rayner CK, Feinle-Bisset C, Jones KL, Horowitz M. Gastrointestinal hormonal dysfunction in gastroparesis and functional dyspepsia. *Neurogastroenterol Motil* 2010; **22**: 1270-1278 [PMID: 20939851 DOI: 10.1111/j.1365-2982.2010.01609.x]
70 **Allescher HD**, Ahmad S. Postulated physiological and pathophysiological roles on motility. Neuropeptide Function in the Gastrointestinal Tract 1991; Boca Raton: CRC Press: 311-371
71 **Owyang C**. Physiological mechanisms of cholecystokinin action on pancreatic secretion. *Am J Physiol* 1996; **271**: G1-G7 [PMID: 8760100 DOI: 10.1152/ajpgi.1996.271.1.G1]
72 **Martindale R**, Levin S, Alfin-Slater R. Effects of caerulein and bombesin on insulin and glucagon secretion from the isolated, perfused rat pancreas. *Regul Pept* 1982; **3**: 313-324 [PMID: 7043666 DOI: 10.1016/0167-0115(82)90136-7]
73 **Hermansen K**. Effects of cholecystokinin (CCK)-4, nonsulfated CCK-8, and sulfated CCK-8 on pancreatic somatostatin, insulin, and glucagon secretion in the dog: studies in vitro. *Endocrinology* 1984; **114**: 1770-1775 [PMID: 6143659 DOI: 10.1210/endo-114-5-1770]
74 **Murphy JA**, Criddle DN, Sherwood M, Chvanov M, Mukherjee R, McLaughlin E, Booth D, Gerasimenko JV, Raraty MG, Ghaneh P, Neoptolemos JP, Gerasimenko OV, Tepikin AV, Green GM, Reeve JR Jr, Petersen OH, Sutton R. Direct activation of cytosolic Ca2+ signaling and enzyme secretion by cholecystokinin in human pancreatic acinar cells. *Gastroenterology* 2008; **135**: 632-641 [PMID: 18555802 DOI: 10.1053/j.gastro.2008.05.026]
75 **Andrews PL**, Davis CJ, Bingham S, Davidson HI, Hawthorn J, Maskell L. The abdominal visceral innervation and the emetic reflex: pathways, pharmacology, and plasticity. *Can J Physiol Pharmacol* 1990; **68**: 325-345 [PMID: 2178756 DOI: 10.1139/y90-047]
76 **Blackshaw LA**, Grundy D. Effects of 5-hydroxytryptamine (5-HT) on the discharge of vagal mechanoreceptors and motility in the upper gastrointestinal tract of the ferret. *J Auton Nerv Syst* 1993; **45**: 51-59 [PMID: 8227964 DOI: 10.1016/0165-1838(93)90361-W]
77 **Blackshaw LA**, Grundy D. Effects of 5-hydroxytryptamine on discharge of vagal mucosal afferent fibres from the upper gastrointestinal tract of the ferret. *J Auton Nerv Syst* 1993; **45**: 41-50 [PMID: 8227963 DOI: 10.1016/0165-1838(93)90360-7]
78 **Hendrix TR**, Atkinson M, Clifton JA, Ingelfinger FJ. The effect of 5-hydroxytryptamine on intestinal motor function in man. *Am J Med* 1957; **23**: 886-893 [PMID: 13487606 DOI: 10.1016/0002-9343(57)90298-X]
79 **Misiewicz JJ**, Waller SL, Eisner M. Motor responses of human gastrointestinal tract to 5-hydroxytryptamine in vivo and in vitro. *Gut* 1966; **7**: 208-216 [PMID: 18668794 DOI: 10.1136/gut.7.3.208]
80 **Bornstein JC**. Serotonin in the gut: what does it do? *Front Neurosci* 2012; **6**: 16 [PMID: 22347162 DOI: 10.3389/fnins.2012.00016]
81 **Chey WY**, Chang T. Neural hormonal regulation of exocrine pancreatic secretion. *Pancreatology* 2001; **1**: 320-335 [PMID: 12120211 DOI: 10.1159/000055831]
82 **Aja S**. Serotonin-3 receptors in gastric mechanisms of cholecystokinin-induced satiety. *Am J Physiol Regul Integr Comp Physiol* 2006; **291**: R112-R114 [PMID: 16690770 DOI: 10.1152/ajpregu.00159.2006]
83 **Fehmann HC**, Göke R, Göke B. Glucagon-like peptide-1(7-37)/(7-36)amide is a new incretin. *Mol Cell Endocrinol* 1992; **85**: C39-C44 [PMID: 1382025 DOI: 10.1016/0303-7207(92)90118-P]
84 **Schirra J**, Göke B. The physiological role of GLP-1 in human: incretin, ileal brake or more? *Regul Pept* 2005; **128**: 109-115 [PMID: 15780430 DOI: 10.1016/j.regpep.2004.06.018]

85 **Drucker DJ**, Philippe J, Mojsov S, Chick WL, Habener JF. Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci* USA 1987; **84**: 3434-3438 [PMID: 3033647 DOI: 10.1073/pnas.84.10.3434]

86 **Drucker DJ**. Glucagon-like peptides. *Diabetes* 1998; **47**: 159-169 [PMID: 9519708 DOI: 10.2337/diab.47.2.159]

87 **Fehmann HC**, Göke R, Göke B. Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. *Endocr Rev* 1995; **16**: 390-410 [PMID: 7671853 DOI: 10.1210/edrv-16-3-390]

88 **Harada K**, Kitaguchi T, Kamiya T, Aung KH, Nakamura K, Ohta K, Tsuboi T. Lysophosphatidylinositol-induced activation of the cation channel TRPV2 triggers glucagon-like peptide-1 secretion in enteroendocrine L cells. *J Biol Chem* 2017; **292**: 10855-10864 [PMID: 28533434 DOI: 10.1074/jbc.M117.788653]

89 **Nakatani Y**, Maeda M, Matsumura M, Shimizu R, Banba N, Aso Y, Yasu T, Harasawa H. Effect of GLP-1 receptor agonist on gastrointestinal tract motility and residue rates as evaluated by capsule endoscopy. *Diabetes Metab* 2017; **43**: 430-437 [PMID: 28648835 DOI: 10.1016/j.diabet.2017.05.009]

90 **Cork SC**, Richards JE, Holt MK, Gribble FM, Reimann F, Trapp S. Distribution and characterisation of Glucagon-like peptide-1 receptor expressing cells in the mouse brain. *Mol Metab* 2015; **4**: 718-731 [PMID: 26500843 DOI: 10.1016/j.molmet.2015.07.008]

91 **Kolligs F**, Fehmann HC, Göke R, Göke B. Reduction of the incretin effect in rats by the glucagon-like peptide 1 receptor antagonist exendin (9-39) amide. *Diabetes* 1995; **44**: 16-19 [PMID: 7813808 DOI: 10.2337/diab.44.1.16]

92 **Wang Z**, Wang RM, Owji AA, Smith DM, Ghatei MA, Bloom SR. Glucagon-like peptide-1 is a physiological incretin in rat. *J Clin Invest* 1995; **95**: 417-421 [PMID: 7814643 DOI: 10.1172/JCI117671]

93 **Wettergren A**, Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ. Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man. *Dig Dis Sci* 1993; **38**: 665-673 [PMID: 8462365 DOI: 10.1007/BF01316798]

94 **Willms B**, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA. Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients. *J Clin Endocrinol Metab* 1996; **81**: 327-332 [PMID: 8550773 DOI: 10.1210/jcem.81.1.8550773]

95 **Kreymann B**, Williams G, Ghatei MA, Bloom SR. Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet* 1987; **2**: 1300-1304 [PMID: 2890903 DOI: 10.1016/S0140-6736(87)91194-9]

96 **Nauck MA**, Bartels E, Orskov C, Ebert R, Creutzfeldt W. Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7-36) amide infused at near-physiological insulinotropic hormone and glucose concentrations. *J Clin Endocrinol Metab* 1993; **76**: 912-917 [PMID: 8473405 DOI: 10.1210/jcem.76.4.8473405]

97 **Meyer JH**, Gu YG, Jehn D, Taylor IL. Intragastric vs intraintestinal viscous polymers and glucose tolerance after liquid meals of glucose. *Am J Clin Nutr* 1988; **48**: 260-266 [PMID: 3136640 DOI: 10.1093/ajcn/48.2.260]

98 **Thompson DG**, Wingate DL, Thomas M, Harrison D. Gastric emptying as a determinant of the oral glucose tolerance test. *Gastroenterology* 1982; **82**: 51-55 [PMID: 7053335]

99 **Woodyatt RT**, Sansum WD, Wilder RM. Prolonged and accurately timed intravenous injection of sugar. *JAMA* 1915; **65**: 2067-2070 [DOI: 10.1001/jama.1915.02580240023006]

100 **Tambascia MA**, Malerbi DA, Eliaschewitz FG. Influence of gastric emptying on the control of postprandial glycemia: physiology and therapeutic implications. *Einstein* (Sao Paulo) 2014; **12**: 251-253 [PMID: 25003936 DOI: 10.1590/S1679-45082014RB2862]

101 **Vinik A**, Nakave A, Chuecos Mdel P. A break in the brake mechanism in diabetes: a cause of postprandial hyperglycemia. *Diabetes Care* 2008; **31**: 2410-2413 [PMID: 19033422 DOI: 10.2337/dc08-1694]

102 **Fujino K**, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M. Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 2003; **550**: 227-240 [PMID: 12837928 DOI: 10.1113/jphysiol.2003.040600]

103 **Eliasson B**, Björnsson E, Urbanavicius V, Andersson H, Fowelin J, Attvall S, Abrahamsson H, Smith U. Hyperinsulinaemia impairs gastrointestinal motility and slows carbohydrate absorption. *Diabetologia* 1995; **38**: 79-85 [PMID: 7744232 DOI: 10.1007/BF02369356]

104 **Jones KL**, Horowitz M, Carney BI, Wishart JM, Guha S, Green L. Gastric emptying in early noninsulin-dependent diabetes mellitus. *J Nucl Med* 1996; **37**: 1643-1648 [PMID: 8862300]

105 **Sims MA**, Hasler WL, Chey WD, Kim MS, Owyang C. Hyperglycemia inhibits mechanoreceptor-mediated gastrocolonic responses and colonic peristaltic reflexes in healthy humans. *Gastroenterology* 1995; **108**: 350-359 [PMID: 7835576 DOI: 10.1016/0016-5085(95)90060-8]

106 **Schvarcz E**, Palmér M, Aman J, Horowitz M, Stridsberg M, Berne C. Physiological hyperglycemia slows gastric emptying in normal subjects and patients with insulin-dependent diabetes mellitus. *Gastroenterology* 1997; **113**: 60-66 [PMID: 9207262 DOI: 10.1016/S0016-5085(97)70080-5]

107 **Russo A**, Stevens JE, Chen R, Gentilcore D, Burnet R, Horowitz M, Jones KL. Insulin-induced hypoglycemia accelerates gastric emptying of solids and liquids in long-standing type 1 diabetes. *J Clin Endocrinol Metab* 2005; **90**: 4489-4495 [PMID: 15899955 DOI: 10.1210/jc.2005-0513]

108 **Ma J**, Pilichiewicz AN, Feinle-Bisset C, Wishart JM, Jones KL, Horowitz M, Rayner CK. Effects of variations in duodenal glucose load on glycaemic, insulin, and incretin responses in type 2 diabetes. *Diabet Med* 2012; **29**: 604-608 [PMID: 22004512 DOI: 10.1111/j.1464-5491.2011.03496.x]

109 **Liu HP**, Leong SK, Tay SS. Localization of NADPH-diaphorase positive neurons in the pancreas of the mouse, rat, chick, kitten and monkey. *J Hirnforsch* 1994; **35**: 501-510 [PMID: 7533810]

110 **Liu HP**, Tay SS, Leong SK. Nitrergic neurons in the pancreas of newborn guinea pig: their distribution and colocalization with various neuropeptides and dopamine-beta-hydroxylase. *J Auton Nerv Syst* 1996; **61**: 248-256 [PMID: 8988482 DOI: 10.1016/S0165-1838(96)00090-2]

111 **De Giorgio R**, Parodi JE, Brecha NC, Brunicardi FC, Becker JM, Go VL, Sternini C. Nitric oxide producing neurons in the monkey and human digestive system. *J Comp Neurol* 1994; **342**: 619-627 [PMID: 8040367 DOI: 10.1002/cne.903420409]

112 **Shimosegawa T**, Abe T, Satoh A, Asakura T, Yoshida K, Koizumi M, Toyota T. Histochemical demonstration of NADPH-diaphorase activity, a marker for nitric oxide synthase, in neurons of the rat pancreas. *Neurosci Lett* 1992; **148**: 67-70 [PMID: 1284446 DOI: 10.1016/0304-3940(92)90806-I]

113 **Sha L**, Miller SM, Szurszewski JH. Nitric oxide is a neuromodulator in cat pancreatic ganglia: histochemical and electrophysiological study. *Neurosci Lett* 1995; **192**: 77-80 [PMID: 7675325 DOI: 10.1016/0304-3940(95)11614-3]

114 **Umehara K**. [Localization of NADPH-diaphorase activity and NOS immunoreactivity in the pancreas of rat and dog]. *Nihon Shokakibyo Gakkai Zasshi* 1995; **92**: 1161-1168 [PMID: 7563923]

115 **Corbett JA**, Wang JL, Misko TP, Zhao W, Hickey WF, McDaniel ML. Nitric oxide mediates IL-1 beta-induced islet dysfunction and destruction: prevention by dexamethasone. *Autoimmunity* 1993; **15**: 145-153 [PMID: 7692996 DOI: 10.3109/08916939309043889]

116 **Vincent SR**. Nitric oxide and arginine-evoked insulin secretion. *Science* 1992; **258**: 1376-1378 [PMID: 1455235 DOI: 10.1126/science.1455235]

117 **Panagiotidis G**, Akesson B, Alm P, Lundquist I. The nitric oxide system in the endocrine pancreas induces differential effects on secretion of insulin and glucagon. *Endocrine* 1994; **2**: 787-792

118 **Panagiotidis G**, Alm P, Lundquist I. Inhibition of islet nitric oxide synthase increases arginine-induced insulin release. *Eur J Pharmacol* 1992; **229**: 277-278 [PMID: 1283375 DOI: 10.1016/0014-2999(92)90568-O]

119 **Schmidt HH**, Warner TD, Ishii K, Sheng H, Murad F. Insulin

secretion from pancreatic B cells caused by L-arginine-derived nitrogen oxides. *Science* 1992; **255**: 721-723 [PMID: 1371193 DOI: 10.1126/science.1371193]

120 **Salehi A**, Carlberg M, Henningson R, Lundquist I. Islet constitutive nitric oxide synthase: biochemical determination and regulatory function. *Am J Physiol* 1996; **270**: C1634-C1641 [PMID: 8764145 DOI: 10.1152/ajpcell.1996.270.6.C1634]

121 **Gangula PR**, Chinnathambi V, Hale AB, Mukhopadhyay S, Channon KM, Ravella K. Impairment of nitrergic system and delayed gastric emptying in low density lipoprotein receptor deficient female mice. *Neurogastroenterol Motil* 2011; **23**: 773-e335 [PMID: 21414103 DOI: 10.1111/j.1365-2982.2011.01695.x]

122 **Lidums I**, Hebbard GS, Holloway RH. Effect of atropine on proximal gastric motor and sensory function in normal subjects. *Gut* 2000; **47**: 30-36 [PMID: 10861261 DOI: 10.1136/gut.47.1.30]

123 **Azpiroz F**, Malagelada JR. Importance of vagal input in maintaining gastric tone in the dog. *J Physiol* 1987; **384**: 511-524 [PMID: 2888879 DOI: 10.1113/jphysiol.1987.sp016467]

124 **Coulie B**, Tack J, Sifrim D, Andrioli A, Janssens J. Role of nitric oxide in fasting gastric fundus tone and in 5-HT1 receptor-mediated relaxation of gastric fundus. *Am J Physiol* 1999; **276**: G373-G377 [PMID: 9950810 DOI: 10.1152/ajpgi.1999.276.2.G373]

125 **Mihara H**, Suzuki N, Yamawaki H, Tominaga M, Sugiyama T. TRPV2 ion channels expressed in inhibitory motor neurons of gastric myenteric plexus contribute to gastric adaptive relaxation and gastric emptying in mice. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G235-G240 [PMID: 23203157 DOI: 10.1152/ajpgi.00256.2012]

126 **Di Stefano M**, Miceli E, Mazzocchi S, Tana P, Corazza GR. The role of gastric accommodation in the pathophysiology of functional dyspepsia. *Eur Rev Med Pharmacol Sci* 2005; **9**: 23-28 [PMID: 16457126]

127 **Petrakis IE**, Vrachassotakis N, Vassilakis SJ, Sciacca V, Chalkiadakis G. Erythromycin enhances solid-phase gastric emptying in induced-hyperglycemia in patients with truncal vagotomy and pyloroplasty. *Dig Dis Sci* 2000; **45**: 937-945 [PMID: 10795758 DOI: 10.1023/A:1005577125356]

128 **Howlett PJ**, Sheiner HJ, Barber DC, Ward AS, Perez-Avila CA, Duthie HL. Gastric emptying in control subjects and patients with duodenal ulcer before and after vagotomy. *Gut* 1976; **17**: 542-550 [PMID: 964687 DOI: 10.1136/gut.17.7.542]

129 **Chen J**, Koothan T, Chen JD. Synchronized gastric electrical stimulation improves vagotomy-induced impairment in gastric accommodation via the nitrergic pathway in dogs. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G310-G318 [PMID: 19023028 DOI: 10.1152/ajpgi.90525.2008]

130 **Rivera LR**, Poole DP, Thacker M, Furness JB. The involvement of nitric oxide synthase neurons in enteric neuropathies. *Neurogastroenterol Motil* 2011; **23**: 980-988 [PMID: 21895878 DOI: 10.1111/j.1365-2982.2011.01780.x]

131 **Piciucchi M**, Capurso G, Archibugi L, Delle Fave MM, Capasso M, Delle Fave G. Exocrine pancreatic insufficiency in diabetic patients: prevalence, mechanisms, and treatment. *Int J Endocrinol* 2015; **2015**: 595649 [PMID: 25892991 DOI: 10.1155/2015/595649]

132 **Hardt PD**, Brendel MD, Kloer HU, Bretzel RG. Is pancreatic diabetes (type 3c diabetes) underdiagnosed and misdiagnosed? *Diabetes Care* 2008; **31** Suppl 2: S165-S169 [PMID: 18227480 DOI: 10.2337/dc08-s244]

133 **Hardt PD**, Ewald N. Exocrine pancreatic insufficiency in diabetes mellitus: a complication of diabetic neuropathy or a different type of diabetes? *Exp Diabetes Res* 2011; **2011**: 761950 [PMID: 21822421 DOI: 10.1155/2011/761950]

134 **Ewald N**, Raspe A, Kaufmann C, Bretzel RG, Kloer HU, Hardt PD. Determinants of Exocrine Pancreatic Function as Measured by Fecal Elastase-1 Concentrations (FEC) in Patients with Diabetes mellitus. *Eur J Med Res* 2009; **14**: 118-122 [PMID: 19380282]

135 **Chowdhury RS**, Forsmark CE, Davis RH, Toskes PP, Verne GN. Prevalence of gastroparesis in patients with small duct chronic pancreatitis. *Pancreas* 2003; **26**: 235-238 [PMID: 12657948 DOI: 10.1097/00006676-200304000-00005]

136 **Czul F**, Coronel E, Donet JA. [Update on chronic pancreatitis: review article]. *Rev Gastroenterol Peru* 2017; **37**: 146-155 [PMID: 28731995]

137 **Liddle RA**, Morita ET, Conrad CK, Williams JA. Regulation of gastric emptying in humans by cholecystokinin. *J Clin Invest* 1986; **77**: 992-996 [PMID: 3949984 DOI: 10.1172/JCI112401]

138 **Shoji E**, Okumura T, Onodera S, Takahashi N, Harada K, Kohgo Y. Gastric emptying in OLETF rats not expressing CCK-A receptor gene. *Dig Dis Sci* 1997; **42**: 915-919 [PMID: 9149042 DOI: 10.1023/A:1018860313674]

139 **Poulsen JL**, Olesen SS, Malver LP, Frøkjær JB, Drewes AM. Pain and chronic pancreatitis: a complex interplay of multiple mechanisms. *World J Gastroenterol* 2013; **19**: 7282-7291 [PMID: 24259959 DOI: 10.3748/wjg.v19.i42.7282]

140 **Woolf CJ**. Central sensitization: implications for the diagnosis and treatment of pain. *Pain* 2011; **152**: S2-15 [PMID: 20961685 DOI: 10.1016/j.pain.2010.09.030]

141 **Nakamura T**, Takebe K, Ishii M, Kasai F, Arai Y, Tando Y, Yamada N, Terada A, Suda T. Study of gastric emptying in patients with pancreatic diabetes (chronic pancreatitis) using acetaminophen and isotope. *Acta Gastroenterol Belg* 1996; **59**: 173-177 [PMID: 9015926]

142 **Frier BM**. The incidence and impact of hypoglycemia in type 1 and type 2 diabetes. International Diabetes Monitor 2009; **21**: 210-218

143 **Gerich JE**, Langlois M, Noacco C, Karam JH, Forsham PH. Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science* 1973; **182**: 171-173 [PMID: 4581053 DOI: 10.1126/science.182.4108.171]

144 **Siafarikas A**, Johnston RJ, Bulsara MK, O'Leary P, Jones TW, Davis EA. Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes. *Diabetes Care* 2012; **35**: 1757-1762 [PMID: 22699295 DOI: 10.2337/dc11-2010]

P- Reviewer: Mihara H **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3834-3848

DOI: 10.3748/wjg.v24.i34.3834

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

REVIEW

Drug resistance and new therapies in colorectal cancer

Kevin Van der Jeught, Han-Chen Xu, Yu-Jing Li, Xiong-Bin Lu, Guang Ji

Kevin Van der Jeught, Han-Chen Xu, Yu-Jing Li, Xiong-Bin Lu, Guang Ji, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Kevin Van der Jeught, Han-Chen Xu, Yu-Jing Li, Xiong-Bin Lu, Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, United States

Xiong-Bin Lu, Indiana University Melvin and Bren Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, United States

ORCID number: Kevin Van der Jeught (0000-0002-4626-2343); Han-Chen Xu (0000-0003-2335-5421); Yu-Jing Li (0000-0002-3223-769X); Xiong-Bin Lu (0000-0002-7987-9825); Guang Ji (0000-0003-0842-3676).

Author contributions: Van der Jeught K and Xu HC contributed equally to this work; Van der Jeught K, Xu HC, Li YJ, Lu XB and Ji G wrote and edited the manuscript.

Supported by the National Natural Science Foundation of China, No. 81620108030.

Conflict-of-interest statement: The authors declare no competing financial interests.

Manuscript source: Unsolicited manuscript

Correspondence to: Guang Ji, MD, PhD, Chief Doctor, Professor, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, No. 725 South Wanping Road, Shanghai 200032, China. jiliver@vip.sina.com
Telephone: +86-21-64385700
Fax: +86-21-64385700

Received: April 27, 2018
Peer-review started: April 27, 2018
First decision: May 30, 2018
Revised: June 25, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 14, 2018

Abstract

Colorectal cancer (CRC) is often diagnosed at an advanced stage when tumor cell dissemination has taken place. Chemo- and targeted therapies provide only a limited increase of overall survival for these patients. The major reason for clinical outcome finds its origin in therapy resistance. Escape mechanisms to both chemo- and targeted therapy remain the main culprits. Here, we evaluate major resistant mechanisms and elaborate on potential new therapies. Amongst promising therapies is α-amanitin antibody-drug conjugate targeting hemizygous p53 loss. It becomes clear that a dynamic interaction with the tumor microenvironment exists and that this dictates therapeutic outcome. In addition, CRC displays a limited response to checkpoint inhibitors, as only a minority of patients with microsatellite instable high tumors is susceptible. In this review, we highlight new developments with clinical potentials to augment responses to checkpoint inhibitors.

Key words: Colorectal cancer; Therapy resistance; Antibody-drug conjugates; α-amanitin; Tumor microenvironment; Immunotherapy; Checkpoint inhibitors; Microbiome

Core tip: Therapy resistance has been a culprit for colorectal cancer (CRC) treatment. Here, we review a novel therapeutic approach using α-amanitin antibody-drug conjugates inhibiting RNA polymerase Ⅱ against

CRC with hemizygous loss of p53. Since its mechanism of cell killing is independent of the generally used tubulin inhibitors and chemotherapy drugs, this approach shows the promise to overcome common drug resistance. In addition, we summarize the sensitivity of CRC to newly developed immune checkpoint inhibitors. While patients with microsatellite instability-high CRC remain the sole subgroup responsive to current checkpoint inhibitors so far, we highlight potentially new developments that may lead to promising results in treating patients with microsatellite-stable CRC, which constitutes the majority of this disease.

Van der Jeught K, Xu HC, Li YJ, Lu XB, Ji G. Drug resistance and new therapies in colorectal cancer. *World J Gastroenterol* 2018; 24(34): 3834-3848 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3834.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3834

INTRODUCTION

Colorectal cancer (CRC) is ranked third amongst the most common cancers affecting both men and women worldwide[1]. Over one million new cases are reported and around 600000 patients die from the disease every year[2]. The five-year survival prognosis is highly dependent on the stage of the disease. While displaying over 90 percent survival for patients with stage Ⅰ CRC, it barely reaches 10 percent for patients with stage Ⅳ CRC. Thus, early detection of the disease has been a priority. For patients failing to be screened early enough, late-stage CRC remains an arduous disease to treat. The basis of CRC treatment consists of surgery, targeted therapy, neoadjuvant radiotherapy and adjuvant chemotherapy. Unfortunately, drug-resistance remains one of the deadlocks for the low survival rates of CRC patients. A better understanding in the intrinsic and acquired therapy resistance will be a great asset for drug development. Recently, the impact of the tumor microenvironment (TME) has gained attention in CRC, prompting the extensive analysis of clinical trials to assess immune-cell infiltration as prognostic and predictive markers. In addition, a promising avenue of clinical research for the treatment of CRC is the use of immunotherapy. Currently, encouraging results have been obtained with the use of immune checkpoint inhibitors in CRC in subgroups of patients. Discovery to improve the responsiveness to checkpoint inhibitors is one of the major points of focus for CRC treatment and will be a point of focus during this review.

Drug resistance in CRC

Since the 1950s, 5-fluorouracil (5-FU)-based chemotherapy remains the mainstay of therapy for patients with CRC[3,4]. In recent years, chemotherapy drugs such as oxaliplatin, irinotecan and capecitabine have been developed. Conventional treatment for advanced CRC encompasses the combination of 5-FU and leucovorin with oxaliplatin or irinotecan[5]. The medical treatment in CRC has made great strides with the advent of monoclonal antibodies such as Bevacizumab and Cetuximab. Despite the improvement in response rates with various modulation strategies such as monoclonal antibodies combined with chemotherapy, the five-year survival rate for metastatic CRC (mCRC) is only slightly over 12 percent[1]. One of the major obstacles for this observation is due to the appearance of drug resistance. Nearly half of mCRC patients are resistant to 5-FU-based chemotherapies[6]. With continuous research, multiple drug resistance mechanisms are being unraveled, such as enhanced DNA repair and increased drug metabolism.

Chemotherapy resistance and their potential mechanisms

In addition to the above-mentioned general resistance mechanisms, 5-FU also has its unique drug resistance. 5-FU is a synthetic fluorinated pyrimidine analog that inhibits DNA replication. This leads to the replacement of thymidine by fluorinated nucleotides into the DNA, hereby causing cell death. Therefore, it is not surprising that 5-FU resistance is closely related to the expression of thymidylate synthase (TS). Since TS is the primary target of 5-FU, patients with low TS expression display a better overall survival (OS) than patients with higher TS expression in tumor tissue[7,8]. As TS is encoded by the TYMS gene, the level of TYMS gene expression offers significant prognostic value[9]. Thymidine phosphorylase (TP), uridine phosphorylase (UP), orotate phosphoribosyl transferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) are all involved in the metabolism and degradation of 5-FU. The relationship between their activity and the sensitivity of CRC to 5-FU has been demonstrated in several studies. Higher expression of TP, UP and OPRT levels displayed enhanced sensitivity to 5-FU therapy[10-12]. Similarly, as DPD contributes to the degradation of 5-FU, its expression level was inversely correlated with chemosensitivity[11]. Collectively, inhibition of the activity of these enzymes could allow enhanced sensitivity to 5-FU.

In 1996, irinotecan (CPT-11) was approved by the FDA for CRC treatment. Irinotecan is a semi-synthetic camptothecin derivative that selectively inhibits topoisomerase Ⅰ (Topo Ⅰ). In the cell, CPT-11 undergoes intracellular modifications such as the removal of the C10 group through carboxylesterase catalysis, and then is metabolized to become 7-ethyl-10-hydroxycamptothecin (SN-38). SN-38 possesses 100 to 1000 times stronger anticancer activity than CPT-11[13]. CPT-11 or its active metabolite SN-38 forms a topoisomerase-inhibitor-DNA complex affecting the DNA function. Therefore, the higher the concentration of Topo Ⅰ, the more sensitive the cells becomes to irinotecan[14,15]. Carboxylesterases (CES), uridine diphosphate glucuronosyltransferase (UGT), hepatic cytochrome P-450 enzymes CYP3A, β-glucuronidase and ATP-binding cassette (ABC) transporter protein are involved in the uptake and metabolism of irinotecan.

Consequently, they stand out as major players that determine drug resistance[16,17]. Additionally, targeting the MAPK signal transduction pathway *via* the inhibition of FGF2, FGF9, MECOM, PLA2G4C and PRKACB could also potentially improve responsiveness to irinotecan[18]. Epigenetic changes take part in development of irinotecan resistance. A change in histone acetylation, such as H4K16 acetylation, is associated with the resistance to irinotecan. Combinatory therapy with histone deacetylase (HDAC) inhibitors holds promise in overcoming irinotecan resistance[19].

Oxaliplatin, a platinum-based chemotherapeutic drug, is approved for the treatment of CRC. Itis most commonly combined with 5-FU and leucovorin, a folinic acid. The combination of these drugs as a treatment regimen is referred to as FOLFOX and has been the first-line chemotherapy strategy for mCRC. The chemical structure difference between oxaliplatin and other platinum-based chemotherapeutic drugs is that oxaliplatin possesses a 1,2-diaminocyclohexane ligand (DACH). DACH together with its platinum compound causes DNA to be more difficult to repair, hereby improving its tumor cell killing potential[20]. Oxaliplatin resistance is related to the nucleotide excision repair (NER) pathway. Gene expression levels of ERCC1, XRCC1 and XDP are correlated with resistance to oxaliplatin, and can be used together as a drug sensitivity predictor index[21]. In addition to NER, the WBSCR22 protein represents a novel oxaliplatin resistance biomarker as well as a possible drug target for therapeutic development[22]. Transforming growth factor-β1 (TGF-β1) is secreted abundantly by a variety of cells within the TME. TGF-β1 is thought to help the induction of resistance to oxaliplatin through epithelial to mesenchymal transition (EMT)[23]. Thus, interfering with TGF-β1 to abrogate EMT could potentially sensitize tumor cells towards oxaliplatin cell-mediated killing.

Capecitabine is the first oral chemotherapy drug for CRC. It is metabolized in the body and converted to 5'-deoxy-5-fluorocytidine (5'-OFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR). Hereafter, 5'-DFUR is eventually hydrolyzed by TP to 5-FU, which will exert its cytotoxic effect. Many of the resistance mechanisms involved in 5-FU resistance are shared. In particular, TP, which is an essential enzyme for the conversion of capecitabine to 5-FU, plays a central role in its resistance. Patients with higher expression levels of TP will have better responses to capecitabine, while loss of function confers the resistance[24,25].The multinational phase Ⅲ trial provided evidence for capecitabine and irinotecan combination therapy (XELIRI) with or without Bevacizumab as a second-line treatment option of mCRC[26,27].

In addition to the above described mechanisms, there is tremendous heterogeneity within CRC cells. The discovery of cancer stem cells and their therapy resistance as well as their self-renewal capacity has driven the attention towards this peculiar cell population. This specific subset of tumor cells has been shown to be prognostic for patients[28,29]. So far, CRC stem cells have been reported to be enriched for specific surface markers such as CD133, EphB2^{high}, EpCAM^{high}, CD44^{+}, CD166^{+}, ALDH^{+}, LGR5^{+} and CD44v6^{+}[30]. Aside from surface markers, cancer stem cells can be characterized through molecular features such as hyperactivated β-catenin pathway and functional traits such as self-renewal[31,32]. Another functional phenotype is their expression of efflux pumps such as the ATP binding cassette (ABC) family members, including ABCG2[28]. The presence of efflux pumps promotes the transport of drugs, such as chemotherapeutic compounds, outside the cell. Therefore, cancer stem cells are in part more resistant to chemotherapy. Cancer stem cells have shown an ability to respond to therapy challenges such as chemotherapy, radiotherapy and more recently immunotherapy[33-35].

Taken together, many chemotherapeutic regimens are currently being adopted for the treatment of CRC. However, this disease displays specific mechanisms rendering a lower therapeutic benefit (Figure 1). In-depth study of drug resistance and targeting the cancer stem cell population will eventually improve the clinical outcome.

Hurdles and new avenues for targeted therapy

Targeted therapies including monoclonal antibodies and small molecule inhibitors are effective treatments following chemotherapy. With the apparition of monoclonal antibodies against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), the OS for CRC increased up to three years[36-38]. Targeted therapies display significantly lower side effects as compared to chemotherapy. Bevacizumab is the first anti-angiogenic drug that can precisely target VEGF, leading to reduced tumor growth[39]. Kabbinavar and colleagues showed improved response rates and OS from data obtained from three clinical trials comparing patients treated with fluorouracil/leucovorin alone or in combination with Bevacizumab[40]. However, the survival benefit of anti-VEGF therapy in mCRC patients is limited to a few months due to acquired resistance. During Bevacizumab exposure, VEGF-A is decreased, but increased levels of VEGFR1 result in drug resistance. Decreased hepatocyte growth factor (HGF) levels are observed during acquired resistance, suggesting the potential implementation of strategies to counter HGF-ligand inhibition[41]. Findings by Carbone *et al*[42] propose a role for the transcription factor HOXB9 as one of the key mechanisms of anti-VEGF resistance. Silencing HOXB9 is thought to be a promising approach to modulate this resistance. Despite these few findings, the major mechanism of drug resistance to anti-VEGF therapy is not fully elucidated. Further research on drug resistance mechanisms, as well as on predictive biomarkers, is therefore essential.

EGFR is a key component involved in the regulation of cell proliferation. Anti-EGFR antibodies, such as Cetuximab and Panitumumab, inhibit downstream signaling pathways. This leads to an inhibition of

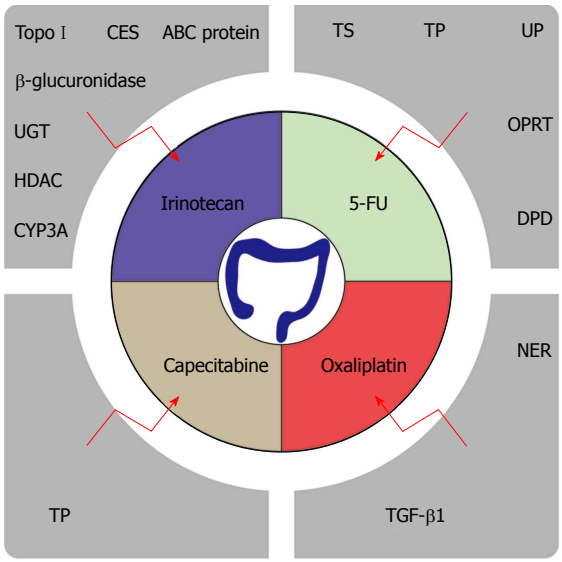


Figure 1 Potential mechanisms of resistance to chemotherapy agents. In this schematic representation, the grey boxes highlight major contributors to chemotherapy resistance of irinotecan, 5-FU, capecitabine and oxaliplatin. Topo Ⅰ: Topoisomerase Ⅰ; CES: Carboxylesterases; UGT: Uridine diphosphate glucuronosyltransferase; CYP3A: Hepatic cytochrome P450 enzymes; HDAC: Histone deacetylase; ABC protein: ATP-binding cassette transporter protein; TP: Thymidine phosphorylase; NER: Nucleotide excision repair; TGF-β1: Transforming growth factor β1; TS: Thymidylate synthase; UP: Uridine phosphorylase; OPRT: Orotate phosphoribosyl transferase; DPD: Dihydropyrimidine dehydrogenase.

proliferation and induction of apoptosis. KRAS or NRAS mutations are well-known predictors of resistance to anti-EGFR therapy[43]. The efficacy of Cetuximab was demonstrated in the CRYSTAL trial for patients with wild type KRAS in combination with FOLFIRI (leucovorin + 5-FU + irinotecan) or FOLFOX regimen. In this trial, the median OS was extended for more than three months. However, patients with CRC carrying KRAS mutations did not benefit from the combination therapy[36,44]. In addition, several clinical trials proved that either Cetuximab or Panitumumab significantly improved the OS in wild-type KRAS patients. The effect was more pronounced in wild-type RAS mCRC, delineating that RAS mutation could be a negative predictive marker for Panitumumab[45-47]. However, the G13D KRAS mutation in exon 2 codon 13 deserves particular attention. In several studies, patients harboring the G13D KRAS mutation as well as wild-type KRAS, significantly benefited from the addition of Cetuximab based on OS[48-50]. Some studies pointed out that BRAF, PTEN and PIK3CA (like KRAS) have predictive value for anti-EGFR treatment efficacy[36,51-54]. All of the clinical trials support KRAS and BRAF mutation assessment for mCRC patients before initiation of treatment with anti-EGFR therapy. In addition to the above-described targeted therapies, Regorafenib, a multikinase inhibitor, is also approved for the treatment of mCRC. It can inhibit the function of fms-related tyrosine kinase 1 (FLT1), kinase insert domain receptor (KDR), TEK receptor tyrosine kinase, KIT proto-oncogene receptor tyrosine kinase, Raf-1 proto-oncogene and serine/threonine kinase[55]. Two phase Ⅲ clinic trails showed that Regorafenib treatment could improve the OS by 1.4 mo and 2.5 mo[56,57]. Even though these drugs are approved, they display limited progression free survival (PFS) and OS due to resistance[44,58]. Their major advantage is that they have limited toxicity side effects as compared to chemotherapeutic drugs.

CRC development and progression is associated with acquired genomic events. The amount of mutations and genomic alterations is extensive. One of the main drivers for cancer development is gene copy number variation, such as the amplification of oncogenes or the deletion of tumor suppressor genes. Over the years, significant work has been done to tackle the tumor suppressor p53 activity in cancer therapies. Usually, promotion of cancer development can occur when a tumor suppressor gene, such as p53, undergoes a two-hit modification, being the mutation of the gene and the hemizygous loss of its other counterpart on the other arm of the chromosome. Unfortunately, no effective drug has reached the clinic due to its complex signaling pathway[59]. We demonstrate that genomic deletion of p53 frequently encompasses neighboring essential genes, rendering cancer cells with hemizygous p53 deletion vulnerable to further suppression of such genes. POLR2A is identified as such a gene that is always co-deleted with p53 in human cancers. Hemizygous loss of p53/POLR2A occurs in 53% of CRC. POLR2A encodes the largest and catalytic subunit of RNA polymerase Ⅱ complex. It is specifically inhibited by α-amanitin, a cyclic 8-aa peptide toxin found in the death cap mushroom (*Amanita phalloides*)[60,61]. Suppression of POLR2A selectively inhibits proliferation, survival and tumorigenic potential of CRC cells with hemizygous p53 loss. Previous clinical applications of α-amanitin have been limited due to its liver toxicity. Free α-amanitin causes apoptosis and necrosis of hepatocytes by interacting with the hepatocyte-specific transporting protein OATP1B3[62]. However, α-amanitin is no longer a substrate for OATP1B3 when coupled to antibodies[63]. Therefore, α-amanitin-based antibody drug conjugates (ADCs) are highly effective therapeutic agents with significantly reduced toxicity. Our study has shown that low doses of α-amanitin-conjugated anti-epithelial cell adhesion molecule (EpCAM) antibody leads to complete tumor regression in murine models of human CRC with hemizygous deletion of POLR2A (Figure 2)[64]. The preclinical studies provide the foundation for future clinical trials. The major advantage for the use of such targeted therapy is that the function of POLR2A is essential for cell survival. Thus, no alternative "escape" pathway can be recruited, leading to drug resistance. In addition, this mode of action is not related to the proliferation of cancer cells. Cancer stem cells would be also targeted *via* this approach, leading to a potentially more pronounced therapeutic benefit. However, hypothetically, resistance could occur if the remaining POLR2A allele would undergo mutations, amplification or transcriptional activation as well as post-transcriptional or post-translation enhancement. In addition, tumor cells could downregulate the EpCAM receptor on their

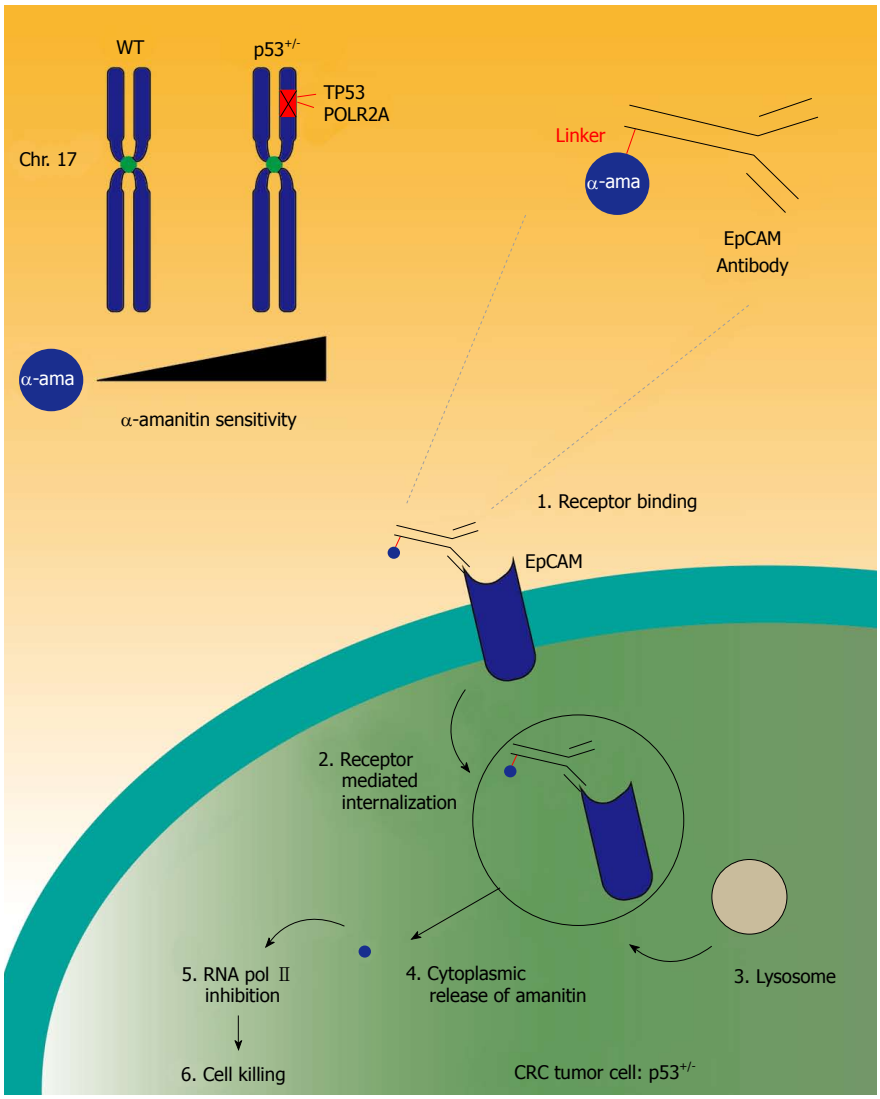


Figure 2 Working model of α-amanitin antibody-drug conjugates. Genomic deletion of p53 frequently encompasses neighboring essential genes such as POLR2A. Colorectal cancer (CRC) cells displaying this loss are vulnerable to α-amanitin. The figure summarizes the different steps in α-amanitin-based antibody drug conjugate (ADC) killing of CRC cells with hemizygous p53 loss. (1) The ADC binds to CRC cells expressing epithelial cell adhesion molecule (EpCAM). (2) Hereafter, the ADC is internalized via receptor-mediated endocytosis. After fusing with lysozyme (3) the α-amanitin is released in the cytoplasm (4), leading to inhibition of the catalytic subunit of RNA polymerase Ⅱ complex (5). Suppression of POLR2A will ultimately lead to cell death (6).

surface, hereby avoiding the binding of the ADC.

TUMOR MICROENVIRONMENT, MICROBE BIOFILM, AND THEIR CONTRIBUTION TO DRUG RESISTANCE AND TUMORIGENESIS

CRC is often diagnosed at a later stage when tumor cell dissemination has taken place. Over the last decades, metastatic disease, occurring in almost half of the patients, has been a challenge for clinicians to treat and remains an incurable disease. Unfortunately, most of the promising preclinical approaches have proven scarce in clinical translation. The metastatic process has been extensively investigated but has yet to be linked with specific genetic alterations of epithelial CRC cells[65]. Nevertheless, it has become clear that one of the key issues relies on the TME[66]. Therefore, blockage of cancer immunity and tumor-promoting inflammation have become hallmarks of cancer[67]. The TME plays a critical role at the different stages of the disease from a physiological colonic epithelium to an adenomatous polyp and eventually to a mCRC. The TME confers to CRC cell survival, immune evasion and a favorable environment to grow and metastasize.

The TME consists of a variety of cells that are continuously interacting with each other in a dynamic manner: Immune cells, extra-cellular matrix, cancer associated fibroblasts, endothelial cells, endothelial progenitor cells, platelets and mesenchymal stem cells, to name a few. The initial attraction of these cell types is mediated through inflammation, leading to the secretion by both the tumor cells and stromal cells of a variety of cytokines and chemokines. Stromal inflammation was shown to promote the evolution of adenomas to adenocarcinomas in nude mice[68]. Ultimately, the

tumor breaches the equilibrium and the tumor becomes uncontrollable. At this stage tumor cells will abuse stromal cells to promote tumor survival, proliferation and metastasis.

Dendritic cells (DCs) are considered the most professional antigen presenting cells (APCs) and are essential to generate a proper adaptive immune response[69]. Ideally, DCs within the TME will engulf tumor associated antigens (TAAs) and migrate towards the draining lymph nodes, where they will elicit T-cell mediated responses. In CRC, no correlation was found between the frequency of DCs in the tumor and patient survival[70]. O'Toole *et al*[71], however, could link the capacity of tumor conditioned media to inhibit the lipopolysaccharide (LPS)-induced maturation of DCs with patient survival. The suppression of DCs was independent of the stage of the disease. Thus, the outcome could be rather linked with the potential functionality of DCs than their presence in the TME. DCs are very versatile based on their environment. In a suppressive environment that hampers their maturation, they become tolerogenic or regulatory DCs, which promote tumor cell survival. On the other hand, well-activated DCs will induce immunity and Th1 immune cell responses.

The role of tumor associated macrophages (TAMs) is of particular interest in CRC. Usually in most types of solid cancers, the infiltration of TAMs is linked with a poor survival and enhanced metastasis. However in CRC, the infiltration of TAMs is linked with better prognosis[72,73]. Recently, Zhang *et al*[74] conducted a meta-analysis of 55 studies with a total of 8692 patients in which they correlated the survival with the infiltration of TAMs using the pan-macrophage marker CD68. Strikingly, only in CRC were favorable clinical outcomes correlated with their infiltration. Moreover, TAM-rich tumors are accompanied with a lower amount of both lymph node and distant metastases[74].

A variety of chemoattractants are involved in the recruitment of monocytes in the TME. Chemokines such as C-C motif chemokine ligand-2 (CCL-2), CCL-5 and C-X-C motif chemokine ligand 12 (CXCL-12), cytokines such as colony stimulating factor1 (CSF-1) and VEGF family members, and complement components such as C5a contribute to the recruitment of macrophages in the TME[75-77]. These factors also lead to a differential activation status of macrophages[78]. TAMs can be subdivided into two categories based on their activation status: M1 (classically activated) or M2 (alternatively activated). Classically activated M1 TAMs are driven by interferon-γ (IFN-γ), whereas alternative M2 TAMs are driven by interleukin-4 (IL-4) and IL-13[75]. This nomenclature is based on the Th1 and Th2 concept, and thus reflects their role in adaptive immunity. Therefore, in most cases M2 TAMs are considered pro-tumorigenic through their contribution to tumor vascularization and dampening of adaptive immune responses, while M1 macrophages possess antitumor activities. To address the potential influence of TAMs in improved patient survival, Malesci *et al*[79] studied the impact of high TAM infiltration in stage Ⅲ CRC patients treated with the adjuvant 5-FU chemotherapeutic drug. Their results showed a clear benefit of TAM infiltration when associated with 5-FU, while no benefit was observed in the untreated group. In addition, the effect of high-density TAM in metastatic lymph nodes is more pronounced in patient survival compared to the high TAM infiltration in the primary tumors. *In vitro* experiments pointed out that treatment with 5-FU resulted in M1 polarization of the macrophages[79]. However, further research into which subtype of macrophages is critical for patient outcome in CRC remains to be defined.

The correlation of T-cell infiltration on the OS at different stages of CRC has been well documented but remains controversial[80-86]. Numerous studies were made to assess the correlation of distinct T-cell populations (Including: $CD3^+$, $CD4^+$, $CD8^+$, $CD45RO^+$ and $FoxP3^+$) with the clinical outcome. Most studies point out that T-cell infiltration is unlikely a predictive factor in CRC patients. The majority of studies encompassed only a low number of patients, which leads to debatable interpretation of the tumor-infiltrating lymphocytes (TILs) and specific subpopulations thereof on the clinical outcome. This has prompted the meta-analysis of TILs and their correlation with survival. Nosho *et al*[87] analyzed 768 CRC cases ranging from stage Ⅰ to Ⅳ. They concluded that the density of memory T cells ($CD45RO^+$) in the tumors was associated with improved survival. In addition, assessment was done on molecular alterations such as microsatellite instability (MSI), CpG island methylator phenotype (CIMP), BRAF, KRAS, PIK3CA and LINE-1 hypomethylation. The high-frequency of MSI (MSI-H) and high LINE-1 methylation were correlated with higher $CD45RO^+$ cell density. These data are in accordance with previously published data showing the relationship between MSI and TILs. MSI is thought to induce truncated peptides that cause immunogenicity of tumor cells[85], which in turn contributes to the stimulation of adaptive immune responses. Taken together, the predictive value of TILs in CRC remains unclear.

The gut microbiome regulates the homeostasis of the digestive tract in a very dynamic way. Disruption in the latter can thus disturb this balance and cause major environmental changes leading to diseases such as inflammatory bowel disease (IBD) and cancer[88]. The gut microbiota consists of trillions of micro-organisms such as bacteria, viruses and fungi[89,90]. Recent studies have investigated the presence and functional roles of certain bacteria in CRC[91-96].

In the last three years, the gut microbiome has emerged as a potential key player in cancer immunotherapy. Initial findings by Vetizou *et al*[97] showed that the checkpoint inhibitor (CPI) ipilimumab (anti-cytotoxic T-lymphocyte antigen-4: Anti-CTLA4) could treat specific pathogen free (SPF) mice, but not germ-free mice. In addition, the anti-tumor effects of ipilimumab could be deteriorated by antibiotics. Analysis of murine

feces revealed significant changes in the microbiome, leading to a decrease in the bacterial species *Bacteroidales* and *Burkholderiales*. Supplementation of these missing microbes could restore the anti-tumor effects of ipilimumab. In the same vein, Sivan *et al*[98] showed that mice obtained from two different providers responded distinctly to anti-programmed death-1 receptor ligand (anti-PD-L1) treatment. These mice were shown to harbor a different microbiome, and fecal transplants could reverse the treatment discrepancies. In their case, *Bifidobacterium* showed a positive correlation with anti-tumor T-cell responses.

Future research could potentially link different species of bacteria with an alternate immune cell infiltration. For instance, certain bacteria could potentially shape macrophage polarization towards a distinct phenotype and thus be used as a potential predictive marker for CRC patients. It could also be that the presence of bacteria species would be distinct based on the stage of the disease. Interestingly, the right or proximal colon more frequently exhibits MSI tumors, whereas the left or distal colon displays more chromosomal instability (CIN)[99]. It might be that the proximal or distal colon display different type of bacteria leading to these genetically different tumors. We expect that in the future, intestinal microbiota might serve as a standard of care biomarker for immunotherapies such as CPIs. Moreover, fecal transplant or supplementation of certain species of bacteria could potentially be co-administered with CPI, leading to improved responses towards CPI. Taken together, better understanding of the microbiota dysbiosis could serve as prognostic and predictive marker in CRC.

Immunotherapy development

Several types of cancers have undergone a complete revolution thanks to immunotherapy. This has led the editors of Science calling cancer immunotherapy the "breakthrough of the year" in 2013[100]. Nonetheless, CRC has so far been a poor candidate for immunotherapy. Initial studies lacked objective clinical responses with nivolumab in unselected patients[101,102]. Previous observations noticed that immunotherapy works better in tumors containing a high mutational load as illustrated in melanoma and lung cancer. To further emphasize the importance of the mutational load in lung cancer, smoking patients displayed a better response rate to CPI compared to non-smokers[103]. Increased amount of mutations is associated with the production of neoantigens, which in turn enhance the tumor immunogenicity[104]. The better predictive value of smoking lung cancer patients to CPI was linked with an increased amount of neoantigens.

Consequently, this has prompted the application of CPI in patients with MSI-H CRC. In MSI, frameshift mutations in protein-coding sequences possess the capacity to generate different peptides with potential neoepitopes recognized as foreign by the immune system[85]. To further illustrate this, Saeterdal *et al*[105] found an immunogenic peptide derived from a frameshift mutation in transforming growth factor β receptor type Ⅱ (TGFβRⅡ) referred to as p538. This peptide is expressed in over 90 percent of tumors with DNA mismatch repair (dMMR), suggesting it is highly applicable in the field. Many of such genes in MSI-H tumors are shared by a majority of patients as they are thought to be part of the carcinogenesis process[105]. Therefore, both prophylactic for patients with a genetic predisposition and therapeutic cancer vaccinations could be done using such peptides.

Interestingly, the number of TILs is increased in MSI tumors compared to microsatellite stable (MSS) tumors[84,106,107]. Furthermore, TILs display an enhanced $CD8^+CD103^+$ phenotype[108]. $CD103^+$ TILs were found in 27-fold higher amounts within the same patient tumor compared to normal epithelium. Increased objective response (OR), stable disease (SD) and PFS were observed in a phase Ⅱ clinical trial using pembrolizumab (anti-PD-1) to treat MSI-H patients. Similarly, when nivolumab (anti-PD-L1) was administered to MSI-H patients, a clear benefit was observed which led to the approval for these selected patients. As for melanoma and other cancers, the combination of ipilimumab and nivolumab is currently being tested for MSI-H metastatic CRC patients[109].

Of note, colon cancer cell lines derived from MSI tumors display a loss in human leukocyte antigen (HLA) class Ⅰ expression[110]. This is due to genetic mutations in the β2m, which is an essential part of the HLA class Ⅰ complex. The presentation of TAAs is considered a prerequisite for successful T-cell responses[111]. Therefore, it is thought that these surviving tumors were exposed to high selection pressure to escape T-cell surveillance. In many cancers, treatment with CPI fails to reach satisfying results[112,113]. Consequently, there is a growing interest in combining CPI together with chemotherapies or targeted therapies (Figure 3). The rationale is that certain chemotherapeutic drugs or targeted therapies could enhance the immunogenicity of the tumors. This process is dependent on induction of immunogenic cell death (ICD) of tumor cells[114]. When killed in an immunogenic way, tumor cells will express surface makers such as calreticulin and will secrete factors such a high-mobility group box 1 (HMGB1) in the extracellular milieu, hereby allowing the spontaneous generation of an adaptive immune response that might benefit from CPI. Preclinical evidence supported the oxaliplatin-induced immunogenic cell death in the murine BALB/c colon carcinoma model CT26[115].

Furthermore, a few ongoing clinical trials hold the promise to improve the outcome of PD1/PD-L1 blockade. VEGF, leading to angiogenesis, is frequently upregulated in CRC and is linked with poor OS. The latter can also influence the maturation of DCs. As described above, the DC maturation capacity was correlated with patient survival[70]. Therefore, blockage of VEGF through Bevacizumab could potentiate immune responses. In a phase Ib clinical trial, the combination

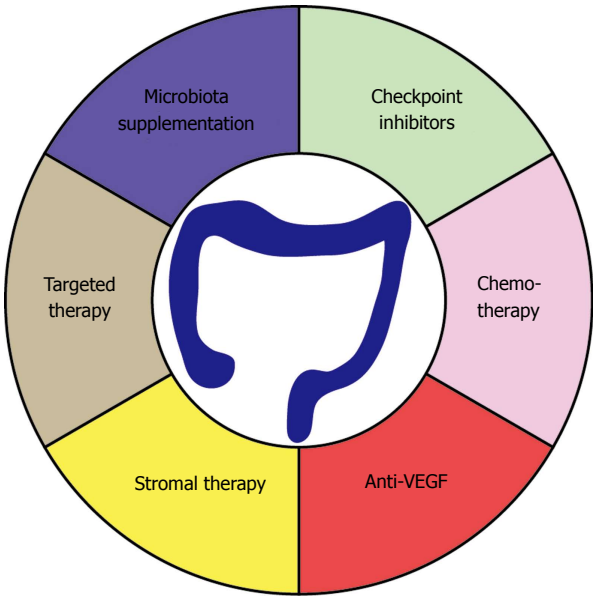


Figure 3 Combination therapies for the development of durable colorectal cancer responses. Durable cancer responses are impeded by a dysfunctional immunological control. Strategies aiming to boost T cell-mediated immune responses will most likely need the combination of therapies that counter the tumor and their environment-mediated escape mechanisms avoiding T-cell recognition as well as down-regulation of T-cell mediated functions. In this figure, we list potential interesting combinations leading to durable T-cell mediated killing for CRC. CRC: Colorectal cancer; VEGF: Vascular endothelial growth factor.

of Bevacizumab and Atezolizumab displayed a clear benefit of the combination therapy for MSI-H patients[116]. Similarly, in the NCT02997228 clinical trial, 439 patients with MSI-H CRC will be treated with either: Atezolizumab as monotherapy; Atezolizumab combined with FOLFOX (a chemotherapy regimen consisting of folinic acid + 5-FU + oxaliplatin) and Bevacizumab; or FOLFOX and Bevacizumab. FOLFOX will also be tested with and without Atezolizumab in over 700 MSI-H CRC stage Ⅲ patients (NCT02912559).

In addition to Bevacizumab, several targeted therapies have been approved for the treatment of CRC. Amongst them, Cetuximab was shown to display antibody-dependent cellular cytoxicity (ADCC). Interestingly, Cetuximab could induce antigen-spreading in head and neck cancer patients[117]. Antigen-spreading is a therapy induced phenomenon where secondary to therapy more antigens are released and can trigger the generation of antigen-specific immune responses against a broader number of antigens[118]. As tumor cells are known to evolve and "protect" themselves against any form of therapy, they will ultimately try to down-regulate immune responses against a single antigen. Therefore, the generation of immune responses against several epitopes could lead to robust and long-lasting immune responses, which encouraged the clinical evaluation for the combination of Cetuximab with Pembrolizumab (NCT02713373).

Unfortunately, the presence of MSI solely accounts for 15% of CRC cases, while the frequency of MSI-H is even lower at 5.9% of the patients[119-122]. The amount of MSI-H varies only slightly based on the stage of the disease (0-I: 5.9%; Ⅱ: 8.9%; Ⅲ: 4.0% and Ⅳ: 3.7%). The overwhelming majority of CRC patients would thus remain out of scope for CPI. Ebert *et al*[123] studied the potential combination of mitogen-activated protein kinase kinase (MEK) inhibition using the MEK inhibitor G-38963, which is considered similar to the clinically used Cobimetinib, together with anti-PD-L1 in CT26 tumor-bearing mice[123-125]. Treatments with only MEK inhibitors lead to an initial delay in tumor growth. However, upon analysis of these tumors, an increased amount of antigen-specific T cells were present with a distinct T-bet^{high} phenotype. Therapeutic combination of MEK inhibition and PD-L1 blockage led to an impressive long-term survival, whereas single agents only displayed an initial delay of tumor growth. This MEK inhibitor was furthermore shown to act on the post-naïve stage of T-cell differentiation. Antigen-specific CD8$^+$ T cells expressed Nur77, which is associated with exhaustive T-cell death. MEK inhibition was shown to counter the expression of Nur77 and thus could rescue T-cell exhaustion. This effect seemed to be in parallel with the PD-1 axis; therefore, a therapeutic rescue might only be possible in the case of blocking the PD1/PD-L1 axis. A potential risk to this strategy might be the prolonged exposure to MEK inhibition. The latter could lead to a depletion of the T-cell population. To address this issue, a period of two days without MEK treatment was introduced, which could bring back the amount of T-bet^{high} antigen-specific T cells to normal. Based on the findings that MEK inhibition leads to enhanced T-cell infiltration, synergy with blockade of the PD-1/PD-L1 axis and an upregulation of MHC class Ⅰ on tumor cells clinical efficacy was assessed[123,126-129]. Phase Ib clinical results (NCT01988896) indicated a potential benefit for the combination of PD-L1 inhibition (Atezolizumab) and Cobimetinib on proficient MMR (pMMR), which is the equivalent of MSS tumors that were previously unresponsive to CPI. These data have prompted the more in depth analysis of this combination and is currently evaluated in a phase Ⅲ clinical trial (NCT02788279). Such combination therapy trying to render MSS tumors sensible for CPI might open the avenue for CRC sensitivity towards CPI, and thus, long-term benefits.

Assessing a change in the TME is one of the major targets for current immunotherapeutic approaches. Depletion of myeloid-derived suppressor cells (MDSCs) using anti-CSF1R and anti-CTLA4 improved the survival of CT26 tumor-bearing mice[130]. Similarly, a decrease in the granulocyte fraction of the MDSCs was found to be a favorable factor for patients treated with FOLFOX and Bevacizumab[131]. Another option would be the local delivery of drugs directly in the TME to reduce the toxicity of the delivered drugs. The effectiveness of the local delivery also possesses multiple advantages[132-134]. Local delivery can induce systemic immune responses, leading to the eradication

of tumors on multiple locations[135]. This phenomenon is also known as the abscopal effect. Aside from targeting cells within the tumors, it is also possible to modulate soluble factors such as cytokines and chemokines[133]. TGF-β, for example, is a very well-studied factor in CRC, which is linked with a poor prognosis[136,137]. Its function is somewhat controversial in CRC. In early stages, it possesses tumor suppressive properties, whereas in a later stage of the disease, it will promote tumor progression. TGF-β will initially lead to a cytostatic effect on epithelial cells. Calon *et al*[136] evidenced that pharmacological inhibition of stromal TGF-β signaling blocks metastasis. Local neutralization of soluble factors such as TGF-β have been previously reported[138]. Currently, intratumoral approaches for CRC are being tested, such as intratumoral injection of autologous activated DCs. The latter is being tested in a phase Ⅰ clinical trial by Northwest Biotherapeutics (NCT01882946) using their DCVax-Direct. Another currently tested approach is the intratumoral injection of the adenoviral vector coding for the human IL-12 (cDNA) by Mount Sinai School of Medicine National Cancer Institute (NCI) for liver metastases secondary to colorectal cancer (NCT00072098).

The major drawback for the local injection remains the involvement of a surgical procedure, thus impairing large scalability of similar immunotherapeutic approaches. An elegant solution to avoid local injection is the use of tumor targeting antibodies linked with the component of interest. In the past, preclinical results were obtained using tumor targeting moieties such as anti-CD20, anti-Neu or anti-EGFR to name a few[139,140]. In CRC, an interesting clinical trial is ongoing using a T-cell bispecific (TCB) antibody targeting carcinoembryonic antigen (CEA) on CRC tumor cells and CD3 on T cells (NCT02324257 and NCT02650713). This molecule simultaneously binds to T-cells and tumor cells. This bispecific antibody is currently being tested both as a monotherapy and in combination with Atezolizumab in a majority of MSS patients. Preliminary results show promising disease control and stable disease with only a minority of progressive disease[141]. A better understanding in cancer immunotherapy drugs leading to an improved survival in the MSS subgroup of CRC will help further development of this currently unfavorable subset of patients constituting the majority of CRC patients.

CONCLUSION

The CRC field has been evolving over the last decades. Nevertheless, therapy resistance and unresponsiveness to immunotherapy remain major obstacles. Recent findings might give hope for targeted therapy resistance thanks to ADCs using RNA polymerase Ⅱ inhibition as a different killing mechanism as compared to tubulin inhibitors. Less resistant mechanisms are expected; however, the future will tell us whether this holds true.

The potential use of CPI to treat CRC has been successful in a minority of patients displaying MSI-H tumors. However, recent findings point towards new avenues leading to potential enlargement of the CPI sensitive pool. Currently, a tremendous effort is being made in understanding the effects of the TME and microbiome on the outcome of CPI therapy. However, it has become clear that combination therapy will lead to tremendous benefits for patients in the future.

ACKNOWLEDGMENTS

The authors would like to thank Michael Frieden for reviewing the manuscript.

REFERENCES

1 **Siegel R**, Desantis C, Jemal A. Colorectal cancer statistics, 2014. *CA Cancer J Clin* 2014; **64**: 104-117 [PMID: 24639052 DOI: 10.3322/caac.21220]
2 **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]
3 **Salonga D**, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 2000; **6**: 1322-1327 [PMID: 10778957]
4 **Showalter SL**, Showalter TN, Witkiewicz A, Havens R, Kennedy EP, Hucl T, Kern SE, Yeo CJ, Brody JR. Evaluating the drug-target relationship between thymidylate synthase expression and tumor response to 5-fluorouracil. Is it time to move forward? *Cancer Biol Ther* 2008; **7**: 986-994 [PMID: 18443433 DOI: 10.4161/cbt.7.7.6181]
5 **Yaffee P**, Osipov A, Tan C, Tuli R, Hendifar A. Review of systemic therapies for locally advanced and metastatic rectal cancer. *J Gastrointest Oncol* 2015; **6**: 185-200 [PMID: 25830038 DOI: 10.3978/j.issn.2078-6891.2014.112]
6 **Douillard JY**, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 2000; **355**: 1041-1047 [PMID: 10744089 DOI: 10.1016/S0140-6736(00)02034-1]
7 **Qiu LX**, Tang QY, Bai JL, Qian XP, Li RT, Liu BR, Zheng MH. Predictive value of thymidylate synthase expression in advanced colorectal cancer patients receiving fluoropyrimidine-based chemotherapy: evidence from 24 studies. *Int J Cancer* 2008; **123**: 2384-2389 [PMID: 18729195 DOI: 10.1002/ijc.23822]
8 **Abdallah EA**, Fanelli MF, Buim ME, Machado Netto MC, Gasparini Junior JL, Souza E Silva V, Dettino AL, Mingues NB, Romero JV, Ocea LM, Rocha BM, Alves VS, Araújo DV, Chinen LT. Thymidylate synthase expression in circulating tumor cells: a new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients. *Int J Cancer* 2015; **137**: 1397-1405 [PMID: 25721610 DOI: 10.1002/ijc.29495]
9 **Zhou JY**, Shi R, Yu HL, Zeng Y, Zheng WL, Ma WL. The association between two polymorphisms in the TS gene and risk of cancer: a systematic review and pooled analysis. *Int J Cancer* 2012; **131**: 2103-2116 [PMID: 22307944 DOI: 10.1002/ijc.27465]
10 **Che J**, Pan L, Yang X, Liu Z, Huang L, Wen C, Lin A, Liu H. Thymidine phosphorylase expression and prognosis in colorectal cancer treated with 5-fluorouracil-based chemotherapy: A meta-analysis. *Mol Clin Oncol* 2017; **7**: 943-952 [PMID: 29285354 DOI: 10.3892/mco.2017.1436]
11 **Sakowicz-Burkiewicz M**, Przybyla T, Wesserling M, Bielarczyk H, Maciejewska I, Pawelczyk T. Suppression of TWIST1 enhances the sensitivity of colon cancer cells to 5-fluorouracil. *Int J Biochem*

Cell Biol 2016; **78**: 268-278 [PMID: 27458056 DOI: 10.1016/j.biocel.2016.07.024]
12 **Yanagisawa Y**, Maruta F, Iinuma N, Ishizone S, Koide N, Nakayama J, Miyagawa S. Modified Irinotecan/5FU/Leucovorin therapy in advanced colorectal cancer and predicting therapeutic efficacy by expression of tumor-related enzymes. *Scand J Gastroenterol* 2007; **42**: 477-484 [PMID: 17454858 DOI: 10.1080/00365520600994418]
13 **Hicks LD**, Hyatt JL, Stoddard S, Tsurkan L, Edwards CC, Wadkins RM, Potter PM. Improved, selective, human intestinal carboxylesterase inhibitors designed to modulate 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (Irinotecan; CPT-11) toxicity. *J Med Chem* 2009; **52**: 3742-3752 [PMID: 19534556 DOI: 10.1021/jm9001296]
14 **Palshof JA**, Høgdall EV, Poulsen TS, Linnemann D, Jensen BV, Pfeiffer P, Tarpgaard LS, Brünner N, Stenvang J, Yilmaz M, Nielsen DL. Topoisomerase Ⅰ copy number alterations as biomarker for irinotecan efficacy in metastatic colorectal cancer. *BMC Cancer* 2017; **17**: 48 [PMID: 28077117 DOI: 10.1186/s12885-016-3001-y]
15 **Meisenberg C**, Gilbert DC, Chalmers A, Haley V, Gollins S, Ward SE, El-Khamisy SF. Clinical and cellular roles for TDP1 and TOP1 in modulating colorectal cancer response to irinotecan. *Mol Cancer Ther* 2015; **14**: 575-585 [PMID: 25522766 DOI: 10.1158/1535-7163.MCT-14-0762]
16 **Nielsen DL**, Palshof JA, Brünner N, Stenvang J, Viuff BM. Implications of ABCG2 Expression on Irinotecan Treatment of Colorectal Cancer Patients: A Review. *Int J Mol Sci* 2017; **18**: [PMID: 28880238 DOI: 10.3390/ijms18091926]
17 **de Man FM**, Goey AKL, van Schaik RHN, Mathijssen RHJ, Bins S. Individualization of Irinotecan Treatment: A Review of Pharmacokinetics, Pharmacodynamics, and Pharmacogenetics. *Clin Pharmacokinet* 2018 ; : [PMID: 29520731 DOI: 10.1007/s40262-018-0644-7]
18 **Makondi PT**, Chu CM, Wei PL, Chang YJ. Prediction of novel target genes and pathways involved in irinotecan-resistant colorectal cancer. *PLoS One* 2017; **12**: e0180616 [PMID: 28749961 DOI: 10.1371/journal.pone.0180616]
19 **Meisenberg C**, Ashour ME, El-Shafie L, Liao C, Hodgson A, Pilborough A, Khurram SA, Downs JA, Ward SE, El-Khamisy SF. Epigenetic changes in histone acetylation underpin resistance to the topoisomerase Ⅰ inhibitor irinotecan. *Nucleic Acids Res* 2017; **45**: 1159-1176 [PMID: 28180300 DOI: 10.1093/nar/gkw1026]
20 **Chaney SG**, Campbell SL, Bassett E, Wu Y. Recognition and processing of cisplatin- and oxaliplatin-DNA adducts. *Crit Rev Oncol Hematol* 2005; **53**: 3-11 [PMID: 15607931 DOI: 10.1016/j.critrevonc.2004.08.008]
21 **Gnoni A**, Russo A, Silvestris N, Maiello E, Vacca A, Marech I, Numico G, Paradiso A, Lorusso V, Azzariti A. Pharmacokinetic and metabolism determinants of fluoropyrimidines and oxaliplatin activity in treatment of colorectal patients. *Curr Drug Metab* 2011; **12**: 918-931 [PMID: 21787270 DOI: 10.2174/138920011798062300]
22 **Yan D**, Tu L, Yuan H, Fang J, Cheng L, Zheng X, Wang X. WBSCR22 confers oxaliplatin resistance in human colorectal cancer. *Sci Rep* 2017; **7**: 15443 [PMID: 29133897 DOI: 10.1038/s41598-017-15749-z]
23 **Mao L**, Li Y, Zhao J, Li Q, Yang B, Wang Y, Zhu Z, Sun H, Zhai Z. Transforming growth factor-β1 contributes to oxaliplatin resistance in colorectal cancer via epithelial to mesenchymal transition. *Oncol Lett* 2017; **14**: 647-654 [PMID: 28693217 DOI: 10.3892/ol.2017.6209]
24 **Stark M**, Bram EE, Akerman M, Mandel-Gutfreund Y, Assaraf YG. Heterogeneous nuclear ribonucleoprotein H1/H2-dependent unsplicing of thymidine phosphorylase results in anticancer drug resistance. *J Biol Chem* 2011; **286**: 3741-3754 [PMID: 21068389 DOI: 10.1074/jbc.M110.163444]
25 **Lin S**, Lai H, Qin Y, Chen J, Lin Y. Thymidine phosphorylase and hypoxia-inducible factor 1-α expression in clinical stage Ⅱ/Ⅲ rectal cancer: association with response to neoadjuvant chemoradiation therapy and prognosis. *Int J Clin Exp Pathol* 2015; **8**: 10680-10688 [PMID: 26617778]
26 **Kotaka M**, Xu R, Muro K, Park YS, Morita S, Iwasa S, Uetake H, Nishina T, Nozawa H, Matsumoto H, Yamazaki K, Han SW, Wang W, Ahn JB, Deng Y, Cho SH, Ba Y, Lee KW, Zhang T, Satoh T, Buyse ME, Ryoo BY, Shen L, Sakamoto J, Kim TW. Study protocol of the Asian XELIRI ProjecT (AXEPT): a multinational, randomized, non-inferiority, phase Ⅲ trial of second-line chemotherapy for metastatic colorectal cancer, comparing the efficacy and safety of XELIRI with or without bevacizumab versus FOLFIRI with or without bevacizumab. *Chin J Cancer* 2016; **35**: 102 [PMID: 28007025 DOI: 10.1186/s40880-016-0166-3]
27 **Garcia-Alfonso P**, Chaves M, Muñoz A, Salud A, García-Gonzalez M, Grávalos C, Massuti B, González-Flores E, Queralt B, López-Ladrón A, Losa F, Gómez MJ, Oltra A, Aranda E; Spanish Cooperative Group for the Treatment of Digestive Tumors (TTD). Capecitabine and irinotecan with bevacizumab 2-weekly for metastatic colorectal cancer: the phase Ⅱ AVAXIRI study. *BMC Cancer* 2015; **15**: 327 [PMID: 25925749 DOI: 10.1186/s12885-015-1293-y]
28 **Hu J**, Li J, Yue X, Wang J, Liu J, Sun L, Kong D. Expression of the cancer stem cell markers ABCG2 and OCT-4 in right-sided colon cancer predicts recurrence and poor outcomes. *Oncotarget* 2017; **8**: 28463-28470 [PMID: 28212529 DOI: 10.18632/oncotarget.15307]
29 **de Sousa E Melo F**, Colak S, Buikhuisen J, Koster J, Cameron K, de Jong JH, Tuynman JB, Prasetyanti PR, Fessler E, van den Bergh SP, Rodermond H, Dekker E, van der Loos CM, Pals ST, van de Vijver MJ, Versteeg R, Richel DJ, Vermeulen L, Medema JP. Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. *Cell Stem Cell* 2011; **9**: 476-485 [PMID: 22056143 DOI: 10.1016/j.stem.2011.10.008]
30 **Zeuner A**, Todaro M, Stassi G, De Maria R. Colorectal cancer stem cells: from the crypt to the clinic. *Cell Stem Cell* 2014; **15**: 692-705 [PMID: 25479747 DOI: 10.1016/j.stem.2014.11.012]
31 **Vermeulen L**, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 2010; **12**: 468-476 [PMID: 20418870 DOI: 10.1038/ncb2048]
32 **Paquet-Fifield S**, Koh SL, Cheng L, Beyit LM, Shembrey C, Mølck C, Behrenbruch C, Papin M, Gironella M, Guelfi S, Nasr R, Grillet F, Prudhomme M, Bourgaux JF, Castells A, Pascussi JM, Heriot AG, Puisieux A, Davis MJ, Pannequin J, Hill AF, Sloan EK, Hollande F. Tight Junction Protein Claudin-2 Promotes Self-Renewal of Human Colorectal Cancer Stem-like Cells. *Cancer Res* 2018; **78**: 2925-2938 [PMID: 29510994 DOI: 10.1158/0008-5472.CAN-17-1869]
33 **Bialkowski L**, Van der Jeught K, Bevers S, Tjok Joe P, Renmans D, Heirman C, Aerts JL, Thielemans K. Immune checkpoint blockade combined with IL-6 and TGF-β inhibition improves the therapeutic outcome of mRNA-based immunotherapy. *Int J Cancer* 2018; **143**: 686-698 [PMID: 29464699 DOI: 10.1002/ijc.31331]
34 **Hu X**, Ghisolfi L, Keates AC, Zhang J, Xiang S, Lee DK, Li CJ. Induction of cancer cell stemness by chemotherapy. *Cell Cycle* 2012; **11**: 2691-2698 [PMID: 22732500 DOI: 10.4161/cc.21021]
35 **Dylla SJ**, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One* 2008; **3**: e2428 [PMID: 18560594 DOI: 10.1371/journal.pone.0002428]
36 **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]
37 **Heinemann V**, von Weikersthal LF, Decker T, Kiani A, Vehling-

Kaiser U, Al-Batran SE, Heintges T, Lerchenmüller C, Kahl C, Seipelt G, Kullmann F, Stauch M, Scheithauer W, Hielscher J, Scholz M, Müller S, Link H, Niederle N, Rost A, Höffkes HG, Moehler M, Lindig RU, Modest DP, Rossius L, Kirchner T, Jung A, Stintzing S. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *Lancet Oncol* 2014; **15**: 1065-1075 [PMID: 25088940 DOI: 10.1016/S1470-2045(14)70330-4]

38 **Schwartzberg LS**, Rivera F, Karthaus M, Fasola G, Canon JL, Hecht JR, Yu H, Oliner KS, Go WY. PEAK: a randomized, multicenter phase Ⅱ study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. *J Clin Oncol* 2014; **32**: 2240-2247 [PMID: 24687833 DOI: 10.1200/JCO.2013.53.2473]

39 **Yancopoulos GD**, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 2000; **407**: 242-248 [PMID: 11001067 DOI: 10.1038/35025215]

40 **Kabbinavar FF**, Hambleton J, Mass RD, Hurwitz HI, Bergsland E, Sarkar S. Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. *J Clin Oncol* 2005; **23**: 3706-3712 [PMID: 15867200 DOI: 10.1200/jco.2005.00.232]

41 **Pineda E**, Salud A, Vila-Navarro E, Safont MJ, Llorente B, Aparicio J, Vera R, Escudero P, Casado E, Bosch C, Bohn U, Pérez-Carrión R, Carmona A, Ayuso JR, Ripollés T, Bouzas R, Gironella M, García-Albéniz X, Feliu J, Maurel J. Dynamic soluble changes in sVEGFR1, HGF, and VEGF promote chemotherapy and bevacizumab resistance: A prospective translational study in the BECOX (GEMCAD 09-01) trial. *Tumour Biol* 2017; **39**: 1010428317705509 [PMID: 28621236 DOI: 10.1177/1010428317705509]

42 **Carbone C**, Piro G, Simionato F, Ligorio F, Cremolini C, Loupakis F, Alì G, Rossini D, Merz V, Santoro R, Zecchetto C, Zanotto M, Di Nicolantonio F, Bardelli A, Fontanini G, Tortora G, Melisi D. Homeobox B9 Mediates Resistance to Anti-VEGF Therapy in Colorectal Cancer Patients. *Clin Cancer Res* 2017; **23**: 4312-4322 [PMID: 28298545 DOI: 10.1158/1078-0432.CCR-16-3153]

43 **Khambata-Ford S**, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, Wong TW, Huang X, Takimoto CH, Godwin AK, Tan BR, Krishnamurthi SS, Burris HA 3rd, Poplin EA, Hidalgo M, Baselga J, Clark EA, Mauro DJ. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007; **25**: 3230-3237 [PMID: 17664471 DOI: 10.1200/jco.2006.10.5437]

44 **Van Cutsem E**, Köhne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pintér T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J, Rougier P. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009; **360**: 1408-1417 [PMID: 19339720 DOI: 10.1056/NEJMoa0805019]

45 **Kim TW**, Elme A, Kusic Z, Park JO, Udrea AA, Kim SY, Ahn JB, Valencia RV, Krishnan S, Bilic A, Manojlovic N, Dong J, Guan X, Lofton-Day C, Jung AS, Vrdoljak E. A phase 3 trial evaluating panitumumab plus best supportive care vs best supportive care in chemorefractory wild-type KRAS or RAS metastatic colorectal cancer. *Br J Cancer* 2016; **115**: 1206-1214 [PMID: 27736842 DOI: 10.1038/bjc.2016.309]

46 **Shitara K**, Yonesaka K, Denda T, Yamazaki K, Moriwaki T, Tsuda M, Takano T, Okuda H, Nishina T, Sakai K, Nishio K, Tokunaga S, Yamanaka T, Boku N, Hyodo I, Muro K. Randomized study of FOLFIRI plus either panitumumab or bevacizumab for wild-type KRAS colorectal cancer-WJOG 6210G. *Cancer Sci* 2016; **107**: 1843-1850 [PMID: 27712015 DOI: 10.1111/cas.13098]

47 **Peeters M**, Oliner KS, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, He P, Yu H, Koukakis R, Terwey JH, Jung AS, Sidhu R, Patterson SD. Analysis of KRAS/NRAS Mutations in a Phase Ⅲ Study of Panitumumab with FOLFIRI Compared with FOLFIRI Alone as Second-line Treatment for Metastatic Colorectal Cancer. *Clin Cancer Res* 2015; **21**: 5469-5479 [PMID: 26341920 DOI: 10.1158/1078-0432.CCR-15-0526]

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

49 **Osumi H**, Shinozaki E, Osako M, Kawazoe Y, Oba M, Misaka T, Goto T, Kamo H, Suenaga M, Kumekawa Y, Ogura M, Ozaka M, Matsusaka S, Chin K, Hatake K, Mizunuma N. Cetuximab treatment for metastatic colorectal cancer with KRAS p.G13D mutations improves progression-free survival. *Mol Clin Oncol* 2015; **3**: 1053-1057 [PMID: 26623049 DOI: 10.3892/mco.2015.602]

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

51 **Pietrantonio F**, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, Cabiddu M, Iacovelli R, Bossi I, Lonati V, Ghilardi M, de Braud F, Barni S. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. *Eur J Cancer* 2015; **51**: 587-594 [PMID: 25673558 DOI: 10.1016/j.ejca.2015.01.054]

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

53 **Kaczirek K**, Ciuleanu TE, Vrbanec D, Marton E, Messinger D, Liegl-Atzwanger B, Wrba F, Knittelfelder R, Lindner E, Zielinski CC, Streubel B, Brodowicz T. FOLFOX4 Plus Cetuximab for Patients With Previously Untreated Metastatic Colorectal Cancer According to Tumor RAS and BRAF Mutation Status: Updated Analysis of the CECOG/CORE 1.2.002 Study. *Clin Colorectal Cancer* 2015; **14**: 91-98 [PMID: 25666295 DOI: 10.1016/j.clcc.2014.12.003]

54 **Karapetis CS**, Jonker D, Daneshmand M, Hanson JE, O' Callaghan CJ, Marginean C, Zalcberg JR, Simes J, Moore MJ, Tebbutt NC, Price TJ, Shapiro JD, Pavlakis N, Gibbs P, Van Hazel GA, Lee U, Haq R, Virk S, Tu D, Lorimer IA; NCIC Clinical Trials Group and the Australasian Gastro-Intestinal Trials Group. PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer--results from NCIC CTG/AGITG CO.17. *Clin Cancer Res* 2014; **20**: 744-753 [PMID: 24218517 DOI: 10.1158/1078-0432.CCR-13-0606]

55 **Wilhelm SM**, Dumas J, Adnane L, Lynch M, Carter CA, Schütz G, Thierauch KH, Zopf D. Regorafenib (BAY 73-4506): a new oral multikinase inhibitor of angiogenic, stromal and oncogenic receptor tyrosine kinases with potent preclinical antitumor activity. *Int J Cancer* 2011; **129**: 245-255 [PMID: 21170960 DOI: 10.1002/ijc.25864]

56 **Grothey A**, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, Humblet Y, Bouché O, Mineur L, Barone C, Adenis A, Tabernero J, Yoshino T, Lenz HJ, Goldberg RM, Sargent DJ, Cihon F, Cupit

L, Wagner A, Laurent D; CORRECT Study Group. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 2013; **381**: 303-312 [PMID: 23177514 DOI: 10.1016/S0140-6736(12)61900-X]

57 **Li J**, Qin S, Xu R, Yau TC, Ma B, Pan H, Xu J, Bai Y, Chi Y, Wang L, Yeh KH, Bi F, Cheng Y, Le AT, Lin JK, Liu T, Ma D, Kappeler C, Kalmus J, Kim TW; CONCUR Investigators. Regorafenib plus best supportive care versus placebo plus best supportive care in Asian patients with previously treated metastatic colorectal cancer (CONCUR): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2015; **16**: 619-629 [PMID: 25981818 DOI: 10.1016/S1470-2045(15)70156-7]

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

59 **Liu Y**, Wang L, Lu X. A new way to target p53-defective colorectal cancer. *Future Oncol* 2015; **11**: 3101-3104 [PMID: 26549592 DOI: 10.2217/fon.15.223]

60 **Bensaude O**. Inhibiting eukaryotic transcription: Which compound to choose? How to evaluate its activity? *Transcription* 2011; **2**: 103-108 [PMID: 21922053 DOI: 10.4161/trns.2.3.16172]

61 **Lindell TJ**, Weinberg F, Morris PW, Roeder RG, Rutter WJ. Specific inhibition of nuclear RNA polymerase Ⅱ by alpha-amanitin. *Science* 1970; **170**: 447-449 [PMID: 4918258 DOI: 10.1126/science.170.3956.447]

62 **Letschert K**, Faulstich H, Keller D, Keppler D. Molecular characterization and inhibition of amanitin uptake into human hepatocytes. *Toxicol Sci* 2006; **91**: 140-149 [PMID: 16495352 DOI: 10.1093/toxsci/kfj141]

63 **Moldenhauer G**, Salnikov AV, Lüttgau S, Herr I, Anderl J, Faulstich H. Therapeutic potential of amanitin-conjugated anti-epithelial cell adhesion molecule monoclonal antibody against pancreatic carcinoma. *J Natl Cancer Inst* 2012; **104**: 622-634 [PMID: 22457476 DOI: 10.1093/jnci/djs140]

64 **Liu Y**, Zhang X, Han C, Wan G, Huang X, Ivan C, Jiang D, Rodriguez-Aguayo C, Lopez-Berestein G, Rao PH, Maru DM, Pahl A, He X, Sood AK, Ellis LM, Anderl J, Lu X. TP53 loss creates therapeutic vulnerability in colorectal cancer. *Nature* 2015; **520**: 697-701 [PMID: 25901683 DOI: 10.1038/nature14418]

65 **Jones S**, Chen WD, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, Traulsen A, Nowak MA, Siegel C, Velculescu VE, Kinzler KW, Vogelstein B, Willis J, Markowitz SD. Comparative lesion sequencing provides insights into tumor evolution. *Proc Natl Acad Sci USA* 2008; **105**: 4283-4288 [PMID: 18337506 DOI: 10.1073/pnas.0712345105]

66 **Hanahan D**, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012; **21**: 309-322 [PMID: 22439926 DOI: 10.1016/j.ccr.2012.02.022]

67 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

68 **Okada F**, Kawaguchi T, Habelhah H, Kobayashi T, Tazawa H, Takeichi N, Kitagawa T, Hosokawa M. Conversion of human colonic adenoma cells to adenocarcinoma cells through inflammation in nude mice. *Lab Invest* 2000; **80**: 1617-1628 [PMID: 11092522 DOI: 10.1038/labinvest.3780172]

69 **Palucka K**, Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* 2012; **12**: 265-277 [PMID: 22437871 DOI: 10.1038/nrc3258]

70 **Michielsen AJ**, Noonan S, Martin P, Tosetto M, Marry J, Biniecka M, Maguire AA, Hyland JM, Sheahan KD, O'Donoghue DP, Mulcahy HE, Fennelly D, Ryan EJ, O'Sullivan JN. Inhibition of dendritic cell maturation by the tumor microenvironment correlates with the survival of colorectal cancer patients following bevacizumab treatment. *Mol Cancer Ther* 2012; **11**: 1829-1837 [PMID: 22675042 DOI: 10.1158/1535-7163.MCT-12-0162]

71 **O'Toole A**, Michielsen AJ, Nolan B, Tosetto M, Sheahan K, Mulcahy HE, Winter DC, Hyland JM, O'Connell PR, Fennelly D, O'Donoghue D, O'Sullivan J, Doherty GA, Ryan EJ. Tumour microenvironment of both early- and late-stage colorectal cancer is equally immunosuppressive. *Br J Cancer* 2014; **111**: 927-932 [PMID: 25058349 DOI: 10.1038/bjc.2014.367]

72 **Cavnar MJ**, Turcotte S, Katz SC, Kuk D, Gönen M, Shia J, Allen PJ, Balachandran VP, D'Angelica MI, Kingham TP, Jarnagin WR, DeMatteo RP. Tumor-Associated Macrophage Infiltration in Colorectal Cancer Liver Metastases is Associated With Better Outcome. *Ann Surg Oncol* 2017; **24**: 1835-1842 [PMID: 28213791 DOI: 10.1245/s10434-017-5812-8]

73 **Koelzer VH**, Canonica K, Dawson H, Sokol L, Karamitopoulou-Diamantis E, Lugli A, Zlobec I. Phenotyping of tumor-associated macrophages in colorectal cancer: Impact on single cell invasion (tumor budding) and clinicopathological outcome. *Oncoimmunology* 2015; **5**: e1106677 [PMID: 27141391 DOI: 10.1080/2162402x.2015.1106677]

74 **Zhang QW**, Liu L, Gong CY, Shi HS, Zeng YH, Wang XZ, Zhao YW, Wei YQ. Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PLoS One* 2012; **7**: e50946 [PMID: 23284651 DOI: 10.1371/journal.pone.0050946]

75 **Mantovani A**, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* 2017; **14**: 399-416 [PMID: 28117416 DOI: 10.1038/nrclinonc.2016.217]

76 **Bonavita E**, Galdiero MR, Jaillon S, Mantovani A. Phagocytes as Corrupted Policemen in Cancer-Related Inflammation. *Adv Cancer Res* 2015; **128**: 141-171 [PMID: 26216632 DOI: 10.1016/bs.acr.2015.04.013]

77 **Bonavita E**, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, Greco C, Feruglio F, Molgora M, Laface I, Tartari S, Doni A, Pasqualini F, Barbati E, Basso G, Galdiero MR, Nebuloni M, Roncalli M, Colombo P, Laghi L, Lambris JD, Jaillon S, Garlanda C, Mantovani A. PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer. *Cell* 2015; **160**: 700-714 [PMID: 25679762 DOI: 10.1016/j.cell.2015.01.004]

78 **Kitamura T**, Qian BZ, Soong D, Cassetta L, Noy R, Sugano G, Kato Y, Li J, Pollard JW. CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. *J Exp Med* 2015; **212**: 1043-1059 [PMID: 26056232 DOI: 10.1084/jem.20141836]

79 **Malesci A**, Bianchi P, Celesti G, Basso G, Marchesi F, Grizzi F, Di Caro G, Cavalleri T, Rimassa L, Palmqvist R, Lugli A, Koelzer VH, Roncalli M, Mantovani A, Ogino S, Laghi L. Tumor-associated macrophages and response to 5-fluorouracil adjuvant therapy in stage Ⅲ colorectal cancer. *Oncoimmunology* 2017; **6**: e1342918 [PMID: 29209561 DOI: 10.1080/2162402X.2017.1342918]

80 **Scurr M**, Gallimore A, Godkin A. T cell subsets and colorectal cancer: discerning the good from the bad. *Cell Immunol* 2012; **279**: 21-24 [PMID: 23041206 DOI: 10.1016/j.cellimm.2012.08.004]

81 **Baxevanis CN**, Papamichail M, Perez SA. Immune classification of colorectal cancer patients: impressive but how complete? *Expert Opin Biol Ther* 2013; **13**: 517-526 [PMID: 23289642 DOI: 10.1517/14712598.2013.751971]

82 **Senovilla L**, Vacchelli E, Galon J, Adjemian S, Eggermont A, Fridman WH, Sautès-Fridman C, Ma Y, Tartour E, Zitvogel L, Kroemer G, Galluzzi L. Trial watch: Prognostic and predictive value of the immune infiltrate in cancer. *Oncoimmunology* 2012; **1**: 1323-1343 [PMID: 23243596 DOI: 10.4161/onci.22009]

83 **Zlobec I**, Terracciano LM, Lugli A. Local recurrence in mismatch repair-proficient colon cancer predicted by an infiltrative tumor border and lack of CD8+ tumor-infiltrating lymphocytes. *Clin Cancer Res* 2008; **14**: 3792-3797 [PMID: 18559598 DOI:

10.1158/1078-0432.CCR-08-0048]
84 **Galon J**, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; **313**: 1960-1964 [PMID: 17008531 DOI: 10.1126/science.1129139]
85 **Deschoolmeester V**, Baay M, Lardon F, Pauwels P, Peeters M. Immune Cells in Colorectal Cancer: Prognostic Relevance and Role of MSI. *Cancer Microenviron* 2011; **4**: 377-392 [PMID: 21618031 DOI: 10.1007/s12307-011-0068-5]
86 **Deschoolmeester V**, Baay M, Van Marck E, Weyler J, Vermeulen P, Lardon F, Vermorken JB. Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. *BMC Immunol* 2010; **11**: 19 [PMID: 20385003 DOI: 10.1186/1471-2172-11-19]
87 **Nosho K**, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA, Giovannucci E, Dranoff G, Fuchs CS, Ogino S. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010; **222**: 350-366 [PMID: 20927778 DOI: 10.1002/path.2774]
88 **Sears CL**, Garrett WS. Microbes, microbiota, and colon cancer. *Cell Host Microbe* 2014; **15**: 317-328 [PMID: 24629338 DOI: 10.1016/j.chom.2014.02.007]
89 **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]
90 **Suau A**, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* 1999; **65**: 4799-4807 [PMID: 10543789]
91 **Sears CL**, Geis AL, Housseau F. Bacteroides fragilis subverts mucosal biology: from symbiont to colon carcinogenesis. *J Clin Invest* 2014; **124**: 4166-4172 [PMID: 25105360 DOI: 10.1172/JCI72334]
92 **Kostic AD**, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M, Garrett WS. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013; **14**: 207-215 [PMID: 23954159 DOI: 10.1016/j.chom.2013.07.007]
93 **Castellarin M**, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012; **22**: 299-306 [PMID: 22009989 DOI: 10.1101/gr.126516.111]
94 **Gao Z**, Guo B, Gao R, Zhu Q, Qin H. Microbiota disbiosis is associated with colorectal cancer. *Front Microbiol* 2015; **6**: 20 [PMID: 25699023 DOI: 10.3389/fmicb.2015.00020]
95 **Kostic AD**, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS, Meyerson M. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* 2012; **22**: 292-298 [PMID: 22009990 DOI: 10.1101/gr.126573.111]
96 **Rubinstein MR**, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. *Cell Host Microbe* 2013; **14**: 195-206 [PMID: 23954158 DOI: 10.1016/j.chom.2013.07.012]
97 **Vétizou M**, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, Rusakiewicz S, Routy B, Roberti MP, Duong CP, Poirier-Colame V, Roux A, Becharef S, Formenti S, Golden E, Cording S, Eberl G, Schlitzer A, Ginhoux F, Mani S, Yamazaki T, Jacquelot N, Enot DP, Bérard M, Nigou J, Opolon P, Eggermont A, Woerther PL, Chachaty E, Chaput N, Robert C, Mateus C, Kroemer G, Raoult D, Boneca IG, Carbonnel F, Chamaillard M, Zitvogel L. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* 2015; **350**: 1079-1084 [PMID: 26541610 DOI: 10.1126/science.aad1329]
98 **Sivan A**, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, Benyamin FW, Lei YM, Jabri B, Alegre ML, Chang EB, Gajewski TF. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* 2015; **350**: 1084-1089 [PMID: 26541606 DOI: 10.1126/science.aac4255]
99 **Söreide K**, Janssen EA, Söiland H, Körner H, Baak JP. Microsatellite instability in colorectal cancer. *Br J Surg* 2006; **93**: 395-406 [PMID: 16555243 DOI: 10.1002/bjs.5328]
100 **Couzin-Frankel J**. Breakthrough of the year 2013. Cancer immunotherapy. *Science* 2013; **342**: 1432-1433 [PMID: 24357284 DOI: 10.1126/science.342.6165.1432]
101 **Brahmer JR**, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; **366**: 2455-2465 [PMID: 22658128 DOI: 10.1056/NEJMoa1200694]
102 **Topalian SL**, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; **366**: 2443-2454 [PMID: 22658127 DOI: 10.1056/NEJMoa1200690]
103 **Hiniker SM**, Maecker HT, Knox SJ. Predictors of clinical response to immunotherapy with or without radiotherapy. *J Radiat Oncol* 2015; **4**: 339-345 [PMID: 26709361 DOI: 10.1007/s13566-015-0219-2]
104 **Schumacher TN**, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* 2015; **348**: 69-74 [PMID: 25838375 DOI: 10.1126/science.aaa4971]
105 **Saeterdal I**, Bjørheim J, Lislerud K, Gjertsen MK, Bukholm IK, Olsen OC, Nesland JM, Eriksen JA, Møller M, Lindblom A, Gaudernack G. Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer. *Proc Natl Acad Sci USA* 2001; **98**: 13255-13260 [PMID: 11687624 DOI: 10.1073/pnas.231326898]
106 **Turksma AW**, Coupé VM, Shamier MC, Lam KL, de Weger VA, Belien JA, van den Eertwegh AJ, Meijer GA, Meijer CJ, Hooijberg E. Extent and Location of Tumor-Infiltrating Lymphocytes in Microsatellite-Stable Colon Cancer Predict Outcome to Adjuvant Active Specific Immunotherapy. *Clin Cancer Res* 2016; **22**: 346-356 [PMID: 26420855 DOI: 10.1158/1078-0432.CCR-13-2462]
107 **Phillips SM**, Banerjea A, Feakins R, Li SR, Bustin SA, Dorudi S. Tumour-infiltrating lymphocytes in colorectal cancer with microsatellite instability are activated and cytotoxic. *Br J Surg* 2004; **91**: 469-475 [PMID: 15048750 DOI: 10.1002/bjs.4472]
108 **Quinn E**, Hawkins N, Yip YL, Suter C, Ward R. CD103+ intraepithelial lymphocytes--a unique population in microsatellite unstable sporadic colorectal cancer. *Eur J Cancer* 2003; **39**: 469-475 [PMID: 12751377 DOI: 10.1016/S0959-8049(02)00633-0]
109 **Overman MJ**, Kopetz S, McDermott RS, Leach J, Lonardi S, Lenz H-J, Morse MA, Desai J, Hill A, Axelson MD, Moss RA, Lin C-S, Goldberg M, Andre T. Nivolumab ± ipilimumab in treatment (tx) of patients (pts) with metastatic colorectal cancer (mCRC) with and without high microsatellite instability (MSI-H): CheckMate-142 interim results. *J Clin Oncol* 2016; **34**: 3501-3501 [DOI: 10.1200/JCO.2016.34.15_suppl.3501]
110 **Branch P**, Bicknell DC, Rowan A, Bodmer WF, Karran P. Immune surveillance in colorectal carcinoma. *Nat Genet* 1995; **9**: 231-232 [PMID: 7773283 DOI: 10.1038/ng0395-231]
111 **Wallich R**, Bulbuc N, Hämmerling GJ, Katzav S, Segal S, Feldman M. Abrogation of metastatic properties of tumour cells

by de novo expression of H-2K antigens following H-2 gene transfection. *Nature* 1985; **315**: 301-305 [PMID: 3873616 DOI: 10.1038/315301a0]

112 **Page DB**, Postow MA, Callahan MK, Allison JP, Wolchok JD. Immune modulation in cancer with antibodies. *Annu Rev Med* 2014; **65**: 185-202 [PMID: 24188664 DOI: 10.1146/annurev-med-092012-112807]

113 **Pardoll DM**. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; **12**: 252-264 [PMID: 22437870 DOI: 10.1038/nrc3239]

114 **Galluzzi L**, Buqué A, Kepp O, Zitvogel L, Kroemer G. Immunogenic cell death in cancer and infectious disease. *Nat Rev Immunol* 2017; **17**: 97-111 [PMID: 27748397 DOI: 10.1038/nri.2016.107]

115 **Tesniere A**, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F, Aymeric L, Michaud M, Apetoh L, Barault L, Mendiboure J, Pignon JP, Jooste V, van Endert P, Ducreux M, Zitvogel L, Piard F, Kroemer G. Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene* 2010; **29**: 482-491 [PMID: 19881547 DOI: 10.1038/onc.2009.356]

116 **Hochster HS**, Bendell JC, Cleary JM, Foster P, Zhang W, He X, Hernandez G, Iizuka K, Eckhardt SG. Efficacy and safety of atezolizumab (atezo) and bevacizumab (bev) in a phase Ib study of microsatellite instability (MSI)-high metastatic colorectal cancer (mCRC). *J Clin Oncol* 2017; **35**: 673-673 [DOI: 10.1200/JCO.2017.35.4_suppl.673]

117 **Srivastava RM**, Lee SC, Andrade Filho PA, Lord CA, Jie HB, Davidson HC, López-Albaitero A, Gibson SP, Gooding WE, Ferrone S, Ferris RL. Cetuximab-activated natural killer and dendritic cells collaborate to trigger tumor antigen-specific T-cell immunity in head and neck cancer patients. *Clin Cancer Res* 2013; **19**: 1858-1872 [PMID: 23444227 DOI: 10.1158/1078-0432.CCR-12-2426]

118 **Gulley JL**, Madan RA, Pachynski R, Mulders P, Sheikh NA, Trager J, Drake CG. Role of Antigen Spread and Distinctive Characteristics of Immunotherapy in Cancer Treatment. *J Natl Cancer Inst* 2017; **109**: [PMID: 28376158 DOI: 10.1093/jnci/djw261]

119 **Fujiyoshi K**, Yamamoto G, Takenoya T, Takahashi A, Arai Y, Yamada M, Kakuta M, Yamaguchi K, Akagi Y, Nishimura Y, Sakamoto H, Akagi K. Metastatic Pattern of Stage Ⅳ Colorectal Cancer with High-Frequency Microsatellite Instability as a Prognostic Factor. *Anticancer Res* 2017; **37**: 239-247 [PMID: 28011498 DOI: 10.21873/anticanres.11313]

120 **Merok MA**, Ahlquist T, Røyrvik EC, Tufteland KF, Hektoen M, Sjo OH, Mala T, Svindland A, Lothe RA, Nesbakken A. Microsatellite instability has a positive prognostic impact on stage Ⅱ colorectal cancer after complete resection: results from a large, consecutive Norwegian series. *Ann Oncol* 2013; **24**: 1274-1282 [PMID: 23235802 DOI: 10.1093/annonc/mds614]

121 **de la Chapelle A**, Hampel H. Clinical relevance of microsatellite instability in colorectal cancer. *J Clin Oncol* 2010; **28**: 3380-3387 [PMID: 20516444 DOI: 10.1200/JCO.2009.27.0652]

122 **Popat S**, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005; **23**: 609-618 [PMID: 15659508 DOI: 10.1200/jco.2005.01.086]

123 **Ebert PJR**, Cheung J, Yang Y, McNamara E, Hong R, Moskalenko M, Gould SE, Maecker H, Irving BA, Kim JM, Belvin M, Mellman I. MAP Kinase Inhibition Promotes T Cell and Anti-tumor Activity in Combination with PD-L1 Checkpoint Blockade. *Immunity* 2016; **44**: 609-621 [PMID: 26944201 DOI: 10.1016/j.immuni.2016.01.024]

124 **Tan N**, Wong M, Nannini MA, Hong R, Lee LB, Price S, Williams K, Savy PP, Yue P, Sampath D, Settleman J, Fairbrother WJ, Belmont LD. Bcl-2/Bcl-xL inhibition increases the efficacy of MEK inhibition alone and in combination with PI3 kinase inhibition in lung and pancreatic tumor models. *Mol Cancer Ther* 2013; **12**: 853-864 [PMID: 23475955 DOI: 10.1158/1535-7163.MCT-12-0949]

125 **Hoeflich KP**, Merchant M, Orr C, Chan J, Den Otter D, Berry L, Kasman I, Koeppen H, Rice K, Yang NY, Engst S, Johnston S, Friedman LS, Belvin M. Intermittent administration of MEK inhibitor GDC-0973 plus PI3K inhibitor GDC-0941 triggers robust apoptosis and tumor growth inhibition. *Cancer Res* 2012; **72**: 210-219 [PMID: 22084396 DOI: 10.1158/0008-5472.CAN-11-1515]

126 **Brea EJ**, Oh CY, Manchado E, Budhu S, Gejman RS, Mo G, Mondello P, Han JE, Jarvis CA, Ulmert D, Xiang Q, Chang AY, Garippa RJ, Merghoub T, Wolchok JD, Rosen N, Lowe SW, Scheinberg DA. Kinase Regulation of Human MHC Class Ⅰ Molecule Expression on Cancer Cells. *Cancer Immunol Res* 2016; **4**: 936-947 [PMID: 27680026 DOI: 10.1158/2326-6066.Cir-16-0177]

127 **Liu L**, Mayes PA, Eastman S, Shi H, Yadavilli S, Zhang T, Yang J, Seestaller-Wehr L, Zhang SY, Hopson C, Tsvetkov L, Jing J, Zhang S, Smothers J, Hoos A. The BRAF and MEK Inhibitors Dabrafenib and Trametinib: Effects on Immune Function and in Combination with Immunomodulatory Antibodies Targeting PD-1, PD-L1, and CTLA-4. *Clin Cancer Res* 2015; **21**: 1639-1651 [PMID: 25589619 DOI: 10.1158/1078-0432.CCR-14-2339]

128 **Pollack BP**, Sapkota B, Cartee TV. Epidermal growth factor receptor inhibition augments the expression of MHC class Ⅰ and Ⅱ genes. *Clin Cancer Res* 2011; **17**: 4400-4413 [PMID: 21586626 DOI: 10.1158/1078-0432.CCR-10-3283]

129 **Mimura K**, Shiraishi K, Mueller A, Izawa S, Kua LF, So J, Yong WP, Fujii H, Seliger B, Kiessling R, Kono K. The MAPK pathway is a predominant regulator of HLA-A expression in esophageal and gastric cancer. *J Immunol* 2013; **191**: 6261-6272 [PMID: 24244023 DOI: 10.4049/jimmunol.1301597]

130 **Holmgaard RB**, Brachfeld A, Gasmi B, Jones DR, Mattar M, Doman T, Murphy M, Schaer D, Wolchok JD, Merghoub T. Timing of CSF-1/CSF-1R signaling blockade is critical to improving responses to CTLA-4 based immunotherapy. *Oncoimmunology* 2016; **5**: e1151595 [PMID: 27622016 DOI: 10.1080/2162402X.2016.1151595]

131 **Limagne E**, Euvrard R, Thibaudin M, Rébé C, Derangère V, Chevriaux A, Boidot R, Végran F, Bonnefoy N, Vincent J, Bengrine-Lefevre L, Ladoire S, Delmas D, Apetoh L, Ghiringhelli F. Accumulation of MDSC and Th17 Cells in Patients with Metastatic Colorectal Cancer Predicts the Efficacy of a FOLFOX-Bevacizumab Drug Treatment Regimen. *Cancer Res* 2016; **76**: 5241-5252 [PMID: 27496709 DOI: 10.1158/0008-5472.CAN-15-3164]

132 **Marabelle A**, Kohrt H, Caux C, Levy R. Intratumoral immunization: a new paradigm for cancer therapy. *Clin Cancer Res* 2014; **20**: 1747-1756 [PMID: 24691639 DOI: 10.1158/1078-0432.CCR-13-2116]

133 **Van der Jeught K**, Bialkowski L, Daszkiewicz L, Broos K, Goyvaerts C, Renmans D, Van Lint S, Heirman C, Thielemans K, Breckpot K. Targeting the tumor microenvironment to enhance antitumor immune responses. *Oncotarget* 2015; **6**: 1359-1381 [PMID: 25682197 DOI: 10.18632/oncotarget.3204]

134 **Van der Jeught K**, Van Lint S, Thielemans K, Breckpot K. Intratumoral delivery of mRNA: Overcoming obstacles for effective immunotherapy. *Oncoimmunology* 2015; **4**: e1005504 [PMID: 26155403 DOI: 10.1080/2162402X.2015.1005504]

135 **Sagiv-Barfi I**, Czerwinski DK, Levy S, Alam IS, Mayer AT, Gambhir SS, Levy R. Eradication of spontaneous malignancy by local immunotherapy. *Sci Transl Med* 2018; **10**: [PMID: 29386357 DOI: 10.1126/scitranslmed.aan4488]

136 **Calon A**, Lonardo E, Berenguer-Llergo A, Espinet E, Hernando-Momblona X, Iglesias M, Sevillano M, Palomo-Ponce S, Tauriello DV, Byrom D, Cortina C, Morral C, Barceló C, Tosi S, Riera A, Attolini CS, Rossell D, Sancho E, Batlle E. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet* 2015; **47**: 320-329 [PMID: 25706628 DOI: 10.1038/ng.3225]

137 **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]

138 **Van der Jeught K**, Joe PT, Bialkowski L, Heirman C, Daszkiewicz L, Liechtenstein T, Escors D, Thielemans K, Breckpot K. Intratumoral administration of mRNA encoding a fusokine consisting of IFN-β and the ectodomain of the TGF-β receptor Ⅱ potentiates antitumor immunity. *Oncotarget* 2014; **5**: 10100-10113 [PMID: 25338019 DOI: 10.18632/oncotarget.2463]

139 **Xuan C**, Steward KK, Timmerman JM, Morrison SL. Targeted delivery of interferon-alpha via fusion to anti-CD20 results in potent antitumor activity against B-cell lymphoma. *Blood* 2010; **115**: 2864-2871 [PMID: 20139095 DOI: 10.1182/blood-2009-10-250555]

140 **Yang X**, Zhang X, Fu ML, Weichselbaum RR, Gajewski TF, Guo Y, Fu YX. Targeting the tumor microenvironment with interferon-β bridges innate and adaptive immune responses. *Cancer Cell* 2014; **25**: 37-48 [PMID: 24434209 DOI: 10.1016/j.ccr.2013.12.004]

141 **Tabernero J**, Melero I, Ros W, Argiles G, Marabelle A, Rodriguez-Ruiz ME, Albanell J, Calvo E, Moreno V, Cleary JM, Eder JP, Karanikas V, Bouseida S, Sandoval F, Sabanes D, Sreckovic S, Hurwitz H, Paz-Ares LG, Suarez JMS, Segal NH. Phase Ia and Ib studies of the novel carcinoembryonic antigen (CEA) T-cell bispecific (CEA CD3 TCB) antibody as a single agent and in combination with atezolizumab: Preliminary efficacy and safety in patients with metastatic colorectal cancer (mCRC). *J Clin Oncol* 2017; **35**: 3002-3002 [DOI: 10.1200/JCO.2017.35.15_suppl.3002]

P- Reviewer: Grizzi F, Kim T, Paoluzi OA, Vynios D
S- Editor: Wang XJ **L- Editor**: Filipodia **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3849-3860

DOI: 10.3748/wjg.v24.i34.3849

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

MINIREVIEWS

Role of two-dimensional shear wave elastography in chronic liver diseases: A narrative review

Jae Yoon Jeong, Young Seo Cho, Joo Hyun Sohn

Jae Yoon Jeong, Joo Hyun Sohn, Department of Internal Medicine, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri 11923, South Korea

Young Seo Cho, Department of Radiology, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri 11923, South Korea

ORCID number: Jae Yoon Jeong (0000-0002-3624-3261); Young Seo Cho (0000-0003-4034-7271); Joo Hyun Sohn (0000-0002-8977-4509).

Author contributions: All the authors participated in the interpretation of the study results, and in the drafting, critical revision, and approval of the final version of the manuscript.

Conflict-of-interest statement: All authors declared they have nothing to disclose concerning this study.

Manuscript source: Invited manuscript

Correspondence to: Joo Hyun Sohn, MD, PhD, Professor of Medicine, Department of Internal Medicine, Hanyang University Guri Hospital, 153 Gyeongchun-ro, Guri 11923, South Korea. sonjh@hanyang.ac.kr
Telephone: +82-31-5602225
Fax: +82-31-5552998

Received: June 27, 2018
Peer-review started: July 2, 2018
First decision: July 12, 2018
Revised: July 18, 2018
Accepted: July 21, 2018
Article in press: July 21, 2018
Published online: September 14, 2018

Abstract

Liver biopsy is the gold standard for evaluating the degree of liver fibrosis in patients with chronic liver disease. However, due to the many limitations of liver biopsy, there has been much interest in the use of noninvasive techniques for this purpose. Among these techniques real-time two-dimensional shear wave elastography (2D-SWE) has the advantage of measuring tissue elasticity with the guidance of B-mode images. Recently, many studies have been conducted on the application of 2D-SWE in patients with various liver diseases, and their validity has been confirmed. Here, we briefly discuss the role of 2D-SWE in patients with chronic liver diseases, particularly aspects of the examination techniques and clinical applications.

Key words: Shear wave elastography; Liver disease; Liver fibrosis; Portal hypertension; Hepatocelluar carcinoma

Core tip: Assessing the degree of liver fibrosis in patients with chronic liver disease is clinically important. Real-time two-dimensional shear wave elastography (2D-SWE) has the advantage of measuring tissue elasticity with the guidance of B-mode images. Recently, many studies have shown that 2D-SWE is a useful tool for evaluating not only liver fibrosis in various liver diseases but also portal hypertension, and for predicting the development of hepatocellular carcinoma. Here, we discuss briefly the role of 2D-SWE in patients with chronic liver diseases, particularly aspects of the examination technique and clinical applications.

Jeong JY, Cho YS, Sohn JH. Role of two-dimensional shear wave elastography in chronic liver diseases: A narrative review. *World J Gastroenterol* 2018; 24(34): 3849-3860 Available from: URL:

http://www.wjgnet.com/1007-9327/full/v24/i34/3849.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3849

INTRODUCTION

Chronic liver diseases are one of the major causes of illness and death worldwide, and a substantial public health issue. Chronic liver diseases can lead to liver fibrosis due to transient or persistent intrahepatic inflammation, and some eventually progress to liver cirrhosis and hepatocellular carcinoma[1]. Therefore, assessing the degree of fibrosis in patients with chronic liver diseases, especially before the advanced stage, is clinically important to allow early care and prevent fatal liver disease[1].

To date, the gold standard for evaluating the degree of liver fibrosis is liver biopsy[2]. However, it has several limitations[3]. Because it is an invasive method, it may cause pain, bleeding and perforation[3], and can uncommonly lead to massive bleeding that requires blood transfusion, or to death[3]. Also, it has limitations for representing the whole liver parenchyma because it evaluates only about 1/50000 of the total liver volume and there is potential for sampling errors and interobserver or intraobserver variability of interpretation[4,5].

Because of these limitations of liver biopsy, there has been much interest in noninvasive techniques for assessing the degree of liver fibrosis[6]. In particular, several ultrasonography-based elastographic methods have been developed in the past decade, and evaluation of liver fibrosis by measuring liver stiffness (LS) has been the main type of noninvasive method[6]. Transient elastography (TE), which was the first method introduced into the market, is a highly reproducible and user-friendly technique for evaluating liver fibrosis, and is also used for assessing portal hypertension and predicting the development of hepatocellular carcinoma (HCC)[7]. However, it has some limitations, including frequent invalid results especially in patients with ascites or severe obesity[7]. Also the attempts to ameliorate diagnostic accuracy, adding to TE the calculation realized by software of quantitative measurements of the Glissonian line, have failed[8].

Real-time two-dimensional shear wave elastography (2D-SWE), which was developed subsequent to TE, can measure tissue elasticity with the real-time guidance of B-mode image. Recently there have been many studies of 2D-SWE, related especially to examination technique and clinical applications. In this article, we review the focusing 2D SWE technique using the Aixplorer ultrasound (US) system (Supersonic Imagine SA, Aix-en-Provence, France).

MEASURING LIVER STIFFNESS

Measurements of liver stiffness (LS) using 2D-SWE are usually performed through right intercostal scans,with the patient in a supine position. Because the sonographic window gets clearer as the intercostal space enlarges, LS is measured with right arm maximal abduction. Deep inspiration is avoided as it increases the measured LS value, and, if possible, LS is measured with a short breath hold for 4 to 5 s and neutral breathing. A trapezoidal color box (3.5 cm × 2.5 cm) is positioned in the liver parenchyma and acquires the elasticity signals. When the elastogram signals in the color box are judged to reach a plateau, *i.e.*, after about 2 or 3 s, the image is frozen. After call-back, the most homogenous areas of elastogram signals among the sequential frames are identified using a cine loop, and a round ROI (also referred to as the Q-box) is positioned in the region of the color box. The brighter the grayscale image obtained without shadowing in the scan, the more uniform the elastogram signal generated. The ROI is located in a homogenous elastogram signal in the liver parenchyma where there is no large vessel or hepatic nodule. To avoid reverberation artifacts, ROIs are located 1 to 2 cm from the liver capsule. The ROI is as large as possible and up to 2 cm in diameter, but its size is reduced if necessary, depending on the measurable areas of the elastogram signal and the location of large vessels. Also, if the measurement depth is too great, a qualitatious elastogram signal is not generated and the signal is less reliable; measurement should preferably be at a depth of less than 6 cm from the capsule. Measured elasticity values are expressed in kilopascal (kPa) and recorded on the image as means and standard deviations (Figure 1).

Technically, measurement of LS using 2D-SWE has several advantages. It is not affected by ascites, because the shear waves are generated by the focused beam inside the liver parenchyma rather than at the surface of the body. Large vessels can be avoided using simultaneous gray scale images, and the sampling volume is larger than in p-SWE. By means of real-time color mapping, an experienced examiner can judge whether measurements are reliable.

Optimal region and number of measurements, and validation

LS was measured in the right lobe in all previous studies. Measurement of LS in the left lobe is inappropriate, because it is affected by cardiac pulsation. Most measurements of LS by 2D-SWE use an intercostal scan, and they are usually made in the right anterior section. When measured in this way, measurement reliability is high and the correlation with histologic hepatic fibrosis staging is good[9,10].

When LS is measured by TE, it is measured 10 times and validated using a success rate of 60% or more and interquartile range/median (IQR/M) < 0.3, and the median value of the measurements is selected as the LS value. However there is no agreement on the objective number of measurements needed or on the quality criteria for validation of 2D-SWE. Most

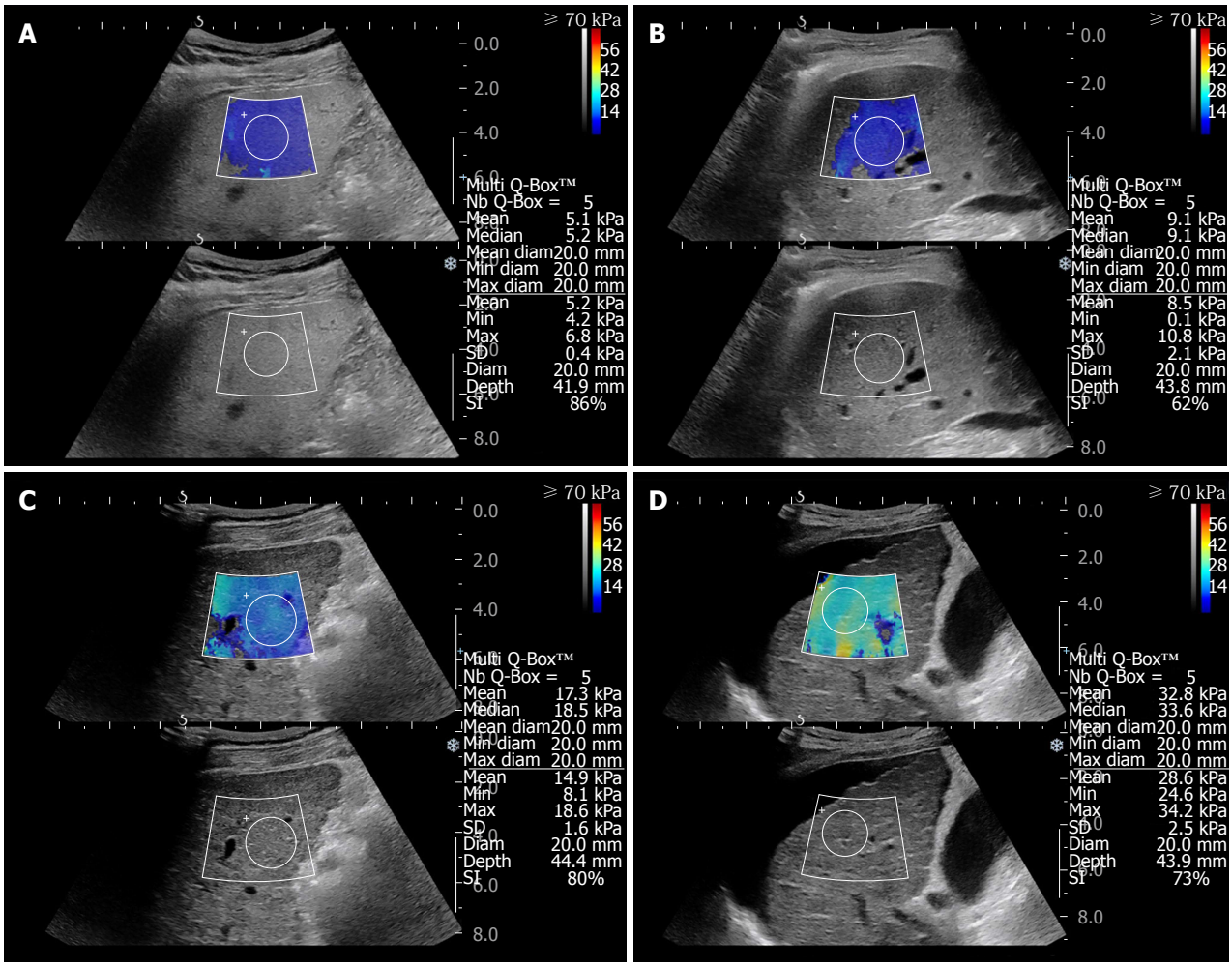


Figure 1 Liver two-dimensional shear wave elastography images. A. 2D-SWE images of a 52-year-old patient without underlying disease with normal range of LS. Ultrasound images show the color-code mapping of 2D-SWE (top) and the corresponding B-mode image (bottom). On the right side of the image, the mean (5.2 kPa) and standard deviation (0.4 kPa) of Young modulus in the ROI have been calculated. And the size and depth of the measured ROI are recorded. The summarized values at the top are the mean and median values of the stiffness values of the previous 4 measurements and the 5th measurement, and the average sizes of the measured ROI. B. A 2D-SWE image of a 58-year-old patient with chronic hepatitis B who was proven as F2 fibrosis in liver biopsy specimen. Increased LS (8.5 kPa) was identified compared to normal patients. C. In 55-year-old patient with chronic hepatitis B and compensated cirrhosis, median LS was 18.5 kPa. D. In 71-year-old patient with chronic hepatitis B and decompensated cirrhosis with ascites, median LS was 33.6 kPa. 2D-SWE: Two-dimensional shear wave elastography; LS: Liver stiffness; ROI: Region of interest.

studies using 2D-SWE have measured LS with 3 to 5 repetitions. According to previous studies of the number of LS measurements, when LS is measured 6 or more times no further increase in intra-class correlation (ICC) is observed[11], and the LS from a 10-repetition protocol is not significantly different from that from a 5-repetition protocol[12]. Another group has concluded that three valid measurements are enough[13]. There is no evidence about whether the mean or median values of repeated measurements correlate better with liver fibrosis. There are quality criteria for LS measurements by 2D-SWE, such as standard deviation (SD), IQR/M and coefficient of variance (CV, SD/mean), but there is no established standard of validation as there is for TE. Therefore, we suggest that three to five measurements of LS by 2D-SWE are appropriate, and in case of validation by IQR/M, five measurements are required.

In LS measurement using 2D-SWE, it is measured faster and more consistently in a patient with a good sonographic window for B-mode images. In the patients with obese and thick abdominal wall, the shadowing occurs in the liver parenchyma and the elasticity signal is not generated well in the color box. In case of poor sonographic window due to severe shrinkage of liver and interposition of omental fat or bowel, the measurement is not successful. And, if the motion is not restricted because the patient is not coordinated, or the liver is affected by cardiac movement, there is a limitation in the measurement. 2D-SWE has more chance to be affected by technical factors because it has larger sampling volume compared to TE or point shear wave elastography. However, the measurement failure rate of 2D-SWE is lower than that of TE when the experienced examiner measures LS[14,15].

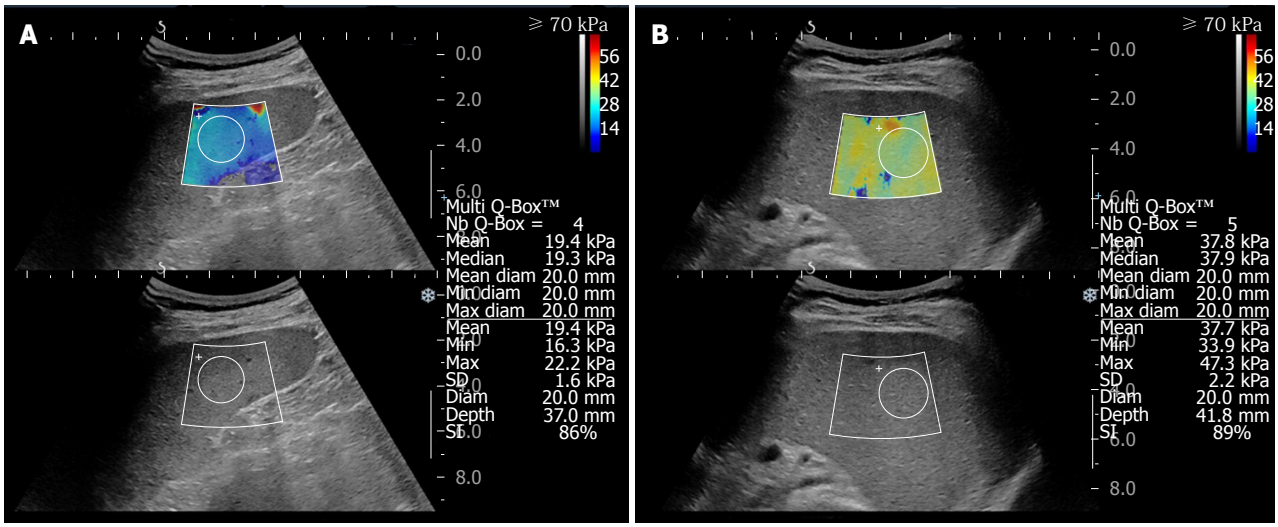


Figure 2 Spleen two-dimensional shear wave elastography images. Spleen 2D-SWE images of a 50-year-old male patient with normal SS (A) and 57-year-old female patient with liver cirrhosis who underwent endoscopic variceal ligation (B). A. The normal patient had a small size and measurable area of spleen. And the SS was measured to 19.4 kPa. B. Patient with liver cirrhosis had relatively large size and measurable area of spleen with good sonographic window. Increased spleen stiffness compared with that of normal patients was identified (37.7 kPa). 2D-SWE: Two-dimensional shear wave elastography; SS: Spleen stiffness.

Reproducibility

The reproducibility of LS measurements by 2D-SWE is high but user-dependent[16]. The intra-observer reproducibility of 2D-SWE in healthy volunteers is excellent (ICC 0.92 to 0.95)[16-18]. Inter-observer agreement is good (0.63 to 0.84[16,18]) and is influenced by operator experience. In the chronic liver disease group, intra-observer reproducibility is excellent, with an ICC of 0.9 to 0.95[11,19,20], and intra-subject reproducibility at short intervals is excellent, with an ICC of 0.83 to 0.9[21]. The inter-observer reproducibility of LS measurements using 2D-SWE is excellent, from 0.83 to 0.94[21,22].

Since 2D-SWE measurement is user-dependent, it is recommended that at least 50 supervised scans and measurements are performed by a novice operator to ensure consistent measurements[23].

Normal values of liver stiffness, and confounders

The LS value using 2D-SWE in healthy volunteers was found to be 4.5-5.5 kPa[17,24]. Food intake increases LS value and IQR[25-27], and may result in over-staging of liver fibrosis and unreliable measurements. According to Mederacke *et al*[28], LS value declines to the normal range by 180 min after food intake; hence it is recommended to measure LS at least 4 h after food consumption, or after overnight fasting. Caffeine intake, smoking, and exercise also increase LS value[29], as do acute hepatic inflammation, obstructive cholestasis, and hepatic congestion[30-36]. The effect of hepatic steatosis on LS value is not yet clear[37-40]. These confounding factors should be avoided when measuring LS, and patient co-morbidities must be considered when interpreting LS values so as to prevent over-staging of hepatic fibrosis.

MEASURING SPLEEN STIFFNESS

According to a recent meta-analysis, Spleen Stiffenss (SS) values measured by 2D-SWE are useful for predicting clinically significant portal hypertension in chronic liver diseases[41]. They are significantly correlated with the presence of esophageal varix, and are superior to LS values[42]. In addition, 2D-SWE can check real-time grayscale images at the time of measurement, so that SS can be measured in the most appropriate region. SS is measured by left intercostal or subcostal scans, and is not fundamentally different from LS measurements (Figure 2). The spleen is smaller than the liver and varies in size, and the measurement success rate is lower than that of LS (over 90%). The success rate of SS in all patient groups according to the meta-analysis was 75.5%[41], and most of the studies included (many) portal hypertension patients with advanced liver cirrhosis. However in a study by Grgurevic *et al*[43], which included many non-cirrhotic chronic liver disease patients, the success rate of SS measurements was only 53.7%. As spleen size increases, the measurement success rate of SS by 2D-SWE also increases, so that the LS and SS success rates are not significantly different in patients with advanced liver cirrhosis versus severe portal hypertension.

ROLE OF 2D-SWE IN ASSESSING LIVER FIBROSIS

Various liver diseases

Several studies have evaluated fibrosis in various liver diseases by 2D-SWE (Table 1)[44,45]. LS measured by 2D-SWE had an excellent diagnostic performance with areas under the curve (AUROCs) of about 0.9 for

Table 1 Diagnostic performance of shear wave elastography for significant fibrosis (F ⩾ 2), advanced fibrosis (F ⩾ 3) and cirrhosis (F4) in patients with various liver diseases

Ref.	Year	Patients (*n*)	F ⩾ 2 (%)	F ⩾ 3 (%)	F = 4 (%)	AUROC	Cutoffs (kPa)	Se (%)	Sp (%)	PPV (%)	NPV (%)
Jeong *et al*[44]	2014	70	78.6			0.915	8.60	78.2	93.3	97.7	53.8
				50		0.913	10.46	88.6	80.0	81.6	87.6
					31.4	0.878	14.00	77.3	85.4	70.8	89.2
Deffieux *et al*[45]	2015	120	48.0			0.890	8.90	77.0	79.0	77.0	79.0
				33		0.880	9.10	85.0	72.0	60.0	90.0
					15.0	0.890	10.20	83.0	76.0	38.0	96.0

AUROC: Area under ROC curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; SWE: Shear wave elastography.

Table 2 Diagnostic performance of shear wave elastography for significant fibrosis (F ⩾ 2), advanced fibrosis (F ⩾ 3) and cirrhosis (F4) in patients with chronic hepatitis C

Ref.	Year	Patients (*n*)	F ⩾ 2 (%)	F ⩾ 3 (%)	F = 4 (%)	AUROC	Cutoffs (kPa)	Se (%)	Sp (%)	PPV (%)	NPV (%)
Bavu *et al*[46]1	2011	113	55.8			0.950	9.12	81.0	72.0		
				34.5		0.960	10.08	75.0	78.0		
					13.3	0.970	13.30	80.0	87.0		
Ferraioli *et al*[47]	2012	121	58.7			0.920	7.10	90.0	87.5	91.3	85.7
				31.4		0.980	8.70	97.3	95.1	90.0	98.7
					19.8	0.980	10.40	87.5	96.8	87.5	96.8
Tada *et al*[48]	2013	55	32.7			0.940	8.80	88.9	91.9	84.2	94.4
Herrmann *et al*[49]	2018	379	58.3			0.863	7.10	94.7	52.0		
				33.5		0.915	9.20	90.3	76.8		
					18.2	0.929	13.00	85.8	87.8		

[1]The reference fibrosis level is derived from the algorithm proposed by Sebastiani *et al*[79]. AUROC: Area under ROC curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

assessing each stage of fibrosis[44,45]. However, since the burden of fibrosis depends on the dominant disease, the value of LS for a given stage of fibrosis is also dependent on the dominant disease in the patients that are examined. Therefore, the diagnostic performance of 2D-SWE, which was expected to be superior to other noninvasive fibrosis methods such as TE, did not show a statistically significant dependence on stage of fibrosis.

Chronic hepatitis C

Studies of the degree of fibrosis according to the disease involved were the first to evaluate patients with chronic hepatitis C (CHC). The results are summarized in Table 2. LS measured by 2D-SWE showed a significant positive correlation with fibrosis stage evaluated by the METAVIR scoring system in patients with CHC[46-48]. Also, 2D-SWE had a similar or better diagnostic performance than TE for evaluating liver fibrosis[46-48].

Bavu *et al*[46] compared 2D-SWE and TE after classifying fibrosis stage on serology without histological examination. In that study, the AUROCs for diagnoses of significant fibrosis (⩾ F2), advanced fibrosis (⩾ F3) and cirrhosis (F4) were 0.948, 0.962 and 0.968, respectively[46]. Ferraioli *et al*[47] compared 2D-SWE with TE for assessing fibrosis stage using liver biopsy specimens. The AUROCs of 2D-SWE were 0.92 for ⩾ F2, 0.98 for ⩾ F3 and 0.98 for F4, and were similar (⩾ F3 and F4) or significantly higher (⩾ F2) than those of TE[47]. In several studies the optimal cutoff values for each fibrosis stage were 7.1-9.12 kPa for ⩾ F2, 8.7-10.08 kPa for ⩾ F3, and 10.4-13.30 kPa for F4[46-48]. In recently published patient data based on a meta-analysis, the AUROCs for ⩾ F2, ⩾ F3 and F4 of 2D SWE were 0.863, 0.915 and 0.929, respectively, and the proposed cut off values were 7.1 kPa, 9.2 kPa and 13.0 kPa, respectively[49]. However, the diagnostic performance of 2D SWE for each stage of fibrosis was not significantly different from that of TE [49].

2D-SWE can be used to predict the efficacy of antiviral treatment in CHC as well as the degree of fibrosis. Tada *et al*[50] reported that patients with CHC who achieved a sustained virologic response showed an early decrease in LS after administration of a direct acting agent (DAA), and this was the case especially in patients with progressive liver fibrosis. Similarly, Korda *et al*[51] found a significant decrease in LS after DAA treatment in patients with recurrent HCV infection after liver transplantation. Therefore 2D-SWE may be a useful tool in the follow-up after treatment of CHC.

Chronic hepatitis B

So far the disease most studied for assessing degree of fibrosis by 2D-SWE is hepatitis B virus (HBV) infection. Studies of patients with chronic hepatitis B (CHB) have been mainly performed in China, where HBV is endemic. LS measured by 2D-SWE was positively

Table 3 Diagnostic performance of shear wave elastography for significant fibrosis (F ⩾ 2), advanced fibrosis (F ⩾ 3) and cirrhosis (F4) in patients with chronic hepatitis B

Ref.	Year	Patients (*n*)	F ⩾ 2 (%)	F ⩾ 3 (%)	F = 4 (%)	AUROC	Cutoffs (kPa)	Se (%)	Sp (%)	PPV (%)	NPV (%)
Leung *et al*[14]	2013	226	60.2			0.880	7.100	84.70	92.10	85.3	91.7
				35.4		0.930	7.900	89.80	90.30	71.8	97.0
					15.5	0.980	10.100	97.40	93.00	60.1	99.6
Zeng *et al*[54]1	2014	206 (104)	45.7 (45.1)			0.917 (0.907)	7.200	86.36 (85.19)	86.96 (80.85)	88.8 (83.6)	84.2 (82.6)
				69.0 (70.1)		0.945 (0.934)	9.100	91.94 (89.66)	85.71 (80.56)	74.0 (65.0)	96.0 (95.1)
					81.1 (83.7)	0.945 (0.967)	11.700	91.89 (88.24)	89.70 (88.10)	66.7 (60.0)	98.0 (97.4)
Wu *et al*[53]	2016	437	47.2			0.903	8.200	78.16	85.28	82.6	81.4
					14.0	0.926	11.256	91.80	84.31	48.7	98.4
Zhuang *et al*[55]1	2017	304(155)	86.8 (84.6)			0.970 (0.970)	7.600	92.00 (91.6)	90.00 (87.5)	98.4 (96.0)	64.3 (65.0)
				70.4 (67.8)		0.960 (0.970)	9.200	91.60 (88.6)	96.70 (96.0)	98.5 (97.8)	82.9 (80.1)
					54.9 (48.4)	0.980 (0.980)	10.400	94.60 (92.0)	94.90 (95.0)	95.7 (94.5)	93.5 (92.7)
Zeng *et al*[52]	2017	257	46.3			0.882	7.100	88.89	76.38	76.2	89.0
				24.9		0.917	8.300	89.66	76.84	55.9	95.8
					13.2	0.926	11.300	93.55	87.25	52.7	98.9
Herrmann *et al*[49]	2018	379	52.0			0.906	7.100	87.60	73.60		
				29.8		0.931	8.100	94.90	73.10		
					13.0	0.955	11.500	79.90	93.90		

[1]These studies are divided into index cohort and validation cohort and parentheses are index cohort. AUROC: Area under ROC curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

correlated with liver fibrosis stage evaluated by the METAVIR scoring system in patients with CHB, as it was for those with CHC[14,52-55].

Leung *et al*[14] reported that the AUROCs for ⩾ F2, ⩾ F3, and F4 of 2D-SWE were 0.88, 0.93, and 0.98, respectively, and 2D-SWE performed better than TE for predicting all fibrosis stages. In particular, the cutoff value of 7.1 kPa for F2 by SWE had a relatively high specificity of 92.1%, indicating that 2D-SWE is an excellent screening tool for diagnosing significant fibrosis, which is an important starting point for the treatment of chronic viral hepatitis[14]. In addition, as fibrosis progressed, the optimal cut off value had a high negative predictive value, indicating that 2D-SWE is a very reliable tool for excluding cirrhosis[14]. Similar trends were seen in other studies.

Zeng *et al*[54] and Zhuang *et al*[55] analyzed hepatitis B patients using an index cohort and a validation cohort, and showed that SWE had good diagnostic accuracy in predicting each fibrosis stage. Diagnostic performances in patients with CHB are summarized in Table 3. AUROCs for ⩾ F2, ⩾ F3 and F4 were 0.88-0.97, 0.917-0.96 and 0.926-0.98, respectively[14,52-55]. The optimal cutoff values for each fibrosis stage were 7.1-8.2 kPa for ⩾ F2, 7.9-9.1 kPa for ⩾ F3, and 10.1-11.3 kPa for F4[14,52-55]. In addition, the diagnostic performance of 2D-SWE was equivalent or superior to use of non-invasive fibrosis markers including TE in most fibrosis stages[14,52,53,55].

In a recently published patient data-based meta-analysis, the AUROCs for ⩾ F2, ⩾ F3 and F4 of 2D-SWE were 0.906, 0.931, and 0.955, respectively, and the proposed cut off values were 7.1 kPa, 8.1 kPa, and 11.5 kPa, respectively[49]. In addition, 2D-SWE in patients with CHB had a better diagnostic performance than TE in predicting ⩾ F2 and F4, but not ⩾ F3, unlike in patients with CHC[49].

Non-viral liver diseases

One of the most common causes of advanced liver disease worldwide is nonalcoholic fatty liver disease (NAFLD)[56]. It is important to diagnose the fibrosis stage in patients with NAFLD because the degree of fibrosis is the most important prognostic factor in these patients[57]. Three studies on the degrees of fibrosis in NAFLD have recently been published (Table 4)[20,49,58]. LS measurements by 2D-SWE in these patients had a relatively high failure rate (2.7%-13%) because of the higher BMIs in these patients[20,58]. Diagnostic performance in predicting each fibrosis stage was relatively low, and the cut-off values of the fibrosis stages differed between the studies[20,49,58]. This suggests that steatosis may have an effect on liver stiffness measurements, and further studies are needed[58].

The only study of patients with alcoholic liver disease was one performed by Thiele *et al*[19]. In that study, SWE had high diagnostic performances with AUCs of 0.94 and 0.95, respectively, for detecting significant fibrosis (Ishak fibrosis stage ⩾ 3) and cirrhosis (Ishak fibrosis stage ⩾ 5)[19]. In addition, the cutoff values for predicting the fibrosis stages there were higher than in other diseases, particularly in chronic viral hepatitis; liver injury in alcoholic liver disease is associated with relatively high levels of perivenular and pericellular fibrosis with central extension, and this may have resulted in a higher fibrosis burden[19].

There are two recent studies of autoimmune liver disease[59,60]. Because of the low prevalence of this disease, these studies included patients with auto-immune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, and overlap syndrome, all of

Table 4 Diagnostic performance of shear wave elastography for significant fibrosis (F ⩾ 2), advanced fibrosis (F ⩾ 3) and cirrhosis (F4) in patients with non-viral liver diseases

Ref.	Year	Etiology	Patients (*n*)	F ⩾ 2 (%)	F ⩾ 3 (%)	F = 4 (%)	AUROC	Cutoffs (kPa)	Se (%)	Sp (%)	PPV (%)	NPV (%)
Cassinotto *et al*[20]	2016	NAFLD	291	70.8			0.860	8.90	68.0	94.0		
					43.3		0.890	9.30	84.0	83.0		
						16.8	0.880	10.00	95.0	69.0		
Takeuchi *et al*[58]	2018	NAFLD	71	64.8			0.750	11.57	52.0	44.0		
					45.1		0.820	13.07	63.0	57.0		
						7.0	0.900	15.73	100.0	82.0		
Herrmann *et al*[49]	2018	NAFLD	156	58.3			0.855	7.10	93.8	52.0		
					32.1		0.928	9.20	93.1	80.9		
						12.2	0.917	13.00	75.3	87.8		
Thiele *et al*[19]	2016	Alcohol	199	42.0			0.940	10.20	82.0	93.0	90.0	88.0
						18.0	0.950	16.40	94.0	91.0	71.0	99.0
Zeng *et al*[59]	2017	Autoimmune	114	71.9			0.850	9.70	81.7	81.3	91.8	63.4
					41.3		0.850	13.20	83.0	74.6	69.6	86.2
						20.2	0.860	16.30	87.0	80.2	52.6	96.1
Li *et al*[60]	2018	Autoimmune	51	35.2			0.781	9.15	83.3	72.7		

AUROC: Area under ROC curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; NAFLD: Non-alcoholic fatty liver disease

which have different liver damage patterns[59,60]. For this reason, the AUROCs of autoimmune liver disease according to fibrosis stage were lower than those of chronic viral hepatitis[59,60]. Further studies should be performed separately for each disease.

USE OF 2D-SWE FOR ASSESSING PORTAL HYPERTENSION AND ESOPHAGEAL VARICES

Measurement of hepatic venous pressure gradient (HVPG) is considered the reference standard for assessing portal hypertension in liver cirrhosis, which is one of the most powerful prognostic factors in advanced chronic liver disease[61]. However, the use of HVPG is limited because it is unavailable in some centers and because of its invasiveness[62]. Hence, TE was introduced as a noninvasive tool and is known to be strongly correlated with HVPG and excellent for predicting clinically significant portal hypertension (CSPH, HVPG ⩾ 10 mmHg)[61].

There have been many studies aimed at establishing whether LS measured by 2D-SWE can identify portal hypertension. First, Choi *et al*[63] analyzed the association of HVPG with LS by 2D-SWE. They showed that HVPG and LS measured by 2D-SWE were moderately correlated (r = 0.593), and that change in LS and change in HVPG were strongly related (r = 0.863)[63]. As a result of that study, 2D-SWE unlike TE, can be considered a useful method for monitoring hemodynamic responses to drug therapy. Since then, several studies have examined whether LS measured by 2D-SWE can predict CSPH, and they are summarized in Table 5[64-67]. The AUROCs for predicting CSPH ranged from 0.81 to 0.87, which are relatively high diagnostic performances, and optimal cut-off values ranged from 15.2 to 24.6 kPa[64-67]. The different optimal cut-off values in the different studies were probably due to differences between the major forms of disease examined in the studies[64-67]. Therefore, as in the case of degree of fibrosis, studies on the prediction of portal hypertension may need to be carried out separately for each disease.

Efforts have been made to improve the reliability of LS measurements by 2D-SWE for predicting portal hypertension. Procopet *et al*[64] obtained a diagnostic performance with an AUC of 0.939 for predicting CSPH using an SD/median ⩽ 0.10 and/or depth < 5.6 cm. In addition, Elkrief *et al*[65] and Jansen *et al*[67] observed a strong correlation between HVPG and LS by 2D-SWE and an excellent AUROC in predicting CSPH, when the variation coefficient (SD/mean) was < 10%.

There have been attempts to complement LS in predicting CSPH by measuring SS, but the results were unsatisfactory. Procopet *et al*[64] found a 66% success rate for SS measurements and an AUROC of 0.725 for predicting CSPH. In addition, they obtained a high mismatch rate (25%) and indeterminate outcomes (60%) with a method employing a rule-out CSPH cutoff of > 90% sensitivity and a rule-in CSPH cutoff of > 90% specificity[64]. In that study, a small spleen was the most common reason for the inability to measure SS[64]. Elkrief *et al*[65] achieved a success rate of 97% for SS measurements but the AUC of SS in predicting CSPH was only 0.64, a moderate diagnostic performance. Unlike other studies, Jansen *et al*[67] had a success rate of 81.2% for SS measurements and a relatively good diagnostic performance with an AUROC of 0.84 in predicting CSPH. Based on this finding, they proposed a combined algorithm consisting of a rule-in algorithm and a rule-out algorithm, and the diagnostic accuracy of the algorithm was 91.6%[67]. Therefore they suggested that only those patients who were indeterminate in this algorithm would need to undergo invasive HVPG measurements[67]. Recently, Elkrief *et al*[68] performed

Table 5 Diagnostic performance of shear wave elastography for detecting clinically significant portal hypertension (HVPG ⩾ 10 mmHg)

Ref.	Year	Patients (*n*)	Study design	Prevalence (%)	Site	Success rate (%)	Cutoffs (kPa)	AUROC	Se (%)	Sp (%)	PPV (%)	NPV (%)
Procopet *et al*[64]	2015	88	Restospective	55.0	LS	99.0	17.0	0.859	80.8	82.1		
							15.4[1]	0.948	91.3	90.9		
					SS	66.0		0.725				
Elkrief *et al*[65]	2015	79	Prospective	90.9	LS	97.0	24.5	0.870	81.0	88.0	98.0	35.0
					SS	97.0	34.7	0.640	40.0	100.0	100.0	18.0
Kim *et al*[66]	2015	92	Prospective	83.7	LS	98.3	15.2	0.819	85.7	80.0	95.7	52.2
							21.6[2]	0.867	83.3	80.8	91.7	65.6
Jansen *et al*[67]	2017	109	Prospective multicenter	67.9	LS	100.0	24.6	0.860	68.3	80.4	87.7	55.4
					SS	81.2	26.3	0.840	79.7	84.2	90.8	68.0

[1]Highly reliable and reliable measurements (n = 45): SD/median > 0.10 or depth ⩾ 5.6 cm; [2]Severe portal hypertension (HVPG ⩾ 12 mmHg). AUROC: Area under ROC curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; LS: Liver stiffness; SS: Spleen stiffness.

an external validation of the algorithm. When it was used in 191 patients with liver cirrhosis, the negative predictive value for rule-out was estimated to be 60% and the positive predictive value for rule-in was 87% for predicting CSPH[68]. Thus the algorithm was not good enough to diagnose CSPH[68].

There have been three studies on the use of 2D-SWE for predicting esophageal varices (EV). Elkrief *et al*[65] compared the diagnostic performance of LS and SS in predicting high risk EV. They detected no difference in LS and SS between patients with high risk EV and without high risk EV, and the AUROCs of the LS and SS values for predicting high risk EV were 0.54 and 0.64, respectively[65]. This outcome was probably due to the small number of patients tested (n = 35) most of whom had high HVPG and/or decompensated cirrhosis[65]. On the other hand, Stefanescu *et al*[69] studied the use of LS and SS in predicting EV in 73 patients with compensated liver cirrhosis. The AUROCs of LS, SS and platelet count (PLT) were 0.753, 0.747, and 0.773, respectively, and the best cut-off values of LS, SS and PLT gave moderate diagnostic performances of 19 kPa, 38 kPa, and 100 × 10^3/mL, respectively[69]. When this result was used to apply the Baveno IV recommendations and stepwise approaches (LS < 19 kPa and PLT < 100 × 10^3/mL = no EV, LS > 19 kPa and PLT > 100 × 10^3/mL = probable EV; in the Grey zone, SS < 38 kPa = no EV, SS ⩾ 38 kPa = probable EV), it had an accuracy of 83.07% for ruling out EV[69]. However, when the algorithm was used with the platelet counts to predict EV it did not improve the diagnostic accuracy of the rule out algorithm proposed by Jansen *et al*[70]. Similarly, Kim *et al*[71] evaluated the predictive performance of LS for presence of EV and high risk EV in 103 patients with compensated liver cirrhosis. The AUROCs of LS for presence of EV and high risk EV were 0.887 and 0.880, respectively, and the best cut-off values were 13.9 kPa and 16.1 kPa, respectively[71].

ROLE OF 2D-SWE IN PREDICTING THE DEVELOPMENT OF HCC

TE is a useful predictor of HCC development in patients with CHB[72]. In particular, it is known to identify patients with CHB who do not have clinical cirrhosis but who rather have so-called subclinical cirrhosis with a high risk of developing HCC[73]. There have been two studies on the role of 2D-SWE in predicting the development of HCC. Jeong *et al*[74] followed up 291 compensated hepatitis B patients for 35.8 months and examined the use of measurements of LS by 2D-SWE for predicting HCC development. Patients with LS ⩾ 10kPa by 2D-SWE had a 4-fold higher risk of developing HCC than those with LS < 10 kPa. Lee *et al*[75] investigated the role of SWE in the prognosis of HCC after radiofrequency ablation (RFA). In 134 patients who underwent RFA as a curative treatment for HCC, LS by 2D-SWE was a significant predictor of overall survival and recurrence-free survival, and the optimal cutoff value was 13.3 kPa[75].

ROLE OF 2D-SWE IN ASSESSING FOCAL LIVER LESIONS

Focal lesions are often seen in US examinations, but benign focal lesions and malignant focal lesions are difficult to distinguish by conventional US. In such cases additional Doppler or contrast US has been used. Unlike TE, 2D-SWE can measure the stiffness of focal liver lesions (FLLs) under B-mode guidance. Several groups have reported that stiffness measured by 2D-SWE helps distinguish intrahepatic focal lesions[76-78]. The stiffness value of malignant lesions was significantly higher than that of benign lesions[76,78]. In benign lesions, the stiffness of focal nodular hyperplasia was significantly higher than that of hepatocellular adenoma[77]. In malignant lesions, the stiffness of metastatic tumors was significantly higher than that of HCC[76].

Recently, Grgurevic *et al*[78] analyzed 196 patients with 259 FLLs and found that the best performing cut-off value for malignancy was 22.3 kPa (sensitivity 83%, specificity 86%, positive predictive value 91.5%, negative predictive value 73%). In addition, a Liver Elastography Malignancy Prediction (LEMP) score was constructed by combining lesion stiffness, lesion/liver stiffness ratio and lesion stiffness variability[78]. The

accuracy of this score was 96.1% for distinguishing between benign and malignant FLL[78].

CONCLUSION

Assessing liver fibrosis by noninvasive methods is always an important issue in the management of chronic liver diseases. In this article, we have summarized evidence that 2D-SWE is a promising tool for evaluating liver fibrosis in various liver diseases. It is also a useful method for evaluating portal hypertension and predicting HCC development. However, it cannot completely replace invasive methods for managing these patients because of the complexity of liver diseases and the variety of factors that affect liver stiffness. In addition, the data on some aspects of chronic liver diseases based on studies of LS by 2D-SWE are still inadequate. In that context, larger, prospective and multicenter studies of 2D-SWE are needed.

REFERENCES

1 **Castera L**. Invasive and non-invasive methods for the assessment of fibrosis and disease progression in chronic liver disease. *Best Pract Res Clin Gastroenterol* 2011; **25**: 291-303 [PMID: 21497746 DOI: 10.1016/j.bpg.2011.02.003]

2 **Dienstag JL**. The role of liver biopsy in chronic hepatitis C. *Hepatology* 2002; **36**: S152-S160 [PMID: 12407589 DOI: 10.1053/jhep.2002.36381]

3 **Bravo AA**, Sheth SG, Chopra S. Liver biopsy. *N Engl J Med* 2001; **344**: 495-500 [PMID: 11172192 DOI: 10.1056/NEJM200102153440706]

4 **Bedossa P**, Dargère D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003; **38**: 1449-1457 [PMID: 14647056 DOI: 10.1016/j.hep.2003.09.022]

5 **Regev A**, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, Feng ZZ, Reddy KR, Schiff ER. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol* 2002; **97**: 2614-2618 [PMID: 12385448 DOI: 10.1111/j.1572-0241.2002.06038.x]

6 **Lupsor-Platon M**, Badea R, Gersak M, Maniu A, Rusu I, Suciu A, Vicas C, Stefănescu H, Urs R, Al Hajjar N. Noninvasive Assessment of Liver Diseases using 2D Shear Wave Elastography. *J Gastrointestin Liver Dis* 2016; **25**: 525-532 [PMID: 27981309 DOI: 10.15403/jgld.2014.1121.254.lup]

7 **Friedrich-Rust M**, Poynard T, Castera L. Critical comparison of elastography methods to assess chronic liver disease. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 402-411 [PMID: 27273167 DOI: 10.1038/nrgastro.2016.86]

8 **Borro P**, Dellepiane SG, Pellicano R, Gemme L, Fagoonee S, Testino G. Quantification of ultrasound imaging in the staging of hepatic fibrosis. *Panminerva Med* 2018; **60**: 44-51 [PMID: 29442483 DOI: 10.23736/S0031-0808.18.03416-X]

9 **Samir AE**, Dhyani M, Vij A, Bhan AK, Halpern EF, Méndez-Navarro J, Corey KE, Chung RT. Shear-wave elastography for the estimation of liver fibrosis in chronic liver disease: determining accuracy and ideal site for measurement. *Radiology* 2015; **274**: 888-896 [PMID: 25393946 DOI: 10.1148/radiol.14140839]

10 **Ling W**, Lu Q, Quan J, Ma L, Luo Y. Assessment of impact factors on shear wave based liver stiffness measurement. *Eur J Radiol* 2013; **82**: 335-341 [PMID: 23116805 DOI: 10.1016/j.ejrad.2012.10.004]

11 **Yoon JH**, Lee JM, Han JK, Choi BI. Shear wave elastography for liver stiffness measurement in clinical sonographic examinations: evaluation of intraobserver reproducibility, technical failure, and unreliable stiffness measurements. *J Ultrasound Med* 2014; **33**: 437-447 [PMID: 24567455 DOI: 10.7863/ultra.33.3.437]

12 **Choi SH**, Jeong WK, Kim Y, Lim S, Kwon JW, Kim TY, Kim MY, Sohn JH. How many times should we repeat measuring liver stiffness using shear wave elastography?: 5-repetition versus 10-repetition protocols. *Ultrasonics* 2016; **72**: 158-164 [PMID: 27529140 DOI: 10.1016/j.ultras.2016.08.005]

13 **Sporea I**, Grădinaru-Taşcău O, Bota S, Popescu A, Şirli R, Jurchiş A, Popescu M, Dănilă M. How many measurements are needed for liver stiffness assessment by 2D-Shear Wave Elastography (2D-SWE) and which value should be used: the mean or median? *Med Ultrason* 2013; **15**: 268-272 [PMID: 24286089 DOI: 10.11152/mu.2013.2066.154.isp2]

14 **Leung VY**, Shen J, Wong VW, Abrigo J, Wong GL, Chim AM, Chu SH, Chan AW, Choi PC, Ahuja AT, Chan HL, Chu WC. Quantitative elastography of liver fibrosis and spleen stiffness in chronic hepatitis B carriers: comparison of shear-wave elastography and transient elastography with liver biopsy correlation. *Radiology* 2013; **269**: 910-918 [PMID: 23912619 DOI: 10.1148/radiol.13130128]

15 **Poynard T**, Munteanu M, Luckina E, Perazzo H, Ngo Y, Royer L, Fedchuk L, Sattonnet F, Pais R, Lebray P, Rudler M, Thabut D, Ratziu V. Liver fibrosis evaluation using real-time shear wave elastography: applicability and diagnostic performance using methods without a gold standard. *J Hepatol* 2013; **58**: 928-935 [PMID: 23321316 DOI: 10.1016/j.jhep.2012.12.021]

16 **Ferraioli G**, Tinelli C, Zicchetti M, Above E, Poma G, Di Gregorio M, Filice C. Reproducibility of real-time shear wave elastography in the evaluation of liver elasticity. *Eur J Radiol* 2012; **81**: 3102-3106 [PMID: 22749107 DOI: 10.1016/j.ejrad.2012.05.030]

17 **Suh CH**, Kim SY, Kim KW, Lim YS, Lee SJ, Lee MG, Lee J, Lee SG, Yu E. Determination of normal hepatic elasticity by using real-time shear-wave elastography. *Radiology* 2014; **271**: 895-900 [PMID: 24555633 DOI: 10.1148/radiol.14131251]

18 **Hudson JM**, Milot L, Parry C, Williams R, Burns PN. Inter- and intra-operator reliability and repeatability of shear wave elastography in the liver: a study in healthy volunteers. *Ultrasound Med Biol* 2013; **39**: 950-955 [PMID: 23453379 DOI: 10.1016/j.ultrasmedbio.2012.12.011]

19 **Thiele M**, Detlefsen S, Sevelsted Møller L, Madsen BS, Fuglsang Hansen J, Fialla AD, Trebicka J, Krag A. Transient and 2-Dimensional Shear-Wave Elastography Provide Comparable Assessment of Alcoholic Liver Fibrosis and Cirrhosis. *Gastroenterology* 2016; **150**: 123-133 [PMID: 26435270 DOI: 10.1053/j.gastro.2015.09.040]

20 **Cassinotto C**, Boursier J, de Lédinghen V, Lebigot J, Lapuyade B, Cales P, Hiriart JB, Michalak S, Bail BL, Cartier V, Mouries A, Oberti F, Fouchard-Hubert I, Vergniol J, Aubé C. Liver stiffness in nonalcoholic fatty liver disease: A comparison of supersonic shear imaging, FibroScan, and ARFI with liver biopsy. *Hepatology* 2016; **63**: 1817-1827 [PMID: 26659452 DOI: 10.1002/hep.28394]

21 **Woo H**, Lee JY, Yoon JH, Kim W, Cho B, Choi BI. Comparison of the Reliability of Acoustic Radiation Force Impulse Imaging and Supersonic Shear Imaging in Measurement of Liver Stiffness. *Radiology* 2015; **277**: 881-886 [PMID: 26147680 DOI: 10.1148/radiol.2015141975]

22 **Cassinotto C**, Charrie A, Mouries A, Lapuyade B, Hiriart JB, Vergniol J, Gaye D, Hocquelet A, Charbonnier M, Foucher J, Laurent F, Chermak F, Montaudon M, de Ledinghen V. Liver and spleen elastography using supersonic shear imaging for the non-invasive diagnosis of cirrhosis severity and oesophageal varices. *Dig Liver Dis* 2015; **47**: 695-701 [PMID: 25959234 DOI: 10.1016/j.dld.2015.04.008]

23 **Ferraioli G**, Parekh P, Levitov AB, Filice C. Shear wave elastography for evaluation of liver fibrosis. *J Ultrasound Med* 2014; **33**: 197-203 [PMID: 24449721 DOI: 10.7863/ultra.33.2.197]

24 **Huang Z**, Zheng J, Zeng J, Wang X, Wu T, Zheng R. Normal liver stiffness in healthy adults assessed by real-time shear wave elastography and factors that influence this method. *Ultrasound Med Biol* 2014; **40**: 2549-2555 [PMID: 25282481 DOI: 10.1016/j.ultrasmedbio.2014.05.008]

25 **Arena U**, Lupsor Platon M, Stasi C, Moscarella S, Assarat A, Bedogni G, Piazzolla V, Badea R, Laffi G, Marra F, Mangia A, Pinzani M. Liver stiffness is influenced by a standardized meal in patients with chronic hepatitis C virus at different stages of fibrotic evolution. *Hepatology* 2013; **58**: 65-72 [PMID: 23447459 DOI: 10.1002/hep.26343]

26 **Berzigotti A**, De Gottardi A, Vukotic R, Siramolpiwat S, Abraldes JG, García-Pagan JC, Bosch J. Effect of meal ingestion on liver stiffness in patients with cirrhosis and portal hypertension. *PLoS One* 2013; **8**: e58742 [PMID: 23520531 DOI: 10.1371/journal.pone.0058742]

27 **Lemoine M**, Shimakawa Y, Njie R, Njai HF, Nayagam S, Khalil M, Goldin R, Ingiliz P, Taal M, Nyan O, Corrah T, D'Alessandro U, Thursz M. Food intake increases liver stiffness measurements and hampers reliable values in patients with chronic hepatitis B and healthy controls: the PROLIFICA experience in The Gambia. *Aliment Pharmacol Ther* 2014; **39**: 188-196 [PMID: 24308698 DOI: 10.1111/apt.12561]

28 **Mederacke I**, Wursthorn K, Kirschner J, Rifai K, Manns MP, Wedemeyer H, Bahr MJ. Food intake increases liver stiffness in patients with chronic or resolved hepatitis C virus infection. *Liver Int* 2009; **29**: 1500-1506 [PMID: 19732330 DOI: 10.1111/j.1478-3231.2009.02100.x]

29 **Gersak MM**, Sorantin E, Windhaber J, Dudea SM, Riccabona M. The influence of acute physical effort on liver stiffness estimation using Virtual Touch Quantification (VTQ). Preliminary results. *Med Ultrason* 2016; **18**: 151-156 [PMID: 27239647 DOI: 10.11152/mu.2013.2066.182.vtq]

30 **Coco B**, Oliveri F, Maina AM, Ciccorossi P, Sacco R, Colombatto P, Bonino F, Brunetto MR. Transient elastography: a new surrogate marker of liver fibrosis influenced by major changes of transaminases. *J Viral Hepat* 2007; **14**: 360-369 [PMID: 17439526 DOI: 10.1111/j.1365-2893.2006.00811.x]

31 **Sagir A**, Erhardt A, Schmitt M, Häussinger D. Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. *Hepatology* 2008; **47**: 592-595 [PMID: 18098325 DOI: 10.1002/hep.22056]

32 **Arena U**, Vizzutti F, Corti G, Ambu S, Stasi C, Bresci S, Moscarella S, Boddi V, Petrarca A, Laffi G, Marra F, Pinzani M. Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 2008; **47**: 380-384 [PMID: 18095306 DOI: 10.1002/hep.22007]

33 **Millonig G**, Reimann FM, Friedrich S, Fonouni H, Mehrabi A, Büchler MW, Seitz HK, Mueller S. Extrahepatic cholestasis increases liver stiffness (FibroScan) irrespective of fibrosis. *Hepatology* 2008; **48**: 1718-1723 [PMID: 18836992 DOI: 10.1002/hep.22577]

34 **Millonig G**, Friedrich S, Adolf S, Fonouni H, Golriz M, Mehrabi A, Stiefel P, Pöschl G, Büchler MW, Seitz HK, Mueller S. Liver stiffness is directly influenced by central venous pressure. *J Hepatol* 2010; **52**: 206-210 [PMID: 20022130 DOI: 10.1016/j.jhep.2009.11.018]

35 **Colli A**, Pozzoni P, Berzuini A, Gerosa A, Canovi C, Molteni EE, Barbarini M, Bonino F, Prati D. Decompensated chronic heart failure: increased liver stiffness measured by means of transient elastography. *Radiology* 2010; **257**: 872-878 [PMID: 20935077 DOI: 10.1148/radiol.10100013]

36 **Karlas TF**, Pfrepper C, Rosendahl J, Benckert C, Wittekind C, Jonas S, Moessner J, Tröltzsch M, Tillmann HL, Berg T, Keim V, Wiegand J. Acoustic radiation force impulse (ARFI) elastography in acute liver failure: necrosis mimics cirrhosis. *Z Gastroenterol* 2011; **49**: 443-448 [PMID: 21476180 DOI: 10.1055/s-0029-1245690]

37 **Petta S**, Maida M, Macaluso FS, Di Marco V, Cammà C, Cabibi D, Craxì A. The severity of steatosis influences liver stiffness measurement in patients with nonalcoholic fatty liver disease. *Hepatology* 2015; **62**: 1101-1110 [PMID: 25991038 DOI: 10.1002/hep.27844]

38 **Macaluso FS**, Maida M, Cammà C, Cabibbo G, Cabibi D, Alduino R, Di Marco V, Craxì A, Petta S. Steatosis affects the performance of liver stiffness measurement for fibrosis assessment in patients with genotype 1 chronic hepatitis C. *J Hepatol* 2014; **61**: 523-529 [PMID: 24815874 DOI: 10.1016/j.jhep.2014.04.045]

39 **Yoneda M**, Yoneda M, Mawatari H, Fujita K, Endo H, Iida H, Nozaki Y, Yonemitsu K, Higurashi T, Takahashi H, Kobayashi N, Kirikoshi H, Abe Y, Inamori M, Kubota K, Saito S, Tamano M, Hiraishi H, Maeyama S, Yamaguchi N, Togo S, Nakajima A. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Liver Dis* 2008; **40**: 371-378 [PMID: 18083083 DOI: 10.1016/j.dld.2007.10.019]

40 **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kowo M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454-462 [PMID: 20101745 DOI: 10.1002/hep.23312]

41 **Deng H**, Qi X, Zhang T, Qi X, Yoshida EM, Guo X. Supersonic shear imaging for the diagnosis of liver fibrosis and portal hypertension in liver diseases: a meta-analysis. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 91-98 [PMID: 29186994 DOI: 10.1080/17474124.2018.1412257]

42 **Ma X**, Wang L, Wu H, Feng Y, Han X, Bu H, Zhu Q. Spleen Stiffness Is Superior to Liver Stiffness for Predicting Esophageal Varices in Chronic Liver Disease: A Meta-Analysis. *PLoS One* 2016; **11**: e0165786 [PMID: 27829057 DOI: 10.1371/journal.pone.0165786]

43 **Grgurevic I**, Puljiz Z, Brnic D, Bokun T, Heinzl R, Lukic A, Luksic B, Kujundzic M, Brkljacic B. Liver and spleen stiffness and their ratio assessed by real-time two dimensional-shear wave elastography in patients with liver fibrosis and cirrhosis due to chronic viral hepatitis. *Eur Radiol* 2015; **25**: 3214-3221 [PMID: 25903706 DOI: 10.1007/s00330-015-3728-x]

44 **Jeong JY**, Kim TY, Sohn JH, Kim Y, Jeong WK, Oh YH, Yoo KS. Real time shear wave elastography in chronic liver diseases: accuracy for predicting liver fibrosis, in comparison with serum markers. *World J Gastroenterol* 2014; **20**: 13920-13929 [PMID: 25320528 DOI: 10.3748/wjg.v20.i38.13920]

45 **Deffieux T**, Gennisson JL, Bousquet L, Corouge M, Cosconea S, Amroun D, Tripon S, Terris B, Mallet V, Sogni P, Tanter M, Pol S. Investigating liver stiffness and viscosity for fibrosis, steatosis and activity staging using shear wave elastography. *J Hepatol* 2015; **62**: 317-324 [PMID: 25251998 DOI: 10.1016/j.jhep.2014.09.020]

46 **Bavu E**, Gennisson JL, Couade M, Bercoff J, Mallet V, Fink M, Badel A, Vallet-Pichard A, Nalpas B, Tanter M, Pol S. Noninvasive in vivo liver fibrosis evaluation using supersonic shear imaging: a clinical study on 113 hepatitis C virus patients. *Ultrasound Med Biol* 2011; **37**: 1361-1373 [PMID: 21775051 DOI: 10.1016/j.ultrasmedbio.2011.05.016]

47 **Ferraioli G**, Tinelli C, Dal Bello B, Zicchetti M, Filice G, Filice C; Liver Fibrosis Study Group. Accuracy of real-time shear wave elastography for assessing liver fibrosis in chronic hepatitis C: a pilot study. *Hepatology* 2012; **56**: 2125-2133 [PMID: 22767302 DOI: 10.1002/hep.25936]

48 **Tada T**, Kumada T, Toyoda H, Ito T, Sone Y, Okuda S, Tsuji N, Imayoshi Y, Yasuda E. Utility of real-time shear wave elastography for assessing liver fibrosis in patients with chronic hepatitis C infection without cirrhosis: Comparison of liver fibrosis indices. *Hepatol Res* 2015; **45**: E122-E129 [PMID: 25580959 DOI: 10.1111/hepr.12476]

49 **Herrmann E**, de Lédinghen V, Cassinotto C, Chu WC, Leung VY, Ferraioli G, Filice C, Castera L, Vilgrain V, Ronot M, Dumortier J, Guibal A, Pol S, Trebicka J, Jansen C, Strassburg C, Zheng R, Zheng J, Francque S, Vanwolleghem T, Vonghia L, Manesis EK, Zoumpoulis P, Sporea I, Thiele M, Krag A, Cohen-Bacrie C, Criton A, Gay J, Deffieux T, Friedrich-Rust M. Assessment of biopsy-proven liver fibrosis by two-dimensional shear wave elastography: An individual patient data-based meta-analysis. *Hepatology* 2018; **67**: 260-272 [PMID: 28370257 DOI: 10.1002/hep.29179]

50 **Tada T**, Kumada T, Toyoda H, Mizuno K, Sone Y, Kataoka

S, Hashinokuchi S. Improvement of liver stiffness in patients with hepatitis C virus infection who received direct-acting antiviral therapy and achieved sustained virological response. *J Gastroenterol Hepatol* 2017; **32**: 1982-1988 [PMID: 28299813 DOI: 10.1111/jgh.13788]

51 **Korda D**, Lenard ZM, Gerlei Z, Jakab Z, Haboub-Sandil A, Wagner L, Varga M, Cseprekal O, Marton A, Horvathy D, Takacs S, Doros A, Mathe Z. Shear-wave elastography for the assessment of liver fibrosis in liver transplant recipients treated for hepatitis C virus recurrence. *Eur J Gastroenterol Hepatol* 2018; **30**: 27-32 [PMID: 29049126 DOI: 10.1097/MEG.0000000000001003]

52 **Zeng J**, Zheng J, Huang Z, Chen S, Liu J, Wu T, Zheng R, Lu M. Comparison of 2-D Shear Wave Elastography and Transient Elastography for Assessing Liver Fibrosis in Chronic Hepatitis B. *Ultrasound Med Biol* 2017; **43**: 1563-1570 [PMID: 28483579 DOI: 10.1016/j.ultrasmedbio.2017.03.014]

53 **Wu T**, Wang P, Zhang T, Zheng J, Li S, Zeng J, Kudo M, Zheng R. Comparison of Two-Dimensional Shear Wave Elastography and Real-Time Tissue Elastography for Assessing Liver Fibrosis in Chronic Hepatitis B. *Dig Dis* 2016; **34**: 640-649 [PMID: 27750231 DOI: 10.1159/000448825]

54 **Zeng J**, Liu GJ, Huang ZP, Zheng J, Wu T, Zheng RQ, Lu MD. Diagnostic accuracy of two-dimensional shear wave elastography for the non-invasive staging of hepatic fibrosis in chronic hepatitis B: a cohort study with internal validation. *Eur Radiol* 2014; **24**: 2572-2581 [PMID: 25027837 DOI: 10.1007/s00330-014-3292-9]

55 **Zhuang Y**, Ding H, Zhang Y, Sun H, Xu C, Wang W. Two-dimensional Shear-Wave Elastography Performance in the Noninvasive Evaluation of Liver Fibrosis in Patients with Chronic Hepatitis B: Comparison with Serum Fibrosis Indexes. *Radiology* 2017; **283**: 873-882 [PMID: 27982760 DOI: 10.1148/radiol.2016160131]

56 **Rinella ME**. Nonalcoholic fatty liver disease: a systematic review. *JAMA* 2015; **313**: 2263-2273 [PMID: 26057287 DOI: 10.1001/jama.2015.5370]

57 **Dulai PS**, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, Sebastiani G, Ekstedt M, Hagstrom H, Nasr P, Stal P, Wong VW, Kechagias S, Hultcrantz R, Loomba R. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. *Hepatology* 2017; **65**: 1557-1565 [PMID: 28130788 DOI: 10.1002/hep.29085]

58 **Takeuchi H**, Sugimoto K, Oshiro H, Iwatsuka K, Kono S, Yoshimasu Y, Kasai Y, Furuichi Y, Sakamaki K, Itoi T. Liver fibrosis: noninvasive assessment using supersonic shear imaging and FIB4 index in patients with non-alcoholic fatty liver disease. *J Med Ultrason (2001)* 2018; **45**: 243-249 [PMID: 29128938 DOI: 10.1007/s10396-017-0840-3]

59 **Zeng J**, Huang ZP, Zheng J, Wu T, Zheng RQ. Non-invasive assessment of liver fibrosis using two-dimensional shear wave elastography in patients with autoimmune liver diseases. *World J Gastroenterol* 2017; **23**: 4839-4846 [PMID: 28765706 DOI: 10.3748/wjg.v23.i26.4839]

60 **Li C**, Dhyani M, Bhan AK, Grajo JR, Pratt DS, Gee MS, Samir AE. Diagnostic Performance of Shear Wave Elastography in Patients With Autoimmune Liver Disease. *J Ultrasound Med* 2018; : [PMID: 29761535 DOI: 10.1002/jum.14668]

61 **Castera L**, Pinzani M, Bosch J. Non invasive evaluation of portal hypertension using transient elastography. *J Hepatol* 2012; **56**: 696-703 [PMID: 21767510 DOI: 10.1016/j.jhep.2011.07.005]

62 **Procopet B**, Berzigotti A. Diagnosis of cirrhosis and portal hypertension: imaging, non-invasive markers of fibrosis and liver biopsy. *Gastroenterol Rep* (Oxf) 2017; **5**: 79-89 [PMID: 28533906 DOI: 10.1093/gastro/gox012]

63 **Choi SY**, Jeong WK, Kim Y, Kim J, Kim TY, Sohn JH. Shear-wave elastography: a noninvasive tool for monitoring changing hepatic venous pressure gradients in patients with cirrhosis. *Radiology* 2014; **273**: 917-926 [PMID: 25025464 DOI: 10.1148/radiol.14140008]

64 **Procopet B**, Berzigotti A, Abraldes JG, Turon F, Hernandez-Gea V, García-Pagán JC, Bosch J. Real-time shear-wave elastography: applicability, reliability and accuracy for clinically significant portal hypertension. *J Hepatol* 2015; **62**: 1068-1075 [PMID: 25514554 DOI: 10.1016/j.jhep.2014.12.007]

65 **Elkrief L**, Rautou PE, Ronot M, Lambert S, Dioguardi Burgio M, Francoz C, Plessier A, Durand F, Valla D, Lebrec D, Vilgrain V, Castéra L. Prospective comparison of spleen and liver stiffness by using shear-wave and transient elastography for detection of portal hypertension in cirrhosis. *Radiology* 2015; **275**: 589-598 [PMID: 25469784 DOI: 10.1148/radiol.14141210]

66 **Kim TY**, Jeong WK, Sohn JH, Kim J, Kim MY, Kim Y. Evaluation of portal hypertension by real-time shear wave elastography in cirrhotic patients. *Liver Int* 2015; **35**: 2416-2424 [PMID: 25875718 DOI: 10.1111/liv.12846]

67 **Jansen C**, Bogs C, Verlinden W, Thiele M, Möller P, Görtzen J, Lehmann J, Vanwolleghem T, Vonghia L, Praktiknjo M, Chang J, Krag A, Strassburg CP, Francque S, Trebicka J. Shear-wave elastography of the liver and spleen identifies clinically significant portal hypertension: A prospective multicentre study. *Liver Int* 2017; **37**: 396-405 [PMID: 27569696 DOI: 10.1111/liv.13243]

68 **Elkrief L**, Ronot M, Andrade F, Dioguardi Burgio M, Issoufaly T, Zappa M, Roux O, Bissonnette J, Payancé A, Lebrec D, Francoz C, Soubrane O, Valla D, Durand F, Vilgrain V, Castera L, Rautou PE. Non-invasive evaluation of portal hypertension using shear-wave elastography: analysis of two algorithms combining liver and spleen stiffness in 191 patients with cirrhosis. *Aliment Pharmacol Ther* 2018; **47**: 621-630 [PMID: 29322599 DOI: 10.1111/apt.14488]

69 **Stefanescu H**, Allegretti G, Salvatore V, Piscaglia F. Bidimensional shear wave ultrasound elastography with supersonic imaging to predict presence of oesophageal varices in cirrhosis. *Liver Int* 2017; **37**: 1405 [PMID: 28294520 DOI: 10.1111/liv.13418]

70 **Jansen C**, Thiele M, Verlinden W, Krag A, Francque S, Trebicka J. Prediction of presence of oesophageal varices just by shear-wave elastography of the liver and spleen. *Liver Int* 2017; **37**: 1406-1407 [PMID: 28845618 DOI: 10.1111/liv.13446]

71 **Kim TY**, Kim TY, Kim Y, Lim S, Jeong WK, Sohn JH. Diagnostic Performance of Shear Wave Elastography for Predicting Esophageal Varices in Patients With Compensated Liver Cirrhosis. *J Ultrasound Med* 2016; **35**: 1373-1381 [PMID: 27208198 DOI: 10.7863/ultra.15.07024]

72 **Jung KS**, Kim SU, Ahn SH, Park YN, Kim DY, Park JY, Chon CY, Choi EH, Han KH. Risk assessment of hepatitis B virus-related hepatocellular carcinoma development using liver stiffness measurement (FibroScan). *Hepatology* 2011; **53**: 885-894 [PMID: 21319193 DOI: 10.1002/hep.24121]

73 **Kim MN**, Kim SU, Kim BK, Park JY, Kim DY, Ahn SH, Song KJ, Park YN, Han KH. Increased risk of hepatocellular carcinoma in chronic hepatitis B patients with transient elastography-defined subclinical cirrhosis. *Hepatology* 2015; **61**: 1851-1859 [PMID: 25643638 DOI: 10.1002/hep.27735]

74 **Jeong JY**, Sohn JH, Sohn W, Park CH, Kim TY, Jun DW, Kim Y, Jeong WK. Role of Shear Wave Elastography in Evaluating the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B. *Gut Liver* 2017; **11**: 852-859 [PMID: 28651307 DOI: 10.5009/gnl16521]

75 **Lee DH**, Lee JM, Yoon JH, Kim YJ, Lee JH, Yu SJ, Han JK. Liver Stiffness Measured by Two-Dimensional Shear-Wave Elastography: Prognostic Value after Radiofrequency Ablation for Hepatocellular Carcinoma. *Liver Cancer* 2018; **7**: 65-75 [PMID: 29662834 DOI: 10.1159/000484445]

76 **Park HS**, Kim YJ, Yu MH, Jung SI, Jeon HJ. Shear Wave Elastography of Focal Liver Lesion: Intraobserver Reproducibility and Elasticity Characterization. *Ultrasound Q* 2015; **31**: 262-271 [PMID: 26086459 DOI: 10.1097/RUQ.0000000000000175]

77 **Brunel T**, Guibal A, Boularan C, Ducerf C, Mabrut JY, Bancel B, Boussel L, Rode A. Focal nodular hyperplasia and hepatocellular adenoma: The value of shear wave elastography for differential diagnosis. *Eur J Radiol* 2015; **84**: 2059-2064 [PMID: 26299323 DOI: 10.1016/j.ejrad.2015.07.029]

78 **Grgurevic I**, Bokun T, Salkic NN, Brkljacic B, Vukelić-Markovic M, Stoos-Veic T, Aralica G, Rakic M, Filipec-Kanizaj T, Berzigotti

A. Liver elastography malignancy prediction score for noninvasive characterization of focal liver lesions. *Liver Int* 2018; **38**: 1055-1063 [PMID: 29028279 DOI: 10.1111/liv.13611]

79 **Sebastiani G**, Halfon P, Castera L, Pol S, Thomas DL, Mangia A, Di Marco V, Pirisi M, Voiculescu M, Guido M, Bourliere M, Noventa F, Alberti A. SAFE biopsy: a validated method for large-scale staging of liver fibrosis in chronic hepatitis C. *Hepatology* 2009; **49**: 1821-1827 [PMID: 19291784 DOI: 10.1002/hep.22859]

P- Reviewer: Abenavoli L, Pellicano R **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3861-3870

DOI: 10.3748/wjg.v24.i34.3861

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

ORIGINAL ARTICLE

Basic Study

Delta-like ligand 4 in hepatocellular carcinoma intrinsically promotes tumour growth and suppresses hepatitis B virus replication

Areerat Kunanopparat, Jiraphorn Issara-Amphorn, Asada Leelahavanichkul, Anapat Sanpavat, Suthiluk Patumraj, Pisit Tangkijvanich, Tanapat Palaga, Nattiya Hirankarn

Areerat Kunanopparat, Jiraphorn Issara-Amphorn, Asada Leelahavanichkul, Nattiya Hirankarn, Center of Excellence in Immunology and Immune Mediated Diseases, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Anapat Sanpavat, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Suthiluk Patumraj, Center of Excellence for Microcirculation, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Pisit Tangkijvanich, Research Unit of Hepatitis and Liver Cancer, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

ORCID number: Areerat Kunanopparat (0000-0001-6311-7255); Jiraphorn Issara-Amphorn (0000-0002-7421-2749); Asada Leelahavanichkul (0000-0002-5566-6403); Anapat Sanpavat (0000-0002-6425-3379); Suthiluk Patumraj (0000-0002-8346-2205); Pisit Tangkijvanich (0000-0002-2926-8671); Tanapat Palaga (0000-0001-8734-6626); Nattiya Hirankarn (0000-0003-2224-6856).

Author contributions: Kunanopparat A, Palaga T and Hirankarn N conceived and designed experiments; Kunanopparat A, Issara-Amphorn J, Leelahavanichkul A, Patumraj S and Tangkijvanich P conducted the experiments; Kunanopparat A and Sanpavat A analysed the data; Kunanopparat A, Palaga T and Hirankarn N wrote the manuscript.

Supported by National Research Council of Thailand 2013; the Ratchadaphiseksomphot Matching Fund from the Faculty of Medicine, Chulalongkorn University; the International Research Integration, Chula Research Scholar, Ratchadaphisek somphot Endowment Fund, Center of Excellence in Immunology and Immune-mediated Diseases; and the Rachadapisaek Sompote Post-Doctoral Fund, Chulalongkorn University.

Institutional review board statement: The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Institutional animal care and use committee statement: All protocols were carried out in accordance with relevant guidelines and regulations.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Nattiya Hirankarn, MD, PhD, Lecturer, Professor, Center of Excellence in Immunology and Immune Mediated Diseases Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. nattiya.H@chula.ac.th
Telephone: +66-2-2564132
Fax: +66-2-2525952

Received: May 25, 2018
Peer-review started: May 25, 2018

First decision: June 21, 2018
Revised: July 5, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 14, 2018

Abstract

AIM

To investigate the role of Delta-like ligand 4 (DLL4) on tumour growth in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) *in vivo*.

METHODS

We suppressed *DLL4* expression in an HBV expressing HCC cell line, HepG2.2.15 and analysed the growth ability of cells as subcutaneous tumours in nude mice. The expression of tumour angiogenesis regulators, VEGF-A and VEGF-R2 in tumour xenografts were examined by western blotting. The tumour proliferation and neovasculature were examined by immunohistochemistry. The viral replication and viral protein expression were measured by quantitative PCR and western blotting, respectively.

RESULTS

Eighteen days after implantation, tumour volume in mice implanted with shDLL4 HepG2.2.15 was significantly smaller than in mice implanted with control HepG2.2.15 ($P < 0.0001$). The levels of angiogenesis regulators, VEGF-A and VEGF-R2 were significantly decreased in implanted tumours with suppressed *DLL4* compared with the control group ($P < 0.001$ and $P < 0.05$, respectively). Furthermore, the suppression of DLL4 expression in tumour cells reduced cell proliferation and the formation of new blood vessels in tumours. Unexpectedly, increased viral replication was observed after suppression of DLL4 in the tumours.

CONCLUSION

This study demonstrates that DLL4 is important in regulating the tumour growth of HBV-associated HCC as well as the neovascularization and suppression of HBV replication.

Key words: Hepatocellular carcinoma; Notch signalling; Delta-like ligand 4; HepG2.2.15

Core tip: We demonstrated that Delta-like ligand 4 (DLL4) is important for tumour growth of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) in a xenograft model. We found that the level of angiogenesis regulators, VEGF-A and VEGF-R2 were significantly decreased in HCC xenograft tumours with suppressed DLL4 compared with the control group. Consistent with these findings, the suppression of DLL4 expression in the tumour cells reduced cell proliferation and the formation of new blood vessels in the tumour. Furthermore, this is the first report that DLL4 in an HBV expressing HCC cell line plays a key role in regulating tumour growth, angiogenesis, and viral replication in a mouse model of xenograft transplantation.

Kunanopparat A, Issara-Amphorn J, Leelahavanichkul A, Sanpavat A, Patumraj S, Tangkijvanich P, Palaga T, Hirankarn N. Delta-like ligand 4 in hepatocellular carcinoma intrinsically promotes tumour growth and suppresses hepatitis B virus replication. *World J Gastroenterol* 2018; 24(34): 3861-3870 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3861.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3861

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-associated mortality. Approximately 80% of HCC is associated with chronic hepatitis viral infections[1]. Hepatitis B virus (HBV) infection is the most prevalent cause of HCC in developing countries. Although an HBV vaccine has successfully prevented HBV infection, there are still a large number of chronic hepatitis B patients who are at a high risk (maximum 100-fold increase over healthy individuals) of developing liver cancer[2,3]. The molecular mechanisms of HBV-associated HCC are poorly understood[4]. To date, sorafenib is the recommended drug for the treatment of HCC patients. However, the therapeutic outcome is still limited because liver cancer is often detected at advanced stages[5]. Therefore, a better understanding of the molecular mechanisms of tumour initiation and progression is needed for the further development of HCC therapy.

Notch signalling is an evolutionarily conserved pathway that regulates cell fate decision, embryonic development, tissue homeostasis, differentiation, proliferation, and apoptosis[6,7]. In mammals, the Notch pathway comprises of four Notch receptors (Notch1, 2, 3, 4) and five Notch ligands (Jagged1, 2, and DLL1, 3, 4). Activation of Notch signalling requires contact between a Notch ligand from the signal sending cells and a receptor on signal receiving cells to activate proteolytic cleavage and the subsequent translocation of the Notch intracellular domain to the nucleus where it translates target genes[8]. Dysregulation of Notch signalling has been reported in many types of cancer as either a tumour suppressor or tumour promoter depending on the type of cancer[9-11]. In HCC, the role of Notch signalling is still controversial. Many studies reported that Notch receptors were highly expressed in HCC compared with the adjacent human tumour tissue and that tumour growth was suppressed after the inhibition of Notch either by a gamma secretase inhibitor or by suppression of Notch target genes[12-16]. Several studies have also suggested that Notch is a tumour suppressor in HCC[15,17-19]. However, more evidence supports the pro-tumourigenic role of Notch in HCC carcinogenesis and progression, especially in HBV-associated HCC[20,21]. We previously reported that HBV regulatory protein HBx promoted HBV-associated HCC

proliferation through Delta-like ligand 4 (DLL4) *via* the NF-κB pathway in HepG2, an HBV expressing HCC cell line[22].

Strong evidence indicates that DLL4 regulates angiogenesis and controls the balance of endothelial tip and stalk cell differentiation induced by VEGF[23]. DLL4 is highly expressed in tumour endothelial cells for tumour angiogenesis, which is the primary signal for tumour progression[24]. The inhibition of DLL4 in tumour endothelial cells suppressed tumour growth by inducing non-productive angiogenesis[25]. Currently, a DLL4 neutralizing antibody has been developed and is being tested in a clinical trial for anticancer therapy in various cancers[26,27]. However, the effect of DLL4 inhibition in HCC has not been explored. In this study, we investigated the role of DLL4 on tumour growth in HCC associated with HBV in a xenograft model and detailed the molecular mechanism of HCC.

MATERIALS AND METHODS

Cell culture

The HBV-expressing HCC cell line (HepG2.2.15) and the HepG2 cell line were obtained from Professor Antonio Bertoletti [Singapore Institute for Clinical Sciences at Agency for Science, Technology and Research (A*Star)]. Cells were cultured in high glucose DMEM medium (Gibco, Carlsbad, CA, United States) supplemented with 10% foetal bovine serum (Gibco), 150 μg/mL of G418 (Gibco), and 1% of penicillin-streptomycin (Invitrogen, Carlsbad, CA, United States). Cultures were maintained at 37 ℃ in a 5% CO_2 humidified incubator.

Generation of stable DLL4 knockdown cell lines

HepG2.2.15 cells line was transfected with a set of DLL4 shRNA (Origene Technologies, MD, United States) targeting four shDLL4 cassettes in the pGFP-V-RS Vector (TG304977). The transient transfection used Lipofectamine 2000 (Invitrogen) at 2.5 μL for 1 μg of shRNA vector into 1×10^5 cells per well in a 12-well plate. After 48 h, DLL4 mRNA expression was determined in transfected cells. The highest efficacy of shRNA (5′-ACCAGAAGAAGGAGCTGGAAGTGGACTGT-3′) vector was used to generate stably transfected cells. For stably transfected cell lines, transiently transfected cells were plated into 96-well plates by limiting dilution and selected by the addition of 0.3 μg/mL puromycin to the culture medium for 4-5 wk. Puromycin-resistant clones with suppressed DLL4 were expanded, and DLL4 expression was analysed by western blot analysis and compared with the control. The clones with the highest degree of DLL4 suppression were used for tumour xenografts.

In vivo study and tumour xenograft

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. All protocols were carried out in accordance with relevant guidelines and regulations. Male BALB/cMlac-nu mice aged four weeks were purchased from the National Laboratory Animal Center (Mahidol University, Thailand) and were acclimatized for two weeks before experimentation. Mice were maintained under 12 h light-dark cycle with 50% humidity and with free access to food and water. The shDLL4 HepG2.2.15 and control HepG2.2.15 cells were trypsinized at a concentration of 1×10^7 cells/mL. One millilitre of the cells was centrifuged and resuspended in 100 μL of Matrigel (Corning, NY, United States). The cell suspension was subcutaneously injected into the back left and right flanks of nude mice (n = 4-6). The tumour volume (cm^3) was measured every three days until 18 d and 30 d using Vernier calipers and calculated using the formula: (length × width^2)/6. The mice were weighed every three days and monitored for activity and mortality. All animals were euthanized by barbiturate overdose for tumour collection.

Western blotting analysis

Total cell lysates were prepared in RIPA buffer (Cell Signaling Technology, MA, United States) containing protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, MA, United States). After sonication, 20 μg of cell lysates were blotted and probed with primary antibodies to anti-DLL4, anti-cleaved Notch 1, anti-VEGFR2, anti-β actin (Cell Signaling Technology; 1:1000), anti-VEGF, anti-PreS1 HBV antigen, and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, United States; 1:1000). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology) and goat anti-mouse immunoglobulin (Cell Signaling Technology) were used as secondary antibodies. Immunoblot detection was performed using Super Signal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Fisher Scientific). The protein intensity was estimated by the densitometry of scanned immunoblot bands using Image Studio Lite version 5.2 software (LI-COR Biosciences).

Immunohistochemistry analysis

After the end of the experiment, the tumours were collected, fixed in 10% formalin solution, and embedded in a paraffin block. The tissue sections were cut with a microtome to obtain 4 μm thick paraffin sections, then deparaffinized and rehydrated in a series of xylenes and alcohols followed by retrieval of the antigenic epitopes. Antigen retrieval was performed in citrate buffer (pH 6, 100 ℃ for 20 min). The tissue sections were treated with 3% H_2O_2 for 15 min and blocked with normal serum for 30 min, then incubated with primary antibody in a humidity chamber at 4 ℃ overnight. The primary antibodies included anti-CD31 (Santa Cruz Biotechnologies; at a dilution of 1:500), and anti-Ki-67 (Ventana Medical Systems, Inc.; AZ, United States) (ready to use). ZytoChem Plus (HRP) Polymer anti-Rabbit (Zytomed Systems, Berlin, Germany) (ready to use) and rabbit anti-goat immunoglobulin-HRP (Dako; CA, United States), were used for the detection of primary antibodies. The immunoreaction was visualized

Table 1 The primer sequences used in this study

Genes	Primer sequence
β-actin	F-5′ACCAACTGGGACGACATGGAGAA-3′
	R-5′GTGGTGGTGAAGCTGTAGCC-3′
IFN-α	F-5′GCTTTACTGATGGTCCTGGTGGTG-3′
	R-5′GAGATTCTGCTCATTTGTGCCAG-3′
IFN-β	F-5′GAATGGGAGGCTTGAATACTGCCT-3′
	R-5′TAGCAAAGATGTTCTGGAGCATCTC-3′
TNF-α	F-5′CTTCTCCTTCCTGATCGTGG-3′
	R-5′GCTGGTTATCTCTCAGCTCCA-3′
HBx	F-5′CACCTCTCTTTACGCGGACT-3′
	R-5′GGTCGTTGACATTGCAGAGA-3′
HBV PreS1	F-5′GGGTCACCATATTCTTGGGAAC-3′
	R-5′CCTGAGCCTGAGGGCTCCAC-3′

IFN: Interferon; TNF: Tumour necrosis factor; HBV: Hepatitis B virus; HBx: Hepatitis B virus X gene-encoded protein.

with ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc.). The nuclei were counterstained with Mayer's haematoxylin. Immunoreactions were measured in five microscopic fields per sample with 20 × objective magnification (Nikon Eclipse50i, Japan). The percentage of Ki67 was analysed by the ImmunoRatio web application[28].

Tumour vasculature imaging

Tumour vasculature imaging was performed as previously described[29]. Briefly, mice were anesthetised with an intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). A catheter was inserted into the jugular vein for the application of fluorescence tracers. Then, the dorsal skin-fold chamber was removed, and the skin area around the chamber was fixed with modelling wax on a plate. To visualise the vascular lumen, a bolus of 0.1 mL of 5% fluorescein isothiocyanate-labelled dextran (FITC-dextran) was injected into the jugular vein. The tumour vasculature was visualised under a confocal microscope.

Quantitative gene expression

Total RNA was extracted from cell culture or xenograft tumour tissues using the RNeasy Mini kit (Qiagen, Hilden, Germany). One microgram of RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA, United States). Quantitative PCR amplification was performed with SYBR green (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System for 40 cycles. Derivation of the 2-ddCT method was applied for the relative quantification of mRNA expression. Beta-actin was used as an endogenous control. The primers used in this study are shown in Table 1.

HBV viral DNA analysis

Genomic DNA was extracted from cell culture or xenograft tumour tissues using the QIAamp DNA Mini Kit (Qiagen). Fifty nanograms of DNA of all samples were amplified to determine preS1 HBV gene expression relative to a standard copy number of HBV. The quantitative PCR amplification was performed with SYBR green (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System.

Statistical analysis

One-way ANOVA and *t*-test were applied using Prism 5 software (GraphPad Software Inc., San Diego, CA, United States). The results are shown as the mean ± SD, and differences of $P < 0.05$ were accepted as the level of significance.

RESULTS

Suppression of DLL4 delays HCC tumour growth and reduces VEGF factor.

To investigate the role of DLL4 in HCC, we transfected a DLL4 suppression construct, shDLL4 to knockdown DLL4 expression or control pGFP-V-RS plasmids into the HepG2.2.15 cell line. Clones of transfected cells with stable knockdown of DLL4 were selected with puromycin by limiting dilution assay. Various clones were selected and DLL4 expression was determined by western blot and compared with the control (Figure 1A). Clones with the strongest DLL4 suppression were chosen for xenograft transplantation. At 18 d and 30 d after shDLL4 HepG2.2.15 transplantation, mice showed significantly reduced tumour growth (Figure 1B) for both tumour volume (n = 4-6, $P < 0.0001$) and tumour weight (n = 4-6, $P < 0.05$ at 18 d and $P < 0.01$ at 30 d) compared with mice transplanted with control HepG2.2.15 cells (Figure 1C and D).

We monitored the expression of DLL4 and cleaved Notch1 expression in tumours taken from xenograft mice. Interestingly, the expression of DLL4 recovered in one mouse (#5 Figure 1E) and cleaved Notch1 was not significantly different in the implanted HepG2.2.15 tumours with shDLL4 compared with controls (Figure 1E and F).

DLL4 is mainly expressed in vascular endothelium and is related to VEGF expression, which promotes tumour angiogenesis[30-32]. To examine whether DLL4-expressing HCC is associated with VEGF expression, we analysed VEGF expression from the tumour xenograft. Interestingly, at 18 d after transplantation, the expression of VEGF was significantly decreased in implanted HepG2.2.15 tumours with shDLL4 compared with the control tumour ($P < 0.001$) (Figure 1E and F). VEGFR2 and CD31, tumour vasculature markers, were also significantly reduced in shDLL4 HepG2.2.15 compared with control HepG2.2.15 ($P < 0.05$ and $P < 0.001$, respectively) (Figures 1E and F, 2A and B, respectively). CD31 expression, which indicates neovascularization, was also decreased consistent with the Ki67 expression.

Next, we analysed cell proliferation by Ki67 expression in tumour xenografts using immunohistochemistry. The suppression of DLL4 reduced the percentage of cells with Ki67 nuclear positivity as shown at day 18 post transplantation in Figure 2B and 2D ($P < 0.05$). The tumour

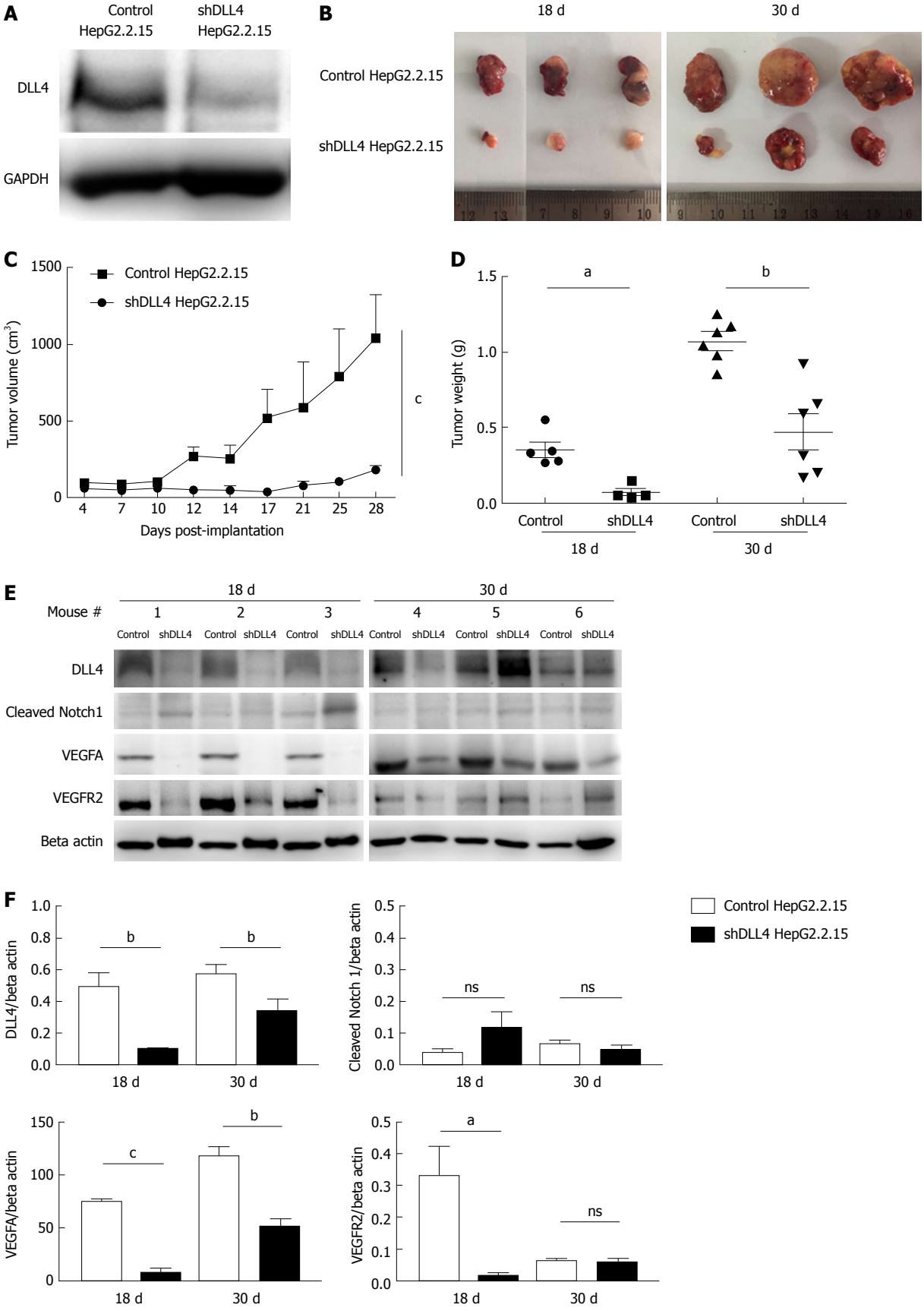


Figure 1 Delta-like ligand 4 expression promotes hepatitis B virus-associated hepatocellular carcinoma tumour growth *in vivo*. A: Western blot analyses of DLL4 expression in HepG2.2.15 stably transfected with shDLL4 or control vector. GAPDH was used as the loading control. B-D: HepG2.2.15 transfected with shDLL4 or control vector were subcutaneously injected into athymic nude mice (B) (1×10^7 cells per mouse, n = 4-6). Tumour volume (C) and tumour weights (D) are shown. At 18 d and 30 d after implantation, tumours were collected and analysed for DLL4, cleaved Notch1, VEGFA, and VEGFR2 by western blot. Beta-actin was used for the loading control. The blots cropped from different parts of the same gel (E). Band intensities from (E) were measured and the results are presented as the mean ± SD of three independent experiments (F). $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$.

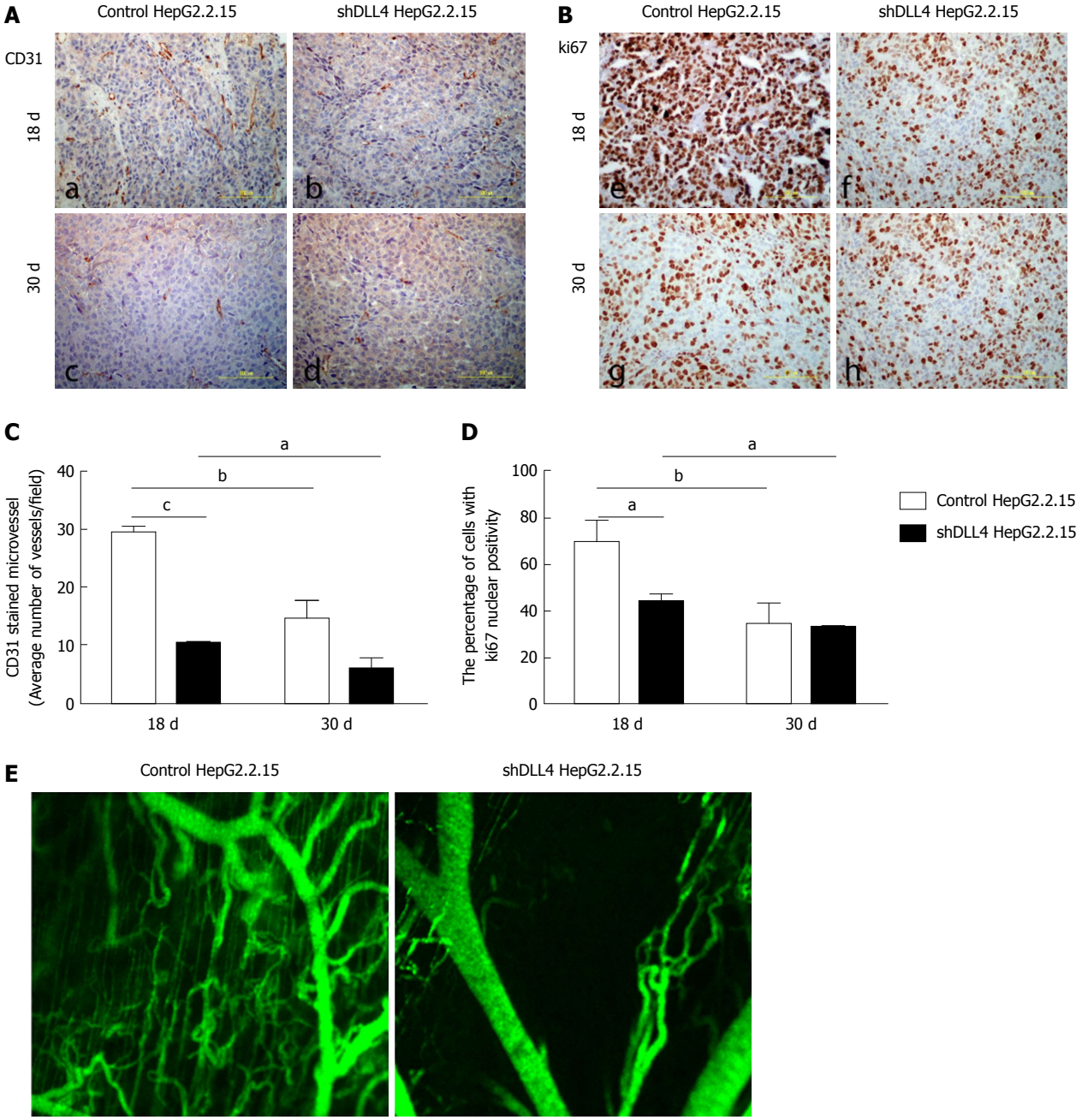


Figure 2 Suppression of Delta-like ligand 4 reduces tumour proliferation and neovasculature at the initiation stage of implantation. A and B: Immunohistochemical staining of CD31 (a-d) and Ki67 (e-h) shows mouse neovessels and tumour cell proliferation in paraffin sections of tumour xenografts, respectively. Five fields of each section were quantified for the amount of CD31 staining (C), and the percentage of Ki67 positive cells (D) in tumours transfected with shDLL4 or control vector at 18 d and 30 d after implantation. The tumour vasculature was measured after tumour implantation with shDLL4 HepG2.2.15 or control cells at 30 d (E). The data represent the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

vasculature was also decreased in shDLL4 HepG2.2.15 compared with control HepG2.2.15 (Figure 2E).

Interestingly, at 30 d after transplantation, the expression of VEGFR2, CD31, and Ki67 were not significantly different between shDLL4 HepG2.2.15 and control tumours. These data suggested that DLL4 may have an important role at the initiation stage of tumour proliferation.

Suppression of DLL4 increases HBV viral production in vivo

We have previously shown that *in vitro* HBV activated Notch signalling by increasing DLL4 had no effect on HBV viral replication[22]. To confirm our observation *in vivo*, we monitored viral production in the tumour xenograft. Unexpectedly, we found that HBV viral DNA and HBx mRNA expression were significantly increased in shDLL4 HepG2.2.15 compared with control HepG2.2.15 ($P < 0.05$ and $P < 0.01$, respectively). HBV preS1 protein was also increased in shDLL4 HepG2.2.15 at 18 d and 30 d after implantation (Figure 3A-C). We therefore measured the amount of type I interferon and found no difference in the level of IFN-α, IFN-β, or TNF-α (Figure 3D-F). Taken together, we found that a decrease in DLL4 expression in

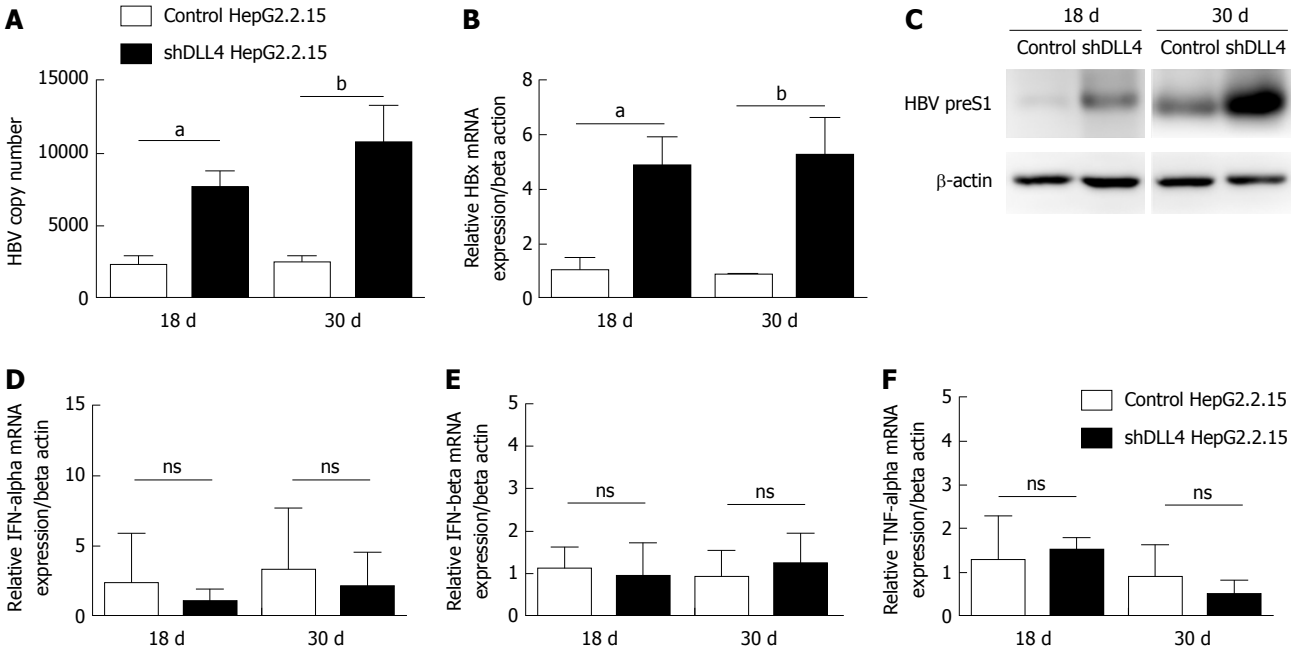


Figure 3 Suppression of Delta-like ligand 4 enhances hepatitis B virus viral replication (A), HBx mRNA expression (B), and HBs protein expression (C) *in vivo*. DNA, RNA, and proteins were extracted from tumour xenografts to analyse HBV viral components at 18 d and 30 d after implantation. The mRNA levels of human IFN-alpha (D), IFN-beta (E), and TNF-alpha (F) from tumours transfected with shDLL4 or control vectors were measured by quantitative RT-PCR and normalized to beta-actin mRNA expression. The data are represented by the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$.

the HBV expressing HCC cell line *in vivo* reduced tumour cell proliferation and increased viral replication.

DISCUSSION

In this study, we followed up on our previous observation that HBx induced DLL4 in the HCC cell line and regulated cell survival at least *via* the activation of Notch1[22]. The effects of DLL4 suppression in an HCC cell line was observed at two main levels: (1) was the effect on tumour growth and (2) was the effect on viral replication. As expected, HepG2.2.15 with reduced DLL4 expression grew poorly in immunocompromised nude mice, compared with the siRNA transfected control. The suppression effect of *DLL4* remained intact 18 d after implantation but it was diminished in some, but not all mice, after 30 d. Interestingly, the level of cleaved Notch1 was not reduced in all tumours even when *DLL4* was successfully suppressed. Similarly, the level of *Hes1* and *Hey1*, two well-characterized Notch target genes, were also not affected by *DLL4* suppression in tumours (data not shown). It is possible that other Notch receptors besides Notch1 and Notch ligands, such as JAG1 or DLL3, are activated in the tumours. Indeed, HBx expression induced JAG1 expression in the HCC cell line[33]. In one study, suppressing both DLL4 and Jagged1 increased the inhibitory effect on the proliferation and invasiveness of human gastric carcinoma[34]. Although we cannot rule out the possibility that Notch receptors/ligands are activated in the tumour, our observation clearly showed that DLL4 is important for the tumour growth of the implanted HepG2.2.15 cell line. However, there are studies that suggested that a high level of DLL4 is associated with inhibition of tumour growth and metastasis in HCC[35,36]. Notch receptors have been suggested to play a role in both oncogenes and tumour-suppressor genes in different cell types[37,38]. We hypothesized that DLL4 may act as an oncogene in the initiation stage of tumour development, and then act as a tumour suppressor in the late stage depending on the DLL4 isoform or other tumour microenvironments. However, the dual function of DLL4 as a tumour-suppressor and oncogene needs to be further clarified.

The most striking effect of DLL4 knockdown on HepG2.2.15 *in vivo* was the reduction of angiogenesis factors, VEGFA and VEGFR2 (Figure 1E). In our study, we detected VEGFA of tumour (human) origin and VEGFR2 of host (mouse) origin. When the tumour vasculature was visualized, a reduced vasculature was observed in DLL4 knockdown tumours, consistent with the reduced expression of angiogenesis factors. CD31, an endothelial cell marker, was also reduced. DLL4 was reported to be involved in tumour angiogenesis[39]. Suppressing DLL4 in tumours resulted in non-productive angiogenesis and the suppression of tumour growth[40]. Our observation is in line with these reports and confirmed the importance of DLL4 in angiogenesis during tumour growth. DLL4 on tumour cells interacts with Notch receptors on host stromal/endothelial cells and helps tumour angiogenesis, thus improving tumour vascular function[41]. This event leads to increased tumour growth in some, but not all, types of cancer cells such as glioblastoma and prostate cancer. Our result is consistent with this observation, and we have added HCC as another tumour cell that

relies on DLL4 for vasculature formation. One intriguing observation from our study is that tumour (human) derived VEGF induces vasculature in the host (mouse).

Recent comprehensive and integrative genomic characterisation of hepatocellular carcinoma was performed on various HCC of different aetiologies. Although Notch receptors/ligands and the associated signalling molecules did not stand out as the prime mutated genes, the core Notch signalling was one of the pathways in HCC with enriched frequencies of functionally impactful mutations (ranked as No. 71)[42]. In HCV-related HCC, the Notch tumour signature genes (activation and deregulation) were found in 31.8% of patients (n = 91), suggesting a partial role of Notch signalling in promoting HCC in HCV infection[16]. This analysis highlighted the fact that Notch signalling may be involved in certain, but not all, subsets of HCC. In addition, it is not known whether HCC arising from other non-viral infections causes, but DLL4 has been linked to liver fibrosis and non-alcoholic steatohepatitis pathogenesis[43].

Tumour cell proliferation was significantly reduced when *DLL4* was suppressed. This effect was determined by the reduction in Ki67, which was robust at an early stage (18 d post transplantation). There are two likely scenarios for the effect of DLL4 on tumour cell growth. One possibility is that reducing DLL4 expression decreased Notch signalling and as a result, cell growth *in vivo* was compromised. Many studies have reported the cell proliferation promoting effect of Notch in HCC[44-46]. Suppression of *DLL4* in HepG2.2.15 reduced cell viability and interfered with cell cycle progression *in vitro*[22]. Another possibility is that the defect in angiogenesis within tumours played a key role in reducing cell proliferation. This effect together with reduced angiogenesis may contribute to severe growth retardation *in vivo*.

Unexpectedly, increased viral replication was observed in HepG2.2.15 upon DLL4 suppression *in vivo*. We previously reported that suppressing *DLL4* in HepG2.2.15 *in vitro* did not alter viral replication[22]. This discrepancy highlights the more complex multi-cellular interactions *in vivo*. It is unclear how DLL4 suppression promoted HBV viral replication. However, there may be two possibilities: the extrinsic and/or the intrinsic effect. If suppressing DLL4 created an environment that was friendly to viral replication, such as by inducing less anti-viral cytokines IFNα/β or promoting skewed helper T cell polarization, then this is considered an extrinsic effect. In contrast, what we observed is that there was no significant difference in IFNα/β level between control and tumour with suppressed DLL4 and the mice lacked adaptive immune response. Thus, we concluded that the viral replication promoting effect must be intrinsic. Namely the intracellular environment with reduced DLL4 levels allowed the virus to replicate better. Currently, there is no evidence that this effect is dependent upon reduced Notch signalling.

There are several reports on the effect of viral infection and Notch ligand expression. In Dengue virus infection, DLL1 and DLL4 were upregulated in antigen presenting cells *via* the IFN-β signalling pathway, which in turn influenced helper T cell responses[47]. Respiratory syncytial virus also induced DLL4 expression in dendritic cells to direct helper T cell polarization[48]. In another report, Kaposi sarcoma herpesvirus induced the expression of DLL4 and JAG1 to alter cell cycle regulating genes in neighbouring cells[49]. However, there has been no report on the impact of DLL4 expression on HBV replication. Our observation that suppressing DLL4 decreased angiogenesis indicates it might induce hypoxic conditions within the tumour. Indeed, various studies have linked the expression of hypoxia-inducible factor and viral replication during carcinogenesis[50,51]. If hypoxia and cellular stress due to defective angiogenesis caused by DLL4 suppression is the cause for enhanced viral replication, then it is speculated that suppressing DLL4 expression may promote viral replication in other cell types as well. Whether increased HBV replication is the cause for reduced tumour growth is not determined.

Taken together, we report the novel findings that DLL4 in an HBV expressing HCC cell line regulated tumour growth, angiogenesis, and viral replication in a mouse model of xenograft transplantation. Therefore, DLL4 may be a good candidate for HCC therapy.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) has been studied for many decades. However, the molecular mechanism is still unclear. Notch signaling in HCC pathogenesis is controversial, but we found that HBx promoted HBV-associated HCC proliferation through Delta-like ligand 4 (DLL4) (Notch ligand) in an *in vitro* study. However, the effect of DLL4 inhibition in HCC has not been explored.

Research motivation

DLL4 has a potential function for angiogenesis that supports tumour growth. The understanding of DLL4 mechanism might lead to identifying a new target for HCC therapy.

Research objective

We investigated the role of DLL4 on tumour growth in HCC associated with HBV in a xenograft model and detailed the molecular mechanism of HCC.

Research methods

We inhibited the DLL4 expression in HBV-associated HCC, and then subcutaneously implanted in nude mice. We analysed the ability for tumour growth, angiogenesis regulators (VEGF-A, VEGF-R2) expression, neovasculature, and HBV expression in tumour xenografts.

Research results

The tumour volume, VEGF-A, and VEGF-R2 were significantly decreased in mice implanted with suppressed DLL4 HCC compared with the control group. The suppression of DLL4 expression in tumour cells reduced cell proliferation and the formation of new blood vessels in tumours. Unexpectedly, viral replication increased in DLL4 suppressed tumours.

Research conclusions

This study demonstrates that DLL4 is important in regulating the tumour growth and neovascularization in HBV-associated HCC, as well as suppressing HBV replication *in vivo*.

Research perspective

This study showed that DLL4 is essential for the tumour growth of the implanted HBV-associated HCC cell line, especially in the initiation stage of tumour growth. However, the role of DLL4 as a tumour oncogene and tumour suppressor gene in HCC needs to further clarification.

REFERENCES

1 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]

2 **El-Serag HB**. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; **142**: 1264-1273.e1 [PMID: 22537432 DOI: 10.1053/j.gastro.2011.12.061]

3 **Yang HI**, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ; Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med* 2002; **347**: 168-174 [PMID: 12124405 DOI: 10.1056/NEJMoa013215]

4 **Benhenda S**, Cougot D, Buendia MA, Neuveut C. Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. *Adv Cancer Res* 2009; **103**: 75-109 [PMID: 19854353 DOI: 10.1016/S0065-230X(09)03004-8]

5 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

6 **Hori K**, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci* 2013; **126**: 2135-2140 [PMID: 23729744 DOI: 10.1242/jcs.127308]

7 **Lai EC**. Notch signaling: control of cell communication and cell fate. *Development* 2004; **131**: 965-973 [PMID: 14973298 DOI: 10.1242/dev.01074]

8 **Yamamoto S**, Schulze KL, Bellen HJ. Introduction to Notch signaling. *Methods Mol Biol* 2014; **1187**: 1-14 [PMID: 25053477 DOI: 10.1007/978-1-4939-1139-4_1]

9 **Koch U**, Radtke F. Notch and cancer: a double-edged sword. *Cell Mol Life Sci* 2007; **64**: 2746-2762 [PMID: 17687513 DOI: 10.1007/s00018-007-7164-1]

10 **South AP**, Cho RJ, Aster JC. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol* 2012; **23**: 458-464 [PMID: 22309843 DOI: 10.1016/j.semcdb.2012.01.017]

11 **Timmerman LA**, Grego-Bessa J, Raya A, Bertrán E, Pérez-Pomares JM, Díez J, Aranda S, Palomo S, McCormick F, Izpisúa-Belmonte JC, de la Pompa JL. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* 2004; **18**: 99-115 [PMID: 14701881 DOI: 10.1101/gad.276304]

12 **Gao J**, Dong Y, Zhang B, Xiong Y, Xu W, Cheng Y, Dai M, Yu Z, Xu H, Zheng G. Notch1 activation contributes to tumor cell growth and proliferation in human hepatocellular carcinoma HepG2 and SMMC7721 cells. *Int J Oncol* 2012; **41**: 1773-1781 [PMID: 22922832 DOI: 10.3892/ijo.2012.1606]

13 **Gramantieri L**, Giovannini C, Lanzi A, Chieco P, Ravaioli M, Venturi A, Grazi GL, Bolondi L. Aberrant Notch3 and Notch4 expression in human hepatocellular carcinoma. *Liver Int* 2007; **27**: 997-1007 [PMID: 17696940 DOI: 10.1111/j.1478-3231.2007.01544.x]

14 **Hu L**, Xue F, Shao M, Deng A, Wei G. Aberrant expression of Notch3 predicts poor survival for hepatocellular carcinomas. *Biosci Trends* 2013; **7**: 152-156 [PMID: 23836039]

15 **Ning L**, Wentworth L, Chen H, Weber SM. Down-regulation of Notch1 signaling inhibits tumor growth in human hepatocellular carcinoma. *Am J Transl Res* 2009; **1**: 358-366 [PMID: 19956448]

16 **Villanueva A**, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, Rodriguez-Carunchio L, Solé M, Thung S, Stanger BZ, Llovet JM. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. *Gastroenterology* 2012; **143**: 1660-1669.e7 [PMID: 22974708 DOI: 10.1053/j.gastro.2012.09.002]

17 **Qi R**, An H, Yu Y, Zhang M, Liu S, Xu H, Guo Z, Cheng T, Cao X. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* 2003; **63**: 8323-8329 [PMID: 14678992]

18 **Viatour P**, Ehmer U, Saddic LA, Dorrell C, Andersen JB, Lin C, Zmoos AF, Mazur PK, Schaffer BE, Ostermeier A, Vogel H, Sylvester KG, Thorgeirsson SS, Grompe M, Sage J. Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. *J Exp Med* 2011; **208**: 1963-1976 [PMID: 21875955 DOI: 10.1084/jem.20110198]

19 **Wang C**, Qi R, Li N, Wang Z, An H, Zhang Q, Yu Y, Cao X. Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human hepatocellular carcinoma cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression. *J Biol Chem* 2009; **284**: 16183-16190 [PMID: 19376776 DOI: 10.1074/jbc.M109.002105]

20 **Sun Q**, Wang R, Luo J, Wang P, Xiong S, Liu M, Cheng B. Notch1 promotes hepatitis B virus X protein-induced hepatocarcinogenesis *via* Wnt/β-catenin pathway. *Int J Oncol* 2014; **45**: 1638-1648 [PMID: 25017705 DOI: 10.3892/ijo.2014.2537]

21 **Wang F**, Zhou H, Yang Y, Xia X, Sun Q, Luo J, Cheng B. Hepatitis B virus X protein promotes the growth of hepatocellular carcinoma by modulation of the Notch signaling pathway. *Oncol Rep* 2012; **27**: 1170-1176 [PMID: 22218807 DOI: 10.3892/or.2012.1620]

22 **Kongkavitoon P**, Tangkijvanich P, Hirankarn N, Palaga T. Hepatitis B Virus HBx Activates Notch Signaling *via* Delta-Like 4/Notch1 in Hepatocellular Carcinoma. *PLoS One* 2016; **11**: e0146696 [PMID: 26766040 DOI: 10.1371/journal.pone.0146696]

23 **Hellström M**, Phng LK, Gerhardt H. VEGF and Notch signaling: the yin and yang of angiogenic sprouting. *Cell Adh Migr* 2007; **1**: 133-136 [PMID: 19262131 DOI: 10.4161/cam.1.3.4978]

24 **Patel NS**, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL. Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res* 2005; **65**: 8690-8697 [PMID: 16204037 DOI: 10.1158/0008-5472.CAN-05-1208]

25 **Ridgway J**, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo JA, de Sauvage F, Plowman G, Yan M. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 2006; **444**: 1083-1087 [PMID: 17183323 DOI: 10.1038/nature05313]

26 **Chiorean EG**, LoRusso P, Strother RM, Diamond JR, Younger A, Messersmith WA, Adriaens L, Liu L, Kao RJ, DiCioccio AT, Kostic A, Leek R, Harris A, Jimeno A. A Phase I First-in-Human Study of Enoticumab (REGN421), a Fully Human Delta-like Ligand 4 (Dll4) Monoclonal Antibody in Patients with Advanced Solid Tumors. *Clin Cancer Res* 2015; **21**: 2695-2703 [PMID: 25724527 DOI: 10.1158/1078-0432.CCR-14-2797]

27 **Smith DC**, Eisenberg PD, Manikhas G, Chugh R, Gubens MA, Stagg RJ, Kapoun AM, Xu L, Dupont J, Sikic B. A phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (anti-DLL4) in patients with previously treated solid tumors. *Clin Cancer Res* 2014; **20**: 6295-6303 [PMID: 25324140 DOI: 10.1158/1078-0432.CCR-14-1373]

28 **Tuominen VJ**, Ruotoistenmäki S, Viitanen A, Jumppanen M, Isola J. ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. *Breast Cancer Res* 2010; **12**: R56 [PMID: 20663194 DOI: 10.1186/bcr2615]

29 **Patumraj S**, Yoysungnoen P, Kachonrattanadet P, Wirachwong P. Tumor neocapillary density in hepatocellular carcinoma cells implanted nude mice model. *Clin Hemorheol Microcirc* 2005; **33**: 137-144 [PMID: 16151261]

30 **Miles KM**, Seshadri M, Ciamporcero E, Adelaiye R, Gillard B, Sotomayor P, Attwood K, Shen L, Conroy D, Kuhnert F, Lalani AS, Thurston G, Pili R. Dll4 blockade potentiates the anti-tumor effects of VEGF inhibition in renal cell carcinoma patient-derived xenografts.

PLoS One 2014; **9**: e112371 [PMID: 25393540 DOI: 10.1371/journal.pone.0112371]

31 **Vincent F**, Bonnin P, Clemessy M, Contrerès JO, Lamandé N, Gasc JM, Vilar J, Hainaud P, Tobelem G, Corvol P, Dupuy E. Angiotensinogen delays angiogenesis and tumor growth of hepatocarcinoma in transgenic mice. *Cancer Res* 2009; **69**: 2853-2860 [PMID: 19318581 DOI: 10.1158/0008-5472.CAN-08-2484]

32 **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 DOI: 10.1158/1078-0432.CCR-08-2780]

33 **Gao J**, Chen C, Hong L, Wang J, Du Y, Song J, Shao X, Zhang J, Han H, Liu J, Fan D. Expression of Jagged1 and its association with hepatitis B virus X protein in hepatocellular carcinoma. *Biochem Biophys Res Commun* 2007; **356**: 341-347 [PMID: 17359939 DOI: 10.1016/j.bbrc.2007.02.130]

34 **Sun HW**, Wu C, Tan HY, Wang QS. Combination DLL4 with Jagged1-siRNA can enhance inhibition of the proliferation and invasiveness activity of human gastric carcinoma by Notch1/VEGF pathway. *Hepatogastroenterology* 2012; **59**: 924-929 [PMID: 22020917 DOI: 10.5754/hge11484]

35 **Chen H**, Yang L, Zang S, Zhuo L, Fang X, Zhang Y, Li K, Song K, A H. High level of Delta-like ligand 4 suppresses the metastasis of hepatocellular carcinoma. *Int J Clin Exp Pathol* 2016; **9**: 2989-2997

36 **Liu X**, Zhou J, Zhou N, Zhu J, Feng Y, Miao X. SYNJ2BP inhibits tumor growth and metastasis by activating DLL4 pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res* 2016; **35**: 115 [PMID: 27440153 DOI: 10.1186/s13046-016-0385-0]

37 **Lobry C**, Oh P, Mansour MR, Look AT, Aifantis I. Notch signaling: switching an oncogene to a tumor suppressor. *Blood* 2014; **123**: 2451-2459 [PMID: 24608975 DOI: 10.1182/blood-2013-08-355818]

38 **Aster JC**, Pear WS, Blacklow SC. The Varied Roles of Notch in Cancer. *Annu Rev Pathol* 2017; **12**: 245-275 [PMID: 27959635 DOI: 10.1146/annurev-pathol-052016-100127]

39 **Li JL**, Harris AL. Crosstalk of VEGF and Notch pathways in tumour angiogenesis: therapeutic implications. *Front Biosci (Landmark Ed)* 2009; **14**: 3094-3110 [PMID: 19273260 DOI: 10.2741/3438]

40 **Noguera-Troise I**, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 2006; **444**: 1032-1037 [PMID: 17183313 DOI: 10.1038/nature05355]

41 **Li JL**, Sainson RC, Shi W, Leek R, Harrington LS, Preusser M, Biswas S, Turley H, Heikamp E, Hainfellner JA, Harris AL. Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. *Cancer Res* 2007; **67**: 11244-11253 [PMID: 18056450 DOI: 10.1158/0008-5472.CAN-07-0969]

42 **Cancer Genome Atlas Research Network.** Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

43 **Kawaguchi K**, Honda M, Kaneko S. The Role of Notch Signaling in Liver Diseases: Contribution to Development and Cancer. *Int J Cancer Clin Res* 2017; **4**: 079 [DOI: 10.23937/2378-3419/1410079]

44 **Lu J**, Xia Y, Chen K, Zheng Y, Wang J, Lu W, Yin Q, Wang F, Zhou Y, Guo C. Oncogenic role of the Notch pathway in primary liver cancer. *Oncol Lett* 2016; **12**: 3-10 [PMID: 27347091 DOI: 10.3892/ol.2016.4609]

45 **Strazzabosco M**, Fabris L. Notch signaling in hepatocellular carcinoma: guilty in association! *Gastroenterology* 2012; **143**: 1430-1434 [PMID: 23099244 DOI: 10.1053/j.gastro.2012.10.025]

46 **Zhu B**, Sun L, Luo W, Li M, Coy DH, Yu L, Yu W. Activated Notch signaling augments cell growth in hepatocellular carcinoma *via* up-regulating the nuclear receptor NR4A2. *Oncotarget* 2017; **8**: 23289-23302 [PMID: 28423575 DOI: 10.18632/oncotarget.15576]

47 **Li Y**, Wu S, Pu J, Huang X, Zhang P. Dengue virus up-regulates expression of notch ligands Dll1 and Dll4 through interferon-β signalling pathway. *Immunology* 2015; **144**: 127-138 [PMID: 25041739 DOI: 10.1111/imm.12357]

48 **Schaller MA**, Neupane R, Rudd BD, Kunkel SL, Kallal LE, Lincoln P, Lowe JB, Man Y, Lukacs NW. Notch ligand Delta-like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines. *J Exp Med* 2007; **204**: 2925-2934 [PMID: 17998388 DOI: 10.1084/jem.20070661]

49 **Emuss V**, Lagos D, Pizzey A, Gratrix F, Henderson SR, Boshoff C. KSHV manipulates Notch signaling by DLL4 and JAG1 to alter cell cycle genes in lymphatic endothelia. *PLoS Pathog* 2009; **5**: e1000616 [PMID: 19816565 DOI: 10.1371/journal.ppat.1000616]

50 **Cuninghame S**, Jackson R, Zehbe I. Hypoxia-inducible factor 1 and its role in viral carcinogenesis. *Virology* 2014; **456-457**: 370-383 [PMID: 24698149 DOI: 10.1016/j.virol.2014.02.027]

51 **Wilson GK**, Tennant DA, McKeating JA. Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. *J Hepatol* 2014; **61**: 1397-1406 [PMID: 25157983 DOI: 10.1016/j.jhep.2014.08.025]

P- Reviewer: Abd-Elsalam A, Abid S, Ahn KS, Li W
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3871-3883

DOI: 10.3748/wjg.v24.i34.3871

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

ORIGINAL ARTICLE

Basic Study

Optimal immunosuppressor induces stable gut microbiota after liver transplantation

Jian-Wen Jiang, Zhi-Gang Ren, Hai-Feng Lu, Hua Zhang, Ang Li, Guang-Ying Cui, Jun-Jun Jia, Hai-Yang Xie, Xin-Hua Chen, Yong He, Li Jiang, Lan-Juan Li

Jian-Wen Jiang, Zhi-Gang Ren, Jun-Jun Jia, Hai-Yang Xie, Xin-Hua Chen, Yong He, Li Jiang, Department of Hepatobiliary and Pancreatic Surgery, First Affiliated Hospital, School of Medicine, Zhejiang University, Key Laboratory of Combined Multi-organ Transplantation, Ministry of Public Health, Hangzhou 310003, Zhejiang Province, China

Jian-Wen Jiang, Zhi-Gang Ren, Hai-Feng Lu, Hua Zhang, Ang Li, Guang-Ying Cui, Jun-Jun Jia, Hai-Yang Xie, Xin-Hua Chen, Lan-Juan Li, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Hai-Feng Lu, Hua Zhang, Ang Li, Lan-Juan Li, State Key Laboratory for Diagnosis and Treatment of Infectious Disease, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Zhi-Gang Ren, Ang Li, Guang-Ying Cui, Department of Infectious Diseases, Precision Medicine Center, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Jian-Wen Jiang, Health Management Center, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

ORCID number: Jian-Wen Jiang (0000-0003-3745-1254); Zhi-Gang Ren (0000-0002-0913-0130); Hai-Feng Lu (0000-0003-2886-5615); Hua-Zhang (0000-0001-7333-9548); Ang Li (0000-0003-4685-4752); Guang-Ying Cui (0000-0003-3656-8100); Jun-Jun Jia (0000-0002-1495-2390); Hai-Yang Xie (0000-0002-6913-9284); Xin-Hua Chen (0000-0002-4573-9111); Yong He (0000-0003-4814-7880); Li Jiang (0000-0002-6461-3354); Lan-Juan Li (0000- 0001-6945-0593).

Author contributions: Li LJ designed the experiments; Jiang JW, Ren ZG, Lu HF, Zhang H, Jia JJ, Xie HY, He Y, Jiang L performed the experiments; Lu HF, Ren ZG, Li A, Chen XH analyzed the data; Ren ZG, Jiang JW wrote the paper; All authors reviewed and approved the manuscript.

Supported by the National Natural Science Foundation of China, No. 81672422, No. 81600506, and No. 81702757; Open Project in State Key Laboratory for Diagnosis and Treatment of Infectious Disease, No. 2015KF03; National S&T Major Project of China, No. 2018ZX10301201; Natural Science Foundation of Zhejiang Province, No. LY15H160033; China Postdoctoral Science Foundation, No. 2017464; Zhejiang Province Health Department Program, No. 2014KYB081, and No. 2017KY322; Academician Jieshou Li Mucosal Barrier Fund, No. 201208.

Institutional animal care and use committee statement: This study was reviewed and approved by the Research Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University.

Conflict-of-interest statement: All authors declare no conflict of interest exists.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Lan-Juan Li, MD, PhD, Academic Research, Doctor, Professor, Senior Researcher, State Key Laboratory for Diagnosis and Treatment of Infectious Disease, First Affiliated Hospital, School of Medicine, Zhejiang University, 79# Qingchun Road, Hangzhou 310003, Zhejiang Province, China. ljli@zju.edu.cn
Telephone: +86-571-87236466
Fax: +86-571-87236466

Received: June 21, 2018
Peer-review started: June 21, 2018

First decision: July 18, 2018
Revised: July 24, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 14, 2018

Abstract

AIM

To study the influence of different doses of tacrolimus (FK506) on gut microbiota after liver transplantation (LT) in rats.

METHODS

Specific pathogen-free Brown Norway (BN) rats and Lewis rats were separated into five groups: (1) Tolerance group (BN-BN LT, n = 8); (2) rejection group (Lewis-BN LT, n = 8); (3) high dosage FK506 (FK506-H) group (Lewis-BN LT, n = 8); (4) middle dosage FK506 (FK506-M) group (Lewis-BN LT, n = 8); and (5) low dosage FK506 (FK506-L) group (Lewis-BN LT, n = 8). FK506 was administered to recipients at a dose of 1.0 mg/kg, 0.5 mg/kg, and 0.1 mg/kg body weight for 29 d after LT to the FK506-H, FK506-M, and FK506-L groups, respectively. On the 30th day after LT, all rats were sampled and euthanized. Blood samples were harvested for liver function and plasma endotoxin testing. Hepatic graft and ileocecal tissues were collected for histopathology observation. Ileocecal contents were used for DNA extraction, Real-time quantitative polymerase chain reaction (RT-PCR) and digital processing of denaturing gradient gel electrophoresis (DGGE) profiles and analysis.

RESULTS

Compared to the FK506-H and FK506-L groups, FK506-M was optimal for maintaining immunosuppression and inducing normal graft function; the FK506-M maintained gut barrier integrity and low plasma endotoxin levels; furthermore, DGGE results showed that FK506-M induced stable gut microbiota. Diversity analysis indicated that FK506-M increased species richness and rare species abundance, and cluster analysis confirmed the stable gut microbiota induced by FK506-M. Phylogenetic tree analysis identified crucial bacteria associated with FK506-M; seven of the nine bacteria that were decreased corresponded to *Bacteroidetes*, while increased bacteria were of the *Bifidobacterium* species. FK506-M increased *Faecalibacterium prausnitzii* and *Bifidobacterium spp*. and decreased *Bacteroides-Prevotella* and *Enterobacteriaceae*, as assessed by RT-PCR, which confirmed the crucial bacterial alterations identified through DGGE.

CONCLUSION

Compared to the low or high dosage of FK506, an optimal dosage of FK506 induced immunosuppression, normal graft function and stable gut microbiota following LT in rats. The stable gut microbiota presented increased probiotics and decreased potential pathogenic endotoxin-producing bacteria. These findings provide a novel strategy based on gut microbiota for immunosuppressive dosage assessment for recipients following LT.

Key words: Liver transplantation; Graft function; Gut microbiota; Immunosuppressor; Tacrolimus; Rejection; Denaturing gradient gel electrophoresis

Core tip: This is the first study to illustrate the effects of different dosages of Tacrolimus (FK506) on gut microbiota following liver transplantation (LT) and indicates that an optimal dosage of FK506 induces effective immunosupression, good graft function and stable gut microbiota after LT in rats. Based on the relationship between gut microbiota and the immunosuppressive dosage in this study, we can not only illustrate precise changes of gut microbiota given by different dosages of FK-506 following LT, but we also provide a novel monitoring strategy based on changes in gut microbiota for immunosuppressive dosage assessment in patients following LT.

Jiang JW, Ren ZG, Lu HF, Zhang H, Li A, Cui GY, Jia JJ, Xie HY, Chen XH, He Y, Jiang L, Li LJ. Optimal immunosuppressor induces stable gut microbiota after liver transplantation. *World J Gastroenterol* 2018; 24(34): 3871-3883 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3871.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3871

INTRODUCTION

Liver transplantation (LT) is considered the only definitive therapy for end-stage liver diseases, including liver cirrhosis, liver failure and hepatocellular carcinoma[1,2]. Currently, the 1-year survival rate in patients following LT has reached 90% and the 5-year survival has reached 75%[3]. However, rejection after LT remains the major cause of hepatic graft dysfunction and is a life-threatening complication[4]. Treatment with inadequate immunosuppression might lead to a higher risk of rejection and even graft dysfunction, whereas excessive immunosuppression has been closely associated with a greater incidence of infection, sepsis, drug toxicity, cancer, chronic graft dysfunction, renal dysfunction, and even increased mortality[5-7], with bacterial infection being the most prevalent type of infection[8]. Thus, the optimal dosage of immunosuppressive medication is crucial for the prevention of rejection, lessening of immunosuppression toxicity and treatment of patients following LT[9].

The gut microbiota has been considered the most important micro-ecosystem that possesses a symbiotic relationship within the body[10-12]. The gut microbiota

plays a crucial role in the development of fatty liver disease[13], liver cirrhosis[14], hepatocellular carcinoma[15], liver ischemic injury[16] and liver graft rejection[17]. Our previous study indicated that the healing of hepatic injury could ameliorate gut barrier function and promote gut microbial restoration following LT in rats[16]. We also found that graft rejection after LT could induce gut microbial alterations, thereby inducing subsequent gut barrier dysfunction, which presented a decrease of fecal secretory immunoglobulin A (sIgA) and an increase in blood endotoxin and tumor necrosis factor-α. RT-PCR results showed that the genus *Faecalibacterium prausnitzii* and Lactobacillus were decreased, whereas *Clostridium bolteae* was increased, which might in turn aggravate hepatic rejection[18].

Nevertheless, the influence of immunosuppressive medication on gut microbiota following LT remains unclear, and the association between the dosage of immunosuppressants and gut microbial alterations requires urgent elucidation. In this study, we studied the influence of different dosages of FK506 on hepatic graft function and gut microbiota following LT in rats. Furthermore, we identified the gut microbial profile and crucial bacterial community constituents using Denaturing gradient gel electrophoresis (DGGE), and further verified the alterations of dominant gut bacterial populations with RT-PCR.

MATERIALS AND METHODS

Animals

Specific pathogen-free (SPF) male inbred Brown Norway (BN) and Lewis rats (weight 220-250 g, age 12-15 wk) were purchased from Beijing Vital River Laboratories (Beijing, China). They were housed in a clean-level animal house located at the First Affiliated Hospital, School of Medicine, Zhejiang University. All rats were housed at 22-24 ℃ in 12 h light/dark cycles and fed sterilized standard rat chow and water.

Experimental design and protocol

The study animals were divided into five groups as follows: (1) Tolerance group (BN-BN LT, n = 8); (2) rejection group (Lewis-BN LT, n = 8); (3) high dosage FK506 (FK506-H) group (Lewis-BN LT, n = 8); (4) middle dosage FK506 (FK506-M) group (Lewis-BN LT, n = 8); (5) low dosage FK506 (FK506-L) group (Lewis-BN LT, n = 8). In the Tolerance group, both donors and recipients were BN rats; in the other four groups, both donors were Lewis and recipients were BN rats. FK506 was administered to recipients at a dose of 1.0, 0.5, and 0.1 mg/kg weight for 1 mo after LT to the FK506-H, FK506-M, and FK506-L groups, respectively. To mimic the clinical application, FK506 was administrated *via* abdominal subcutaneous injection once every 12 h for 7 d after LT, and then *via* intragastric administration, once per day for the following 8-29 d. The sustained-release FK506 was administered intragastrically to maintain a consistent dosage effect for 24 h.

The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23, revised 1985). The experimental protocol was approved by the Animal Care and Use Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University.

LT surgical procedures and sample collection

The LT surgery was performed according to our previous methods[16,19], with slight modifications. The anesthesia was performed by intraperitoneal injection of Ketamine Hydrochloride (100 mg/kg) and Atropine (1 mg/kg) (Shanghai No. 1 Biochemical and Pharmaceutical, China), and then ether was inhaled to maintain anesthesia[6]. All recipients were revived shortly after the procedure and no further treatment was administered.

All rats were sampled on the 30th day after LT. The abdominal aorta was punctured, and blood samples were harvested for liver function and plasma endotoxin testing. Hepatic graft and ileum tissues near the ileocecus were collected for morphological observation. Ileocecal contents were harvested and stored at -80 ℃ for gut microbial analysis. All rats were then euthanized *via* an overdose of anesthetic.

Liver function and plasma endotoxin testing

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected using an automatic biochemical analyzer (Hitachi 7600, Tokyo, Japan).Plasma endotoxin level was determined using a colorimetric Limulus Test (Shanghai Yihua Medical Technology Co., Ltd, China) in accordance with the manufacturer's instructions.

Histopathology and transmission electron microscopy evaluation

The hepatic graft and an ileum tissue sample taken 3 cm from the ileocecus were fixed in 40 g/L neutral formaldehyde and embedded in paraffin, cut into 4-μm slices, stained with hematoxylin and eosin (HE), and then analyzed under light microscopy. At the same time, approximately 1 mm^3 of the same samples were fixed in a 2.5% glutaraldehyde solution and prepared in accordance with the standard technical procedures for transmission electron microscopy (TEM), as previously described[20]. Hepatic and ileal ultrastructures were analyzed at the Imaging Facility of Core Facilities, Zhejiang University School of Medicine. Histopathology and TEM evaluation were investigated by a pathologist blinded to the treatment group.

DNA extraction of ileocecal contents

DNA extraction of a frozen aliquot of each ileocecal fecal sample was performed as in our previous studies[16]. DNA concentration was measured by NanoDrop (Thermo Scientific), and DNA integrity was verified using agarose gel electrophoresis.

Real-time quantitative PCR

The primers used for quantitative RT-PCR (RT-qPCR) are listed in Supplementary Table 1. All oligonucleotide primers were synthesized by TAKARA (Dalian, China). RT-qPCR was performed as reported previously[21]. The copy number of 16s rDNA operons per microliter of crude DNA template was determined by comparing serially diluted plasmid DNA standards running on the same plate.

DGGE profiling

The V3 variable region of 16S rDNA was amplified using a hot-start touchdown protocol with specific primers for the conserved regions of 16S rDNA. DGGE was performed using the D-Code universal mutation detection system apparatus (Bio-Rad, Hercules, CA, United States) with 16 cm × 18 cm × 1.5 mm gels according to the manufacturer's protocol. On each DGGE gel, standard references in the middle and at each end were used for digital gel normalization and comparison of gels.

Digital processing of DGGE profiles

DGGE profiles were digitally processed using BioNumerics software version 6.01 (Applied Maths, St-Martens-Latem, Belgium) in a multistep procedure following the manufacturer's instructions. The parameters for band-class allocation followed those of our previous study[16]. Quantitative information of a given band was calculated using the Gel-Pro analyzer 4 software (Media Cybernetics, United States). Diversity was calculated using Shannon's diversity, Simpson index, species richness, Chao-1, Fisher alpha, and Menhinick index with Past software (http://folk.uio.no/ohammer/past/)[21]. Cluster analysis of the DGGE was conducted with an unweighted pair-group method with arithmetic means (UPGMA) based on the Dice similarity coefficient (band-based). Multidimensional scaling (MDS) and principal components analysis (PCA) were utilized following the instructions of the BioNumerics software.

Sequencing of DGGE bands

Specific DGGE bands of interest were excised and sequenced. Positive clones were verified and sequenced using Sanger's method on an ABI 3730 automated sequencing system (Invitrogen, Shanghai, China). Homology searches of the GenBank DNA database were performed using the BLAST tool. Reference sequences of phylogenetic neighbor species (up to 97% similarity) were included to construct a phylogenetic tree with MEGA 5.0 program based on the neighbor-joining method[15].

Statistical analysis

Continuous variables were summarized as mean ± standard deviation (SD). One-way variance analysis followed by Dunn's multiple comparison tests was utilized to evaluate statistical differences among different groups. Student *t*-test was used to examine parametric data between the two groups. Statistical analyses were performed using SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, United States). A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

A middle dosage of FK506 is optimal for maintaining liver transplant immunosuppression

An overdose or insufficient dosage of FK506 may lead to graft dysfunction, thus we first observed survival of all recipients. All rats following LT survived except one LT recipient in the FK506-H group that died of severe abdomen infection, which suggested an over-suppression of the immune status, thereby inducing infection and increasing mortality.

To illustrate the influence of different dosages of FK506 on hepatic grafts, we observed graft morphology. Under light microscopy (Figure 1A), hepatic grafts in the tolerance group presented a regular structure with well-arranged hepatocyte cords. In the rejection group, graft histology showed no obvious hepatocyte cords, accumulation of numerous red blood cells, and marked hepatocyte necrosis. However, FK506-M treatment preserved an approximately normal hepatic structure without hepatic acute rejection. Notably, hepatic graft resulted in hepatocyte cord disruption, widened sinusoids, and middle rejection injury in the FK506-L dosage group, suggesting an insufficient suppression of the immune status, thereby inducing rejection. Thus, compared to the FK506-H and FK506-L groups, FK506-M was the optimal dosage for maintaining immunosuppression in rats following LT.

We next observed hepatic graft ultrastructure with TEM (Figure 1B). In the tolerance group, a normal hepatocyte structure with intact mitochondria and endoplasmic reticulum was observed. Hepatic graft presented significant karyopyknosis, marked mitochondrial vacuolar degeneration, and organelle breakdown in the rejection group. Nevertheless, FK506-M treatment noticeably improved hepatocyte structure, mitochondria morphology, and other organelle structures. Notably, hepatocyte apoptosis and mild organelle damage were noted in the FK506-L group. Plasma ALT and AST levels reflected graft function. Compared with the rejection group, plasma ALT and AST were significantly decreased in the FK506-M treatment group (both $P < 0.001$). In addition, plasma ALT and AST were reduced in the FK506-M group versus the FK506-L group (both $P < 0.05$). There was no statistically significant difference between the tolerance and FK506-M-treated groups (Figure 1C).

An optimal dosage of FK506 keeps the gut barrier intact and maintains low endotoxin levels following LT

The liver-gut circulation and the gut-liver axis are closely associated with gut and liver function following LT. Thus,

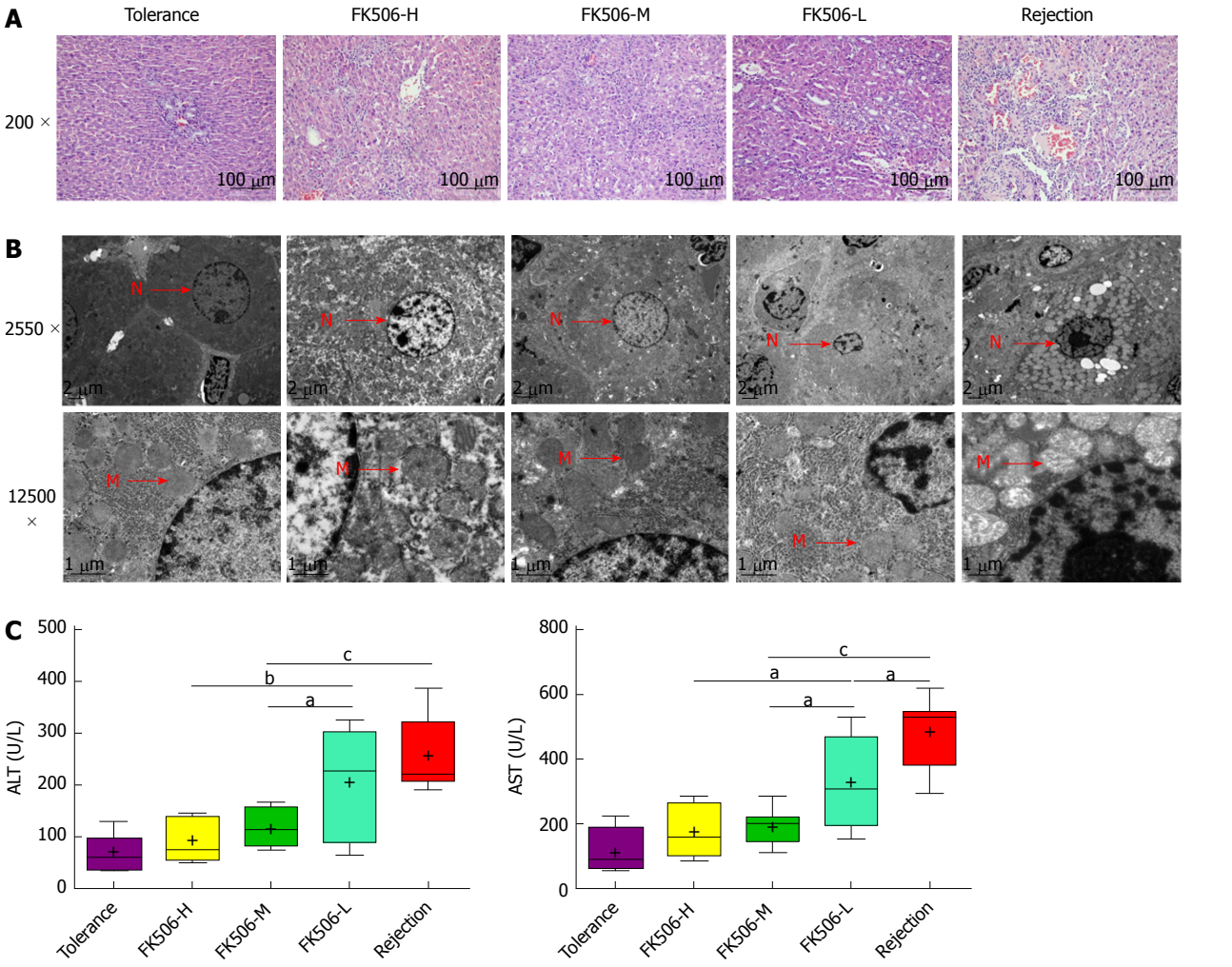


Figure 1 Middle dosage of FK506 is optimal for maintaining immunosuppression after liver transplantation. A: Representative image of the hepatic graft pathological structure stained with hematoxylin and eosin (HE, 200 ×); B: Representative hepatocyte ultrastructure obtained via transmission electron microscopy (TEM, 2550 ×, 12500 ×); C: Assessment of hepatic graft function by plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in different treatment groups. N: Cell nucleus; M: Mitochondria. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

we next observed the ileal mucosa ultrastructure using TEM (Figure 2A). In the tolerance group, intestinal epithelial cells presented a normal morphology with many homogenously distributed microvilli and integrated tight junctions. In contrast, microvilli loss, tight junction damage, and bacterial invasion in epithelial cells were observed in the rejection group. However, FK506-M treatment significantly improved intestinal epithelial structure and kept microvilli and tight junctions intact. Notably, epithelial cell damage, microvilli disruption, and wider lateral spaces between neighboring cells were observed in the FK506-L treatment group.

Plasma endotoxin reflects gut barrier function. Compared with the tolerance and FK506-M groups, plasma endotoxin was significantly increased in the FK506-H group ($P < 0.01$ and 0.05, respectively), suggesting that over-suppression of immune status might lead to higher endotoxin levels. Meanwhile, plasma endotoxins were remarkably elevated in the rejection and FK506-L groups compared to the tolerance group (both $P < 0.001$). However, FK506-M administration significantly decreased endotoxin levels compared to the rejection and FK506-L groups (both $P < 0.001$) (Figure 2B).

An optimal dosage of FK506 induces a stable gut microbiota as determined by DGGE

Endotoxins are the product of common gram-negative bacteria and can initiate various pathophysiological cascades[22]. Plasma endotoxin changes are mainly derived from changes in the gut microbiota. Based on the DGGE method, we analyzed the distribution of the ileocecal bacterial community (Figure 3). To characterize the DGGE profiles, we utilized the Dice coefficient and UPGMA as a cluster method to indicate band similarity. These profiles formed two primary clusters. The left cluster mainly consisted of samples from the tolerance group, in which total similarity was 80.1%, with the similarity among lanes ranging from 80.1% to 91.1%. The other cluster contained samples mainly from the rejection group and the different doses of the FK506 groups. Importantly, the similarity among lanes ranged

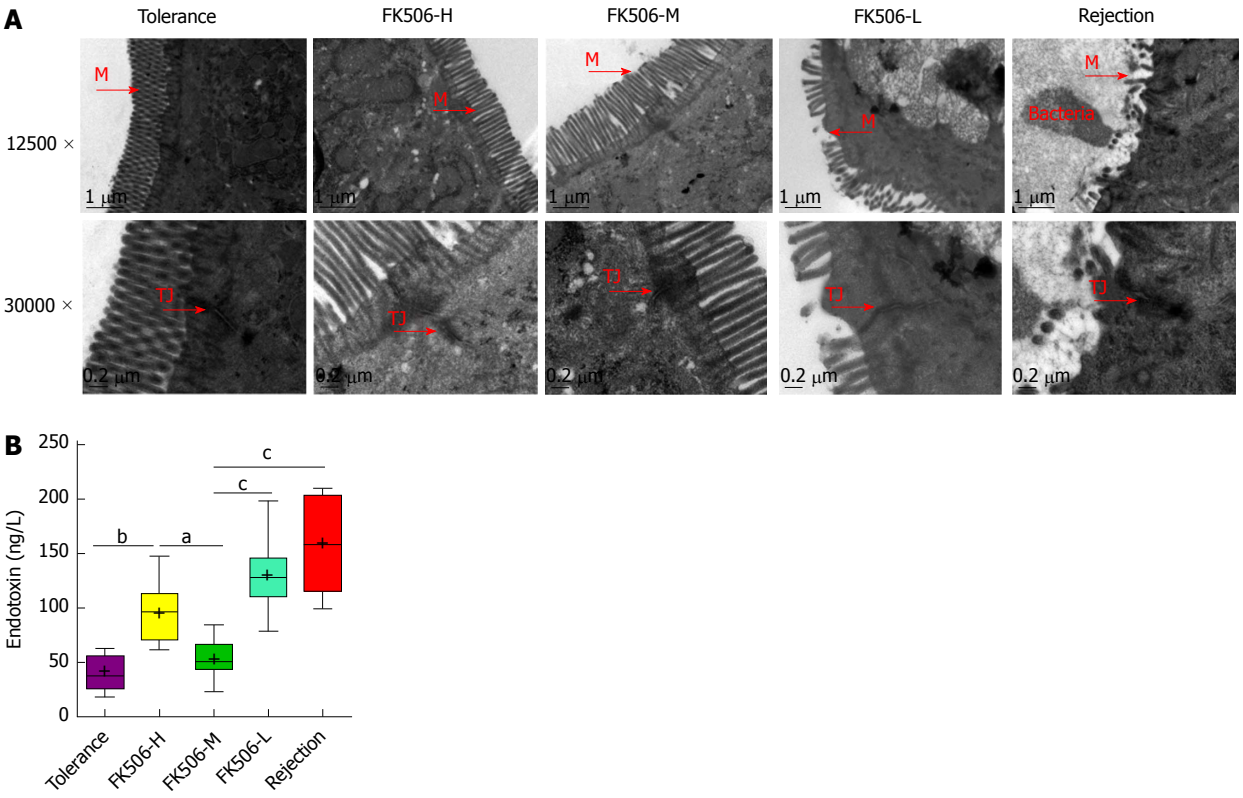


Figure 2 An optimal dosage of FK506 maintained the gut barrier and resulted in low endotoxin levels after liver transplantation. A: Representative intestinal mucosal ultrastructure shown by transmission electron microscopy for the different treatment groups (12500 ×, 30000 ×); B: Levels of plasma endotoxins in the different groups. M: Microvilli; TJ: Tight junction. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

from 82.4% to 96.7% in the rejection group and from 82.7% to 95.4% in the FK506-M treatment group, suggesting a significant uniqueness and stability of the gut microbiota.

In contrast, the DGGE lanes from the FK506-H and FK506-L treatment groups presented an inordinate distribution and an evident difference. The similarity of these lanes was relatively low and could not be clustered together. These results based on the DGGE profile indicated that an optimal dosage of FK506 induced a unique and stable gut microbiota in rats following LT.

Diversity analysis and cluster analysis of gut microbiota using DGGE profiles

Gut microbial diversity and species richness are important indexes used to evaluate gut microbial balance and stability. Thus, we calculated the gray amount of each band in each lane of the DGGE profiles with Gel-Pro analyzer to compare microbial diversity. There were no statistical differences in the Shannon and Simpson indexes among the five groups (Figure 4A). Compared to the tolerance group, species richness was significantly decreased in the FK506-H, FK506-L, and rejection groups (all $P < 0.05$). In contrast, FK506-M treatment markedly increased species richness compared to the FK506-H, FK506-L, and rejection groups ($P < 0.01$, 0.05 and 0.001, respectively) (Figure 4A).

We also analyzed the abundance of rare species, estimated using the Chao-1, Fisher alpha, and Menhinick indexes. Compared with the tolerance group, Chao-1, Fisher alpha and Menhinick indexes were remarkably reduced in the FK506-H, FK506-L, and rejection groups (all $P < 0.05$). However, FK506-M treatment significantly increased the abundance of rare species in contrast to the FK506-H, FK506-L, and rejection groups (all $P < 0.05$) (Figure 4B).

Furthermore, based on DGGE profiles, we conducted MDS and PCA analysis. The Euclidean distance between two points indicates the similarity between the two. Gut microbial structures from samples of the tolerance, FK506-M, and rejection groups were respectively clustered together, and showed separation from each other using MDS analysis (Dim 1, Dim 2, and Dim 3) (Figure 4C), suggesting a relatively unique and stable gut microbiota. PCA is an alternative approach to visualizing relationships among lanes using lane data (band classes). Our PCA analysis also gave similar results based on PCA axes X/Y/Z (13.7%, 11.2%, and 10.0%, respectively) (Figure 4D).

Phylogenetic tree analysis of sequences based on DGGE profiles

Of the 49 PCR-DGGE bands analyzed in the study, 48 band classes were selected. To identify crucial bacterial populations induced by FK506-M treatment, we compared band intensity between the FK506-M and the rejection groups. Of the 41 band classes selected,

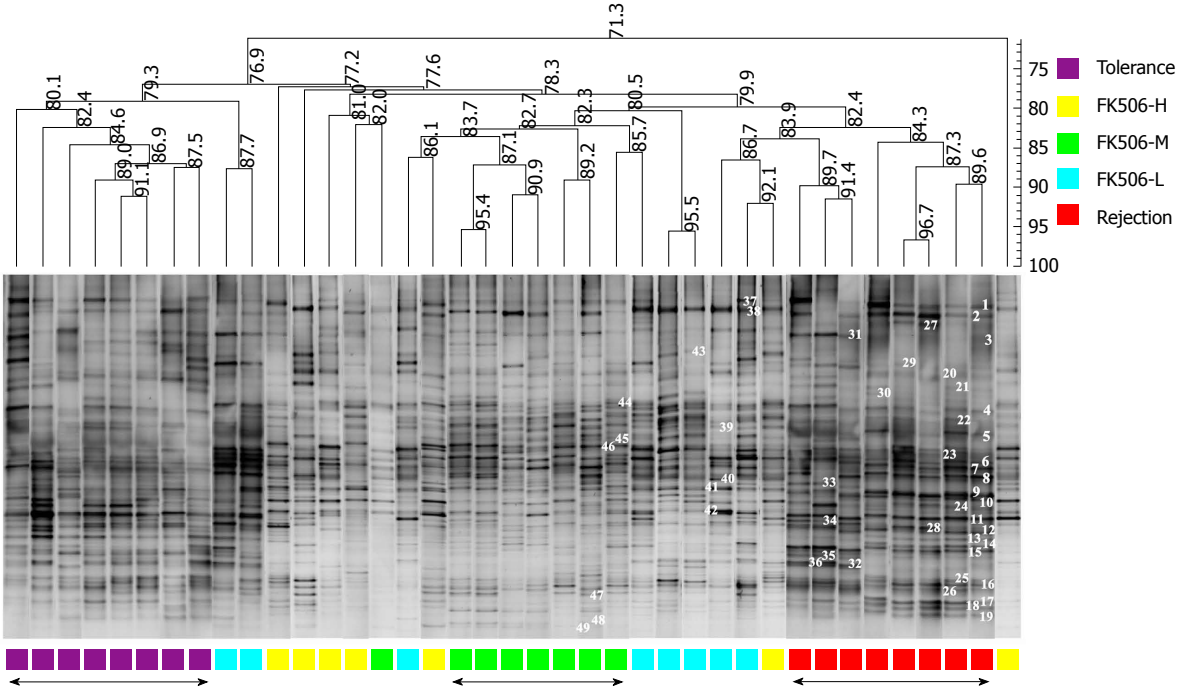


Figure 3 Optimal dosage of FK506 induced a stable gut microbiota as determined using denaturing gradient gel electrophoresis. Based on the DGGE method, the composition and distribution of ileocecal bacterial communities from samples was obtained in rats following LT. The Dice similarity coefficient (band-based) and unweighted pair-group method with arithmetic means (UPGMA) as a cluster method were utilized to analyze the similarity of each band in the different groups. Metric scale denotes the degree of similarity. Each band represents a bacterial clone. Band numbers indicate the position of bands excised for sequence analyses. DGGE: Denaturing gradient gel electrophoresis.

28 showed slight variation in band intensity between the two groups. In contrast to the rejection group, the intensities of nine band classes [18.2 (band 1), 19.9 (band 2), 61.0 (band 9), 72.9 (band 15), 81.0 (band 16), 82.0 (band 26), 85.3 (band 17), 86.5 (band 18), and 88.2 (band 19)] were significantly decreased (Figure 5A), while four band classes [33.7 (band 20), 41.8 (band 39), 56.8 (band 41), and 82.7 (band 47)] were increased in the FK506-M treated group (Figure 5B).

To determine the bacterial phylogenetic relationships and identify key bacteria involved in the microbial changes induced by FK506-M, the phylogenetic tree of sequences derived from DGGE bands was analyzed using the MEGA 5 software (Figure 6). Almost all the matched bacteria of the 48 band classes were assigned to three phyla: *Bacteroidetes* (50.0%), *Firmicutes* (39.6%), and *Gammaproteobacteria* (10.4%). The closest matched bacteria shown on the phylogenetic tree corresponded to 13 crucial band classes associated with FK506-M treatment. Details of the 13 crucial band classes are presented in Supplementary Table 2. In these crucial bacteria associated with FK506-M treatment, 7 of the 9 decreased band classes were matched to *Bacteroidetes bacterium*, belonging to phylum *Bacteroidetes*, but the increased band classes were matched to the *Bifidobacterium* species.

Quantitative verification of the predominant bacterial community in fecal microbiota using RT-qPCR

To verify crucial bacteria alterations induced by FK506-M treatment, we further analyzed dominant bacterial populations using RT-qPCR (Figure 7). Compared with the tolerance group, *Faecalibacterium prausnitzii* and *Bifidobacterium spp.* were significantly decreased, whereas the *Bacteroides-Prevotella* group and *Enterobacteriaceae* were increased in the FK506-L treated and rejection groups (all $P < 0.05$). However, FK506-M treatment significantly increased *Faecalibacterium prausnitzii* and *Bifidobacterium spp.* and decreased the *Bacteroides-Prevotella* group and *Enterobacteriaceae* when compared to the FK506-L and rejection groups (all $P < 0.05$) (Figure 7A). These results essentially verified the changes observed in the levels of 13 crucial bacteria associated with FK506-M treatment based on the DGGE profiles.

Moreover, in the rejection group, the *Clostridium clusters* I was elevated, whereas the *Clostridium clusters* XI was reduced, in contrast to the other groups (Figure 7B). *Enterococcus faecalis* was specifically decreased in the tolerance group (all $P < 0.001$). There were no statistical differences in the *Clostridium cluster* XIV*ab* and *Lactobacillus* between the different groups (Figure 7C).

DISCUSSION

Liver transplantation is a life-saving technique for patients with end-stage liver disease. Appropriate dosage of immunosuppressive medications including FK506 could effectively prevent and treat allograft rejection

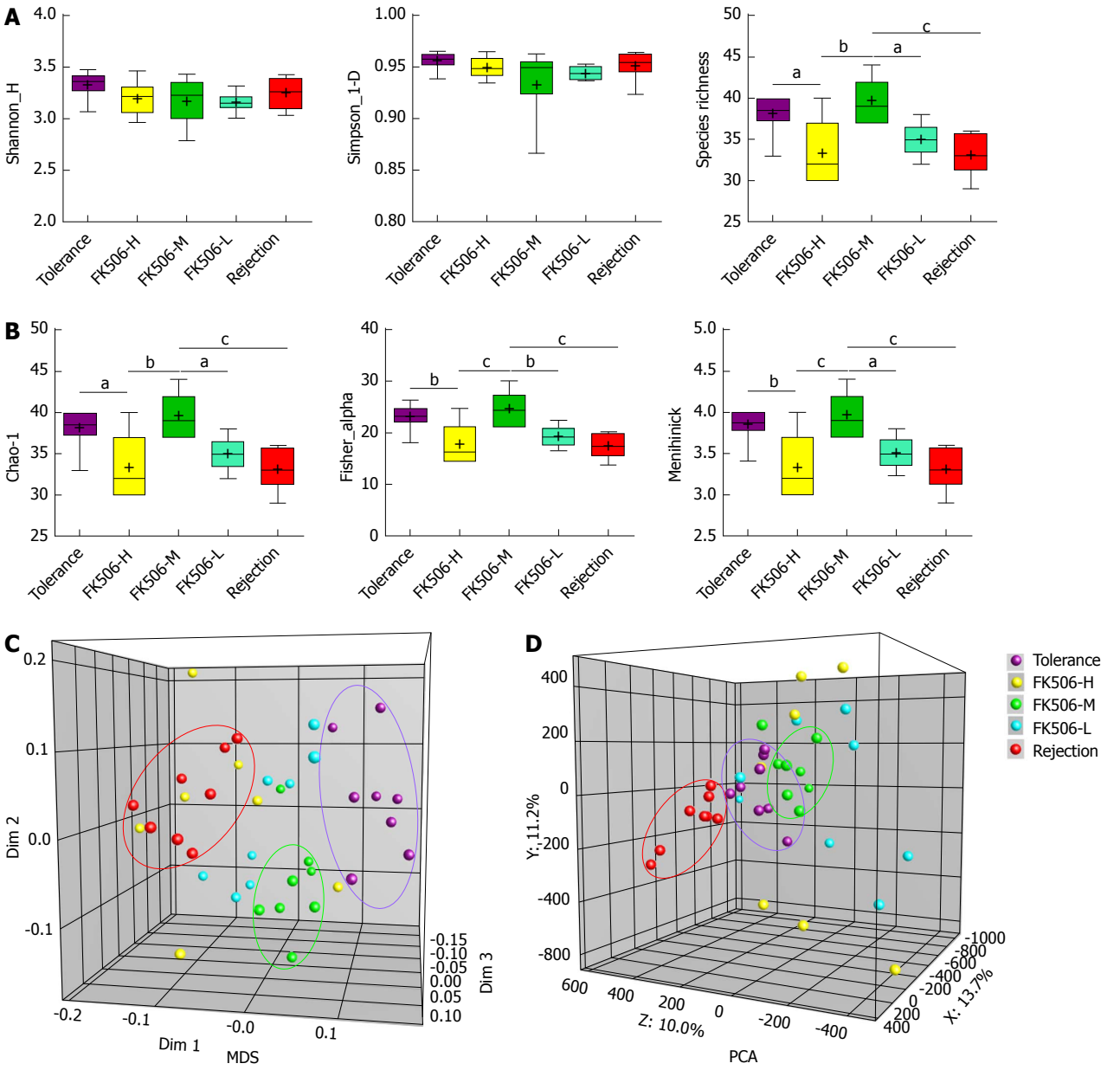


Figure 4 Diversity analysis and cluster analysis of denaturing gradient gel electrophoresis profiles based on Dice's coefficient and the unweighted pair-group method with arithmetic means method. A: Based on amount of gray area of each band of each lane in the denaturing gradient gel electrophoresis (DGGE) profiles quantified using the Gel-Pro analyzer, the gut microbial diversity and species richness were assessed with the Shannon index and Simpson index in the different groups; B: The abundance and distribution of the rare species were estimated using the Chao-1 index, Fisher alpha index, and Menhinick index in the different groups; C: Multidimensional scaling (MDS) analysis of each sample of the DGGE profiles. The plot is an optimized three-dimensional representation of the similarity matrix obtained from BioNumerics software. The Euclidean distance between the two points reflects similarity; D: Principal components analysis (PCA) of fecal microbiota based on DGGE fingerprinting. The plot is orientated to maximize the variation among lanes along the first three principal components (with contributions of 13.7, 11.2 and 10.0, respectively) obtained from BioNumerics software. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

in patients following LT. FK506 is a calcineurin inhibitor, which has become the most used immunosuppressant following LT[23]. Compared to cyclosporine, FK506 is more effective in reducing rejection, leading to better graft function and overall survival in LT patients[24]. Insufficient dosage of FK506 may lead to rejection and even graft dysfunction, while over dosage is closely associated with infection, increased complications, and even mortality. Unfortunately, FK506-related toxicity, mainly due to overload usage is frequent in LT patients[25].

In patients following LT, an optimal immunosuppressive therapy should balance the risk of infection, cancer, and drug toxicity caused by excessive immunosuppression, and the risk of rejection caused by an inadequately suppressed immune system[5]. To explore the optimal immunosuppressive dosage is very important for the long-term survival of the recipient and the liver graft. Jia *et al*[9] proved that compared to high FK506 blood concentration (10-15 ng/L) with adverse effects such as infection, early renal impairment, hepatocellular carcinoma recurrence, even

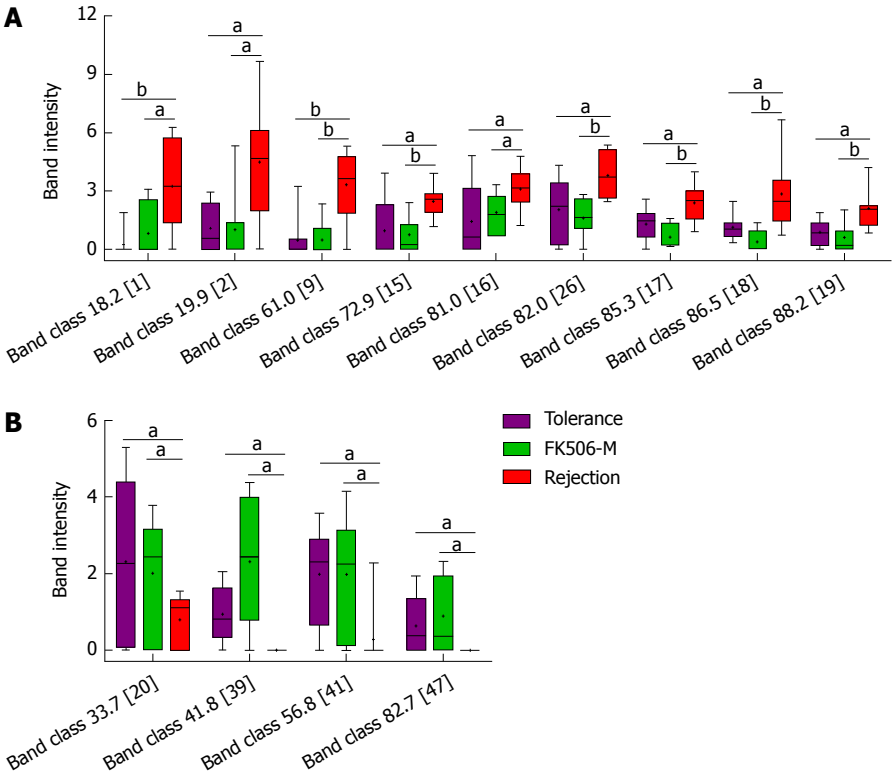


Figure 5 Identification of crucial bands of gut microbial changes induced by middle dosage tacrolimus treatment. A: Compared to the rejection group, the intensities of nine band classes including 18.2 (band 1), 19.9 (band 2), 61.0 (band 9), 72.9 (band 15), 81.0 (band 16), 82.0 (band 26), 85.3 (band 17), 86.5 (band 18), and 88.2 (band 19), were significantly decreased in the FK506-M-treated and tolerance groups; B: Compared with the rejection group, four band classes, including 33.7 (band 20), 41.8 (band 39), 56.8 (band 41), and 82.7 (band 47), showed increased intensities in the FK506-M-treated and tolerance groups. $^{a}P < 0.05$, $^{b}P < 0.01$.

death, reasonably low FK506 blood concentrations (5-10 ng/mL) by less oral dosage especially in the early phase, namely "Minimizing tacrolimus", could prevent graft rejection, avoid related toxicity, protect the graft function, and promote long-term survival of the recipient. Moreover, Geng[26] proved that lower FK506 trough levels (< 5 ng/mL) in the late period of living donor LT are safe and improve the long-term outcomes.

Our previous studies indicated that bacterial community alterations in the gut were associated with liver cirrhosis[14], hepatic graft rejection[18], hepatic injury[19], and pancreatic carcinoma[27]. However, the influence of immunosuppressive medication on the gut microbiota and the association between the dosage of immunosuppressive drugs and alterations in gut microbiota have not been reported to date. In our study, all recipients survived following LT, except one recipient in the FK506-H dosage group that died of severe abdomen infection, suggesting an over-suppression of the immune status. Hepatic graft presented intermediate rejection injury in the FK506-L group, suggesting insufficient immunosuppression. In contrast, the FK506-M dosage maintained approximately normal hepatic structure and function. Survival results and graft morphology indicated that FK506-M treatment was optimal for maintaining immunosuppression. Meanwhile, the FK506-M dosage kept the gut barrier intact and maintained low levels of plasma endotoxin. Thus, the FK506-M dosage was considered optimal for maintaining immunosuppression in rats following LT.

Furthermore, we observed the influence of FK506 with different dosages on the gut microbiota. We further analyzed gut microbiota using a DGGE approach and found that the FK506-M dosage induced a unique and stable gut microbiota. MDS and PCA analysis validated the stable gut microbiota identified by DGGE. Phylogenetic tree analysis identified crucial bacteria associated with the FK506-M treatment. In addition, treatment with FK506-M significantly increased the growth of *Faecalibacterium prausnitzii* and *Bifidobacterium spp*. and decreased that of the *Bacteroides-Prevotella* group and *Enterobacteriaceae* as determined *via* RT-qPCR, which essentially verified the alterations in the population of crucial bacteria observed from the DGGE profiles.

Endotoxin, also known as lipopolysaccharide (LPS), is the main product of common gram-negative bacteria and is a crucial factor for the association of gut microbiota with liver inflammation[22]. In a pathogen-associated molecular pattern of microbiota-liver axis, LPS may possess the capacity to activate inflammation and initiate various pathophysiological cascades. High levels of LPS activate the NF-κB pathway, produce proinflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1], and lead to liver inflammation and aggravation of hepatic oxidative damage[28]. In our study, plasma endotoxins were remarkably elevated in the rejection and FK506-L

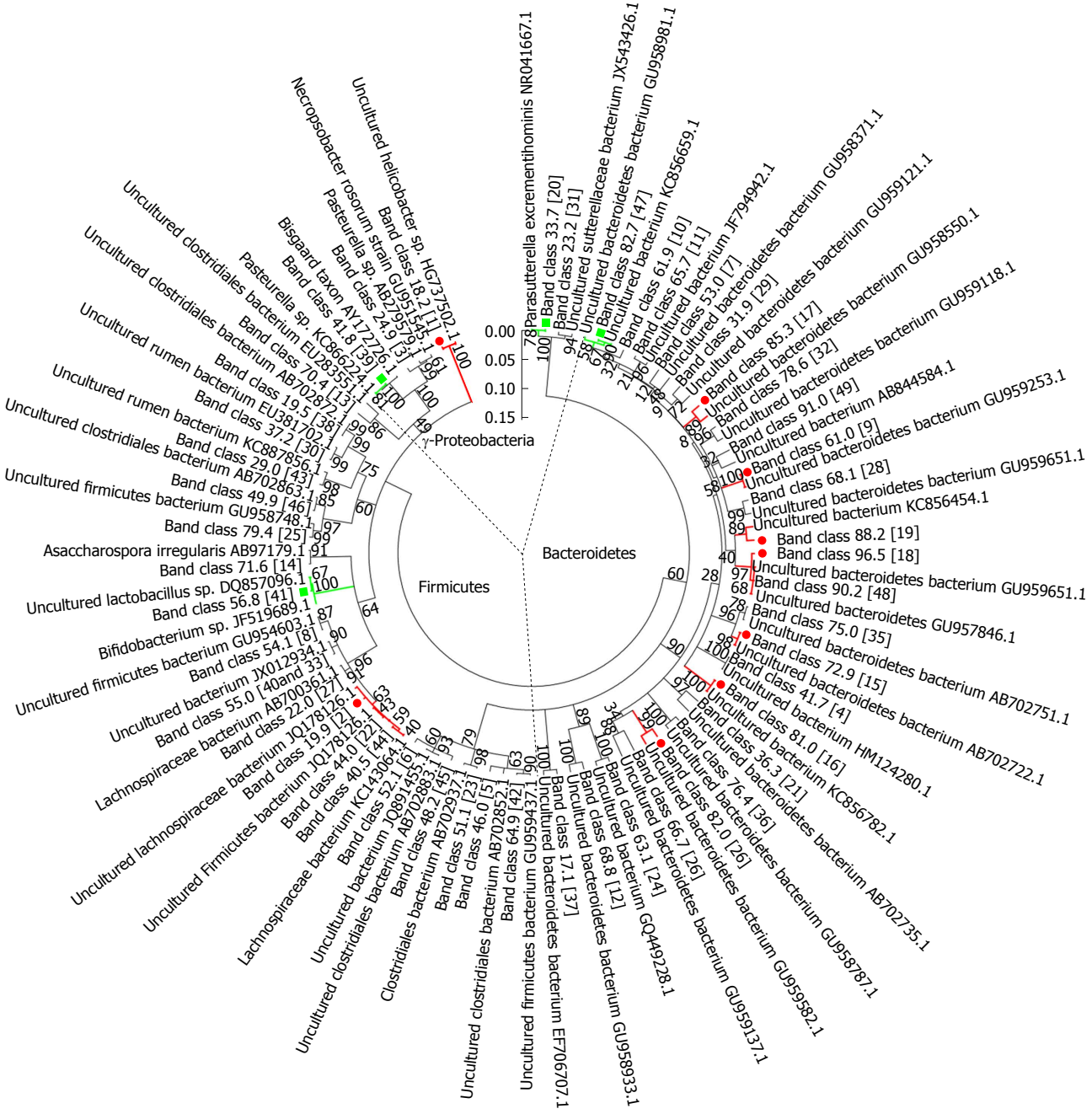


Figure 6 Phylogenetic tree analysis of sequences in the denaturing gradient gel electrophoresis profiles. Phylogenetic tree analysis of sequences from denaturing gradient gel electrophoresis (DGGE) profiles using the neighbor-joining method was conducted using MEGA 5 software. Almost all matched bacteria of the 48 DGGE band classes were assigned to three phyla: Bacteroidetes (50.0%), Firmicutes (39.6%), and Gammaproteobacteria (10.4%). The fragment sequences were defined according to their positions in gels using the band-matching tool with BioNumerics software version 6.01 (Applied Maths). The numbers in the brackets were consistent with the numbers shown in DGGE profiling. Nine band classes (labeled with a red dot) were decreased and four band classes (labeled with the green square) were higher in the FK506-M group than the rejection group. The plot was obtained using MEGA5 software (http://en.wikipedia.org/wiki/MEGA,_Molecular_Evolutionary_Genetics_Analysis).

dosage groups, but the FK506-M dosage significantly decreased endotoxin levels. To explore the causes of the observed endotoxin changes, we analyzed gut microbial alterations and identified the crucial bacteria involved. Importantly, compared to the rejection and FK506-L dosage groups, RT-qPCR results verified that FK506-M treatment significantly increased *Faecalibacterium prausnitzii* and *Bifidobacterium spp*. and decreased the growth of the *Bacteroides-Prevotella* group and Enterobacteriaceae, which are the main common gram-negative bacteria producing LPS. Thus, we speculated that the optimal dosage of FK506 might lead to an increase in probiotics and a decrease in the potential pathogenic endotoxin-producing bacteria.

In patients following LT, blood concentrations of immunosuppressant and its stability are important. To mimic clinical application, FK506 was administered *via* abdominal subcutaneous injection for 7 d and then

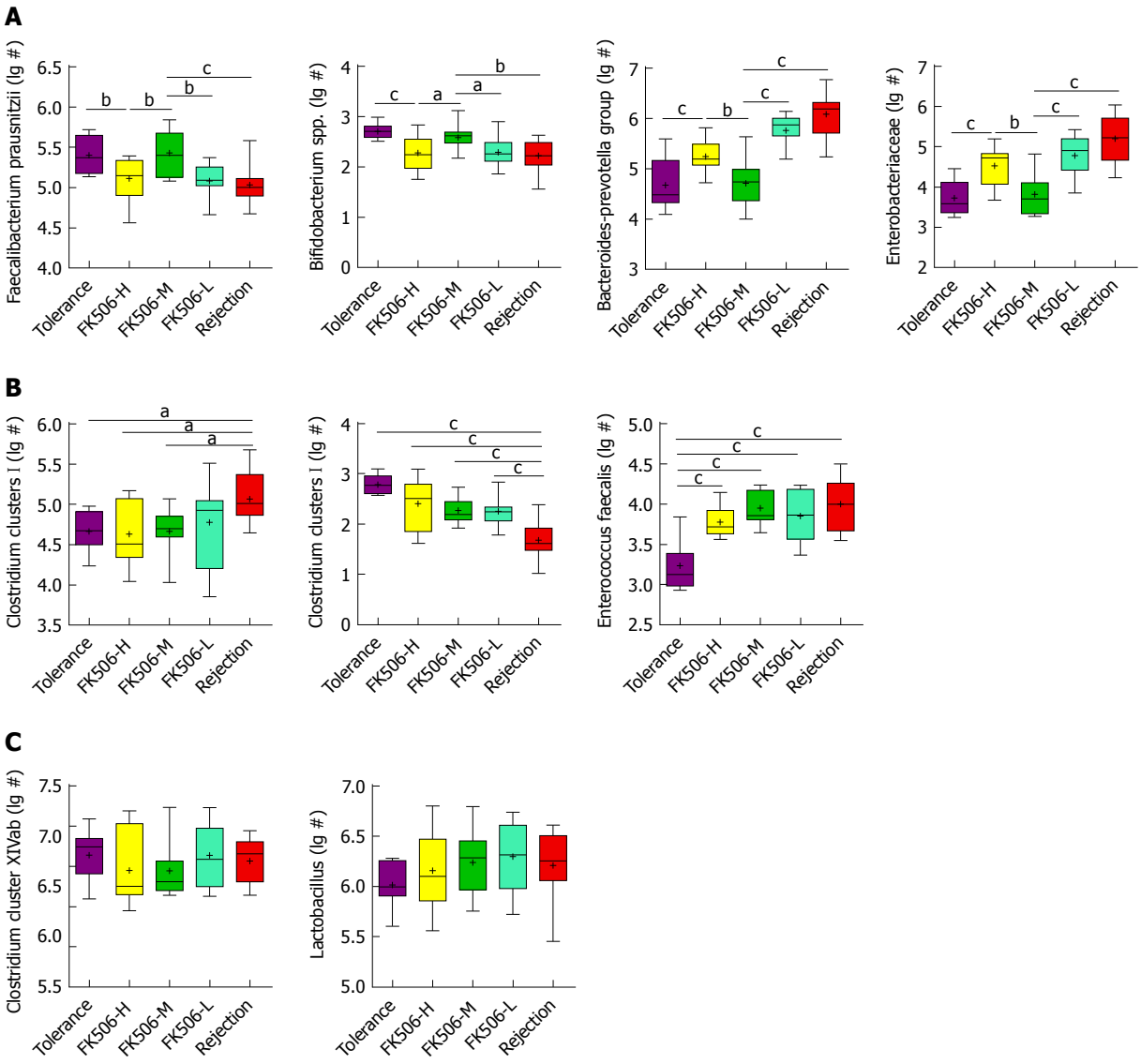


Figure 7 Quantitative verification of predominant bacterial community in fecal microbiota using RT-qPCR. Gut dominant bacterial populations were analyzed with RT-qPCR for the different groups. The dominant bacteria mainly included *Faecalibacterium prausnitzii, Bifidobacterium spp., Bacteroides-Prevotella* group, *Enterobacteriaceae, Clostridium clusters* Ⅰ, *Clostridium clusters* XI, *Enterococcus faecalis, Clostridium cluster* XIV*ab, Lactobacillus*. Statistical analysis was performed with one-way ANOVA. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. [1]Log_{10} copies/g: log_{10} No. of 16S rDNA gene copies per gram feces (wet weight).

via intragastric administration during the subsequent 8-28 d after LT. In patients, routine monitoring of blood tacrolimus concentration (TC) was performed using the PRO-TracTM Ⅱ Tacrolimus Elisa Kit (Diasorin, United States), however, there is no kit for rat blood TC testing. According to our experimental experience, it may be due to immunological reasons, as the blood TC in rats can not be tested by the above kit used for humans. Fortunately, according to clinical practice of LT, different dosages of FK506 are positively correlated to different serum concentrations across a specific dosage range, meanwhile, intragastric dosages of FK506 were more feasible and easier to control the blood TC. In this experiment, the over-low dosage of FK506 led to a higher risk of rejection, whereas over-high dosage was closely associated with a greater incidence of infection, sepsis, and even increased mortality. Therefore, treatment with different dosages of FK506 instead of relying on the examination of blood TC was feasible and credible in rats after LT.

Gut microbiota alterations are closely associated with liver function in health and disease due to liver-gut circulation and the gut microbiota-liver axis[29]. Gut microbiota is involved in a variety of human liver diseases, such as alcoholic liver disease[30,31], non-alcoholic fatty liver disease[32], non-alcoholic steatohepatitis[33], hepatitis B infection[21], liver cirrhosis[14,34], and hepatocellular carcinoma[15,28,35]. Liver diseases always reflect changes due to alterations in intestinal permeability and gut microbial composition[29], while an imbalance in the gut microbiota in turn, has been reported to be an important contributor to the progression of liver disease or liver injury[36]. Notably, the improvement of hepatic injury might ameliorate gut

barrier function and promote gut microbial restorations following LT, which might further benefit hepatic graft by positive feedback of the "gut-liver axis"[16,18]. Thus, the gut microbiota becomes an additional therapeutic target to improve hepatic injury after LT. In the present study, the optimal dosage of FK506 led to good hepatic graft function, kept the gut barrier intact, and induced a unique and stable gut microbiota, which further verify the effects of the "gut microbiota-liver" axis.

In conclusion, an optimal dosage of FK506 induces effective immunosuppression, normal graft function and stable gut microbiota after LT in rats. A stable gut microbiota resulted in increased probiotics, including *Faecalibacterium prausnitzii* and *Bifidobacterium spp*. and decreased potential pathogenic endotoxin-producing bacteria, such as the *Bacteroides-Prevotella* group and Enterobacteriaceae. These findings provide a novel strategy involving the use of the gut microbiota for the assessment of the dosage of immunosuppressive medications and its effects in recipients following LT.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation (LT) is the only definitive therapy for end-stage liver diseases. The optimal dosage of immunosuppressive medication is crucial to prevent rejection, lessen the side effects of immunosuppressors (IS) and treatment of patients following LT. The gut microbiota plays a crucial role in the development of obesity, diabetes, liver diseases and so on. Nevertheless, the influence of IS on gut microbiota following LT remains unclear, the association between the dosage of IS and gut microbial alterations requires urgent elucidation.

Research motivation

Acute rejection is still a leading cause of hepatic graft dysfunction following LT. Each recipient of LT must take IS to prevent acute rejection, but the effect of IS on gut microbiota remains unclear. Tacrolimus (FK506) is the main IS following LT, so we studied the influence of different dosages of FK506 on gut microbiota after LT in rat.

Research objectives

In this study, we studied the influence of different dosages of FK506 on hepatic graft function and gut microbiota following LT in rats. Furthermore, we will identify the precise changes of the gut microbiota given by different doses of FK-506 and provide a new monitoring strategy for IS dosage assessment in recipients after LT.

Research methods

We performed LT experiments in rats by taking different dosage of FK506 for 29 d, and on the 30th day after LT, all rats were sampled and then euthanized. We studied the hepatic graft function using serum alanine aminotransferase and aspartate aminotransferase examination and morphology change using histopathology and transmission electron microscopy evaluation. We also identified the gut microbial profile and crucial bacterial community constituents using digital processing of denaturing gradient gel electrophoresis (DGGE), and further verified the alterations of dominant gut bacterial populations with RT-PCR.

Research results

Compared to the FK506-H and FK506-L groups, FK506-M was optimal for maintaining immunosuppression and induced normal graft function; the FK506-M maintained the integrity of gut barrier and low plasma endotoxin levels. Furthermore, FK506-M induced stable gut microbiota, increased species richness and rare species abundance by DGGE and Cluster analysis. The FK506-M increased *Faecalibacterium prausnitzii* and *Bifidobacterium spp*. and decreased *Bacteroides-Prevotella* and Enterobacteriaceae by Phylogenetic tree analysis and RT-PCR verification.

Research conclusions

An optimal dosage of FK506 induces effective immunosuppression, normal graft function and stable gut microbiota after LT in rat. A stable gut microbiota resulted in increased probiotics and decreased potential pathogenic endotoxin-producing bacteria. These findings provide a novel strategy involving the use of the gut microbiota for the assessment of the dosage of immunosuppressive medications and their effects on recipients following LT.

Research perspectives

This study is the first to illustrate the effects of FK506 with different dosages on gut microbiota following LT and indicates that an optimal dosage of FK506 induces effective immunosuppression, good graft function and stable gut microbiota after LT in rats. Based on the relationship between gut microbiota and immunosuppressive dosage in this study, we can not only illustrate precise changes of gut microbiota given by FK-506 with different dosages following LT, but we also provide a novel monitoring strategy based on changes in gut microbiota for IS assessment in recipients following LT. With the improvement of metagenomics and metabolomics techniques, the integrative study can further reveal how the gut microbiota participates in the side effects of IS on recipients and gut microbiota may be a target to reduce side effects of IS. In addition, in order to further study the mechanism of the involvement of gut microbiota on the side effects of IS on recipients, it is essential to do clinical research.

REFERENCES

1 **Hübscher SG**. What is the long-term outcome of the liver allograft? *J Hepatol* 2011; **55**: 702-717 [PMID: 21426919 DOI: 10.1016/j.jhep.2011.03.005]

2 **Sayegh MH**, Carpenter CB. Transplantation 50 years later--progress, challenges, and promises. *N Engl J Med* 2004; **351**: 2761-2766 [PMID: 15616214 DOI: 10.1056/NEJMon043418]

3 **Wiesner RH**, Menon KV. Late hepatic allograft dysfunction. *Liver Transpl* 2001; **7**: S60-S73 [PMID: 11689778 DOI: 10.1053/jlts.2001.29094]

4 **Hu J**, Wang Z, Tan CJ, Liao BY, Zhang X, Xu M, Dai Z, Qiu SJ, Huang XW, Sun J, Sun QM, He YF, Song K, Pan Q, Wu Y, Fan J, Zhou J. Plasma microRNA, a potential biomarker for acute rejection after liver transplantation. *Transplantation* 2013; **95**: 991-999 [PMID: 23466638 DOI: 10.1097/TP.0b013e31828618d8]

5 **Ravaioli M**, Neri F, Lazzarotto T, Bertuzzo VR, Di Gioia P, Stacchini G, Morelli MC, Ercolani G, Cescon M, Chiereghin A, Del Gaudio M, Cucchetti A, Pinna AD. Immunosuppression Modifications Based on an Immune Response Assay: Results of a Randomized, Controlled Trial. *Transplantation* 2015; **99**: 1625-1632 [PMID: 25757214 DOI: 10.1097/TP.0000000000000650]

6 **Jiang JW**, Ren ZG, Cui GY, Zhang Z, Xie HY, Zhou L. Chronic bile duct hyperplasia is a chronic graft dysfunction following liver transplantation. *World J Gastroenterol* 2012; **18**: 1038-1047 [PMID: 22416178 DOI: 10.3748/wjg.v18.i10.1038]

7 **Zhang W**, Fung J. Limitations of current liver transplant immunosuppressive regimens: renal considerations. *Hepatobiliary Pancreat Dis Int* 2017; **16**: 27-32 [PMID: 28119255 DOI: 10.1016/S1499-3872(16)60167-4]

8 **Humar A**, Michaels M; AST ID Working Group on Infectious Disease Monitoring. American Society of Transplantation recommendations for screening, monitoring and reporting of infectious complications in immunosuppression trials in recipients of organ transplantation. *Am J Transplant* 2006; **6**: 262-274 [PMID: 16426310 DOI: 10.1111/j.1600-6143.2005.01207.x]

9 **Jia JJ**, Lin BY, He JJ, Geng L, Kadel D, Wang L, Yu DD, Shen T, Yang Z, Ye YF, Zhou L, Zheng SS. "Minimizing tacrolimus" strategy and long-term survival after liver transplantation. *World J Gastroenterol* 2014; **20**: 11363-11369 [PMID: 25170223 DOI: 10.3748/wjg.v20.i32.11363]

10 **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]
11 **Nenci A**, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, Huth M, Nikolaev A, Neufert C, Madison B, Gumucio D, Neurath MF, Pasparakis M. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 2007; **446**: 557-561 [PMID: 17361131 DOI: 10.1038/nature05698]
12 **Cani PD**, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* 2009; **15**: 1546-1558 [PMID: 19442172 DOI: 10.2174/138161209788168164]
13 **Shen F**, Zheng RD, Sun XQ, Ding WJ, Wang XY, Fan JG. Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. *Hepatobiliary Pancreat Dis Int* 2017; **16**: 375-381 [PMID: 28823367 DOI: 10.1016/S1499-3872(17)60019-5]
14 **Chen Y**, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L. Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 2011; **54**: 562-572 [PMID: 21574172 DOI: 10.1002/hep.24423]
15 **Dapito DH**, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, Khiabanian H, Adeyemi A, Bataller R, Lefkowitch JH, Bower M, Friedman R, Sartor RB, Rabadan R, Schwabe RF. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* 2012; **21**: 504-516 [PMID: 22516259 DOI: 10.1016/j.ccr.2012.02.007]
16 **Ren Z**, Cui G, Lu H, Chen X, Jiang J, Liu H, He Y, Ding S, Hu Z, Wang W, Zheng S. Liver ischemic preconditioning (IPC) improves intestinal microbiota following liver transplantation in rats through 16s rDNA-based analysis of microbial structure shift. *PLoS One* 2013; **8**: e75950 [PMID: 24098410 DOI: 10.1371/journal.pone.0075950]
17 **Xie Y**, Luo Z, Li Z, Deng M, Liu H, Zhu B, Ruan B, Li L. Structural shifts of fecal microbial communities in rats with acute rejection after liver transplantation. *Microb Ecol* 2012; **64**: 546-554 [PMID: 22430504 DOI: 10.1007/s00248-012-0030-1]
18 **Ren Z**, Jiang J, Lu H, Chen X, He Y, Zhang H, Xie H, Wang W, Zheng S, Zhou L. Intestinal microbial variation may predict early acute rejection after liver transplantation in rats. *Transplantation* 2014; **98**: 844-852 [PMID: 25321166 DOI: 10.1097/TP.0000000000000334]
19 **Ren ZG**, Liu H, Jiang JW, Jiang L, Chen H, Xie HY, Zhou L, Zheng SS. Protective effect of probiotics on intestinal barrier function in malnourished rats after liver transplantation. *Hepatobiliary Pancreat Dis Int* 2011; **10**: 489-496 [PMID: 21947722 DOI: 10.1016/S1499-3872(11)60083-0]
20 **Ren Z**, Chen X, Cui G, Yin S, Chen L, Jiang J, Hu Z, Xie H, Zheng S, Zhou L. Nanosecond pulsed electric field inhibits cancer growth followed by alteration in expressions of NF-κB and Wnt/β-catenin signaling molecules. *PLoS One* 2013; **8**: e74322 [PMID: 24069295 DOI: 10.1371/journal.pone.0074322]
21 **Lu H**, Wu Z, Xu W, Yang J, Chen Y, Li L. Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. Intestinal microbiota of HBV cirrhotic patients. *Microb Ecol* 2011; **61**: 693-703 [PMID: 21286703 DOI: 10.1007/s00248-010-9801-8]
22 **Lamping N**, Dettmer R, Schröder NW, Pfeil D, Hallatschek W, Burger R, Schumann RR. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J Clin Invest* 1998; **101**: 2065-2071 [PMID: 9593762 DOI: 10.1172/JCI2338]
23 **US Multicenter FK506 Liver Study Group**. A comparison of tacrolimus (FK 506) and cyclosporine for immunosuppression in liver transplantation. *N Engl J Med* 1994; **331**: 1110-1115 [PMID: 7523946 DOI: 10.1056/NEJM199410273311702]
24 **Haddad EM**, McAlister VC, Renouf E, Malthaner R, Kjaer MS, Gluud LL. Cyclosporin versus tacrolimus for liver transplanted patients. *Cochrane Database Syst Rev* 2006; : CD005161 [PMID: 17054241 DOI: 10.1002/14651858.CD005161]
25 **Wiesner RH**, Fung JJ. Present state of immunosuppressive therapy in liver transplant recipients. *Liver Transpl* 2011; **17** Suppl 3: S1-S9 [PMID: 21850697 DOI: 10.1002/lt.22410]
26 **Geng L**, Wang LD, Huang JJ, Shen T, Wang ZY, Lin BY, Ye YF, Zheng SS. Lower tacrolimus trough levels in the late period after living donor liver transplantation contribute to improvements in long-term clinical outcomes. *Hepatobiliary Pancreat Dis Int* 2018; **17**: 204-209 [PMID: 29807766 DOI: 10.1016/j.hbpd.2018.05.001]
27 **Ren Z**, Jiang J, Xie H, Li A, Lu H, Xu S, Zhou L, Zhang H, Cui G, Chen X, Liu Y, Wu L, Qin N, Sun R, Wang W, Li L, Wang W, Zheng S. Gut microbial profile analysis by MiSeq sequencing of pancreatic carcinoma patients in China. *Oncotarget* 2017; **8**: 95176-95191 [PMID: 29221120 DOI: 10.18632/oncotarget.18820]
28 **Darnaud M**, Faivre J, Moniaux N. Targeting gut flora to prevent progression of hepatocellular carcinoma. *J Hepatol* 2013; **58**: 385-387 [PMID: 22940407 DOI: 10.1016/j.jhep.2012.08.019]
29 **Chassaing B**, Etienne-Mesmin L, Gewirtz AT. Microbiota-liver axis in hepatic disease. *Hepatology* 2014; **59**: 328-339 [PMID: 23703735 DOI: 10.1002/hep.26494]
30 **Yan AW**, Fouts DE, Brandl J, Stärkel P, Torralba M, Schott E, Tsukamoto H, Nelson KE, Brenner DA, Schnabl B. Enteric dysbiosis associated with a mouse model of alcoholic liver disease. *Hepatology* 2011; **53**: 96-105 [PMID: 21254165 DOI: 10.1002/hep.24018]
31 **Hartmann P**, Chen P, Wang HJ, Wang L, McCole DF, Brandl K, Stärkel P, Belzer C, Hellerbrand C, Tsukamoto H, Ho SB, Schnabl B. Deficiency of intestinal mucin-2 ameliorates experimental alcoholic liver disease in mice. *Hepatology* 2013; **58**: 108-119 [PMID: 23408358 DOI: 10.1002/hep.26321]
32 **Le Roy T**, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, Martin P, Philippe C, Walker F, Bado A, Perlemuter G, Cassard-Doulcier AM, Gérard P. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* 2013; **62**: 1787-1794 [PMID: 23197411 DOI: 10.1136/gutjnl-2012-303816]
33 **Zhu L**, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, Gill SR. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology* 2013; **57**: 601-609 [PMID: 23055155 DOI: 10.1002/hep.26093]
34 **Qin N**, Yang F, Li A, Prifti E, Chen Y, Shao L, Guo J, Le Chatelier E, Yao J, Wu L, Zhou J, Ni S, Liu L, Pons N, Batto JM, Kennedy SP, Leonard P, Yuan C, Ding W, Chen Y, Hu X, Zheng B, Qian G, Xu W, Ehrlich SD, Zheng S, Li L. Alterations of the human gut microbiome in liver cirrhosis. *Nature* 2014; **513**: 59-64 [PMID: 25079328 DOI: 10.1038/nature13568]
35 **Zhang HL**, Yu LX, Yang W, Tang L, Lin Y, Wu H, Zhai B, Tan YX, Shan L, Liu Q, Chen HY, Dai RY, Qiu BJ, He YQ, Wang C, Zheng LY, Li YQ, Wu FQ, Li Z, Yan HX, Wang HY. Profound impact of gut homeostasis on chemically-induced pro-tumorigenic inflammation and hepatocarcinogenesis in rats. *J Hepatol* 2012; **57**: 803-812 [PMID: 22727732 DOI: 10.1016/j.jhep.2012.06.011]
36 **Schnabl B**, Brenner DA. Interactions between the intestinal microbiome and liver diseases. *Gastroenterology* 2014; **146**: 1513-1524 [PMID: 24440671 DOI: 10.1053/j.gastro.2014.01.020]

P- Reviewer: Cholongitas E, Venu RP **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3884-3897

DOI: 10.3748/wjg.v24.i34.3884

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

ORIGINAL ARTICLE

Basic Study

Formin-like 3 regulates RhoC/FAK pathway and actin assembly to promote cell invasion in colorectal carcinoma

Yuan-Feng Zeng, Yi-Sheng Xiao, Yong Liu, Xiao-Jiang Luo, Li-Dan Wen, Qian Liu, Min Chen

Yuan-Feng Zeng, Yong Liu, Qian Liu, Min Chen, Department of Pathology, Jiangxi Provincial People's Hospital, Nanchang 330006, Jiangxi Province, China

Yi-Sheng Xiao, Teaching and Researching Section of Morphology, College of Basic Medicine, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, Jiangxi Province, China

Xiao-Jiang Luo, Department of General Surgery, Jiangxi Provincial People's Hospital, Nanchang 330006, Jiangxi Province, China

Li-Dan Wen, Clinical Medical Sciences Institute, Jiangxi Provincial People's Hospital, Nanchang 330006, Jiangxi Province, China

ORCID number: Yuan-Feng Zeng (0000-0003-4174-3575); Yi-Sheng Xiao (0000-0002-9487-0175); Yong Liu (0000-0002-6162-6168); Xiao-Jiang Luo (0000-0002-4894-4575); Li-Dan Wen (0000-0003-1531-1904); Qian Liu (0000-0003-1149-0650); Min Chen (0000-0002-5345-0991).

Author contributions: Zeng YF, Xiao YS, Liu Y, Luo XJ, Wen LD, Liu Q and Chen M carried out the experiment; Zeng YF conceived experiments and analyzed data; all authors were involved in writing this paper and had final approval of the submitted and published versions.

Supported by the National Natural Science Foundation of China, No. 81201972; the China Postdoctoral Science Foundation, No. 2013M531555; and the Postdoctoral Science Foundation of Jiangxi province, No. 2013KY44.

Institutional review board statement: The study was reviewed and approved by Ethical Committee of Jiangxi Provincial People's Hospital.

Conflict-of-interest statement: The authors declare no conflicts of interest in the present study.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Yuan-Feng Zeng, PhD, Chief Doctor, Department of Pathology, Jiangxi Provincial People's Hospital, 152 Patriotic Road, Donghu District, Nanchang 330006, Jiangxi Province, China. zyf760928@163.com
Telephone: +86-791-86895672
Fax: +86-791-86895672

Received: May 5, 2018
Peer-review started: May 7, 2018
First decision: May 23, 2018
Revised: June 16, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: September 14, 2018

Abstract

AIM

To clarify the underlying mechanism of formin-like 3 (FMNL3) in the promotion of colorectal carcinoma (CRC) cell invasion.

METHODS

The *in vitro* biological function analyses of FMNL3 were performed by gain- and loss-of function approaches. Changes in the F-actin cytoskeleton were detected by the technologies of phalloidin-TRITC labeling and confocal microscopy. The signaling pathway mediated by FMNL3 was explored by western blot, gelatin zymograph assay, co-immunoprecipitation (co-IP), immunofluorescence co-localization, and glutathione S-transferase (GST) pull-down assay.

RESULTS

The *in vitro* experimental results showed that FMNL3 significantly promoted the proliferation, invasion, and migration of CRC cells ($P < 0.05$ and $P < 0.01$). Moreover, FMNL3 regulated the remodeling of actin-based protrusions such as filopodia and lamellipodia in a RhoC-dependent manner. The western blot and gelatin zymograph assay results indicated that FMNL3 was involved in the RhoC/focal adhesion kinase (FAK) pathway and acted as an effector of RhoC to activate the downstream signaling of p-FAK as well as p-MAPK and p-AKT. This resulted in the increased expression of matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9) and vascular endothelial growth factor (VEGF), and the subsequent promotion of CRC cell invasion. The results of TAE226, U0126 or Ly294002 treatment confirmed an essential role of FMNL3 in activation of the RhoC/FAK pathway and the subsequent promotion of CRC invasion. Co-IP, co-localization and GST pull-down assays showed the direct interaction of FMNL3 with RhoC *in vivo* and *in vitro*.

CONCLUSION

FMNL3 regulates the RhoC/FAK signaling pathway and RhoC-dependent remodeling of actin-based protrusions to promote CRC invasion.

Key words: Formin-like 3; Colorectal carcinoma; Invasion; RhoC/FAK pathway; Actin assembly

Core tip: Formin-like 3 (FMNL3) belongs to the subfamily of diaphanous-related formins, which govern the actin-dependent processes, including cell motility and invasion. The increased expression of FMNL3 in colorectal carcinoma (CRC) was shown to contribute to metastasis and poor prognosis of patients in previous studies, however its regulatory mechanism remains unclear. This work reveals that FMNL3 plays a positive role in CRC cell proliferation, invasion and migration. Moreover, FMNL3 activates the RhoC/FAK signaling pathway, and also regulates RhoC-dependent remodeling of actin-based protrusion, such as filopodia and lamellipodia, to promote CRC cell invasion. FMNL3 can be applied as a promising specific biomarker for CRC progression and metastasis.

Zeng YF, Xiao YS, Liu Y, Luo XJ, Wen LD, Liu Q, Chen M. Formin-like 3 regulates RhoC/FAK pathway and actin assembly to promote cell invasion in colorectal carcinoma. *World J Gastroenterol* 2018; 24(34): 3884-3897 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3884.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3884

INTRODUCTION

Colorectal carcinoma (CRC) is the third most common diagnosis and second deadliest malignancy, and metastasis remains the major cause of mortality in patients with CRC[1,2]. Deregulated cell motility and invasion is a key initial step in metastasis[3]. Invasive cell migration involves movement through tissues, dynamic interactions with the extracellular matrix, rearrangements of cell-to-cell contacts and the cytoskeleton[4]. Further understanding of the underlying regulatory mechanisms may provide novel therapeutic regimes for reducing cancer cell dissemination, blocking metastatic progression, and prolonging life expectancy of patients with CRC.

Diaphanous-related formins (DRFs) are ubiquitously expressed proteins and known to govern cell shape, adhesion, and motility by remodeling the actin cytoskeleton[5-8]. The DRF protein contains a Rho-GTPase binding domain (GBD) in the NH2-terminus. Upon binding to a Rho-GTPase, the bound NH2-terminal diaphanous inhibitory domain is dissociated from the C-terminal diaphanous autoregulatory domain. This, in turn, results in the release of inactive DRF autoinhibition, and subsequently allows the formin homology 2 (FH2) domain to function as the regulator of actin assembly. Three members of the DRFs (DRF1-DRF3) were reported to be associated with invadopodia formation and the invasion of breast tumor cells[9]. As the largest family of Rho GTPase effectors, DRFs regulate cytoskeletal remodeling and cancer cell invasion downstream of Rho GTPase signaling. The DRF protein formin-like 2 (FMNL2) drives actin-based protrusion and migration downstream of CDC42 in melanoma cells[10], and drives the amoeboid invasive cell motility downstream of RhoC[3]. Positive feedback between Dia1, LARG, and RhoA regulates cancer cell morphology and invasion by affecting actin assembly[11].

Formin-like 3 (FMNL3), another novel member of the DRF family, has been recently identified[12]. Several studies have demonstrated the role of FMNL3 in cytoskeletal remodeling and cell migration. FMNL3 participates in filopodia assembly, microtubule acetylation and cell-cell adhesion[13-15], as well as induces protein N-myristoylation required for cellular morphological changes[16]. FMNL3 is also required for the polarized trafficking of podocalyxin to the early apical surface in vascular lumenogenesis, and is a crucial regulator of angiogenesis[17,18]. Recent studies have demonstrated upregulation of FMNL3 in cutaneous melanoma and nasopharyngeal cancer[19,20], as well as its promotion of cancer cell invasion and migration in nasopharyngeal, esophageal carcinoma and neuroblastoma[20-22]. Our previous study also indicated that increased expression of FMNL3 contributes to metastasis and poor prognosis in patients with CRC[23]. Although literature on FMNL3 expression and function in multiple tumors has been presented, the underlying molecular mechanism of FMNL3-promoting tumor progression and metastasis remains to be elucidated.

Hence, in this study we investigate the effects of FMNL3 on CRC cell proliferation, invasion and migration *in vitro* using gain- and loss-of-function approaches. Moreover, we reveal an essential role for FMNL3 in regulating

the RhoC/FAK pathway and actin assembly dynamics, and the subsequent promotion of CRC invasion.

MATERIALS AND METHODS

Cell lines and reagents

All four CRC cell lines (LOVO, SW620, SW480 and HCT116) and the 293T cell line were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured at 37℃ in a 50 mL/L CO_2-humidified atmosphere with the appropriate medium according to the requirements of the Cell Bank. Anti-(p-) Pyk2 (proline-rich tyrosine kinase 2), anti-(p-) FAK, anti-(p-) MAPK (Mitogen activated protein kinase), anti-(p-) AKT and anti-RhoC antibodies were purchased from Cell Signaling Technology. Anti-flag, anti-VEGF (vascular endothelial growth factor) and anti-FMNL3 antibodies were obtained from Abbkine, Inc (Redlands, CA, United States) and Abnova (Taiwan, China), respectively. For inhibitor treatment, 1 μmol/L TAE226 (Selleck), 20 μmol/L U0126 (Selleck) or 20 μmol/L Ly294002 (Selleck) was added to the cultured cells for 48 h, respectively.

Construction of plasmids and transfection

Two groups of specific RNA interference sequences targeting the coding regions of FMNL3 and Pyk2 genes were designed as in the previous study[24,25]. The ones were separately cloned into the GV102 plasmid (Genechem Biotechnology, Shanghai, China) to construct FMNL3-silenced cell lines, named "FMNL3/shRNA1" and "FMNL3/shRNA2". A scrambled shRNA, which has no homology with the mammalian mRNA sequences, was inserted into the GV102 vector and served as the control. The same method was used to construct the Pyk2-silenced cell lines, named "Pyk2/shRNA1" and "Pyk2/shRNA2". To obtain an active mutant construct of RhoC-V14, the wild-type coding region of RhoC was amplified by polymerase chain reaction (PCR) and inserted into the expression plasmid pGEX-4T-1. The mutant construct was then generated with the KOD-Plus-Mutagenesis Kit (TOYOBO, Japan). The primers were designed as follows: 5'-GCTGCAATCCGAAAGAAGCTGGTGA-3' or 5' –TCAGAGAATGGGACAGCCCCTCCGA-3'. DNA was purified with a Mini plasmid Purification Kit (Qiagen, Japan) and digested with suitable restriction enzymes. DNA fragments were electrophoresed on 1% agarose to verify the insertion of sequences. Cells were plated into 6-well plates using 1×10^6 cells/well to grow overnight to 90% confluence, and transiently transfected with 3 μg of plasmid using 2 μL Lipofectamine™ 2000 (Invitrogen, United States) according to the instructions. Cells were incubated for 48 h until they were ready for further assays.

Establishment of cell lines stably expressing FMNL3

Commercialization of the viral particles that express the coding region of the *FMNL3* gene, fused EGFP and three flag genes were purchased from GeneCopoeia, Inc (Guangzhou, China). The *FMNL3* gene was amplified by PCR and then inserted into the plasmid pcDNA3 (Invitrogen, Forster City, CA, United States). The primers used were as follows: forward 5'-TCCGATTCATTCTTAC-3', reverse 5'-CCGCCTCAACTCTGCTATT-3'. The PCR conditions were as follows: 95℃ for 3 min, followed by 35 cycles of amplification (94 ℃ for 30 s, 55 ℃ for 40 s, 72℃ for 2 min). The fragment was inserted into the pGC-FU-EGFP-3FLAG lentiviral vector. The FMNL3 overexpression vector was transfected into lentiviral packaging 293T cells. The culture supernatant containing viral particles was harvested 48h after transfection of 293T cells. The day before the infection of viral particles, CRC cells were seeded into 24-well plates using 1×10^4 cells/well. The next day, 2×10^{12} TU/L of viral supernatant containing 5 μg/mL of polybrene was added to the cells. After 72 h, 2.5 mg/L puromycin (Sigma, United States) was added to the culture for screening. On approximately day 14, puromycin-resistant cell pools were established by selection. Following amplification culture, real-time PCR and Western blot were performed to validate the upregulation of FMNL3.

MTT assay

Cells were inoculated into 96-well plates (1×10^2 cells/well) with 100 ul/well medium and cultured for 5 d. Every 24 h, MTT (20 μL, 5 mg/mL; Promega) was added to the cells to incubate for 4 h until purple precipitates were visible. Precipitates were then dissolved with 150 μL of DMSO. The absorbance value of each well was measured with a microplate reader set at 570 nm. The experiment was repeated three times and the average value was calculated.

In vitro invasive assay

The *in vitro* invasive ability was tested by Boyden chamber assay. The invasion chamber was equipped with 8 μm pores in polyethylene terephthalate membrane coated with matrigel (BD Biosciences, Foster City, CA, United States). First, 1.5×10^5 tumor cells in serum-free RPMI 1640 medium were added to the upper chamber, and the RPMI 1640 with 10% fetal bovine serum was added to the lower chamber as the chemotactic factor. Each cell group was plated in three replicate wells. After incubation for 24 h, the noninvasive cells were gently removed with a cotton swab. Cells that invaded the membrane were fixed with methanol and stained with Giemsa. The number of invaded cells was counted under a light microscope in five random visual fields. The experiment was repeated three times and the average value was calculated.

In vitro scratch assay

The *in vitro* scratch assay is an easy, low-cost and well-developed method to measure cell migration *in vitro*[26]. Cells were seeded into a 24-well plate. When the cells were cultured to confluence, the cell monolayer was scraped in the form of a cross with a

plastic pipette tip. Then the three "wound" areas were marked for orientation and photographed by a phase-contrast microscope both immediately and after 24 h of incubation. The experiment was repeated three times.

F-actin staining and observation

Cells were seeded into 14 mm Confocal Petri dishes and cultured for 24 h. The cells were then fixed with 40 g/L formaldehyde for 30 min, permeabilized by 0.1% Triton X-100 for 10 min, and then blocked with 1% BSA for 30 min, followed by incubation with 5 μg/mL rhodamine-conjugated phalloidin (Sigma, United States) for 1 h. After counter-staining with DAPI, F-actin images were acquired with an Olympus FV1000 confocal microscope (Olympus, Japan) using a 100 × oil immersion objective. The length of filopodia and the cells with broad lamellipodia were quantified as in the previous study[18,24]. The experiment was repeated three times and the average value was calculated.

Western blot assay

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed using ice Lysis buffer containing 0.1% protease inhibitors and 0.5% phenylmethanesulfonyl fluoride (Keygen, China). The proteins in the cells were quantified using the bicinchoninic acid method. Fifty micrograms of proteins were loaded onto 10% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then electro-transferred onto PVDF membranes (Millipore) and blocked in 5% nonfat dry milk in tris-buffered saline. Membranes were immunoblotted overnight at 4 ℃ with anti-FMNL3 antibody (Abnova), anti-RhoC, anti-Pyk2 (or -p-Pyk2), anti-MAPK (or -p-MAPK), anti-AKT (or -p-AKT) (Cell signaling technology), anti-VEGF or anti-GAPDH antibody (Abbkine), respectively, and followed by respective horseradish peroxidase-conjugated secondary antibodies (Abbkine). Signals were detected by BeyoECL Plus (Beyotime Biotechnology, China).

Gelatin zymograph assay

Cells were seeded into 6-well plates and incubated in serum-free medium for 48 h. The cell supernatant was then collected, and the protein concentration was quantified. The cell supernatant was mixed with 5 × SDS loading buffer followed by electrophoresis on 10% SDS-PAGE containing 0.1% gelatin at 4 ℃. The gel was washed with the eluent (containing 2.5% Triton X-100, 50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.6) for 80 min and rinsed (50 mmol/L Tris-HCl, 5 mmol/L CaCl2, pH7.6) for 40 min. The cells were then incubated in the reaction buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 0.02% Brij-35, pH 7.6) at 37 ℃ for 42 h, stained with 0.05% coomassie brilliant blue for 3 h, and then destained with buffer containing 30% methanol and 10% acetic acid for 2 h. The image of each band was finally photographed.

Immunofluorescence co-localization assay

For fluorescence staining, cells were fixed with 40 g/L formaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 1% BSA in PBS for 30 min, followed by incubation overnight at 4 ℃ with both anti-flag and anti-RhoC antibodies. The cells were washed three times with PBS for 5 min, incubated with DyLight™ 488 conjugated Goat anti-Mouse IgG along with DyLight™ 549 conjugated Goat anti-Rabbit IgG for 30 min, and then nuclear stained with 1 mg/L 4, 6-diamidino-2-phenylindole (DAPI, Roche, Germany). The fluorescence images were acquired with an Olympus FV1000 confocal microscope (Olympus, Japan) using a 100 × oil immersion objective.

Co-immunoprecipitation assay

Cell lysates from the stably-expressing FMNL3-3 flag cells were prepared in lysis buffer (FNN0021, Life technologies). Dynabeads-Ab compound was prepared with rotation overnight at 4 ℃ (Dynabeads Protein G: 10004D, Life technologies; anti-flag antibody: Abbkine, Inc. Redlands, CA, United States; anti-RhoC antibody: Cell Signaling Technology). Dynabeads-mouse IgG (M30016, Ab-mart) was used as control. Then the co-incubated Dynabeads-Ab with cell lysates (adjusted the total protein concentration to 1 g/L before co-incubation, added 500 μL) was used to form Dynabeads-Ab-Ag compound. After this, 30 μL of 1 × SDS-PAGE loading buffer was added to the Dynabeads-Ab-Ag compound, and then boiled for western blot detection.

GST pull-down assay

The recombinant pGEX-4T-1-RhoC-V14 plasmids were transformed into colibacillus BL21 (DE3) and induced for expression by IPTG. SDS-PAGE was used for detection and analysis. Glutathione-Sepharose 4B (GE Hearthcare, Little Chalfont, United Kingdom) affinity chromatography was performed to purify GST-RhoC-V14 or GST protein according to the manufacturer's instructions. The purified proteins were then incubated with 293T cell lysates for 2 h at 4 ℃ (293T cells were transfected with the FMNL3-EGFP-3FLAG fusion gene). The Glutathione-Sepharose 4B beads were then washed with ice-cold PBS, and then bound proteins were eluted and subjected to both electrophoresis and detection with the indicated antibodies.

Statistical analysis

In vitro studies and the quantity of filopodia and lamellipodia were tested using One-Way ANOVAs or *t*-tests. SPSS Statistics 17.0.1 software (SPSS, Chicago, IL, United States) was used for all statistical analyses. $P < 0.05$ was considered as statistically significant differences.

RESULTS

FMNL3 promotes CRC cell proliferation, invasion and migration in vitro

Our previous study showed lower expression of FMNL3 in low metastatic potential cell lines (HCT116, HT29, LS174T and SW480) than in high metastatic potential

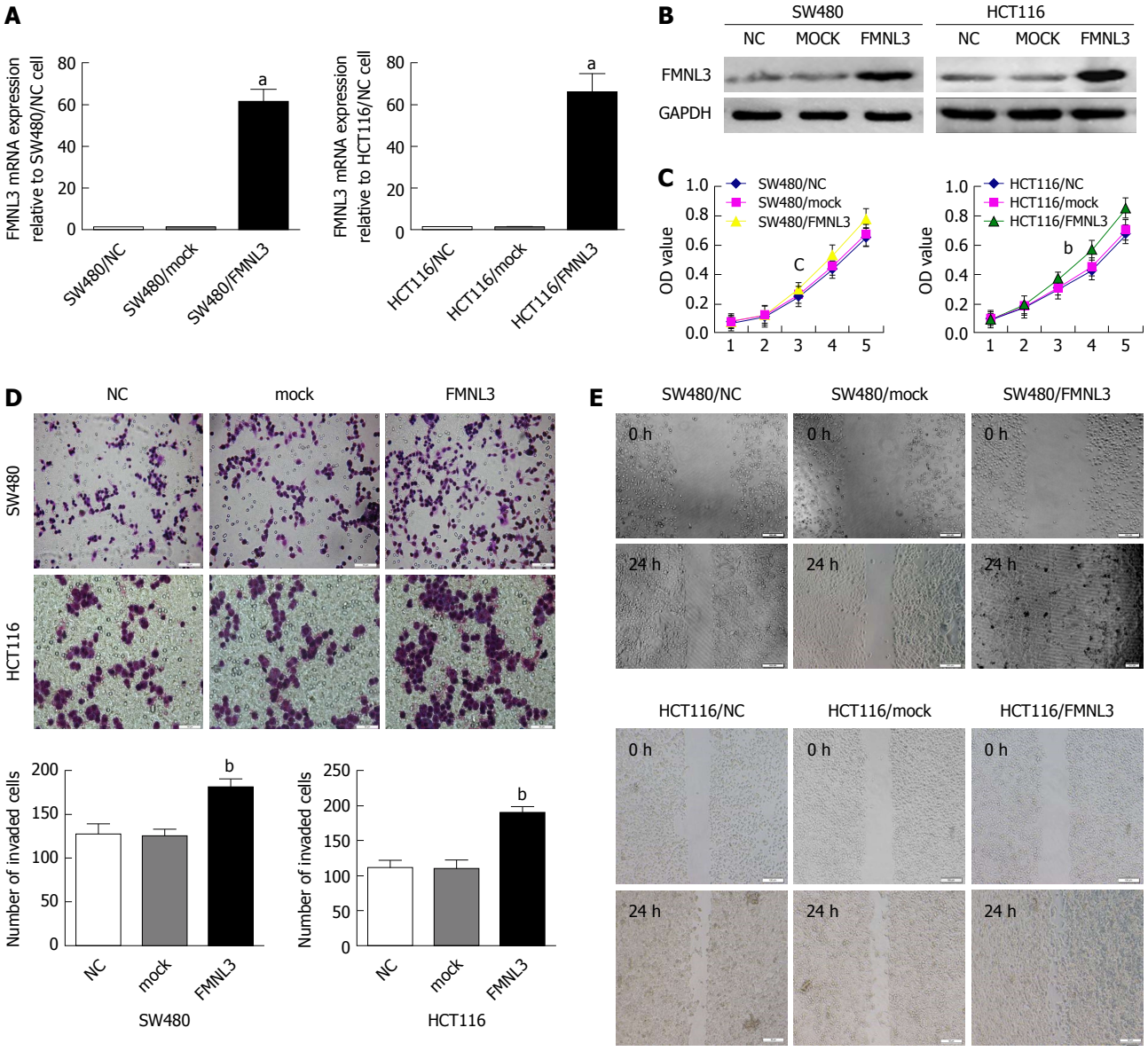


Figure 1 Forced expression of FMNL3 promotes colorectal carcinoma cell proliferation, invasion, and migration *in vitro*. A and B: Identification of FMNL3 expression in FMNL3-overexpressing cells by real-time quantitative PCR and western blot. C: Effects of FMNL3 overexpression on cell proliferation by MTT assay. D: Effects of FMNL3 overexpression on invasive abilities by Boyden chamber assay. Morphological comparison of cell penetration into the artificial basement membrane is shown. E: Effects of FMNL3 overexpression on migratory abilities by scratch assay *in vitro*. Scale bars represent 50 μm (cell invasion assay) or 100 μm (cell migration assay), respectively. $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$ *vs* NC or mock group. Error bars indicate mean ± SD.

cell lines (LOVO and SW620)[23]. Hence, we chose LOVO and SW620 to construct stable FMNL3-knockdown cell lines, as well as HCT116 and SW480 for stable FMNL3-overexpressing cell lines (Figure 1 and Supplementary Figure 1A and B). Then, a series of *in vitro* assays were performed to detect the effect of FMNL3 expression or silencing on CRC cell proliferation, invasion and migration. MTT assays showed that forced expression of FMNL3 caused a significant increase in the proliferation rate of SW480 and HCT116 cells (Figure 1C). Overexpression of FMNL3 also markedly enhanced CRC cell invasion (Figure 1D) and migration (Figure 1E) by the Boyden chamber assay and scratch assays *in vitro*, respectively. In contrast, FMNL3-depletion showed the opposite effects (Supplementary Figure 1). These data suggest that FMNL3 promotes CRC cell proliferation, invasion and migration *in vitro*.

FMNL3 regulates the assembly of actin-based protrusions

Next, we observed the effects of FMNL3 overexpression or silencing on the actin cytoskeleton within filopodia and lamellipodia in CRC cells by analysing the rhodamine-phalloidin staining of F-actin. We found that the filopodia were remarkably more abundant and longer, however the lamellipodia were more narrow in FMNL3-overexpressing cells compared with mock cells (Figure 2A and B). On the contrary, the filopodia were fewer and shorter but the lamellipodia were wider in FMNL3-depleted cells compared with scrambled cells (Figure

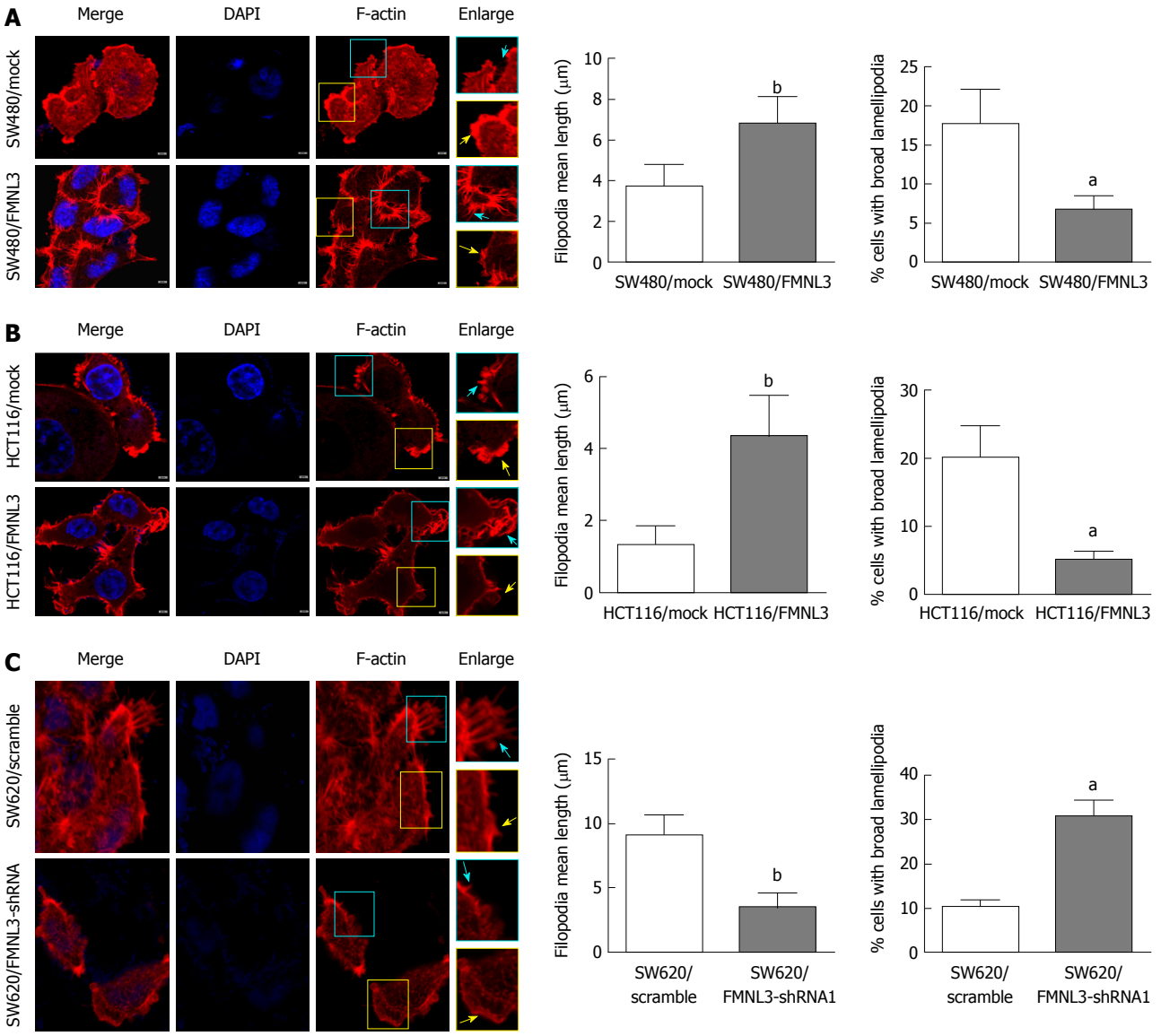


Figure 2 Effects of formin-like 3 overexpression (A and B) and depletion (C) on filopodia and lamellipodia in colorectal carcinoma cells. Cells are displayed using tritc-phalloidine (F-actin, Red) and DAPI (nuclear, blue) staining, and laser scanning confocal microscopy detection. Enlarged views of the boxed regions are shown on the right side of the figures. Blue arrows indicate filopodia, yellow arrows indicate lamellipodia. Scale bars represent 5 μm. $^{a}P < 0.001$, and $^{b}P < 0.01$ *vs* mock or scramble group. Error bars indicate mean ± SD. FMNL3: Formin-like 3.

2C). These results were consistent with the findings from other cells in previous studies[13,18,24], and indicated that FMNL3 plays an important role in regulating the assembly of actin-based protrusions.

FMNL3 plays an essential role in the RhoC/FAK pathway to promote CRC cell invasion

To further gain insight into the signaling pathways by which FMNL3 promotes invasive phenotypes, we prepared cell lysates from FMNL3-overexpressing cells, FMNL3-depleted cells and the corresponding control cells. As the invasive and metastatic abilities of tumor cells were often correlated with the product of matrix metalloproteinases (MMPs) and VEGF[27,28], we measured the expression of these proteins by gelatin zymograph assay and western blot, respectively. Forced expression of FMNL3 significantly caused up-regulation of MMP-2, MMP-9 and VEGF (Figure 3A), and vice versa when FMNL3 was suppressed (Figure 3B). Therefore, our results showed that the invasive phenotypes induced by FMNL3 in CRC cells were partly due to the improved expression of MMP-2, MMP-9 and VEGF.

The levels of MMP-2 and MMP-9 were regulated by phosphorylated MAPK and AKT[29,30], which were activated by RhoC[31-33] or in sequence activated by FAK, Pyk2 and RhoC[25]. Moreover, the expression of VEGF and MMP-9 were inhibited by the down-regulation of RhoC[28]. More importantly, FMNL3 acts as a downstream effector of RhoC[24]. We thus speculated that FMNL3 participates in a RhoC-dependent signaling pathway. To validate this speculation, the expression of p-MAPK, p-AKT, p-FAK, p-Pyk2 and RhoC in CRC cells was measured by western

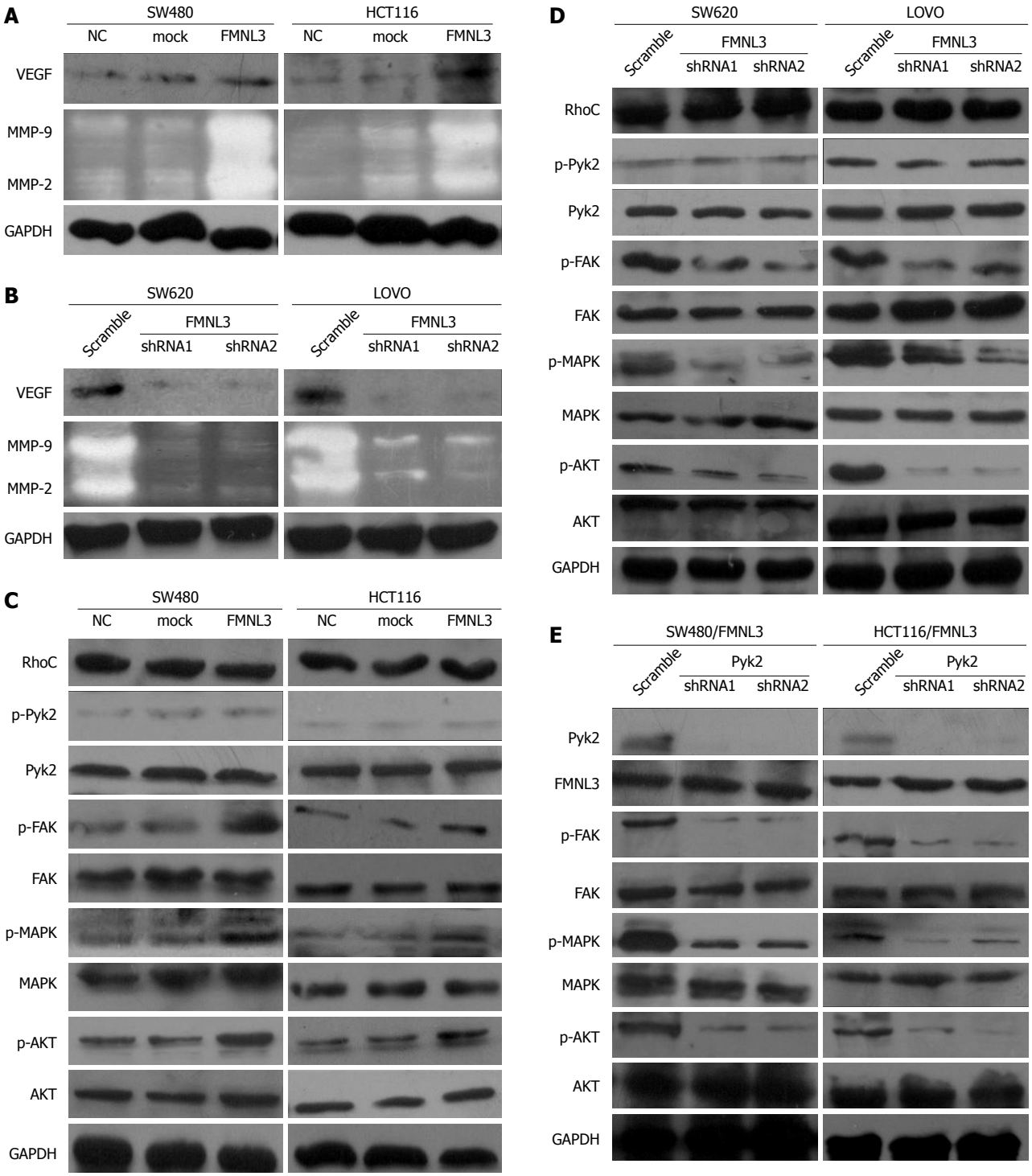


Figure 3 Formin-like 3 regulates the RhoC/FAK signaling pathway to promote colorectal carcinoma invasion. A and B: Analysis of VEGF, MMP-2 and MMP-9 expression in FMNL3-overexpressing or -depleted colorectal carcinoma cells by western blot and gelatin zymography experiments, respectively. C and D: Analysis of the effects of FMNL3 overexpression or depletion on the expression of RhoC, (p-)Pyk2, (p-)FAK, (p-)MAPK and (p-)AKT by western blot. E: Effects of Pyk2 silencing on the expression of FMNL3, (p-)FAK, (p-)MAPK and (p-)AKT in FMNL3-expressing cells by western blot. FMNL3: Formin-like 3; VEGF: vascular endothelial growth factor; MMP: matrix metalloprotein; Pyk2: Proline rich tyrosine kinase 2; FAK: Focal adhesion kinase; MAPK: Mitogen activated protein kinases; AKT: Protein kinase B.

blot. As shown in Figure 3C, the overexpression of FMNL3 strongly increased the expression of p-MAPK, p-AKT, p-FAK, while FMNL3 silencing generated opposite results (Figure 3D). There were no significant differences in the total amounts of these proteins (Figure 3C and D). However, neither overexpression nor depletion of FMNL3 led to any changes in the expressions of p-Pyk2 and RhoC. These results suggested that FMNL3 may play an essential role in the RhoC signaling pathway, and act downstream of RhoC and p-pyk2 as well as upstream of p-FAK, p-MAPK and p-AKT. However, the inhibition of Pyk2 did not affect the expression of FMNL3, although it resulted in the downregulation of p-FAK, p-MAPK and p-AKT as expected (Figure 3E). This suggested that

FMNL3 may not work downstream of p-Pyk2, but only downstream of RhoC. Indeed, when FMNL3/shRNA1 and RhoC genes were transfected simultaneously into SW480 and HCT116 cells, the RhoC-dependent upregulations of MMPs and VEGF were partly blocked by FMNL3 silencing (Figure 4A). These results confirmed the notion that FMNL3 acts as a downstream effector of RhoC. Thus, FMNL3 may act downstream of RhoC (but not downstream of RhoC and p-Pyk2) and upstream of p-FAK, p-MAPK and p-AKT.

We also investigated the effects of the co-transfection of FMNL3/shRNA1 and RhoC genes on the actin cytoskeleton and invasive abilities of CRC cells. We found that the RhoC-dependent restriction of lamellipodial broadening, promotion of filopodia elongation and enhancement of cell invasion were partly inhibited by FMNL3 depletion (Figure 4B and C). These results suggested that FMNL3 regulates the assembly of actin-based protrusions and cell invasion of CRC in a RhoC-dependent manner.

To further validate the above data, we treated FMNL3-overexpressing cells with TAE226 (a FAK-specific inhibitor), U0126 (a MAPK/ERK-specific inhibitor) or Ly294002 (a PI3K/AKT-specific inhibitor) separately. Then the expressions of MMP2, MMP-9 and VEGF, as well as the difference of cell invasive ability, were measured using the same methods as above. We found that inhibition of FAK, MAPK/ERK or PI3K/AKT indeed significantly blocked the effects of FMNL3-induced increases in these three proteins and invasion in CRC cells (Figure 5). These results strongly confirmed the essential role of FMNL3 in the RhoC/FAK signaling pathway.

Taken together, FMNL3 regulates the RhoC/FAK signaling pathway and RhoC-dependent remodeling of actin-based protrusions to promote CRC invasion.

FMNL3 interacts directly with RhoC

Finally, we explored the partner of FMNL3 in the RhoC/FAK signaling pathway. Since FMNL3 belongs to the DRF subfamily and contains a GBD domain in the NH2-terminus, it provides the structural basis for the activation by Rho-GTPases via direct binding. The possibility of the interaction between FMNL3 and RhoC was therein tested by co-immunoprecipitation, immunofluorescence-based confocal microscopy and GST-pull down assays. Indeed, we found that FMNL3 and RhoC co-localized in the cytoplasm (Figure 6A), and immunoprecipitated with each other by one or the other antibody (Figure 6B). The results of GST pull-down assays confirmed the direct binding of FMNL3 to RhoC *in vitro* (Figure 6C). These results demonstrated that FMNL3 interacts directly with RhoC *in vivo* and *in vitro*, which were in accordance with the findings of the Vega FM group regarding the interaction between FMNL3 and RhoC *in vitro*[24].

DISCUSSION

We have shown in previous studies that increased FMNL3 expression contributes to metastasis and poor prognosis in patients with CRC[23]. However, the underlying molecular mechanism remains unclear. In this study, we explored the possible signaling pathway responsible for CRC cell invasion and migration induced by FMNL3. We first determined the biological effects of FMNL3 on CRC cells *in vitro.* Our results showed the positive roles of FMNL3 in CRC cell proliferation, migration and invasion *in vitro*, which were inconsistent with the promotion of FMNL3 in tumor growth and metastasis *in vivo* found in our previous study[23]. Recent studies have also reported the relevant function of FMNL3 in tumor cell growth and proliferation[34]. Other DRF members were also involved in cell proliferation and division through cell cycle regulation[35] or microtubule stabilization in a cell type-selective manner[36]. Moreover, FMNL3 has been shown to promote cell invasion, migration and metastasis in various cell types[19-22], confirming our previous and present study results both *in vivo* and *in vitro*[23].

Previous studies have reported that the reorganization of the actin cytoskeleton is responsible for enhanced cell motility that is necessary for cancer cell invasion and metastasis[4]. As a Rho-GTPase-binding protein, DRF possesses conserved function in actin cytoskeletal dynamics exerted through the formin homology 2 (FH2) domain[37]. DRF contains a NH2-terminal GBD domain, where upon binding to a Rho-GTPase, the bound NH2-terminal diaphanous inhibitory domain dissociates from the COOH-terminal diaphanous autoregulatory domain. This, in turn, results in the release of inactive DRF auto-inhibition and subsequently allows the FH2 domain to function as a direct regulator of actin polymerization[37]. DRFs are major actin filament nucleators, which can bundle linear actin filaments and generate membrane protrusions such as filopodia and lamellipodia[38,39]. Here, we found that FMNL3 overexpression promotes the elongation of filopodia and restricts the broadening of lamellipodia. Some researchers have also reported the assembly of filopodia and lamellipodia by FMNL3[13,22,24] and verified the structure of the FMNL3 FH2/actin complex-mediated actin nucleation and elongation[40].

Evidence has shown that DRFs regulate the assembly of actin-related structures and cancer cell invasion downstream of Rho GTPases[9-11,41]. Rho family GTPases, including RhoA, RhoB, RhoC, Rac and Cdc42, are key regulators of actin cytoskeletal dynamics associated with cell motility and invasion, and their expression and activation generally increase with tumor progression[42,43]. RhoC is the best-characterized Rho GTPase among them, and its overexpression has recently been shown to be closely linked with highly invasive and metastatic forms of many human cancers[44]. Recent studies have also reported that RhoC promotes polarized migration through FMNL3 by restricting the lamellipodia broadening in prostate cancer[24]. Our results also showed that FMNL3 could regulate the actin-based protrusions of filopodia and lamelipodia in a RhoC-dependent manner to accelerate CRC cell invasion. These results were consistent with the findings of the Higgs HN and Ridley AJ groups regarding the roles of FMNL3 in the regulation of

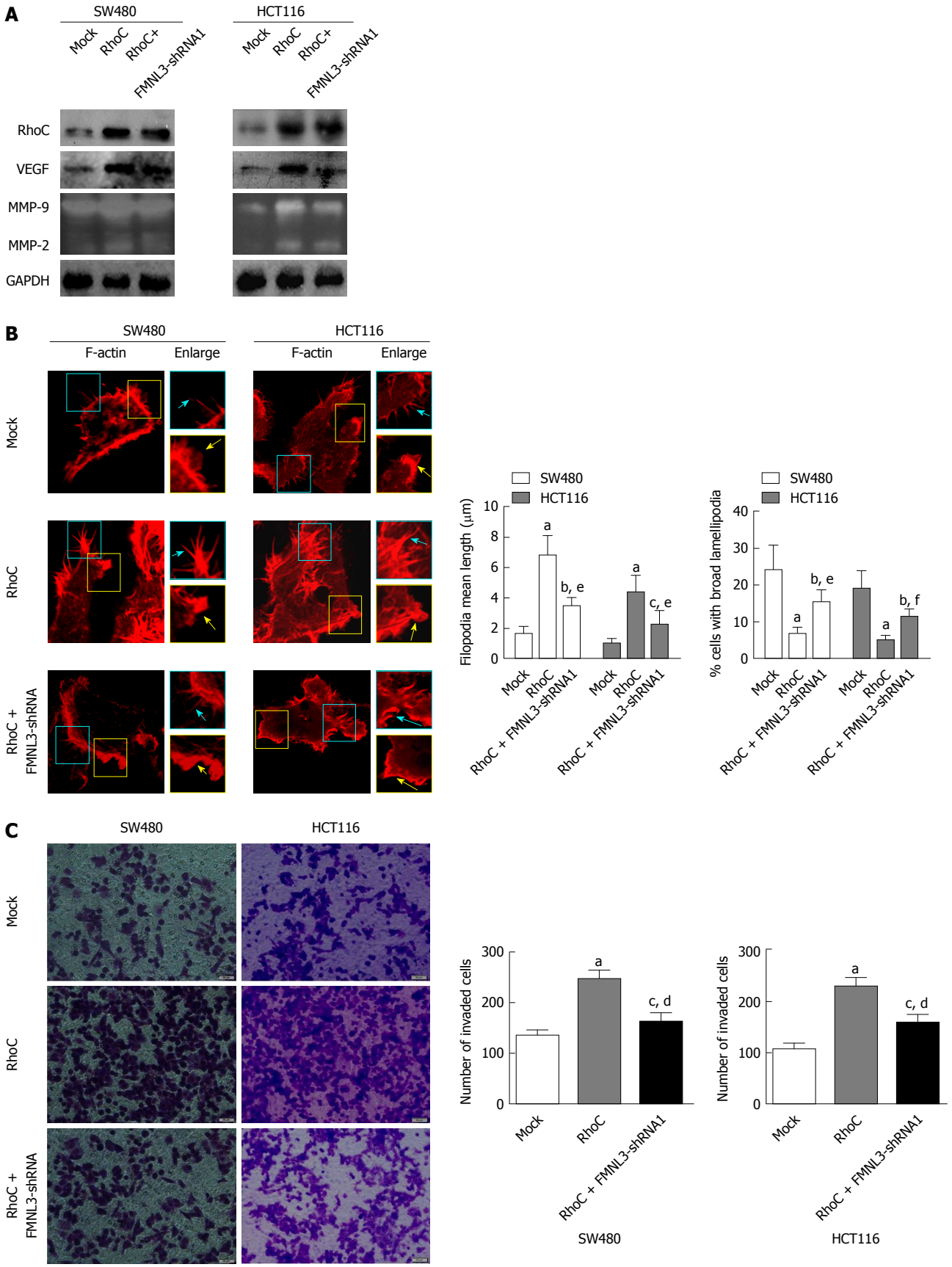


Figure 4 Formin-like 3 depletion blocks RhoC-dependent increases of matrix metalloproteins and vascular endothelial growth factor (A), assembly of actin-based protrusions (B), and invasion (C) in colorectal carcinoma cells. MMPs and VEGF were detected by gelatin zymography experiments and western blot. F-actin is displayed using tritc-phalloidine (Red) staining and laser scanning confocal microscopy detection. Enlarged views of the boxed regions are shown on the right side of the figures. Blue arrows indicate filopodia, yellow arrows indicate lamellipodia. Cell invasion was compared using the Boyden chamber assay. Scale bars represent 5 μm (F-actin) or 50 μm (cell invasion assay), respectively. $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$ *vs* Mock group, $^{d}P < 0.001$, $^{e}P < 0.01$ and $^{f}P < 0.05$ *vs* RhoC-overexpressing group. Error bars indicate mean ± SD. FMNL3: Formin-like 3; MMP: Matrix metalloprotein; VEGF: Vascular endothelial growth factor.

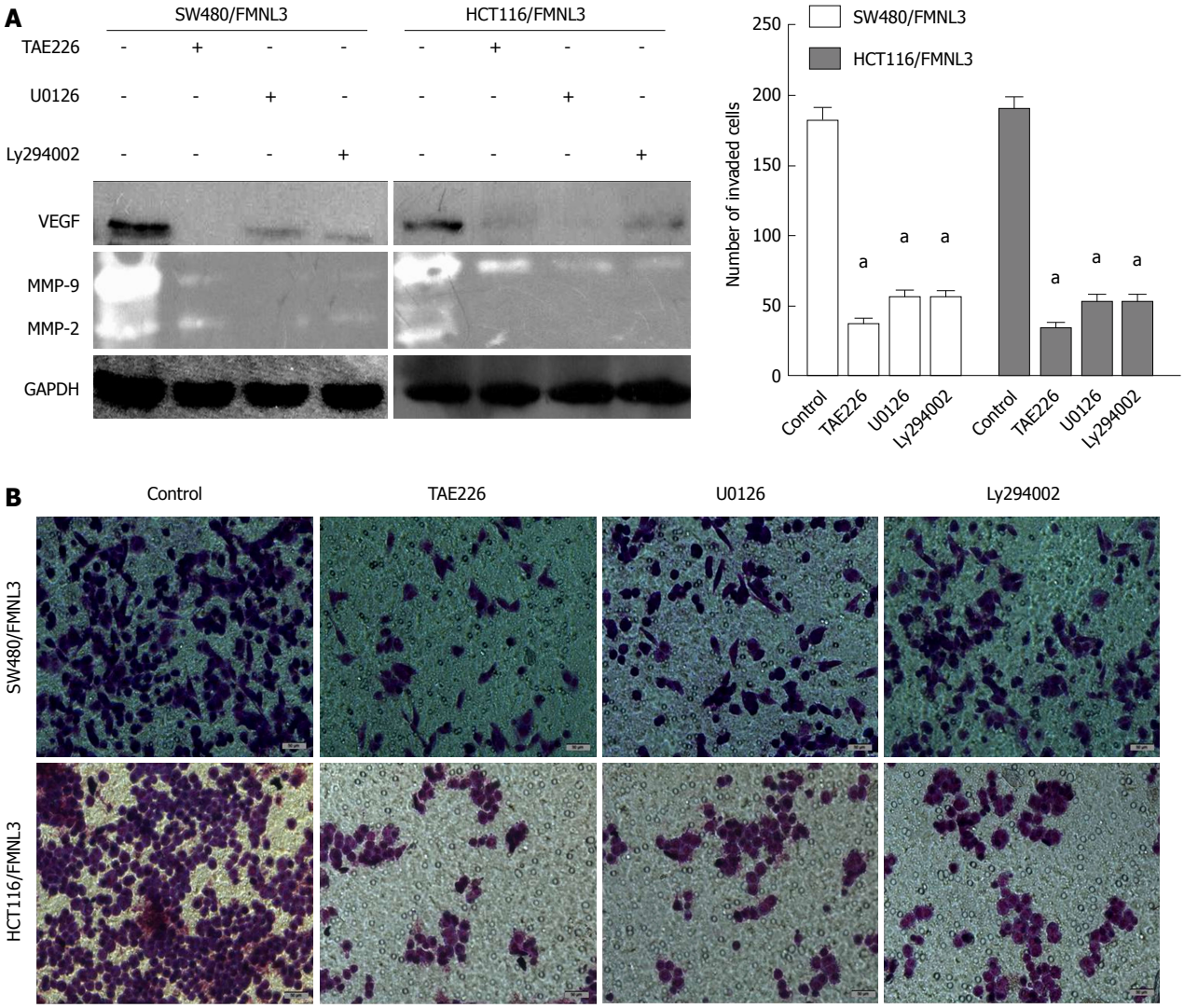


Figure 5 Effects of TAE226, U0126 and Ly294002 treatments, respectively, on the expression of matrix metalloproteins and vascular endothelial growth factor by gelatin zymography experiments and western blot (A), as well as cell invasion by Boyden chamber assay (B and C). Morphological comparison of cells penetrating into the artificial basement membrane is also shown. Scale bars represent 50 μm. $^{a}P < 0.001$ *vs* control group. Error bars indicate mean ± SD. MMP: Matrix metalloprotein; VEGF: Vascular endothelial growth factor.

filopodia and lamellipodia that contribute to the enhanced migratory and invasive potential in other cell types[13,18,24].

The molecular mechanism by which FMNL3 promotes tumor progression and metastasis is a fascinating subject. Evidence has shown that RhoC is closely related to the high invasion and metastasis of various types of human cancers[44]. RhoC induces tumor cell motility and invasion via MAPK- and PI3K/AKT-dependent pathways[25,31-33]. However, whether FMNL3 regulates the RhoC-dependent signaling pathway to promote CRC cell invasion and migration needs further investigation.

MMP-2, MMP-9 and VEGF are important effectors of the RhoC-dependent pathway[25,28,45]. Moreover, MMP-2 and MMP-9 are the two key proteases for tumor metastasis[27]. VEGF is one of the important angiogenetic factors required for tumor angiogenesis[46]. Our results provide evidence for the role of FMNL3 in activation of the two proteases and VEGF in CRC cells, suggesting a possible role of FMNL3 in RhoC-dependent pathway activation during CRC cell invasion.

Both MMP-2 and MMP-9 were shown to be activated by phosphorylated MAPK and AKT[29,30]. In addition, MAPK and AKT signaling pathways were activated by RhoC[31-33] or in sequence by FAK, Pyk2 and RhoC[25]. Moreover, RhoC expression levels are correlated with the expressions levels of VEGF and MMP9[28]. In addition, p-FAK regulates VEGFR2 transcription in angiogenesis[47]. Our study shows that FMNL3 induced the phosphorylation of FAK and subsequent phosphorylation of MAPK and AKT, resulting in the upregulation of MMP-2, MMP-9 and VEGF, and the subsequent promotion of enhanced CRC cell invasion. FAK is a protein tyrosine kinase that was first identified within the extracellular matrix and at integrin receptor cell adhesion sites, and is a key regulator of cell movement[48]. Resent studies showed increased expression of p-FAK in the nuclei of cells in laryngeal cancer and four digestive cancers, including colorectal

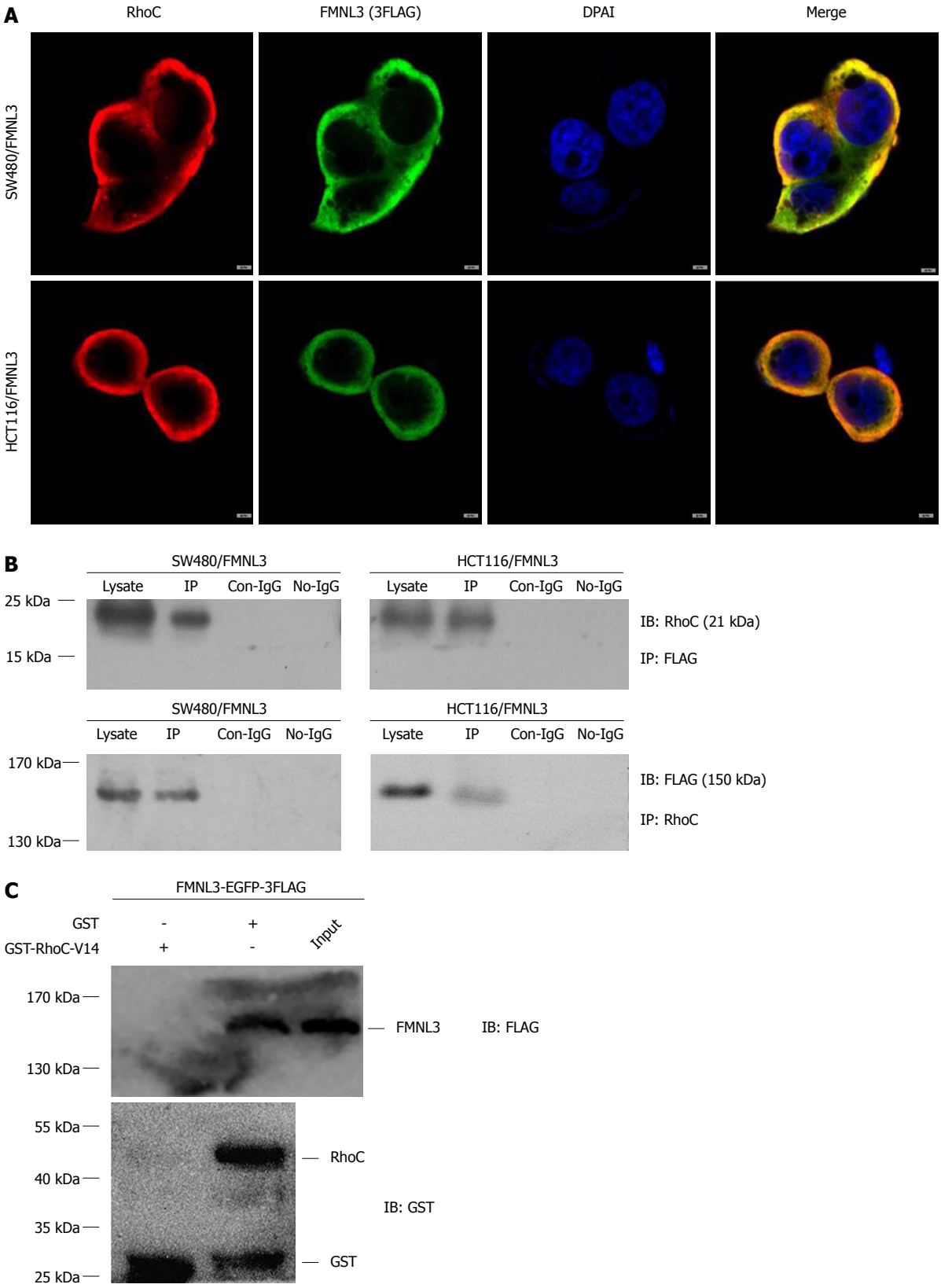


Figure 6 Interaction of formin-like 3 with RhoC. A: FMNL3 co-localizes with RhoC in the cytoplasm, detected by immunofluorescence staining and laser confocal microscope. B and C: FMNL3 interacts directly with RhoC in co-immunoprecipitation experiments and GST-pull down assays *in vitro*. Scale bars represent 5 μm. IP: Immunoprecipitation; GST: Glutathionine-S-transferase; FMNL3: Formin-like 3.

cancer[49,50]. Nuclear FAK promotes cell proliferation and survival through enhanced P53 degradation[51], suggesting an association between p-FAK and abnormal cell proliferation. In addition, nuclear expression of p-FAK is also associated with poor prognosis in colorectal cancer[52]. In this study, we found that the phosphorylation of FAK (triggered by RhoC/FMNL3 signaling) induced the activation of MAPK and AKT

and the subsequent upregulation of MMPs and VEGF, and contributed to the invasive potential of CRC cells. Of course, it may also be correlated with the nuclear location of p-FAK, which is triggered by RhoC/FMNL3 signaling. Other studies also presented the correlation of DRF members with MAPK, AKT and/or FAK, as well as Rho signaling[3,10,53,54]. The DRF FMNL2 enhanced CRC cell invasion via the MAPK/ERK and PI3K/AKT pathways[53], and regulated the invasive cell motility and migration downstream of RhoC and Cdc42[3,10]. Another DRF member, mDia1, acts downstream of RhoA and upstream of MAPK, ERK and FAK to induce intestinal epithelial cell migration[54].

Finally, we uncovered RhoC as the partner of FMNL3 in the RhoC/FAK signaling pathway by co-IPs, immunofluorescence co-localization experiments and GST pull-down assays. This finding was also supported by the structural basis of the GBD domain in the N-terminus of FMNL3, and has also been verified by the GST pull-down assay from the Ridley AJ group[24]. However, other groups reported the interaction of FMNL3 with Cdc42 or RhoJ, and proposed that FMNL3 is a downstream effector of Cdc42 or RhoJ to promote the filopodial outgrowth during endothelial lumen formation[17,55]. Unfortunately, there was a limitation to our present study, as there may be cross-talk between RhoC and other small GTPase-binding proteins such as Cdc42 and RhoJ during FMNL3-dependent CRC cell invasion. This thus needs further investigation.

In conclusion, FMNL3 plays a positive role in CRC cell proliferation, invasion and migration. In addition, FMNL3 activates the RhoC/FAK signaling pathway via its interaction with RhoC, and regulates RhoC-dependent remodeling of actin-based protrusions, such as filopodia and lamellipodia, to promote CRC cell invasion. FMNL3 can be applied as a promising specific biomarker for CRC progression and metastasis.

ARTICLE HIGHLIGHTS

Research background

Formin-like 3 (FMNL3) is a novel member of the diaphanous-related formins subfamily, which act as downstream effectors of Rho-GTPase signaling and regulate actin-dependent processes, such as cell motility and invasion. Increased expression of FMNL3 has been identified to contribute to metastasis and the poor prognosis of colorectal carcinoma (CRC). However, the exact molecular mechanism by which FMNL3 promotes CRC cell invasion and metastasis remains ambiguous. Therefore, elucidation of the underlying mechanism may help to block the metastatic progression and improve the survival rate of patients with CRC.

Research motivation

It is necessary to explore whether FMNL3 regulates Rho GTPase signaling to affect cytoskeletal organization and subsequent CRC cell invasion. Recent studies have demonstrated FMNL3 in reorganization of actin-dependent protrusions, such as filopodia and lamellipodia. The potential role of FMNL3 in tumor cell invasion and metastasis has also been reported in several tumor types. Moreover, FMNL3 acts as a downstream effector of RhoC to promote prostate cancer invasion by controlling lamellipodia. These findings give us a good lead for further study regarding the mechanism of FMNL3 regulation during CRC cell invasion and metastasis.

Research objectives

In this study, we investigate the effects of FMNL3 on CRC cell proliferation, invasion and migration *in vitro* by gain- and loss-of –function approaches. Moreover, we explore the role of FMNL3 in the RhoC-dependent signaling pathway and actin assembly dynamics, and the relation with CRC cell invasion. Our study provides significant insights into the signaling mechanism of FMNL3 during CRC invasion that may contribute to the future design of more effective metastasis-related therapies.

Research methods

Experiments using gene transfection or silencing were conducted to construct FMNL3 stably-expressed or –depleted cell lines to complete the following functional studies. A series of *in vitro* experiments, such as MTT, transwell chamber and scratch assays, were performed to explore the effects of FMNL3 on cell proliferation, invasion and migration. Rhodamine-conjugated phalloidin staining and confocal microscopy was used to display and observe F-actin dependent protrusions, such as filopodia and lamellipodia. Western blots and gelatin zymography assays were carried out to explore how the signaling pathway of FMNL3 is involved in CRC invasion. The key inhibitors of the RhoC/FAK pathway were used to treat CRC cells to verify the reliability of the signaling mechanism. In addition, the experiments involving immunofluorescence co-localization, co-immunoprecipitation and GST-pull downs were performed to unveil the partner of FMNL3 in the signaling pathway.

Research results

The results of *in vitro* experiments showed a positive role of FMNL3 in cell proliferation, invasion and migration of CRC. Rhodamine-conjugated phalloidin staining and confocal microscopy-based observations demonstrated that FMNL3 induced the elongation of filopodia, but inhibited the broadening of lamellipodia in a RhoC-dependent manner to enhance the invasive abilities of CRC cells. In addition, the results of western blots and gelatin zymography assays suggested that FMNL3 was involved in the RhoC/FAK signaling pathway and acted as an effector of RhoC to activate the downstream signaling of p-FAK as well as p-MAPK and p-AKT. This was followed by the increased expression of MMP-2, MMP-9 and VEGF, resulting in the promotion of CRC cell invasion. The results of inhibitor treatments confirmed the essential role of FMNL3 in the activation of the RhoC/FAK pathway and the subsequent promotion of CRC cell invasion. A direct interaction of FMNL3 with RhoC *in vivo* and *in vitro* was displayed by co-IP, co-localization and GST-pull down analyses.

Research conclusions

In conclusion, FMNL3 plays a positive role in CRC cell proliferation, invasion and migration. In addition, FMNL3 activates the RhoC/FAK signaling pathway via its interaction with RhoC. FMNL3 also regulates RhoC-dependent remodeling of actin-based protrusions, such as filopodia and lamellipodia, to promote CRC cell invasion. FMNL3 can be applied as a promising specific biomarker for CRC progression and metastasis.

Research perspectives

Our study illuminates the role and molecular mechanism of FMNL3 in the regulation of CRC invasion, and revealed RhoC's involvement in the RhoC/FAK signaling pathway as the partner of FMNL3. Other groups reported the interaction of FMNL3 with Cdc42 or RhoJ, and proposed that FMNL3 acted as a downstream effector of Cdc42 or RhoJ to promote the filopodia outgrowth during endothelial lumen formation. There may be cross-talk between RhoC and other small GTPase proteins, such as Cdc42 and RhoJ, to coordinate the regulation of FMNL3-dependent CRC cell invasion. This, however, needs further investigation.

REFERENCES

1 **Recio-Boiles A**, Cagir B. Cancer, Colon. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2018 [PMID: 29262132]
2 **Viganò L**, Ferrero A, Lo Tesoriere R, Capussotti L. Liver surgery for colorectal metastases: results after 10 years of follow-up. Long-term survivors, late recurrences, and prognostic role of morbidity. *Ann Surg Oncol* 2008; **15**: 2458-2464 [PMID: 18463927 DOI:

10.1245/s10434-008-9935-9]
3 **Kitzing TM**, Wang Y, Pertz O, Copeland JW, Grosse R. Formin-like 2 drives amoeboid invasive cell motility downstream of RhoC. *Oncogene* 2010; **29**: 2441-2448 [PMID: 20101212 DOI: 10.1038/onc.2009.515]
4 **Kedrin D**, van Rheenen J, Hernandez L, Condeelis J, Segall JE. Cell motility and cytoskeletal regulation in invasion and metastasis. *J Mammary Gland Biol Neoplasia* 2007; **12**: 143-152 [PMID: 17557195 DOI: 10.1007/s10911-007-9046-4]
5 **Faix J**, Grosse R. Staying in shape with formins. *Dev Cell* 2006; **10**: 693-706 [PMID: 16740473 DOI: 10.1016/j.devcel.2006.05.001]
6 **Eisenmann KM**, Harris ES, Kitchen SM, Holman HA, Higgs HN, Alberts AS. Dia-interacting protein modulates formin-mediated actin assembly at the cell cortex. *Curr Biol* 2007; **17**: 579-591 [PMID: 17398099 DOI: 10.1016/j.cub.2007.03.024]
7 **Wodarz A**, Näthke I. Cell polarity in development and cancer. *Nat Cell Biol* 2007; **9**: 1016-1024 [PMID: 17762893 DOI: 10.1038/ncb433]
8 **Higashida C**, Miyoshi T, Fujita A, Oceguera-Yanez F, Monypenny J, Andou Y, Narumiya S, Watanabe N. Actin polymerization-driven molecular movement of mDia1 in living cells. *Science* 2004; **303**: 2007-2010 [PMID: 15044801 DOI: 10.1126/science.1093923]
9 **Lizárraga F**, Poincloux R, Romao M, Montagnac G, Le Dez G, Bonne I, Rigaill G, Raposo G, Chavrier P. Diaphanous-related formins are required for invadopodia formation and invasion of breast tumor cells. *Cancer Res* 2009; **69**: 2792-2800 [PMID: 19276357 DOI: 10.1158/0008-5472.CAN-08-3709]
10 **Block J**, Breitsprecher D, Kühn S, Winterhoff M, Kage F, Geffers R, Duwe P, Rohn JL, Baum B, Brakebusch C, Geyer M, Stradal TE, Faix J, Rottner K. FMNL2 drives actin-based protrusion and migration downstream of Cdc42. *Curr Biol* 2012; **22**: 1005-1012 [PMID: 22608513 DOI: 10.1016/j.cub.2012.03.064]
11 **Kitzing TM**, Sahadevan AS, Brandt DT, Knieling H, Hannemann S, Fackler OT, Grosshans J, Grosse R. Positive feedback between Dia1, LARG, and RhoA regulates cell morphology and invasion. *Genes Dev* 2007; **21**: 1478-1483 [PMID: 17575049 DOI: 10.1101/gad.424807]
12 **Katoh M**, Katoh M. Identification and characterization of human FMNL1, FMNL2 and FMNL3 genes in silico. *Int J Oncol* 2003; **22**: 1161-1168 [PMID: 12684686 DOI: 10.3892/ijo.22.5.1161]
13 **Harris ES**, Gauvin TJ, Heimsath EG, Higgs HN. Assembly of filopodia by the formin FRL2 (FMNL3). *Cytoskeleton (Hoboken)* 2010; **67**: 755-772 [PMID: 20862687 DOI: 10.1002/cm.20485]
14 **Thurston SF**, Kulacz WA, Shaikh S, Lee JM, Copeland JW. The ability to induce microtubule acetylation is a general feature of formin proteins. *PLoS One* 2012; **7**: e48041 [PMID: 23110170 DOI: 10.1371/journal.pone.0048041]
15 **Gauvin TJ**, Young LE, Higgs HN. The formin FMNL3 assembles plasma membrane protrusions that participate in cell-cell adhesion. *Mol Biol Cell* 2015; **26**: 467-477 [PMID: 25428984 DOI: 10.1091/mbc.E14-07-1247]
16 **Moriya K**, Yamamoto T, Takamitsu E, Matsunaga Y, Kimoto M, Fukushige D, Kimoto C, Suzuki T, Utsumi T. Protein N-myristoylation is required for cellular morphological changes induced by two formin family proteins, FMNL2 and FMNL3. *Biosci Biotechnol Biochem* 2012; **76**: 1201-1209 [PMID: 22790947 DOI: 10.1271/bbb.120069]
17 **Richards M**, Hetheridge C, Mellor H. The Formin FMNL3 Controls Early Apical Specification in Endothelial Cells by Regulating the Polarized Trafficking of Podocalyxin. *Curr Biol* 2015; **25**: 2325-2331 [PMID: 26299518 DOI: 10.1016/j.cub.2015.07.045]
18 **Hetheridge C**, Scott AN, Swain RK, Copeland JW, Higgs HN, Bicknell R, Mellor H. The formin FMNL3 is a cytoskeletal regulator of angiogenesis. *J Cell Sci* 2012; **125**: 1420-1428 [PMID: 22275430 DOI: 10.1242/jcs.091066]
19 **Gardberg M**, Heuser VD, Koskivuo I, Koivisto M, Carpén O. FMNL2/FMNL3 formins are linked with oncogenic pathways and predict melanoma outcome. *J Pathol Clin Res* 2016; **2**: 41-52 [PMID: 27499915 DOI: 10.1002/cjp2.34]
20 **Wu Y**, Shen Z, Wang K, Ha Y, Lei H, Jia Y, Ding R, Wu D, Gan S, Li R, Luo B, Jiang H, Jie W. High FMNL3 expression promotes nasopharyngeal carcinoma cell metastasis: role in TGF-β1-induced epithelia-to-mesenchymal transition. *Sci Rep* 2017; **7**: 42507 [PMID: 28198387 DOI: 10.1038/srep42507]
21 **Gao X**, Wang X, Cai K, Wang W, Ju Q, Yang X, Wang H, Wu H. MicroRNA-127 is a tumor suppressor in human esophageal squamous cell carcinoma through the regulation of oncogene FMNL3. *Eur J Pharmacol* 2016; **791**: 603-610 [PMID: 27645894 DOI: 10.1016/j.ejphar.2016.09.025]
22 **Lynch J**, Meehan MH, Crean J, Copeland J, Stallings RL, Bray IM. Metastasis suppressor microRNA-335 targets the formin family of actin nucleators. *PLoS One* 2013; **8**: e78428 [PMID: 24223803 DOI: 10.1371/journal.pone.0078428]
23 **Zeng YF**, Xiao YS, Lu MZ, Luo XJ, Hu GZ, Deng KY, Wu XM, Xin HB. Increased expression of formin-like 3 contributes to metastasis and poor prognosis in colorectal carcinoma. *Exp Mol Pathol* 2015; **98**: 260-267 [PMID: 25758200 DOI: 10.1016/j.yexmp.2015.03.008]
24 **Vega FM**, Fruhwirth G, Ng T, Ridley AJ. RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. *J Cell Biol* 2011; **193**: 655-665 [PMID: 21576392 DOI: 10.1083/jcb.201011038]
25 **Iiizumi M**, Bandyopadhyay S, Pai SK, Watabe M, Hirota S, Hosobe S, Tsukada T, Miura K, Saito K, Furuta E, Liu W, Xing F, Okuda H, Kobayashi A, Watabe K. RhoC promotes metastasis via activation of the Pyk2 pathway in prostate cancer. *Cancer Res* 2008; **68**: 7613-7620 [PMID: 18794150 DOI: 10.1158/0008-5472.CAN-07-6700]
26 **Liang CC**, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2007; **2**: 329-333 [PMID: 17406593 DOI: 10.1038/nprot.2007.30]
27 **Deryugina EI**, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 2006; **25**: 9-34 [PMID: 16680569 DOI: 10.1007/s10555-006-7886-9]
28 **Zhao Y**, Zong ZH, Xu HM. RhoC expression level is correlated with the clinicopathological characteristics of ovarian cancer and the expression levels of ROCK-I, VEGF, and MMP9. *Gynecol Oncol* 2010; **116**: 563-571 [PMID: 20022093 DOI: 10.1016/j.ygyno.2009.11.015]
29 **González MN**, de Mello W, Butler-Browne GS, Silva-Barbosa SD, Mouly V, Savino W, Riederer I. HGF potentiates extracellular matrix-driven migration of human myoblasts: involvement of matrix metalloproteinases and MAPK/ERK pathway. *Skelet Muscle* 2017; **7**: 20 [PMID: 29017538 DOI: 10.1186/s13395-017-0138-6]
30 **Lv C**, Yang S, Chen X, Zhu X, Lin W, Wang L, Huang Z, Wang M, Tu G. MicroRNA-21 promotes bone mesenchymal stem cells migration in vitro by activating PI3K/Akt/MMPs pathway. *J Clin Neurosci* 2017; **46**: 156-162 [PMID: 28844616 DOI: 10.1016/j.jocn.2017.07.040]
31 **van Golen KL**, Bao LW, Pan Q, Miller FR, Wu ZF, Merajver SD. Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. *Clin Exp Metastasis* 2002; **19**: 301-311 [PMID: 12090470 DOI: 10.1023/A:1015518114931]
32 **Ruth MC**, Xu Y, Maxwell IH, Ahn NG, Norris DA, Shellman YG. RhoC promotes human melanoma invasion in a PI3K/Akt-dependent pathway. *J Invest Dermatol* 2006; **126**: 862-868 [PMID: 16470169 DOI: 10.1038/sj.jid.5700211]
33 **Yang H**, Zhou J, Mi J, Ma K, Fan Y, Ning J, Wang C, Wei X, Zhao H, Li E. HOXD10 acts as a tumor-suppressive factor via inhibition of the RHOC/AKT/MAPK pathway in human cholangiocellular carcinoma. *Oncol Rep* 2015; **34**: 1681-1691 [PMID: 26260613 DOI: 10.3892/or.2015.4194]
34 **Martín-Rufián M**, Segura JA, Lobo C, Matés JM, Márquez J, Alonso FJ. Identification of genes downregulated in tumor cells expressing antisense glutaminase mRNA by differential display. *Cancer Biol Ther* 2006; **5**: 54-58 [PMID: 16294018 DOI: 10.4161/cbt.5.1.2238]
35 **Carreira S**, Goodall J, Denat L, Rodriguez M, Nuciforo P, Hoek

KS, Testori A, Larue L, Goding CR. Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. *Genes Dev* 2006; **20**: 3426-3439 [PMID: 17182868 DOI: 10.1101/gad.406406]

36 **Ju R**, Cirone P, Lin S, Griesbach H, Slusarski DC, Crews CM. Activation of the planar cell polarity formin DAAM1 leads to inhibition of endothelial cell proliferation, migration, and angiogenesis. *Proc Natl Acad Sci USA* 2010; **107**: 6906-6911 [PMID: 20351293 DOI: 10.1073/pnas.1001075107]

37 **Goode BL**, Eck MJ. Mechanism and function of formins in the control of actin assembly. *Annu Rev Biochem* 2007; **76**: 593-627 [PMID: 17373907 DOI: 10.1146/annurev.biochem.75.103004.142647]

38 **Schirenbeck A**, Bretschneider T, Arasada R, Schleicher M, Faix J. The Diaphanous-related formin dDia2 is required for the formation and maintenance of filopodia. *Nat Cell Biol* 2005; **7**: 619-625 [PMID: 15908944 DOI: 10.1038/ncb1266]

39 **Yang C**, Czech L, Gerboth S, Kojima S, Scita G, Svitkina T. Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. *PLoS Biol* 2007; **5**: e317 [PMID: 18044991 DOI: 10.1371/journal.pbio.0050317]

40 **Thompson ME**, Heimsath EG, Gauvin TJ, Higgs HN, Kull FJ. FMNL3 FH2-actin structure gives insight into formin-mediated actin nucleation and elongation. *Nat Struct Mol Biol* 2013; **20**: 111-118 [PMID: 23222643 DOI: 10.1038/nsmb.2462]

41 **Pellegrin S**, Mellor H. The Rho family GTPase Rif induces filopodia through mDia2. *Curr Biol* 2005; **15**: 129-133 [PMID: 15668168 DOI: 10.1016/j.cub.2005.01.011]

42 **Sahai E**, Marshall CJ. RHO-GTPases and cancer. *Nat Rev Cancer* 2002; **2**: 133-142 [PMID: 12635176 DOI: 10.1038/nrc725]

43 **Wheeler AP**, Ridley AJ. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp Cell Res* 2004; **301**: 43-49 [PMID: 15501444 DOI: 10.1016/j.yexcr.2004.08.012]

44 **Hakem A**, Sanchez-Sweatman O, You-Ten A, Duncan G, Wakeham A, Khokha R, Mak TW. RhoC is dispensable for embryogenesis and tumor initiation but essential for metastasis. *Genes Dev* 2005; **19**: 1974-1979 [PMID: 16107613 DOI: 10.1101/gad.1310805]

45 **Yang H**, Liang J, Zhou J, Mi J, Ma K, Fan Y, Ning J, Wang C, Wei X, Li E. Knockdown of RHOC by shRNA suppresses invasion and migration of cholangiocellular carcinoma cells via inhibition of MMP2, MMP3, MMP9 and epithelial-mesenchymal transition. *Mol Med Rep* 2016; **13**: 5255-5261 [PMID: 27108649 DOI: 10.3892/mmr.2016.5170]

46 **Hori Y**, Ito K, Hamamichi S, Ozawa Y, Matsui J, Umeda IO, Fujii H. Functional Characterization of VEGF- and FGF-induced Tumor Blood Vessel Models in Human Cancer Xenografts. *Anticancer Res* 2017; **37**: 6629-6638 [PMID: 29187438 DOI: 10.21873/anticanres.12120]

47 **Sun S**, Wu HJ, Guan JL. Nuclear FAK and its kinase activity regulate VEGFR2 transcription in angiogenesis of adult mice. *Sci Rep* 2018; **8**: 2550 [PMID: 29416084 DOI: 10.1038/s41598-018-20930-z]

48 **Mitra SK**, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* 2005; **6**: 56-68 [PMID: 15688067 DOI: 10.1038/nrm1549]

49 **Aronsohn MS**, Brown HM, Hauptman G, Kornberg LJ. Expression of focal adhesion kinase and phosphorylated focal adhesion kinase in squamous cell carcinoma of the larynx. *Laryngoscope* 2003; **113**: 1944-1948 [PMID: 14603053 DOI: 10.1097/00005537-200311000-00017]

50 **Murata T**, Naomoto Y, Yamatsuji T, Okawa T, Shirakawa Y, Gunduz M, Nobuhisa T, Takaoka M, Sirmali M, Nakajima M, Ohno Y, Tanaka N. Localization of FAK is related with colorectal carcinogenesis. *Int J Oncol* 2008; **32**: 791-796 [PMID: 18360706]

51 **Lim ST**, Chen XL, Lim Y, Hanson DA, Vo TT, Howerton K, Larocque N, Fisher SJ, Schlaepfer DD, Ilic D. Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol Cell* 2008; **29**: 9-22 [PMID: 18206965 DOI: 10.1016/j.molcel.2007.11.031]

52 **Albasri A**, Fadhil W, Scholefield JH, Durrant LG, Ilyas M. Nuclear expression of phosphorylated focal adhesion kinase is associated with poor prognosis in human colorectal cancer. *Anticancer Res* 2014; **34**: 3969-3974 [PMID: 25075018]

53 **Liang L**, Li X, Zhang X, Lv Z, He G, Zhao W, Ren X, Li Y, Bian X, Liao W, Liu W, Yang G, Ding Y. MicroRNA-137, an HMGA1 target, suppresses colorectal cancer cell invasion and metastasis in mice by directly targeting FMNL2. *Gastroenterology* 2013; **144**: 624-635.e4 [PMID: 23201162 DOI: 10.1053/j.gastro.2012.11.033]

54 **Chaturvedi LS**, Marsh HM, Basson MD. Role of RhoA and its effectors ROCK and mDia1 in the modulation of deformation-induced FAK, ERK, p38, and MLC motogenic signals in human Caco-2 intestinal epithelial cells. *Am J Physiol Cell Physiol* 2011; **301**: C1224-C1238 [PMID: 21849669 DOI: 10.1152/ajpcell.00518.2010]

55 **Wakayama Y**, Fukuhara S, Ando K, Matsuda M, Mochizuki N. Cdc42 mediates Bmp-induced sprouting angiogenesis through Fmnl3-driven assembly of endothelial filopodia in zebrafish. *Dev Cell* 2015; **32**: 109-122 [PMID: 25584797 DOI: 10.1016/j.devcel.2014.11.024]

P- Reviewer: Abdel-Rahman WM, Jeong KY, Lin JM
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3898-3907

DOI: 10.3748/wjg.v24.i34.3898

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

ORIGINAL ARTICLE

Basic Study

Low expression of CDK5RAP3 and DDRGK1 indicates a poor prognosis in patients with gastric cancer

Jian-Xian Lin, Xin-Sheng Xie, Xiong-Feng Weng, Chao-Hui Zheng, Jian-Wei Xie, Jia-Bin Wang, Jun Lu, Qi-Yue Chen, Long-Long Cao, Mi Lin, Ru-Hong Tu, Ping Li, Chang-Ming Huang

Jian-Xian Lin, Xin-Sheng Xie, Xiong-Feng Weng, Chao-Hui Zheng, Jian-Wei Xie, Jia-Bin Wang, Jun Lu, Qi-Yue Chen, Long-Long Cao, Mi Lin, Ru-Hong Tu, Ping Li, Chang-Ming Huang, Department of Gastric Surgery, Fujian Medical University Union Hospital, Fuzhou 350001, Fujian Province, China

Jian-Xian Lin, Xin-Sheng Xie, Xiong-Feng Weng, Chao-Hui Zheng, Jian-Wei Xie, Jia-Bin Wang, Jun Lu, Qi-Yue Chen, Long-Long Cao, Mi Lin, Ru-Hong Tu, Ping Li, Chang-Ming Huang, Key Laboratory of Ministry of Education of Gastrointestinal Cancer, Fujian Medical University, Fuzhou 350108, Fujian Province, China

Jian-Xian Lin, Xin-Sheng Xie, Xiong-Feng Weng, Chao-Hui Zheng, Jian-Wei Xie, Jia-Bin Wang, Jun Lu, Ping Li, Chang-Ming Huang, Fujian Key Laboratory of Tumor Microbiology, Fujian Medical University, Fuzhou 350108, Fujian Province, China

ORCID number: Jian-Xian Lin (0000-0002-5006-4454); Xin-Sheng Xie (0000-0003-0126-0376); Xiong-Feng Weng (0000-0002-5513-0588); Chao-Hui Zheng (0000-0003-0157-5167); Jian-Wei Xie (0000-0001-9000-5638); Jia-Bin Wang (0000-0002-2023-0183); Jun Lu (0000-0002-8459-4867); Qi-Yue Chen (0000-0001-6391-4043); Long-Long Cao (0000-0003-3144-3050); Mi Lin (0000-0001-7299-6159); Ru-Hong Tu (0000-0002-7491-3879); Ping Li (0000-0002-9418-9339); Chang-Ming Huang (0000-0002-0019-885X).

Author contributions: Lin JX, Xie XS and Weng XF contributed equally to this article and should be considered co-first authors; Li P and Huang CM conceived and designed the study and are the co-corresponding authors; Lin JX, Xie XS and Weng XF performed the experiments; Zheng CH, Xie JW, Wang JB, Lu J, Chen QY, Cao LL, Lin M and Tu RH analyzed and interpreted the data; Lin JX, Xie XS and Weng XF drafted the manuscript. All authors approved the final version of the article to be published.

Supported by the Science Foundation of the Fujian Province, China, No. 2018J01307; Startup Fund for Scientific Research, Fujian Medical University, No. 2016QH024; Scientific and Technological Innovation Joint Capital Projects of Fujian Province, No. 2016Y9031; Minimally Invasive Medical Center of Fujian Province, No. 2011708#; and the Young and Middle-aged Talent Training Project of the Fujian Provincial Health and Family Planning Commission, No. 2014-ZQNJC-13.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Fujian Medical University Union Hospital.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Chang-Ming Huang, MD, Doctor, Professor, Department of Gastric Surgery, Fujian Medical University Union Hospital, No.29 Xinquan Road, Fuzhou 350001, Fujian Province, China. hcmlr2002@163.com
Telephone: +86-591-83363366
Fax: +86-591-83363366

Received: May 19, 2018
Peer-review started: May 22, 2018
First decision: May 30, 2018
Revised: June 11, 2018
Accepted: June 25, 2018
Article in press: June 25, 2018
Published online: September 14, 2018

Abstract

AIM

To investigate the effects of different levels of expression of CDK5RAP3 and DDRGK1 on long-term survival of patients undergoing radical gastrectomy.

METHODS

The expression of CDK5RAP3 and DDRGK1 was detected by immunohistochemistry in 135 patients who received standard gastrectomy were enrolled in the study. Western Blot was used to detect the expression of CDK5RAP3 and DDRGK1 in gastric cancer and its adjacent tissues and cell lines. The correlations between the expression of CDK5RAP3 and DDRGK1 and clinicopathological factors were analyzed, and the value of each parameter to the prognosis of the patients was compared. Receiver operating characteristic analysis was used to compare the accuracy of the prediction of clinical outcome by the parameters.

RESULTS

CDK5RAP3 and DDRGK1 expression was down-regulated in the gastric cancer compared to its respective adjacent non-tumor tissues. The expression of CDK5RAP3 was closely related to the age of the patients (P = 0.035) and the T stage of the tumor (P = 0.017). The expression of DDRGK1 was correlated with the sex of the patients (P = 0.080), the degree of tumor differentiation (P = 0.036), the histological type (P = 0.036) and the N stage of the tumor (P = 0.014). Low expression CDK5RAP3 or DDRGK1 is a poor prognostic factor for gastric cancer patients. Prognostic analysis showed that the co-expression of CDK5RAP3 and DDRGK1 was an independent prognostic factor correlating with the overall survival of gastric cancer patients. Combined expression analysis of CDK5RAP3 and DDRGK1 may provide a more accurate prognostic value for overall survival.

CONCLUSION

The co-expression of CDK5RAP3 and DDRGK1 is an independent prognostic factor for gastric cancer, which can provide a more accurate model for the long-term prognosis.

Key words: Gastric cancer; CDK5RAP3; DDRGK1; Prognosis

Core tip: The expression of CDK5RAP3 and DDRGK1 was down-regulated in gastric cancer tissues. Low expression CDK5RAP3 or DDRGK1 is a poor prognostic factor for gastric cancer patients. The co-expression of CDK5RAP3 and DDRGK1 is an independent prognostic factor for the overall survival of patients with gastric cancer. Moreover, we also found that co-expression of CDK5RAP3 and DDRGK1 can provide a more accurate model for the long-term prognosis of gastric cancer.

Lin JX, Xie XS, Weng XF, Zheng CH, Xie JW, Wang JB, Lu J, Chen QY, Cao LL, Lin M, Tu RH, Li P, Huang CM. Low expression of CDK5RAP3 and DDRGK1 indicates a poor prognosis in patients with gastric cancer. *World J Gastroenterol* 2018; 24(34): 3898-3907 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3898.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3898

INTRODUCTION

Although the morbidity and mortality of primary gastric cancer has declined in recent decades, it is still the third most common cause of cancer-related deaths worldwide[1-3]. At present, the etiology and pathogenesis of gastric cancer has not yet been fully clarified. There is also a lack of specific and highly effective therapeutic drugs available for use in clinical practice. The symptom specificity of early gastric cancer is not obvious, so most patients are already in advanced stages before receiving medical treatment, which seriously affects the prognosis of patients. Therefore, searching for molecular markers that can be used as an independent prognostic factor for gastric cancer is of great significance for the early diagnosis and targeted treatment of gastric cancer.

The cyclin-dependent kinase 5 activating binding protein (CDK5RAP3, also called C53) was first identified as a binding protein of the cyclin-dependent kinase 5 (CDK5) activators P35 and P39[4]. In recent years, an increasing number of studies have been conducted on the role of CDK5RAP3 in tumors, but its expression and role in different tumors has been found to be different. An *et al*[5] reported that CDK5RAP3 inhibited the phosphorylation and activation of p38 by promoting the binding of p38 and p53-induced protein phosphatase 1 to inhibit tumor proliferation. However, Stav *et al*[6] found that the expression of CDK5RAP3 in most cancer tissues was increased, which is of great significance in the diagnosis of lung cancer. The expression and function of CDK5RAP3 are also controversial in the same types of tumors. Mak *et al*[7] found that CDK5RAP3 was highly expressed in hepatocellular cancer and that it could promote the metastasis of hepatoma cancer cell by activating p21-activated protease 4 and down-regulating the expression of tumor suppressor gene p14. However, Zhao *et al*[8] showed that the expression of CDK5RAP3 protein was down-regulated in hepatocellular cancer and that down-regulation of CDK5RAP3 expression was associated with a poor prognosis.

Recent studies have shown that DDRGK1 interacts with CDK5RAP3[9]. DDRGK1 was cloned from human liver in 2010 by Lemaire *et al*[10] .DDRGK1 is located on the short arm of chromosome 20 (20p13), also known as UFBP1, C20orf116, or dJ1187M17. The DDRGK1 sequence is highly conserved and exists in many tissues and organs. Its N-terminal 1-28 amino acid residue region is highly hydrophobic and is an endoplasmic reticulum anchor sequence; 65-69 amino acid residues

are nuclear localization signals. The 229-273 amino acid residues near the C-terminus are the protein PCI domain[11]. Studies have shown that proteins containing a PCI domain are primarily responsible for the construction and assembly of protein complexes[12]. Xi *et al*[13] found that DDRGK1 interacts with IkBa and regulates its stability, thereby regulating the transcriptional activity of NF-κB. At present, there are few studies on the co-expression of CDK5RAP3 and DDRGK1 in gastric cancer and its impact on prognosis. In this study, we examined the expression of CDK5RAP3 and DDRGK1 in 135 cases of gastric cancer, and analyzed their correlation with clinicopathological features and long-term prognosis of the patients.

MATERIALS AND METHODS

Human gastric tumor tissues

The gastric cancer specimens were obtained from 135 patients with gastric adenocarcinoma, who had undergone D2 lymph node dissection and gastrectomy for gastric cancer at the Department of Gastric Surgery, Fujian Medical University Union Hospital (Fujian, China) with available detailed clinic pathologic parameters, between January 2013 and June 2015. All patients received their first diagnosis of gastric cancer and received no other treatment, such as chemotherapy, before surgery. All diagnoses were confirmed by pathology after surgery. Gastric cancer was confirmed by hematoxylin and eosin (H&E) staining in all cases. The clinicopathological data of the 135 GC patients included age, sex, size of the primary tumor, location of the primary tumor, degree of differentiation, histological type, Borrmann type, depth of invasion, lymph node metastasis, distant metastasis and TNM stage. The pathologic stage of the tumor was re-assessed according to the TNM classification of gastric cancer (eighth edition) of the International Union against Cancer (2016).The clinical and pathological data were recorded prospectively for the retrospective analysis. This study was approved by the ethics committee of Fujian Medical University Union Hospital and written consent was obtained from all patients involved.

Immunohistochemistry

Paraffin sections containing sufficient formalin fixed tumor tissue were sectioned continuously at a thickness of 4 μm and were mounted on silage coated slides for immunohistochemical analysis. The slices were deparaffinized with xylene and rehydrated in 95%, 85% and 75% ethanol. Antigen retrieval was performed by subjecting the slides to high-pressure sterilization at 121 ℃ for 2 min in 0.01 mol/L sodium citrate buffer solutions (pH 6.0). Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 at room temperature for 10 min. The slices were then washed in phosphate buffered saline (PBS) solution and blocked in 10% goat serum (Zhongshan Biotechnology Co. Ltd.) for 30 min. Next, the sections were incubated with diluted rabbit anti-human CDK5RAP3 (ab157203, 1:200 dilution; Abcam) or DDRGK1 (21445-1-AP, 1.50 dilution; Proteintech) overnight in a humidified chamber at 4 ℃. After three washes in PBS, the sections were incubated with the secondary antibody conjugated to horseradish peroxidase at room temperature for 30 min. The signal was developed with diaminobenzidine solution, which was followed by counterstaining in 20% hematoxylin. Finally, all slides were dehydrated and mounted on cover glass. For negative controls, non-specific antibody diluent was substituted for the primary antibody.

Evaluation of immunostaining intensity

The immunohistochemistry (IHC) of the tissue sections were examined by two experienced pathologists, who scored the slides according to the intensity of cell staining and the proportion of positively stained tumor cells. The definition for the evaluation of CDK5RAP3 and DDRGK1 staining intensity was as follows: no staining (score of 0), weak staining (light yellow, score of 1), moderate staining (yellow brown, score of 2) and strong staining (brown, score of 3). The positive proportion of stained tumor cells was scored as follows: ⩽ 5% positive cells (score of 0), 6% to 25% positive cells (score of 1), 26% to 50% positive cells (score of 2), ⩾ 51% positive cells (score of 3). If the total score (intensity x percentage score) was less than 3, the protein expression was considered low, however, if the score was 4 or higher, the protein expression was considered high (Figure 1A).

Western blot

After the cells grew to 90%-100% confluence, the cells were flushed twice with pre-cooled PBS and then extracted with RIPA lysis solution (Thermo Fisher Scientific, Waltham, MA, United States) containing a 10% cocktail (Roche, South San Francisco, CA, United States).Protein samples (40 μg per lane) were separated on 10% polyacrylamide gels by the SDS-PAGE method and transferred to PVDF membranes. Then, at room temperature, 5% skim milk was used to block the PVDF membrane for 1 h. The membrane was then incubated at 4 ℃ with the primary anti-CDK5RAP3 (ab157203, 1:1000 dilution; Abcam), anti-DDRGK1 (21445-1-AP, 1:1000 dilution; Proteintech), or anti-GAPDH (ab8245, 1:5000 dilution; Abcam) and washed with TBS-T 3 times, 5 min each time, then incubated at room temperature with the HRP secondary antibody (Cell Signaling Technology) for 1 h. GAPDH was used as an internal control. Finally, the membrane was washed with TBS-T for 30 min and the protein bands were detected by an enhanced chemiluminescence method (Amersham Corporation, Arlin-gton Heights, IL, United States).

Follow-up

All patients were followed up once every three months

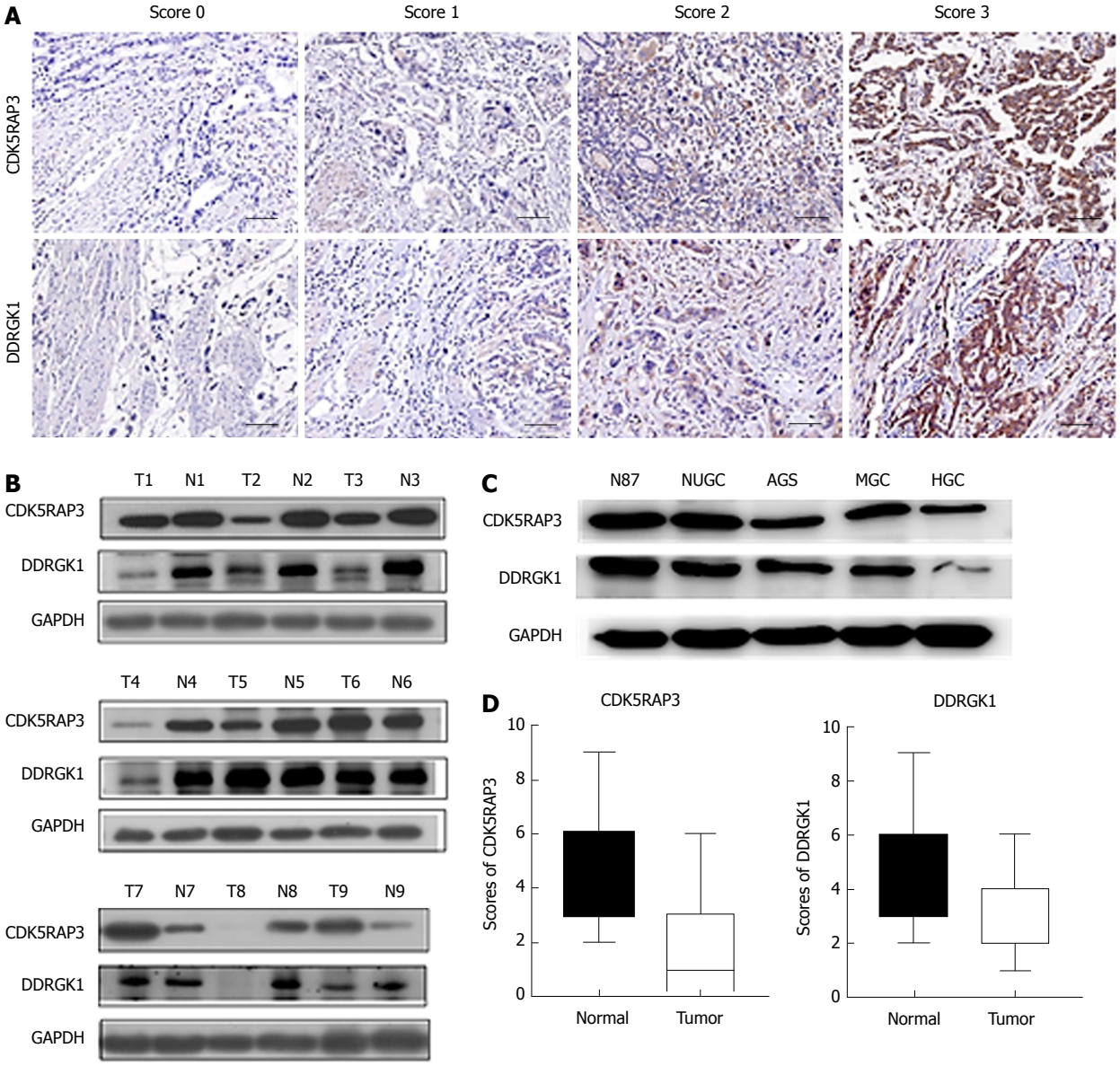


Figure 1 Expression levels of CDK5RAP3 and DDRGK1 in gastric cancer and adjacent non-tumor tissues. A: Immunohistochemical staining of CDK5RAP3 and DDRGK1 expression in gastric cancer tissue and the criteria for immunohistochemistry scores following the intensity of positive signals. Scale bar = 100 μm. B: Western blot of CDK5RAP3 and DDRGK1 in gastric cancer and adjacent non-tumor tissues in nine patients. C: Western blot of CDK5RAP3 and DDRGK1 in five gastric cancer cells. D: CDK5RAP3 and DDRGK1 expression scores are shown as box plots, with the horizontal lines representing the median; the bottom and top of the boxes representing the 25th and 75th percentiles, respectively, and the vertical bars representing the range of data. The expression of CDK5RAP3 and DDRGK1 in gastric tumor tissues and respective adjacent non-tumor tissues was compared using the *t*-test. $n = 135$ ($P < 0.001$).

for the first two years and were then followed up every six months for the next three to five years. The last follow-up time point was January 2018. Follow-up routine examinations, including a physical examination, laboratory tests (CA19-9, CEA and CA72-4), chest X-ray, abdominal CT, B ultrasound, and gastroscopy were performed each year. The total survival time was defined as the time from surgery to the last follow-up, or the time of death, or the expiration of the follow-up database (*e.g.*, lost to follow-up, death from other diseases, *etc*.)

Statistical analysis

All of the data were processed by the SPSS23.0 statistical software package. Appropriate test methods, such as the χ^2 test or Fisher's exact test, were selected according to the type of variables and the purpose of comparison. The survival rate was calculated by the Kaplan-Meier method, and the subsequent survival curve was plotted. The log-rank test was used to compare the survival rates. Cox regression was used to analyze the independent factors that affected the prognosis. The area under the ROC curve was used to compare the prognostic ability of different indexes. The difference was statistically significant when $P < 0.05$.

RESULTS

Expression status of CDK5RAP3 and DDRGK1 in gastric cancer

Of 135 patients with primary gastric cancer, 109 patients (80.7%) had low expression of CDK5RAP3 and 26

Table 1 Relationship between CDK5RAP3 and DDRGK1 protein expression in gastric cancer tissues and various clinicopathological variables

Variables	Total	CDK5RAP3 expression				DDRGK1 expression			
		Low	High	χ^2	*P* value	Low	High	χ^2	*P* value
Gender				1.659	0.198			3.057	0.080
Male	107	84	23			74	33		
Female	28	25	3			24	4		
Age (yr)				4.441	0.035			0.719	0.397
> 60	91	78	13			64	27		
≤ 60	44	31	13			34	10		
Tumor size(cm)				0.125	0.723			0.043	0.835
> 5	53	42	11			39	14		
≤ 5	72	67	15			59	23		
Location of tumor				3.860	0.277			1.537	0.674
Lower 1/3	55	48	7			37	18		
Middle 1/3	20	15	5			16	4		
Upper 1/3	45	36	9			34	11		
More than 1/3	15	10	5			11	4		
Borrmann type				0.285	0.593			1.312	0.252
Ⅰ + Ⅱ Type	31	24	7			25	6		
Ⅲ + Ⅳ Type	104	85	19			73	31		
Degree of differentiation				0.187	0.666			4.414	0.036
Well/moderate	57	47	10			36	21		
Poor and not	78	62	16			62	16		
Histological type				1.271	0.736			8.547	0.036
Papillary	58	48	10			39	19		
Tubular	42	32	10			27	15		
Mucinous	10	9	1			9	1		
Signet-ring cell	25	20	5			23	2		
Depth of invasion				5.674	0.017			1.428	0.232
T1 + T2	21	13	8			13	8		
T3 + T4	114	96	18			85	29		
Lymph node metastasis				0.008	0.927			6.023	0.014
Negative	20	16	4			10	10		
Positive	115	93	22			88	27		
TNM stage				1.383	0.240			2.632	0.105
Ⅰ + Ⅱ	44	33	11			28	16		
Ⅲ + Ⅳ	91	76	15			70	21		
Distant metastasis				1.761	0.184			0.639	0.424
Negative	128	102	26			92	36		
Positive	7	7	0			6	1		

patients (19.3%) had high expression. The DDRGK1 immunohistochemical score showed low expression in 98 cases (72.6%) and high expression in 37 cases (27.4%) (Table 1). Western blotting was used to detect the expression of CDK5RAP3 and DDRGK1 in the tumor and adjacent tissues of nine patients with gastric cancer. It was found that the expression of CDK5RAP3 and DDRGK1 in six patients was higher in respective adjacent non-tumor tissues than that in gastric cancer tissues (Figure 1B). In addition, the expression of CDK5RAP3 and DDRGK1 in gastric cancer cell lines decreased with the decrease of differentiation degree of the gastric cancer cell lines (Figure 1C). We also found that the histological scores of CDK5RAP3 and DDRGK1 in adjacent tissues were higher than those in cancer tissues, with a statistically significant difference (Figure 1D).

Relationships between CDK5RAP3 and DDRGK1 protein expression in gastric cancer tissues and clinicopathological parameters

We analyzed the relationship between CDK5RAP3 and DDRGK1 protein expression in gastric cancer tissues and various clinicopathological data. The expression of CDK5RAP3 in gastric cancer was correlated with the age (P = 0.035) and T stage of the tumor (P = 0.017). However, the expression of DDRGK1 in gastric cancer was closely correlated with the differentiation degree (P = 0.036), the histological type (P = 0.036) and N stage of tumor (P = 0.014), as shown in Table 1. The co-expression level of CDK5RAP3 and DDRGK1 was related to sex (P = 0.024), T stage (P = 0.026), N stage (P = 0.048) and TNM stage (P = 0.016), as shown in Table 2.

Expression levels of CDK5RAP3 and DDRGK1 are correlated with the prognosis of patients with gastric cancer

The median follow-up time was 30.0 mo, and the three-year survival rate was 70.7%. Survival analysis showed that the three-year survival rate of gastric cancer patients with low expression of CDK5RAP3 was 71.1%, which was lower than that of patients with high expression of CDK5RAP3 (90.8%, Figure 2A).

Table 2 Relationship between different CDK5RAP3 and DDRGK1 protein expression status in gastric cancer tissues and various clinicopathological variables

Variables	Total	C53 and DDRGK1 low expression	C53 and/or DDRGK1 high expression	χ^2	*P* value
Gender					
Male	107	59	48	5.077	0.024
Female	28	22	6		
Age (yr)					
> 60	91	55	36	0.022	0.881
≤ 60	44	26	18		
Tumor size (cm)					
> 5	53	32	21	0.005	0.943
≤ 5	82	49	33		
Location of tumor					
Lower 1/3	55	33	22	1.481	0.687
Middle 1/3	20	12	8		
Upper 1/3	45	29	16		
More than 1/3	15	7	8		
Borrmann type					
Ⅰ + Ⅱ Type	31	20	11	0.342	0.559
Ⅲ + Ⅳ Type	104	61	43		
Degree of differentiation					
Well/moderate	57	31	26	1.296	0.255
Poor and not	78	50	28		
Histological type					
Papillary	57	33	25	4.415	0.220
Tubular	42	22	20		
Mucinous	10	8	2		
Signet-ring cell	25	18	7		
Depth of invasion					
T1 + T2	21	8	13	4.972	0.026
T3 + T4	114	73	41		
Lymph node metastasis					
Negative	20	8	12	3.913	0.048
Positive	115	73	42		
TNM stage					
Ⅰ + Ⅱ	44	20	24	5.754	0.016
Ⅲ + Ⅳ	91	61	30		
Distant metastasis					
Negative	126	75	53	2.034	0.154
Positive	7	6	1		

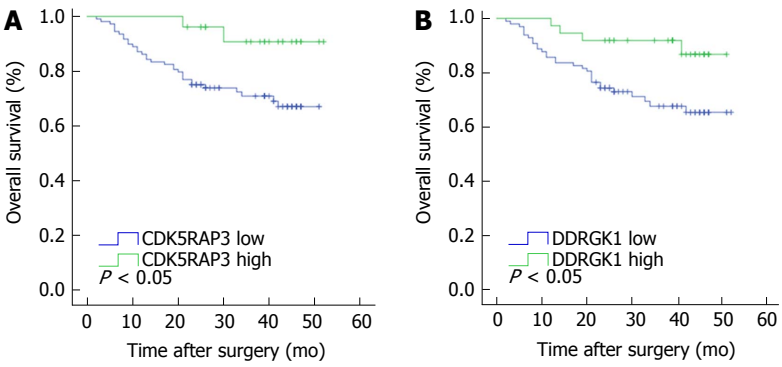


Figure 2 Kaplan-Meier analysis of the correlation between the expression of CDK5RAP3 and DDRGK1 and the overall survival of gastric cancer patients.

The survival time of gastric cancer patients with low expression of DDRGK1 was significantly lower than that of patients with high expression of DDRGK1 (67.7% *vs* 91.9%, Figure 2B). When combining analysis of CDK5RAP3 and DDRGK1, the three-year survival rate of gastric cancer patients with low expression of CDK5RAP3 and DDRGK1 was 64.2%, which was significantly lower than that of the patients with high expression of CDK5RAP3 and DDRGK1 (Figure 3A). We compared the prognostic value of high expression of CDK5RAP3 and low expression of DDRGK1 to low expression of CDK5RAP3 and high expression of DDRGK1, and there were no significant differences between these survival curves (Supplementary Figure

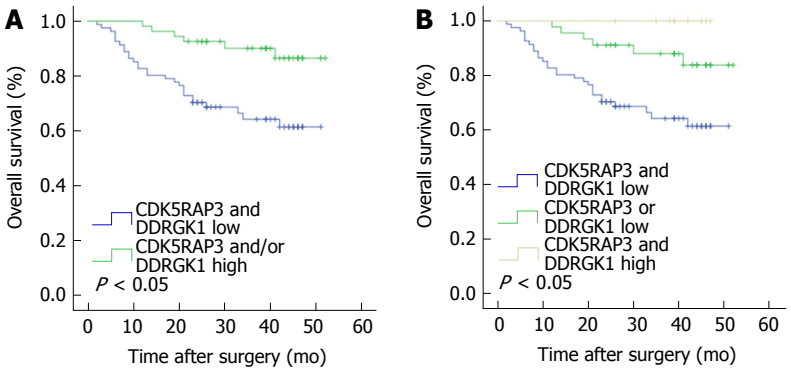


Figure 3 Kaplan-Meier analysis of the correlation between the combined expression of CDK5RAP3 and DDRGK1 with the overall survival of gastric cancer patients.

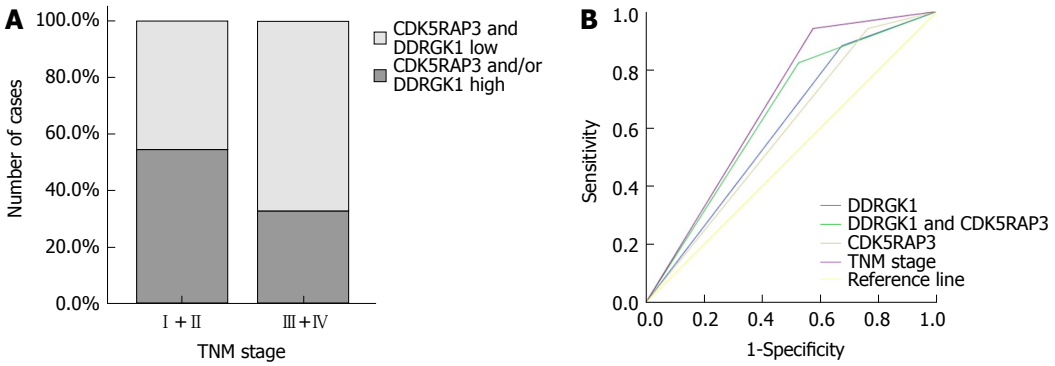


Figure 4 Receiver operating characteristic analysis of the sensitivity and specificity of the predictive value of DDRGK1 expression model, CDK5RAP3 expression model, the combined CDK5RAP3 and DDRGK1 model and the TNM model. A: Co-expression of CDK5RAP3 and DDRGK1 were significantly correlated with TNM stage. B: The area under the ROC curve was 0.649 (0.548-0.751) for the co-expression of CDK5RAP3 and DDRGK1 model, 0.598 (0.486-0.693) for the CDK5RAP3 expression model, 0.605 (0.501-0.708) for the DDRGK1 expression model, and 0.659 (0.562-0.756) for the TNM model.

1). So we combined the two groups into a group for further analysis. Further stratification analysis showed that the prognosis was the best when CDK5RAP3 and DDRGK1 were both highly expressed, and the prognosis was the worse when either CDK5RAP3 or DDRGK1 was highly expressed, while the worst prognosis was correlated with low expression of both CDK5RAP3 and DDRGK1 (Figure 3B). In addition, we used immunoprecipitation combined with mass spectrometry (in an HGC cell line) to find CDK5RAP3 binding protein and potential downstream targets. The results of string analysis show that CDK5RAP3 can bind DDRGK1 (Supplementary Figure 2).

Univariate and multivariate analyses of the prognosis in the entire group

Univariate analysis showed that the overall survival was correlated with the T status (P = 0.026), N status (P = 0.031), M status (P = 0.005), TNM stage (P = 0.001), and the expression level of CDK5RAP3 (P = 0.023) and DDRGK1 (P = 0.015) in gastric cancer tissues and the co-expression level of CDK5RAP3 and DDRGK1 in gastric cancer tissues (P = 0.001) (Table 3). Multivariate Cox prognostic analysis showed that the co-expression levels of CDK5RAP3 and DDRGK1 (P = 0.009) and the TNM stage (P = 0.007) were both independent prognostic factors in gastric cancer patients (Table 4).

The relationship between the expression of CDK5RAP3 and DDRGK1 and TNM stage

As shown in Figure 4, we established a ROC curve to compare the expression of CDK5RAP3 or DDRGK1 alone and the expression of CDK5RAP3 and DDRGK1 together with TNM stage in gastric cancer prognostication. The results showed that the area under the curve of the combination of CDK5RAP3 and DDRGK1 (AUC: 0.649, 95%CI: 0.548-0.751, P = 0.009) was larger than that of CDK5RAP3 or DDRGK1 expression alone (CDK5RAP3: AUC: 0.589, 95%CI: 0.486-0.693, P = 0.120, DDRGK1: AUC: 0.605, 95%CI: 0.501-0.708, P = 0.069). In addition, the prognostic value of the combined expression of CDK5RAP3 and DDRGK1 was closer to that of the TNM stage (AUC: 0.683, 95%CI: 0.591-0.776, P = 0.001).

DISCUSSION

In recent years, although some progress has been made in the treatment of gastric cancer, the prognosis of gastric cancer patients is still not optimistic because the majority of patients are only diagnosed in moderate or advanced stages, and the effect of adjuvant therapy is limited.

Table 3 Univariate analysis of the correlation between clinicopathological parameters and survival of patients with gastric cancer

Clinicopathological parameters	Three-year cumulative survival rate	Log-rank test	*P* value
Gender			
Male	75.4	0.395	0.53
Female	71.4		
Age (yr)			
> 60	75.4	0.163	0.686
⩽ 60	73.8		
Tumor size (cm)			
> 5	68.3	0.988	0.32
⩽ 5	79.3		
Location of tumor			
Lower 1/3	78.2	3.438	0.329
Middle 1/3	83.6		
Upper 1/3	63.3		
More than 1/3	86.7		
Borrmann type			
Ⅰ + Ⅱ	76.9	0.373	0.541
Ⅲ + Ⅳ	74		
Degree of differentiation			
Well/moderate	75.5	0.111	0.739
Poor and not	74.2		
Histological type			
Papillary	70	4.386	0.223
Tubular	85.6		
Mucinous	70		
Signet-ring cell	69.1		
Depth of invasion			
T1 + T2	91.7	4.95	0.026
T3 + T4	71.7		
Lymph node metastasis			
Negative	95	4.629	0.031
Positive	71.2		
TNM stage			
Ⅰ + Ⅱ	92.3	11.424	0.001
Ⅲ + Ⅳ	66.3		
Distant metastasis			
Negative	76.7	8.015	0.005
Positive	42.9		
CDK5RAP3 expression			
Low	71.1	5.168	0.023
High	90.8		
DDRGK1 expression			
Low	67.7	5.971	0.015
High	91.9		
CDK5RAP3/DDRGK1 expression			
CDK5RAP3 and/or DDRGK1 high	90.1	10.415	0.001
CDK5RAP3 and DDRGK1 low	64.2		

Therefore, finding new biomarkers will help to improve earlier diagnosis and treatment of gastric cancer. DDRGK1 is not only a target protein of ufmylation, but is also an integral component of the ufmylation modification system. Ufmylation mediated by DDRGK1 plays an important role in carcinogenesis [14,15]. Shiwaku *et al*[16]found that the amino acid sequence of CDK5RAP3 contained an ubiquitin protein ligase binding region. Wu's study[9] found that CDK5RAP3 can interacted with DDRGK1 and UFL1 (called RCAD in their study) and regulate the stability of CDK5RAP3 and DDRGK1. Therefore, based on these previous reports and our finding, we speculate that the function of CDK5RAP3 and DDRGK1 is related. However, the expression of CDK5RAP3 and DDRGK1 in gastric cancer and their influence on clinicopathological characteristics and prognosis have not previously been reported.

CDK5RAP3 is widely expressed in various tissues and cells of the whole body, including the heart, brain, skeletal muscle, placenta, lung, liver, kidney and pancreas[17]. In early embryonic development, CDK5RAP3 regulates cell cycle progression, epidermal cell adhesion and migration[18]. Recent studies have suggested that CDK5RAP3 plays an important role in various cancers such as lung cancer, liver cancer, and head and neck cancer[6,19,20]. Our study found that the three year survival rate of patients with low expression of CDK5RAP3 was lower than that of patients with high expression of CDK5RAP3 ($P < 0.05$), and the expression of CDK5 RAP3 was correlated with tumor T stage, suggesting that CDK5RAP3 is involved in gastric cancer. It may play a role in suppressing cancer, which is also consistent with our previous research results[21]. In the case of DDRGK1, some studies have shown that DDRGK1 is a tumor suppressor. The ufmylation of DDRGK1 itself is essential for its combination with UFL1 and activation of the UFL1 ubiquitin ligase. If DDRGK1 is unable to undergo ufmylation, it cannot bind and activate UFL1 activity, thereby blocking the ufmylation of the nuclear receptor co-activator ASC1 and, inhibiting the binding of ASC1 and the transcription factors p300 and SRC1 to the downstream target genes of the estrogen receptor ERα[11,14].

In this study, the survival of gastric cancer patients with low expression of DDRGK1 was significantly shorter than that of patients with high expression of DDRGK1 ($P < 0.05$), and the expression of DDRGK1 was related to tumor differentiation, histological type and N stage. It has also been suggested that DDRGK1 may play a role in suppressing the progression of gastric cancer.

In addition, we found that patients with low expression of CDK5RAP3 and DDRGK1 had the worst prognosis while patients with high expression of both proteins had the best prognosis, and the other patients were between them. Further analysis showed that the accuracy of prognostication with a combination of CDK5RAP3 and DDRGK1 was higher than that of CDK5 RAP3 or DDRGK1 alone. We showed that the combined expression of CDK5RAP3 and DDRGK1 had a better ability to predict the overall survival rate of gastric cancer patients. Xi *et al*[13] found that DDRGK1 interacted with IkBa and regulated its stability, thereby regulating the transcriptional activity of NF-κB and its target gene expression. However, Wang's study[9] of CDK5RAP3 found that down-regulation of CDK5RAP3 increased cell invasiveness and increased the transcriptional activity of NF-κB. CDK5RAP3 binds to RelA to inhibit its phosphorylation and increase the binding of HDAC to RERA, thereby inhibiting the transcriptional activity of NF-κB. CDK5RAP3 and DDRGK1 can interact with

Table 4 Multivariate analysis of the correlation between clinicopathological parameters and survival time of patients with gastric cancer

Covariates	Coefficient	Standard error	HR	95%CI for HR	*P* value
CDK5RAP3 expression (high *vs* low)	-1.226	0.735	0.294	0.070-1.239	0.095
DDRGK1 expression (high *vs*. low)	-0.979	0.536	0.376	0.131-1.074	0.068
CDK5RAP3 and DDRGK1 expression (low/low *vs*. high and/or high)	1.178	0.453	3.247	1.336-7.891	0.009
Depth of invasion (T3,T4 *vs* T1,T2)	1.071	1.045	2.920	0.376-22.635	0.305
Lymph node metastasis (positive *vs* negative)	1.538	1.020	1.974	0.631-34.367	0.132
Distant metastasis (positive *vs* negative)	0.861	0.544	2.365	0.815-6.8631	0.113
TNM stage (stage Ⅲ and Ⅳ *vs* Ⅰ and Ⅱ)	-1.630	0.608	7.195	0.060-0.645	0.007

each other, and their roles in the NF-κB pathway are similar. Therefore, we hypothesized that their impact on prognosis may be related to the overlapping of the two tumor suppressing effects. However, the interaction between CDK5RAP3 and DDRGK1 in gastric cancer has not been fully elucidated. Further manipulation of gene expression in different gastric cancer cell lines and investigation of the characteristics and mechanism of these genes effects on gastric cancer are needed in additional studies.

In summary, low expression of CDK5RAP3 and DDRGK1 are closely related to the prognosis of gastric cancer, and the co-expression of CDK5RAP3 and DDRGK1 is an independent prognostic factor correlated with the overall survival of gastric cancer patients.

ARTICLE HIGHLIGHTS

Research background

Although the morbidity and mortality of the primary gastric cancer has declined in recent decades, it is still the third most common cause of cancer-related deaths worldwide. The symptoms of early gastric cancer are not highly specific. Therefore, misdiagnosis and missed diagnosis may occur. Therefore, finding new biomarkers will help to improve earlier diagnosis and treatment of gastric cancer. In recent years, an increasing number of studies on the prognostic indicators of gastric cancer have been published. However, the expression of CDK5RAP3 and DDRGK1 in gastric cancer and its influence on prognosis have not yet been reported.

Research motivation

At present, the etiology and pathogenesis of gastric cancer has not yet been fully clarified. There is also a lack of specific and highly effective therapeutic drugs available for use in clinical practice. The symptom specificity of early gastric cancer is not obvious, so most patients are already in advanced stages before receiving medical treatment, which seriously affects the prognosis of patients. Therefore, searching for molecular markers that can be used as an independent prognostic factor for gastric cancer is of great significance for the early diagnosis and targeted treatment of gastric cancer. A series of studies on tumor prognostic factors is expected to provide a new target for the treatment of gastric cancer while providing new targets for the treatment of gastric cancer. The expression of CDK5RAP3 and DDRGK1 in gastric cancer and their influence on clinicopathological characteristics and prognosis have not previously been discussed.

Research objectives

The aim of this study is to identify novel effective biomarkers to classify patients with low or high survival. This would provide a guide to clinicians to select therapeutic strategies for patients and provide personalized therapy according to the predicted survival rate. In this study, we investigated two interacting proteins, CDK5RAP3 and DDRGK1, which may help determine patient management strategies.

Research methods

We used immunohistochemistry to detect the expression of CDK5RAP3 and DDRGK1 in gastric cancer and adjacent tissues. Western Blot was used to detect the expression of CDK5RAP3 and DDRGK1 in gastric cancer and its adjacent tissues and cell lines. According to immunohistochemistry scores, the patients were divided into CDK5RAP3 high expression group and CDK5RAP3 low expression group, DDRGK1 high expression group and DDRGK1 low expression group, and the relationship between the expression level and clinicopathological data was analyzed. Furthermore, based on the combined expression of CDK5RAP3 and DDRGK1, we classified the patients into three subtypes: CDK5RAP3 and DDRGK1 high (n = 9), CDK5RAP3 or DDRGK1 low (n = 45) and CDK5RAP3 and DDRGK1 low (n = 81). Then, we used the Kaplan-Meier method to analyze the effect of different expression patterns on prognosis.

Research results

Our research found that the expression of CDK5RAP3 and DDRGK1 was down-regulated in gastric cancer. Low expression of CDK5RAP3 or DDRGK1 is a poor prognostic factor for gastric cancer patients. Moreover, prognostic analysis showed that the co-expression of CDK5RAP3 and DDRGK1 was an independent prognostic factor correlating with the overall survival of gastric cancer patients. Combined expression analysis of CDK5RAP3 and DDRGK1 may provide a more accurate prognostic value for overall survival. This study presents two interacting proteins, which may be useful to determine patient management strategies. These makers may predict the prognosis of gastric cancer patients through an analysis of CDK5RAP3 and DDRGK1 protein expression in preoperative biopsy and tumor specimens.

Research conclusions

This study found that low expression of CDK5RAP3 and DDRGK1 are closely related to the poor prognosis of gastric cancer patients, and the co-expression of CDK5RAP3 and DDRGK1 is an independent prognostic factor correlated with the overall survival of gastric cancer patients. These two interacting proteins, CDK5RAP3 and DDRGK1, may be helpful in determining patient management strategies, and to predict the prognosis of gastric cancer patients. We hypothesized that CDK5RAP3 and DDRGK1 were key genes which may participate in the biological regulation of gastric cancer. The mechanism of their role in gastric cancer has not been fully elucidated and further studies are needed. With advances in technology, humans may find more effective and new indicators in the future to guide treatment, improve prognosis, and reduce the recurrence rate and mortality of patients with gastric cancer.

Research perspectives

This study found the prognostic value of two interacting proteins, CDK5RAP3 and DDRGK1, by detecting the expression of both in clinical specimens, combined with detailed clinicopathological data analysis. This study provided ideas for finding new tumor prognosis related molecules. Manipulation of both CDK5RAP3 and DDRGK1 expression in different gastric cancer cell lines, such as overexpression or knockdown, will be needed for future research. Further study is necessary to investigate the characteristics of cancer cells and explore the mechanism of CDK5RAP3 and DDRGK1 affecting the development of

gastric cancer by an *in vitro* cell model and *in vivo* xenograft model.

REFERENCES

1 **Sano T**, Sasako M, Yamamoto S, Nashimoto A, Kurita A, Hiratsuka M, Tsujinaka T, Kinoshita T, Arai K, Yamamura Y, Okajima K. Gastric cancer surgery: morbidity and mortality results from a prospective randomized controlled trial comparing D2 and extended para-aortic lymphadenectomy--Japan Clinical Oncology Group study 9501. *J Clin Oncol* 2004; **22**: 2767-2773 [PMID: 15199090 DOI: 10.1200/JCO.2004.10.184]

2 **Papenfuss WA**, Kukar M, Oxenberg J, Attwood K, Nurkin S, Malhotra U, Wilkinson NW. Morbidity and mortality associated with gastrectomy for gastric cancer. *Ann Surg Oncol* 2014; **21**: 3008-3014 [PMID: 24700300 DOI: 10.1245/s10434-014-3664-z]

3 **Wöhrer SS**, Raderer M, Hejna M. Palliative chemotherapy for advanced gastric cancer. *Ann Oncol* 2004; **15**: 1585-1595 [PMID: 15520058 DOI: 10.1093/annonc/mdh422]

4 **Wang X**, Ching YP, Lam WH, Qi Z, Zhang M, Wang JH. Identification of a common protein association region in the neuronal Cdk5 activator. *J Biol Chem* 2000; **275**: 31763-31769 [PMID: 10915792 DOI: 10.1074/jbc.M004358200]

5 **An H**, Lu X, Liu D, Yarbrough WG. LZAP inhibits p38 MAPK (p38) phosphorylation and activity by facilitating p38 association with the wild-type p53 induced phosphatase 1 (WIP1). *PLoS One* 2011; **6**: e16427 [PMID: 21283629 DOI: 10.1371/journal.pone.0016427]

6 **Stav D**, Bar I, Sandbank J. Usefulness of CDK5RAP3, CCNB2, and RAGE genes for the diagnosis of lung adenocarcinoma. *Int J Biol Markers* 2007; **22**: 108-113 [PMID: 17549666 DOI: 10.1177/172460080702200204]

7 **Mak GW**, Chan MM, Leong VY, Lee JM, Yau TO, Ng IO, Ching YP. Overexpression of a novel activator of PAK4, the CDK5 kinase-associated protein CDK5RAP3, promotes hepatocellular carcinoma metastasis. *Cancer Res* 2011; **71**: 2949-2958 [PMID: 21385901 DOI: 10.1158/0008-5472.CAN-10-4046]

8 **Zhao JJ**, Pan K, Li JJ, Chen YB, Chen JG, Lv L, Wang DD, Pan QZ, Chen MS, Xia JC. Identification of LZAP as a new candidate tumor suppressor in hepatocellular carcinoma. *PLoS One* 2011; **6**: e26608 [PMID: 22028922 DOI: 10.1371/journal.pone.0026608]

9 **Wu J**, Lei G, Mei M, Tang Y, Li H. A novel C53/LZAP-interacting protein regulates stability of C53/LZAP and DDRGK domain-containing Protein 1 (DDRGK1) and modulates NF-kappaB signaling. *J Biol Chem* 2010; **285**: 15126-15136 [PMID: 20228063 DOI: 10.1074/jbc.M110.110619]

10 **Lemaire K**, Moura RF, Granvik M, Igoillo-Esteve M, Hohmeier HE, Hendrickx N, Newgard CB, Waelkens E, Cnop M, Schuit F. Ubiquitin fold modifier 1 (UFM1) and its target UFBP1 protect pancreatic beta cells from ER stress-induced apoptosis. *PLoS One* 2011; **6**: e18517 [PMID: 21494687 DOI: 10.1371/journal.pone.0018517]

11 **Neziri D**, Ilhan A, Maj M, Majdic O, Baumgartner-Parzer S, Cohen G, Base W, Wagner L. Cloning and molecular characterization of Dashurin encoded by C20orf116, a PCI-domain containing protein. *Biochim Biophys Acta* 2010; **1800**: 430-438 [PMID: 20036718 DOI: 10.1016/j.bbagen.2009.12.004]

12 **Hofmann K**, Bucher P. The PCI domain: a common theme in three multiprotein complexes. *Trends Biochem Sci* 1998; **23**: 204-205 [PMID: 9644972 DOI: 10.1016/S0968-0004(98)01217-1]

13 **Xi P**, Ding D, Zhou J, Wang M, Cong YS. DDRGK1 regulates NF-κB activity by modulating IκBα stability. *PLoS One* 2013; **8**: e64231 [PMID: 23675531 DOI: 10.1371/journal.pone.0064231]

14 **Yoo HM**, Kang SH, Kim JY, Lee JE, Seong MW, Lee SW, Ka SH, Sou YS, Komatsu M, Tanaka K, Lee ST, Noh DY, Baek SH, Jeon YJ, Chung CH. Modification of ASC1 by UFM1 is crucial for ERα transactivation and breast cancer development. *Mol Cell* 2014; **56**: 261-274 [PMID: 25219498 DOI: 10.1016/j.molcel.2014.08.007]

15 **Tatsumi K**, Sou YS, Tada N, Nakamura E, Iemura S, Natsume T, Kang SH, Chung CH, Kasahara M, Kominami E, Yamamoto M, Tanaka K, Komatsu M. A novel type of E3 ligase for the Ufm1 conjugation system. *J Biol Chem* 2010; **285**: 5417-5427 [PMID: 20018847 DOI: 10.1074/jbc.M109.036814]

16 **Shiwaku H**, Yoshimura N, Tamura T, Sone M, Ogishima S, Watase K, Tagawa K, Okazawa H. Suppression of the novel ER protein Maxer by mutant ataxin-1 in Bergman glia contributes to non-cell-autonomous toxicity. *EMBO J* 2010; **29**: 2446-2460 [PMID: 20531390 DOI: 10.1038/emboj.2010.116]

17 **Lew J**, Beaudette K, Litwin CM, Wang JH. Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J Biol Chem* 1992; **267**: 13383-13390 [PMID: 1618840]

18 **Mak GW**, Lai WL, Zhou Y, Li M, Ng IO, Ching YP. CDK5RAP3 is a novel repressor of p14ARF in hepatocellular carcinoma cells. *PLoS One* 2012; **7**: e42210 [PMID: 22860085 DOI: 10.1371/journal.pone.0042210]

19 **Wang J**, An H, Mayo MW, Baldwin AS, Yarbrough WG. LZAP, a putative tumor suppressor, selectively inhibits NF-kappaB. *Cancer Cell* 2007; **12**: 239-251 [PMID: 17785205 DOI: 10.1016/j.ccr.2007.07.002]

20 **Wang JB**, Wang ZW, Li Y, Huang CQ, Zheng CH, Li P, Xie JW, Lin JX, Lu J, Chen QY, Cao LL, Lin M, Tu RH, Lin Y, Huang CM. CDK5RAP3 acts as a tumor suppressor in gastric cancer through inhibition of β-catenin signaling. *Cancer Lett* 2017; **385**: 188-197 [PMID: 27793695 DOI: 10.1016/j.canlet.2016.10.024]

21 **Merbl Y**, Refour P, Patel H, Springer M, Kirschner MW. Profiling of ubiquitin-like modifications reveals features of mitotic control. *Cell* 2013; **152**: 1160-1172 [PMID: 23452859 DOI: 10.1016/j.cell.2013.02.007]

P- Reviewer: Chuang SM, Tomizawa M, Zhang J
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**:Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3908-3918

DOI: 10.3748/wjg.v24.i34.3908

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Gastroduodenal ulcer bleeding in elderly patients on low dose aspirin therapy

Koh Fukushi, Keiichi Tominaga, Kazunori Nagashima, Akira Kanamori, Naoya Izawa, Mimari Kanazawa, Takako Sasai, Hideyuki Hiraishi

Koh Fukushi, Keiichi Tominaga, Kazunori Nagashima, Akira Kanamori, Naoya Izawa, Mimari Kanazawa, Hideyuki Hiraishi, Department of Gastroenterology, Dokkyo Medical University, Tochigi 3210293, Japan

Takako Sasai, Department of General Internal Medicine 2, Kawasaki Medical School General Medical Center, Okayama 700850, Japan

ORCID number: Koh Fukushi (0000-0001-8079-3386); Keiichi Tominaga (0000-0002-3716-3386); Kazunori Nagashima (0000-0002-0510-7179); Akira Kanamori (0000-0001-9698-9957); Naoya Izawa (0000-0002-2467-9367); Mimari Kanazawa (0000-0003-2243-4267); Takako Sasai (0000-0002-0596-7013); Hideyuki Hiraishi (0000-0002-0721-5018).

Author contributions: All authors contributed to this research study; Fukushi K contributed to manusucript writing, performing procedures and data analysis; Tominaga K, Sasai T and Hiraishi H contributed to draft conception and design; Nagashima K contributed to data analysis; Nagashima K, Kanamori A, Izawa N and Kanazawa M contributed to writing the manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Dokkyo Medical University.

Informed consent statement: Patients were not required to give informed consent to this study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Keiichi Tominaga, MD, PhD, Doctor, Department of Gastroenterology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Shimotuga, Tochigi 3210293, Japan. tominaga@dokkyomed.ac.jp
Telephone: +81-282-872147
Fax: +81-282-867761

Received: April 4, 2018
Peer-review started: April 4, 2018
First decision: May 29, 2018
Revised: July 12, 2018
Accepted: July 22, 2018
Article in press: July 21, 2018
Published online: September 14, 2018

Abstract

AIM

To determine the clinical characteristics of elderly patients of hemorrhagic gastroduodenal ulcer on low-dose aspirin (LDA) therapy.

METHODS

A total of 1105 patients with hemorrhagic gastroduodenal ulcer treated in our hospital between January 2000 and March 2016 were grouped by age and drugs used, and these groups were compared in several factors. These groups were compared in terms of length of hospital stay, presence/absence of hemoglobin (Hb) decrease, presence/absence of blood transfusion, Forrest Ⅰ, percentage of *Helicobacter pylori* infection, presence/absence of underlying disease, and percentage of severe cases.

RESULTS

The percentage of blood transfusion (62.6% *vs* 47.7 %, $P < 0.001$), Hb decrease (53.8% *vs* 40.8%, $P < 0.001$), and the length of hospital stay (23.5 d *vs* 16.7 d, $P < 0.001$) were significantly greater in those on drug therapy. The percentage of blood transfusion (65.3% *vs* 47.8%, $P < 0.001$), Hb decrease (54.2% *vs* 42.1%, $P < 0.001$), and length of hospital stay (23.3 d *vs* 17.5 d, $P < 0.001$) were significantly greater in the elderly. In comparison with the LDA monotherapy group, the percentage of severe cases was significantly higher in the LDA combination therapy group when elderly patients were concerned (16.1% *vs* 34.0%, $P = 0.030$). Meanwhile, among those on LDA monotherapy, there was no significant difference between elderly and non-elderly (16.1% *vs* 16.0%, $P = 0.985$).

CONCLUSION

A combination of LDA with antithrombotic drugs or non-steroidal anti-inflammatory drugs (NSAIDs) contributes to aggravation. And advanced age is not an aggravating factor when LDA monotherapy is used.

Key words: Hemorrhagic gastroduodenal ulcer; Low-dose aspirin; Antithrombotic drugs; Elderly patients; Proton pump inhibitor

Core tip: A total of 1105 patients with hemorrhagic gastroduodenal ulcer were grouped by age and drugs used, and these groups were compared in several factors. Among the elderly (over 70 years), the rate of severe conditions was significantly higher in patients receiving low-dose aspirin (LDA) combination therapy than in those receiving LDA monotherapy. Meanwhile, in the LDA monotherapy group, no significant difference in the rate of severe conditions was observed between elderly and non-elderly patients. This result suggests LDA combination therapy contributes to the aggravation, and advanced age is not an aggravating factor when LDA monotherapy is used.

Fukushi K, Tominaga K, Nagashima K, Kanamori A, Izawa N, Kanazawa M, Sasai T, Hiraishi H. Gastroduodenal ulcer bleeding in elderly patients on low dose aspirin therapy. *World J Gastroenterol* 2018; 24(34): 3908-3918 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3908.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3908

INTRODUCTION

Japan's population is aging rapidly. According to the White Paper on Aging Society 2016, Cabinet office, Government of Japan, people 65 years of age or older accounted for 27.3% of the total population as of October 1, 2016. Under a situation where cerebrovascular disorder and ischemic heart disease have been increasing, clinical evidence of the usefulness of low-dose aspirin (LDA) as a means of secondary prevention of such diseases has often been reported and the frequency of its use has increased[1-3]. However, Pearson *et al*[4] reported that the use of LDA caused an approximately 20% decrease in cardiovascular events in comparison with the control group, but its use was associated with a 2.7-fold higher risk of gastrointestinal hemorrhage. Serious adverse responses to LDA include gastrointestinal mucosal disorder and gastrointestinal hemorrhage; therefore, there is a concern for an increase and aggravation of these conditions[5-9].

Based on the Special Report of Vital Statistics in Japan issued by the Ministry of Health, Labour and Welfare, data in 1996, when the number of patients with gastric ulcer was the greatest after 1990, and the latest available data in 2014 were compared in regard to the number of patients with gastroduodenal ulcer and the number of deaths from gastroduodenal ulcer. The number of patients and the number of deaths in 1996 were 1124000 and 4514, respectively, whereas the corresponding numbers were 311000 (28% of the number in October 1996) and 2770 (61% of the number in 1996) in October in 2014. Although the number of patients with ulcer was decreased to less than one third, there was no marked decrease in the number of deaths from ulcer. This indicates that the clinical picture of ulcer became more severe, presumably reflecting an increase in the incidence of ulcer due to the increased use of antithrombotic drugs including LDA in the aging society, whereas the rate of infection with *Helicobacter pylori* (*H. pylori*) has decreased, and the rate of *H. pylori* eradication has increased, in the younger generation in recent years[10]. In particular, combined use of LDA and non-steroidal anti-inflammatory drugs (NSAIDs) and advanced age serve as risk factors for the occurrence of LDA-induced ulcer and also increase the risk of hemorrhage and aggravation[11-14]. According to a sub-analysis by Nikolsky *et al*[15], who investigated the presence/absence and prognosis of gastrointestinal hemorrhage within 30 d of hospitalization due to acute coronary syndrome, the overall mortality at 1 year was significantly higher in patients who had gastrointestinal hemorrhage within 30 d of hospitalization than in those who did not. In this study, we paid attention to patients who were on oral LDA therapy, a clinically important issue, among elderly patients with hemorrhagic gastroduodenal ulcer due to oral antithrombotic therapy to elucidate the clinical characteristics of this condition and analyzed patients with hemorrhagic gastroduodenal ulcer treated in our hospital in relation to age and medication.

MATERIALS AND METHODS

Patients

This study included 1105 patients who had hematemesis, melena, or acute anemia symptoms due to hemorrhagic gastroduodenal ulcer [801 (72.5%) cases of gastric ulcer and 304 (27.5%) cases of duodenal ulcer] and who underwent emergency endoscopic hemostasis because

upper gastrointestinal hemorrhage was suspected in Dokkyo Medical University Hospital between January 2000 and March 2016. These 1105 patients comprised inpatients, outpatients at the emergency department, and emergency transport patients.

Patient management

The rules of our response to hemorrhagic gastric and duodenal ulcers are as follows: (1) hemostasis is rapidly and continuously performed by a gastroenterologist; (2) the hemostasis procedure uses clipping or argon plasma coagulation at the operator's discretion, and a local injection of hypertonic saline epinephrine (HSE) and thrombin spray are employed if necessary without restriction to a single technique; (3) blood transfusion is indicated for patients with hemoglobin (Hb) $\leqslant$ 70 g/L or patients in shock; (4) intravenous administration of a proton pump inhibitors (PPIs) is given promptly after endoscopic hemostasis, and it is switched to oral administration after initiation of oral feeding; (5) oral feeding is begun with thin rice gruel if blood test shows no progression of anemia and if no bleeding is found by second-look endoscopy performed within 0-5 d; and (6) when the patient is on antithrombin drug or anticoagulation drug therapy, discontinuation of the drug therapy is considered in consultation with a doctor of the specialty concerned after evaluating the risk of thrombosis, embolism, and bleeding.

Definition

Patients aged 70 years or older were defined as elderly, and those aged younger than 70 years were defined as non-elderly. A significant decrease in the Hb level was defined as a decrease of at least 20 g/L in comparison with the Hb level in the previous blood examination or as an Hb level of 70 g/L or lower in the absence of available data in the previous blood examination. As for *H. pylori* infection, it was possible that the urea breath test would provide a false-negative result because of the PPIs administered. Therefore, *H. pylori*-IgG antibody was measured in all subjects, and antibody titers of 10 U/mL or more were defined as positive. Multiple ulcer was defined by the presence of two or more ulcer lesions. Rebleeding was defined by the endoscopic evidence and additional treatment of hemorrhage within 72 h after the implementation of the initial endoscopic hemostasis. Hemorrhage found after more than 72 h was defined as recurrence. Severe cases were defined as cases with at least two of the following three items: (1) an Hb decrease of 20 g/L or more or blood transfusion; (2) hospital stay of at least 30 d; and (3) rebleeding, surgery, interventional radiology (IVR), or death. The oral drugs examined included antiplatelet drugs, such as LDA, thienopyridines (clopidogrel, ticlopidine, and prasugrel), and cilostazol, and anticoagulation drugs such as warfarin, heparin, and direct oral anticoagulants (DOACs) (dabigatran, rivaroxaban, apixaban, and edoxaban). LDA, administered at doses of 70–330 mg/d, reportedly provides an antiplatelet effect[6,11]. In Japan, LDA is usually prescribed at a dose $\leqslant$ 162 mg/d. This also applies to the present study. In addition, the use of NSAIDs was also examined. The subjects were also examined for the presence/absence of cardiac disease, cerebrovascular disorder, renal disease, peptic ulcer, and diabetes mellitus as possible underlying diseases.

Data analysis

This was a retrospective study. The medical records of the subjects were examined for patient age, sex, Hb level, presence/absence of blood transfusion, Forrest classification, the number of ulcerative lesions, oral drugs, underlying disease, presence/absence of *H. pylori* infection, *etc*. These subjects were divided into those who were on oral drug therapy and those who were not and were also classified as elderly and non-elderly patients. These groups were compared in regard to the percentage of patients with blood transfusion, Hb decrease, rebleeding, surgery, IVR, or fatal outcome, and the length of hospital stay. In addition, among patients on oral drug therapy, attention was focused on LDA; in each of the LDA monotherapy group and LDA combination therapy group, the percentage of severe cases was analyzed in relation to elderly and non-elderly patients. To investigate factors for aggravation of the condition in elderly patients, the elderly group was further divided into those with and without severe conditions for comparison.

Statistical analysis

For statistical analysis, χ^2 test, *t* test, and Mann-Whitney *U* test were used. Logistic regression analysis was also performed using hospital stay of 20 d or more as a dependent variable. SPSS version (IBM SPSS Statistics 21; IBM Japan, Ltd.) was used for statistical analysis processing. This study was approved by the life ethics committee of our institution.

RESULTS

Patient characteristics in each group

The numbers (percentages) of patients with gastric ulcer and duodenal ulcer were 801 (72.5%) and 304 (27.5%), respectively. Table 1 shows the characteristics of the patients with gastroduodenal ulcer examined in this study. These patients were classified into those with oral drug therapy (medicated group) and those without oral drug therapy (non-medicated group). The medicated group comprised 474 (42.9%) patients, whereas the non-medicated group comprised 631 (57.1%) patients. These patients were also divided into elderly and non-elderly patients. There were 436 (39.5%) and 669 (60.5%) elderly and non-elderly patients, respectively. Table 2 shows the patient characteristics of each group.

Comparison between the medicated group and non-medicated group

Types of oral medication included 474 patients (113 cases of LDA monotherapy and 157 cases of NSAIDs

Table 1 Baseline characteristics *n* (%)

Items	All cases (*n* = 1105)
Mean age (yr)	64.37
Sex	
Male	823 (74.5)
Female	282 (25.5)
Mean length of hospital stay (d)	
After endoscopic treatment	19.8
Overall	22.1
Hb (mg/L)	87.4
Hb decrease	509 (46.1)
Blood transfusion	594 (53.8)
H. pylori positive	857 (77.6)
Endoscopic findings	
Ulcer	
Single	759 (68.7)
Multiple	346 (31.3)
Forrest classification	
Ⅰa	88 (8.0)
Ⅰb	171 (15.5)
Ⅱa	525 (47.5)
Ⅱb	142 (12.8)
Ⅲ	179 (16.2)
Rebleeding	82 (7.4)
Recurrence	60 (5.4)
Surgery/IVR/death	32 (2.9)
Prophylactic anti-ulcer medication	
None	749 (67.8)
PPI	88 (8.0)
H2RA	117 (10.6)
MP	152 (13.6)
Comorbidity	
Cardiac disease	254 (30.0)
Cerebrovascular disorder	180 (16.3)
Renal failure	125 (11.3)
DM	198 (17.9)
Orthopedic disorder	162 (14.7)
History of ulcer	317 (28.7)

Hb: Hemoglobin; *H. pylori*: *Helicobacter pylori*; IVR: Interventional radiology; PPI: Proton pump inhibitor; H2RA: Histamine-2 receptor antagonists; MP: Mucosal protectant; DM: Diabetes mellitus.

monotherapy and 113 cases of clopidogrel monotherapy and 10 cases of cilostazol monotherapy and 40 cases of warfarin monotherapy and 4 cases of DOACs monotherapy, and 118 cases of combination therapy). When the medicated and non-medicated groups were compared, the percentage of patients with blood transfusion (62.6% *vs* 47.7%; $P < 0.001$) and the percentage of patients with Hb decrease (53.8% *vs* 40.8%; $P < 0.001$) were significantly higher in the medicated group (Figure 1A). The length of hospital stay after the implementation of endoscopic treatment (23.5 d *vs* 16.7 d; $P < 0.001$) and the overall length of hospital stay (27.0 d *vs* 18.5 d; $P < 0.001$) were significantly longer in the medicated group. There was no significant difference with regard to rebleeding, surgery, IVR, or mortality between the two groups.

Comparison between elderly and non-elderly patients

The results of the comparison between elderly and non-elderly patients are shown in Figure 1B. The percentage of patients with blood transfusion (65.3% *vs* 47.8%; $P < 0.001$), percentage of patients with Hb decrease (54.2% *vs* 42.1%; $P < 0.001$), and percentage of patients with rebleeding, surgery, IVR, or death (11.7% *vs* 6.6%; $P = 0.033$) were significantly higher among elderly patients. The length of hospital stay after the implementation of endoscopic treatment (23.3 d *vs* 17.5 d; $P < 0.001$) and the overall length of hospital stay (26.9 d *vs* 19.0 d; $P < 0.001$) were significantly longer among elderly patients.

Comparison between patients on LDA monotherapy and those on LDA combination therapy

Patients in the medicated group were divided into those with LDA monotherapy or LDA combination therapy and elderly or non-elderly patients, and the percentage of severe cases and the length of hospital stay were examined. Patients in the medicated group were divided into groups A (elderly receiving LDA monotherapy), B (elderly receiving LDA combination therapy), C (non-elderly receiving LDA monotherapy), and D (non-elderly receiving LDA combination therapy). Elderly patients, a population that may have clinical issues, were investigated by comparing groups A and B. In addition, the age-related tendency in the LDA monotherapy group was examined by comparing groups A and C (Table 3). The results are shown in Figures 2 and 3. A comparison between groups A and B revealed that the length of hospital stay tended to be longer in group B than in group A (group A 20.0 d *vs* group B 25.5 d; $P = 0.194$). Rebleeding, surgery, IVR, and death were more frequent in group B than in group A (group A 3.2% *vs* group B 14.6%; $P = 0.038$). In comparison with group A, the percentage of severe cases was significantly higher in group B (group A 16.1% *vs* group B 34.0%; $P = 0.030$). A comparison of groups A and C showed no significant difference in the length of hospital stay or the percentage of severe cases between the two groups.

Risk factor for aggravation in elderly patients

In the elderly group, severe cases defined by rebleeding or fatal outcome were compared with non-severe cases ending in discharge in remission to determine risk factors for aggravation (Table 4). There was a significant inter-group difference in regard to Hb decrease (70.0% *vs* 52.1%; $P = 0.017$), blood transfusion (88.0% *vs* 62.4%; $P < 0.001$), Forrest Ⅰ (45.1% *vs* 22.9%; $P = 0.001$), HSE use (9.6% *vs* 21.6%; $P = 0.010$), and diabetes mellitus (29.4% *vs* 16.9%; $P = 0.030$). Multivariate logistic regression analysis revealed blood transfusion [odds ratio (95%CI): 3.59 (1.42-9.06); $P = 0.007$], Forrest Ⅰ [odds ratio (95%CI): 2.40 (1.07-4.54); $P = 0.007$], and diabetes mellitus [odds ratio (95%CI): 2.02 (1.00-4.06); $P = 0.049$] as independent risk factors.

DISCUSSION

Aspirin exerts an anti-inflammatory action by inhibiting the activity of COX-1 and COX-2 as well as an antiplatelet action by inhibiting intraplatelet COX-1 and suppressing the production of thromboxane A2, a promoter of plate-

Table 2 Characteristics of the medicated *vs* non-medicated groups and the elderly *vs* non-elderly groups

	Medicated group (*n* = 474)	Non-medicated group (*n* = 631)	*P* value	Elderly group (*n* = 436)	Non-elderly group (*n* = 669)	*P* value
Mean age (yr)	69.7	60.4	< 0.001	78.5	54.9	< 0.001
Sex (male:female)	352:122	491:140	< 0.001	271:165	552:117	< 0.001
Hb (mg/L)	82.6	91.1	<0.001	80.8	91.9	< 0.001
H. pylori infection (positive:negative)	73.5% (324:117)	89.3% (533:64)	< 0.001	77.9% (311:88)	85.4% (546:93)	0.002
Single ulcer	61.4% (291:183)	74.2 (468:163)	< 0.001	40.1% (175:261)	25.6% (171:498)	< 0.001
Forrest I	25.7% (122:352)	21.7% (137:494)	0.118	25.5% (11:325)	22.1% (148:521)	0.201
Anti-ulcer medication	52.1% (247:227)	17.3% (109:522)	< 0.001	41.3% (189:256)	26.3% (176:493)	< 0.001
Underlying disease						
Cardiac disease	43.0% (204:270)	7.9% (50:581)	< 0.001	33.9% (148:288)	15.8% (106:563)	< 0.001
Cerebrovascular disorder	25.9% (123:351)	4.0% (25:606)	< 0.001	22.2% (97:339)	7.6% (51:618)	< 0.001
Renal disease	16.5% (78:396)	7.4% (47:584)	< 0.001	13.1% (57:379)	10.2% (68:601)	0.136
Respiratory disease	13.5% (64:410)	8.1% (51:580)	0.003	14.9% (65:371)	7.5% (50:619)	< 0.001
Orthopedic disorder	28.9% (137:337)	4.0% (25:606)	< 0.001	21.6% (94:342)	10.2% (68:601)	< 0.001
History of ulcer	21.9% (104:370)	28.7% (181:450)	< 0.001	18.3% (80:356)	30.6% (205:464)	< 0.001
Hypertension	41.4% (196:278)	25.9% (158:473)	< 0.001	45.2% (197:239)	23.5% (157:512)	< 0.001
DM	21.7% (103:371)	15.1% (95:536)	0.004	18.3% (80:356)	17.6% (118:551)	0.763

Hb: Hemoglobin; *H. pylori: Helicobacter pylori*; DM: Diabetes mellitus.

Table 3 Characteristics of low dose aspirin therapy

	Elderly patients (*n* = 111)		Non-elderly patients (*n* = 99)		*P* (A *vs* B)	*P* (A *vs* C)
	Group A: LDA monotherapy (*n* = 63)	Group B: LDA combination therapy (*n* = 48)	Group C: LDA monotherapy (*n* = 49)	Group D: LDA combination therapy (*n* = 50)		
Mean age (yr)	80.0	80.0	58.3	60.7	0.989	< 0.001
Sex (male:female)	43:20:00	34:14:00	45:04:00	44:06:00	0.770	0.003
Mean length of hospital stay (d)						
After endoscopic treatment	20.0	25.5	20.9	21.9	0.194	0.323
Overall	20.1	28.4	23.0	27.3	0.120	0.685
Hb (mg/L)	84.0	84.0	89.0	86.0	0.948	0.307
Hb decrease (present:absent)	43.5% (27:35)	53.2% (25:22)	49.0% (24:25)	67.4% (31:15)	0.702	0.569
Blood transfusion (present:absent)	61.3% (38:24)	66.0% (31:16)	38.8% (19:30)	58.7% (27:19)	0.690	0.018
H. pylori infection (positive:negative)	77.4% (48:14)	68.3% (28:13)	80.9% (38:9)	68.8% (33:15)	0.303	0.664
Forrest (Ⅰ:Ⅱ, Ⅲ)	23.8% (15:48)	27.1% (13:35)	16.3% (8:41)	20.0% (10:40)	0.694	0.331
Ulcer (multiple:single)	42.9% (27:36)	39.6% (19:29)	22.4% (11:38)	46.0% (23:27)	0.846	0.024
Rebleeding (present:absent)	3.2% (2:61)	12.5% (6:42)	2.0% (1:48)	8.0% (4:46)	0.060	1.000
Rebleeding/surgery/IVR/death (present:absent)	3.2% (2:61)	14.6% (7:41)	4.1% (2:47)	8.0% (4:46)	0.038	1.000
Recurrence (present:absent)	0% (0:63)	4.2% (2:46)	8.2% (4:45)	4.0% (2:48)	0.185	0.034
DM	17.5% (11:51)	25.0% (12:36)	36.7% (18:31)	36.0% (18:32)	0.332	0.021
Cardiac disease	52.4% (33:30)	66.7% (32:16)	46.9% (23:26)	78.0% (39:11)	0.130	0.568
Cerebrovascular disorder	38.1% (24:39)	39.6% (19:29)	22.4% (11:38)	34.0% (17:33)	0.873	0.076
Orthopedic disorder	12.7% (8:55)	25.0% (12:36)	6.1% (3:46)	20.0% (10:40)	0.095	0.342
Respiratory disease	17.5% (11:52)	14.6% (7:41)	4.1% (2:47)	4.0% (2:48)	0.684	0.028
Renal disease	12.7% (8:55)	22.9% (11:37)	18.4% (9:40)	24.0% (12:38)	0.157	0.407
History of peptic ulcer	19.0% (12:51)	12.5% (6:42)	28.6% (14:35)	20.0% (10:40)	0.354	0.236
Hypertension	49.2% (31:32)	52.1% (25:23)	36.7% (18:31)	42.0% (21:29)	0.764	0.187
Preceding anti-ulcer medication (present:absent)	38.1% (24:39)	79.2% (38:10)	44.9% (22:27)	58.0% (29:21)	<0.001	0.468
Preceding PPI medication	11.1% (7:56)	22.9% (11:37)	12.2% (6:43)	16.0% (8:42)	0.095	0.853

Hb: Hemoglobin; LDA: Low dose aspirin; *H. pylori*: *Helicobacter pylori*; IVR: Interventional radiology; PPI: Proton pump inhibitor; DM: Diabetes mellitus.

let aggregation. It is known that aspirin inhibits gastric mucosal protection through COX inhibition. In addition, aspirin takes a lipid-soluble nonionic form under the intragastric acidic condition and accumulates in the cell to cause injury directly, with increased drug permeability. Case-control studies conducted in Europe and North America showed that gastrointestinal mucosal disorder would increase the risk of upper gastrointestinal hemorrhage about 2- to 4-fold[16-17]. Sakamoto *et al*[18] reported based on the results of a case-control study in Japanese people that the odds ratio of upper gastrointestinal hemorrhage due to LDA was 8.2 (95%CI: 3.3-20.7). In addition, studies that examined the prognosis in relation to the presence or absence of the increasingly prevalent gastrointestinal hemorrhage after acute coronary syndrome or acute stroke found

Table 4 Comparison between the severe and non-severe groups in the elderly group

	Elderly group (n = 436)		Univariate analysis	Multivariate analysis	
	Severe cases (n = 51)	Non-severe cases (n = 385)	P value	OR (95%CI)	P value
Mean age (yr)	79.0	78.8	0.846		
Sex (male:female)	64.7% (33:18)	61.8% (238:147)	0.689		
Mean length of hospital stay (d)					
After endoscopic treatment	21.9	23.5	0.699		
Overall	25.9	27.0	0.823		
Hb (mg/L)	76.3	81.4	0.113		
Hb decrease (present:absent)	70.0% (35:15)	52.1% (198:182)	0.017	1.378 (0.693-2.74)	0.361
Blood transfusion (present:absent)	88.0% (44:6)	62.4% (237:143)	< 0.001	3.592 (1.423-9.064)	0.007
H. pylori infection (positive:negative)	69.8% (30:13)	78.9% (281:75)	0.171		
Forrest (Ⅰ:Ⅱ, Ⅲ)	45.1% (23:28)	22.9% (88:297)	0.001	2.395 (1.065-4.537)	0.007
Ulcer (multiple:single)	31.4% (16:35)	41.3% (159:226)	0.174		
HSE use	21.6% (11:40)	9.6% (37:348)	0.010	2.178 (0.975-4.862)	0.058
DM	29.4% (15:36)	16.9% (65:320)	0.030	2.018 (1.002-4.063)	0.049
Cardiac disease	29.4% (15:36)	34.5% (133:252)	0.467		
Cerebrovascular disorder	27.5% (14:37)	21.6% (83:302)	0.342		
Orthopedic disorder	23.5% (12:39)	21.3% (82:303)	0.716		
Respiratory disease	21.6% (11:40)	14.0% (54:331)	0.155		
Renal disease	5.9% (3:48)	14.0% (54:331)	0.105		
History of peptic ulcer	23.5% (12:39)	17.7% (68:317)	0.309		
Hypertension	41.2% (21:30)	45.7% (176:209)	0.541		
Preceding anti-ulcer medication	52.9% (27:24)	39.7% (153:232)	0.072		
Preceding PPI medication	11.8% (6:45)	11.7% (45:340)	0.987		

Hb: Hemoglobin; *H. pylori*: *Helicobacter pylori*; IVR: Interventional radiology; HSE: Hypertonic saline epinephrine; DM: Diabetes mellitus; PPI: Proton pump inhibitor.

that the occurrence of gastrointestinal hemorrhage after acute coronary syndrome or acute stroke would increase the overall mortality at 1 year[15,19]. This should not only alert endoscopists but also alert cardiologists and neurologists. Antiplatelet drugs other than LDA are also associated with the risk of gastrointestinal hemorrhage because they inhibit thrombogenesis, but they cause less injury to the mucosa. Clopidogrel is known to increase the risk of hemorrhage by 1.7-2.8 times; case-control studies with more than 10000 cases showed that its risk of inducing hemorrhage is not statistically significant[16,17,20,21]. Because reports on antiplatelet drugs other than LDA are limited, accumulation of data and additional investigations in the future are awaited. The anticoagulant warfarin significantly increases the risk of upper gastrointestinal hemorrhage by about two to four times[16,20-22]. In recent years, the use of DOACs as a new treatment of venous thromboembolism and atrial fibrillation has increased. In a cohort study, Shimomura *et al*[23] performed a long-term follow-up of 508 patients on oral anticoagulant therapy in whom peptic ulcer and hemorrhage were denied and calculated the incidence rate of gastrointestinal hemorrhage. As a result, acute gastrointestinal hemorrhage occurred in 8.3% of the patients during an average observation period of 31 mo, and the cumulative incidence rates of gastrointestinal hemorrhage at 5 and 10 years were reported to be 13% and 19%, respectively, which were clinically relevant. There was no significant difference in the hemorrhage risk between warfarin and DOACs. In addition, other more recent studies have found no significant difference in the hemorrhage risk between warfarin and DOACs[24,25]. Our present study included only four patients on DOACs therapy, and therefore DOACs were not analyzed. Because the use of DOACs is expected to increase in the future, additional investigations would be necessary.

Hallas *et al*[16] reported that the risk of hemorrhage was increased 1.8-fold by LDA monotherapy, and the risk was further increased by the combined use of LDA with other drugs, *e.g.*, 7.4-fold by combination with clopidogrel and 5.3-fold by combination with warfarin. Several studies have demonstrated bleeding risk in patients treated with a combination of LDA plus antithrombotic drugs[26,27]. The present study showed that the condition was significantly more severe in elderly patients aged 70 years or older on LDA combination therapy than in those on LDA monotherapy. Although a comparison among different drugs was not made, this study indicated that the combined use of drugs would increase the risk of hemorrhage, requiring due caution. In addition, when LDA monotherapy was used, there was no significant difference in the severity of the condition between elderly and non-elderly patients. Although oral LDA therapy poses a risk of ulceration as mentioned previously, the results of this study suggest that LDA monotherapy does not contribute to aggravation of hemorrhage in elderly patients in comparison with non-elderly patients.

Increases in the incidence of rebleeding and mortality in relation to the underlying disease and age have been reported. Rockall *et al*[28] have reported that the fatality rate due to upper gastrointestinal hemorrhage was 14% (584/4412) and that the rate increased with the

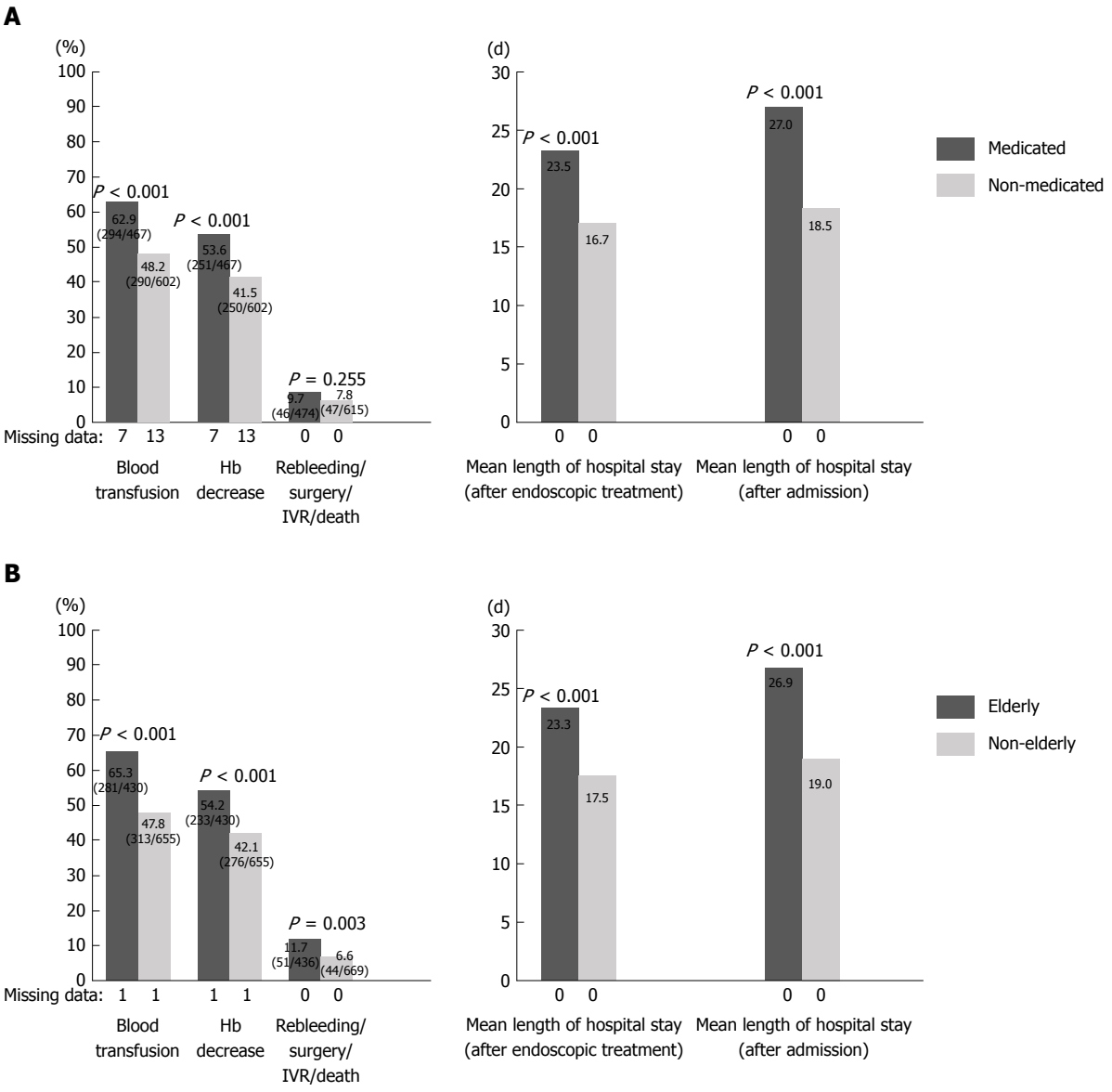


Figure 1 Comparison between 2 groups. A: Medicated and non-medicated groups; B: Elderly and non-elderly groups. Hb: Hemoglobin; IVR: Interventional radiology.

presence of comorbidities such as heart failure, ischemic heart disease, and renal failure. It has also been reported that the mortality within 30 d is proportional to the prevalence of serious comorbidities[29]. In addition, some researchers reported that the Glasgow Blatchford score was the most effective predictive factor for treatment intervention and death[30,31]. Travis *et al*[32] investigated the risk factors for rebleeding after endoscopy and reported that non-use of PPIs, hepatic cirrhosis, heparin, and the use of epinephrine were independent factors. In this study, the condition was more likely to be more severe in elderly patients and medicated patients, indicating that aggressive treatment intervention would be necessary in such patients. In addition, when elderly patients aged 70 years or older were concerned, Hb decrease, implementation of blood transfusion, Forrest Ⅰ, HSE, and a history of diabetes mellitus were found to be risk factors for a severe clinical course (rebleeding, surgery, IVR or other treatment intervention, or death). When multivariate analysis was performed, Hb decrease, implementation of blood transfusion, and a history of diabetes mellitus were identified as independent factors. In these patients, endoscopically and clinically more appropriate management including an adequate endoscopic hemostatic procedure and an aggressive second-look procedure is required.

As for management after hemostasis, both the rebleeding risk due to continued oral antithrombotic medication and the risk of developing thromboembolism due to discontinuation of antithrombotic therapy should be considered. Thus, the method of such management is a clinically relevant issue. Discontinuation of antithrombotic drugs was previously reported to be associated with a significantly higher incidence of thromboembolic events and related deaths[5,16,33-35]. The risk of recurrence of underlying disease associated

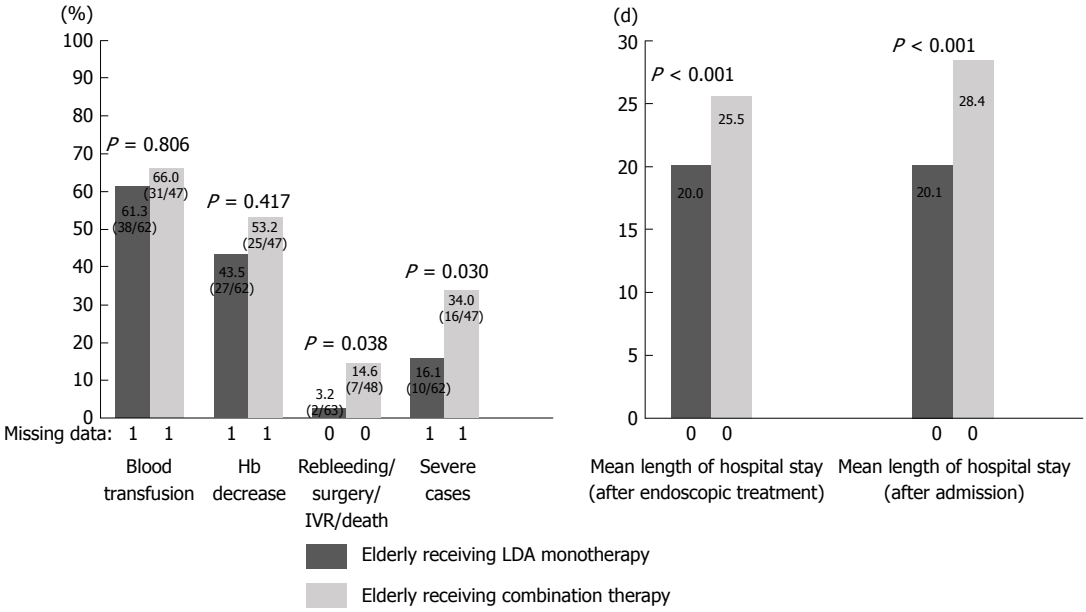


Figure 2 Comparison between the low dose aspirin monotherapy and low dose aspirin combination therapy groups in the elderly. LDA: Low dose aspirin; Hb: Hemoglobin.

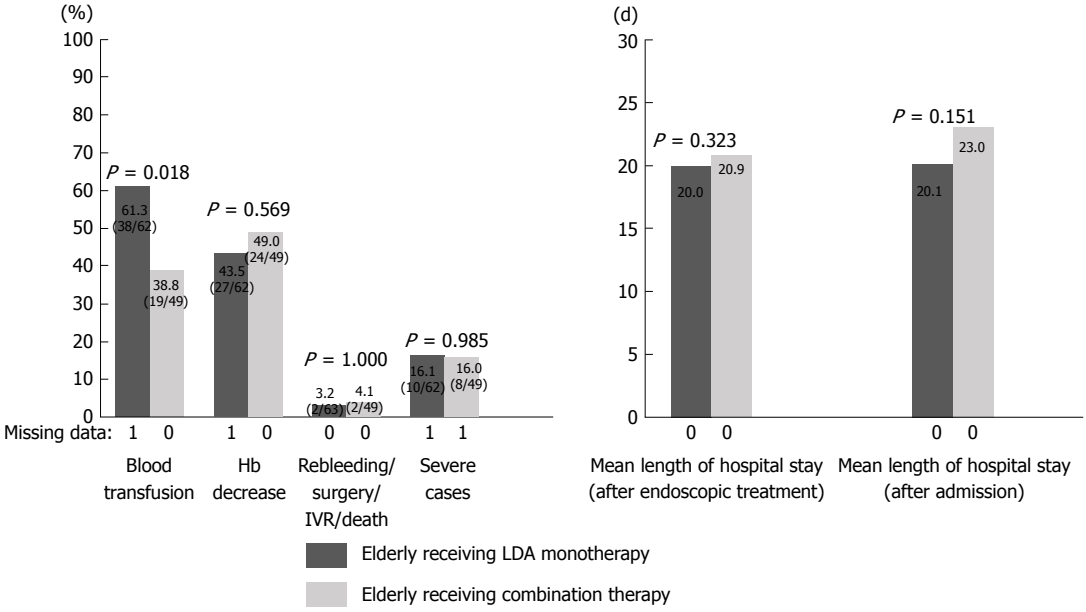


Figure 3 Comparison between the elderly and non-elderly groups in patients receiving low dose aspirin monotherapy. LDA: Low dose aspirin; Hb: Hemoglobin.

with discontinuation of LDA has also been reported to be significantly higher than the risk of recurrence of hemorrhagic gastric ulcer associated with continuation of LDA therapy[36]. Nagata *et al*[37] have reported that a history of thromboembolism, comorbidity score, discontinuation of LDA, discontinuation of antiplatelet drugs other than aspirin, and discontinuation of anticoagulant drugs were identified as risk factors for thromboembolism and that discontinuation of LDA and anticoagulant drugs resulted in a higher risk of thromboembolism. Therefore, when treating patients, it is necessary to consider the propriety of discontinuation of medication and avoid prolonged withdrawal, in consultation with specialists such as a cardiologist or a neurologist. Elderly patients are at a high risk of rebleeding and are more likely to discontinue antithrombotic medication. In such cases, a second-look procedure should be performed aggressively, and antithrombotic medication should be resumed as soon as possible. It is also necessary to ensure that antithrombotic medication is resumed on discharge.

With regard to LDA-induced peptic ulcer, several randomized controlled trails demonstrated the secondary preventive effect of PPIs, and in Japan, an additional indication for the use of PPIs to prevent recurrence of LDA-induced ulcer was approved for the first time in

2010[38-40]. In a randomized controlled trial that included patients aged 60 years or older who had no endoscopic evidence of ulcer, esomeprazole 20 mg proved to be effective for prevention of peptic ulcer (primary prevention)[41]. As mentioned previously, the incidence rates of severe ulcer and drug-induced ulcer are increasing, and administration of more appropriate acid-blocking drugs including PPIs has become important. Japanese guidelines recommend the use of PPIs[42]. However, as demonstrated in the present study, the actual frequency of the use of PPIs is currently low, and therefore further spread of this type of drugs is necessary.

Our present study had several limitations. This was a single-center retrospective study that provided restrictive analysis. Endoscopic skills varied among different endoscopists. The definition of severe ulcer was not based on any well-known scoring system but used unique factors produced from evaluable items. The age difference between elderly and non-elderly subjects was small 54.9 years *vs* 78.5 years. Addition of data and another verification with participation of multiple centers are desirable.

In conclusion, when patients on LDA combination therapy and LDA monotherapy were compared, the percentage of severe cases was high in those on LDA combination therapy among elderly patients, indicating that combined use of LDA with antithrombotic drugs or NSAIDs contributes to the aggravation of hemorrhagic gastroduodenal ulcer. In the LDA monotherapy group, there was no significant difference in the percentage of severe cases between elderly and non-elderly patients, indicating that age is not a risk factor for aggravation of the condition when LDA monotherapy is used.

ARTICLE HIGHLIGHTS

Research background

As the Japanese population ages, the prevalence of cerebrovascular disorders and ischemic heart diseases have been increasing. Under these circumstances, low-dose aspirin (LDA) has increasingly been used for secondary prevention of such conditions in recent years. Severe adverse reactions to LDA include hemorrhagic gastroduodenal ulcer. In the future, the incidences of LDA-induced peptic ulcer and ulcer hemorrhage are expected to rise in the elderly.

Research motivation

As previously reported, the concomitant use of LDA and other antithrombotic drugs increases the risk of ulcer hemorrhage. However, no report of any study that LDA-induced ulcer hemorrhage in elderly patients who expected to become severe. Elucidation of the current status of this condition would thus be useful.

Research objectives

Of patients with hemorrhagic gastroduodenal ulcer caused by oral administration of antithrombotic drugs, those receiving oral LDA, which is likely to be particularly problematic, were targeted. By comparing elderly and non-elderly patients, this study aimed to identify clinical features of the ulcer and factors contributing to its progression to severe conditions. These issues are particularly important in countries that have become aged societies, like Japan, or are aging at a rapid rate.

Research methods

This study included 1105 patients with hemorrhagic gastroduodenal ulcer, who were divided according age (the elderly group consisting of those 70 years of age or older and the non-elderly group consisting of those less than 70 years of age) and orally administered drugs (the LDA monotherapy group and the LDA combination therapy group). We retrospectively compared and analyzed the length of hospital stay, presence or absence of decreased hemoglobin (Hb) level, use of blood transfusion, rate of severe conditions, *etc*.

Research results

When elderly patients were compared between the LDA monotherapy and LDA combination therapy groups, the rate of severe conditions was higher in the LDA combination therapy group. Concomitant use of LDA with antithrombotic drugs or nonsteroidal anti-inflammatory drugs was found to contribute to the progression of severe hemorrhagic gastroduodenal ulcer to severe conditions. Moreover, among the LDA monotherapy group, no significant difference in the rate of severe conditions was observed between elderly and non-elderly patients. Oral administration of LDA alone was not found to be a risk factor for progression to severe conditions in elderly patients.

Research conclusions

This study showed that LDA combination therapy contributes to progression to severe conditions, such as markedly decreased Hb levels, increased frequency of blood transfusion, and prolonged hospital stay, in elderly patients. Meanwhile, in cases receiving LDA monotherapy, advanced age is not a risk factor for progression to severe conditions. Based on these findings, when LDA combination therapy is administered to elderly patients, efforts should be made toward adequate prevention of hemorrhage. In cases with ulcer hemorrhage, while treatment is given, appropriate antithrombotic therapy is required to prevent the occurrence of vascular events. Furthermore, apparently, if LDA monotherapy is administered, even elderly patients may be at a risk of progression to severe conditions similar to that in non-elderly patients.

Research perspectives

The limitations of this study include the single-center retrospective design. In addition, because the analysis in the LDA combination therapy group was not stratified according to the types of antithrombotic drugs used in combination with LDA, the effects of different combinations of drugs on the risk of hemorrhage should be examined in future studies. Although the use of proton pump inhibitors (PPIs) is preferable for prevention of hemorrhage as described in the guidelines, further accumulation of additional data and studies on effects, adverse events, *etc*. are needed to use PPIs appropriately. Furthermore, evidence must be accumulated for the prophylactic effect of novel therapeutic drugs, such as vonoprazan, for ulcers in elderly patients.

ACKNOWLEDGMENTS

The authors appreciate the great support with the statistical analyses provided by Yasuo Haruyama, Department of Public Health, Dokkyo Medical University. The authors also would like to thank all participants and staff members investigators who participated in this study: Yasunaga Suzuki, Yoshihito Watanabe, Kazunari Kanke, Takeshi Oinuma, Yukio Otsuka, Katsuo Morita, Takahiro Mitsuhashi, Michiko Matsuoka, Ayako Aoki, Jun Ishikawa, Etsuko Yonekura, Koji Sudo, Youichiro Fujii, Yutaka Okamoto, Daisuke Arai, Masaya Terauchi, Hayato Takagi, Kenji Yoshida, Takero Koike, Kenichiro Mukawa, Mina Hoshino, Takafumi Hoshino, Rieko Fujii, Genyo Hitomi, Naoto Yoshitake, Yasuyuki Saifuku, Mitsunori Maeda , Makoto Matsumaru, Kohei Tsuchida, Takeshi Sugaya, Masakazu Nakano, Chieko Tsuchida, Yoshimitsu Yamamoto, Kyoko Yamamoto, Misako Tsunemi, Hiroko Sakurai, Naoya Inaba, Takashi Akima, Hitoshi Kino, Yoshihito Kaneko, Atsushi Hoshino, Hidehito Jinnai, Toshinori Komatsubara, Shinji Muraoka, Fumiaki Takahashi, Tsunehiro Suzuki, Mari Iwasaki, Kazuhiro

Takenaka, Keiichiro Abe, and Takahito Minaguchi.

REFERENCES

1 **Antithrombotic Trialists' Collaboration**. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ* 2002; **324**: 71-86 [PMID: 11786451 DOI: 10.1136/bmj.324.7329.71]

2 **Weisman SM**, Graham DY. Evaluation of the benefits and risks of low-dose aspirin in the secondary prevention of cardiovascular and cerebrovascular events. *Arch Intern Med* 2002; **162**: 2197-2202 [PMID: 12390062 DOI: 10.1001/archinte.162.19.2197]

3 **Eidelman RS**, Hebert PR, Weisman SM, Hennekens CH. An update on aspirin in the primary prevention of cardiovascular disease. *Arch Intern Med* 2003; **163**: 2006-2010 [PMID: 14504112 DOI: 10.1001/archinte.163.17.2006]

4 **Pearson TA**, Blair SN, Daniels SR, Eckel RH, Fair JM, Fortmann SP, Franklin BA, Goldstein LB, Greenland P, Grundy SM, Hong Y, Miller NH, Lauer RM, Ockene IS, Sacco RL, Sallis JF Jr, Smith SC Jr, Stone NJ, Taubert KA. AHA Guidelines for Primary Prevention of Cardiovascular Disease and Stroke: 2002 Update: Consensus Panel Guide to Comprehensive Risk Reduction for Adult Patients Without Coronary or Other Atherosclerotic Vascular Diseases. American Heart Association Science Advisory and Coordinating Committee. *Circulation* 2002; **106**: 388-391 [PMID: 12119259 DOI: 10.1161/01.CIR.0000020190.45892.75]

5 **Origasa H**, Goto S, Shimada K, Uchiyama S, Okada Y, Sugano K, Hiraishi H, Uemura N, Ikeda Y; MAGIC Investigators. Prospective cohort study of gastrointestinal complications and vascular diseases in patients taking aspirin: rationale and design of the MAGIC Study. *Cardiovasc Drugs Ther* 2011; **25**: 551-560 [PMID: 21842134 DOI: 10.1007/s10557-011-6328-2]

6 **Hiraishi H**, Oki R, Tsuchida K, Yoshitake N, Tominaga K, Kusano K, Hashimoto T, Maeda M, Sasai T, Shimada T. Frequency of nonsteroidal anti-inflammatory drug-associated ulcers. *Clin J Gastroenterol* 2012; **5**: 171-176 [PMID: 26182316 DOI: 10.1007/s12328-012-0300-y]

7 **Wang Y**, Wang Y, Zhao X, Liu L, Wang D, Wang C, Wang C, Li H, Meng X, Cui L, Jia J, Dong Q, Xu A, Zeng J, Li Y, Wang Z, Xia H, Johnston SC; CHANCE Investigators. Clopidogrel with aspirin in acute minor stroke or transient ischemic attack. *N Engl J Med* 2013; **369**: 11-19 [PMID: 23803136 DOI: 10.1056/NEJMoa1215340]

8 **Uemura N**, Sugano K, Hiraishi H, Shimada K, Goto S, Uchiyama S, Okada Y, Origasa H, Ikeda Y; MAGIC Study Group. Risk factor profiles, drug usage, and prevalence of aspirin-associated gastroduodenal injuries among high-risk cardiovascular Japanese patients: the results from the MAGIC study. *J Gastroenterol* 2014; **49**: 814-824 [PMID: 23754512 DOI: 10.1007/s00535-013-0839-5]

9 **Taha AS**, Angerson WJ, Knill-Jones RP, Blatchford O. Upper gastrointestinal haemorrhage associated with low-dose aspirin and anti-thrombotic drugs - a 6-year analysis and comparison with non-steroidal anti-inflammatory drugs. *Aliment Pharmacol Ther* 2005; **22**: 285-289 [PMID: 16097994 DOI: 10.1111/j.1365-2036.2005.02560.x]

10 **Kamada T**, Haruma K, Ito M, Inoue K, Manabe N, Matsumoto H, Kusunoki H, Hata J, Yoshihara M, Sumii K, Akiyama T, Tanaka S, Shiotani A, Graham DY. Time Trends in Helicobacter pylori Infection and Atrophic Gastritis Over 40 Years in Japan. *Helicobacter* 2015; **20**: 192-198 [PMID: 25581708 DOI: 10.1111/hel.12193]

11 **Lanas A**, Scheiman J. Low-dose aspirin and upper gastrointestinal damage: epidemiology, prevention and treatment. *Curr Med Res Opin* 2007; **23**: 163-173 [PMID: 17257477 DOI: 10.1185/030079907X162656]

12 **Shiotani A**, Nishi R, Yamanaka Y, Murao T, Matsumoto H, Tarumi K, Kamada T, Sakakibara T, Haruma K. Renin-angiotensin system associated with risk of upper GI mucosal injury induced by low dose aspirin: renin angiotensin system genes' polymorphism. *Dig Dis Sci* 2011; **56**: 465-471 [PMID: 20824505 DOI: 10.1007/s10620-010-1382-3]

13 **Antithrombotic Trialists' (ATT) Collaboration**, Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R, Buring J, Hennekens C, Kearney P, Meade T, Patrono C, Roncaglioni MC, Zanchetti A. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet* 2009; **373**: 1849-1860 [PMID: 19482214 DOI: 10.1016/S0140-6736(09)60503-1]

14 **Lanza FL**, Chan FK, Quigley EM; Practice Parameters Committee of the American College of Gastroenterology. Guidelines for prevention of NSAID-related ulcer complications. *Am J Gastroenterol* 2009; **104**: 728-738 [PMID: 19240698 DOI: 10.1038/ajg.2009.115]

15 **Nikolsky E**, Stone GW, Kirtane AJ, Dangas GD, Lansky AJ, McLaurin B, Lincoff AM, Feit F, Moses JW, Fahy M, Manoukian SV, White HD, Ohman EM, Bertrand ME, Cox DA, Mehran R. Gastrointestinal bleeding in patients with acute coronary syndromes: incidence, predictors, and clinical implications: analysis from the ACUITY (Acute Catheterization and Urgent Intervention Triage Strategy) trial. *J Am Coll Cardiol* 2009; **54**: 1293-1302 [PMID: 19778672 DOI: 10.1016/j.jacc.2009.07.019]

16 **Hallas J**, Dall M, Andries A, Andersen BS, Aalykke C, Hansen JM, Andersen M, Lassen AT. Use of single and combined antithrombotic therapy and risk of serious upper gastrointestinal bleeding: population based case-control study. *BMJ* 2006; **333**: 726 [PMID: 16984924 DOI: 10.1136/bmj.38947.697558.AE]

17 **Ibáñez L**, Vidal X, Vendrell L, Moretti U, Laporte JR; Spanish-Italian Collaborative Group for the Epidemiology of Gastrointestinal Bleeding. Upper gastrointestinal bleeding associated with antiplatelet drugs. *Aliment Pharmacol Ther* 2006; **23**: 235-242 [PMID: 16393302 DOI: 10.1111/j.1365-2036.2006.02759.x]

18 **Sakamoto C**, Sugano K, Ota S, Sakaki N, Takahashi S, Yoshida Y, Tsukui T, Osawa H, Sakurai Y, Yoshino J, Mizokami Y, Mine T, Arakawa T, Kuwayama H, Saigenji K, Yakabi K, Chiba T, Shimosegawa T, Sheehan JE, Perez-Gutthann S, Yamaguchi T, Kaufman DW, Sato T, Kubota K, Terano A. Case-control study on the association of upper gastrointestinal bleeding and nonsteroidal anti-inflammatory drugs in Japan. *Eur J Clin Pharmacol* 2006; **62**: 765-772 [PMID: 16821007 DOI: 10.1007/s00228-006-0171-6]

19 **O'Donnell MJ**, Kapral MK, Fang J, Saposnik G, Eikelboom JW, Oczkowski W, Silva J, Gould L, D'Uva C, Silver FL; Investigators of the Registry of the Canadian Stroke Network. Gastrointestinal bleeding after acute ischemic stroke. *Neurology* 2008; **71**: 650-655 [PMID: 18685137 DOI: 10.1212/01.wnl.0000319689.48946.25]

20 **Lanas A**, García-Rodríguez LA, Arroyo MT, Gomollón F, Feu F, González-Pérez A, Zapata E, Bástida G, Rodrigo L, Santolaria S, Güell M, de Argila CM, Quintero E, Borda F, Piqué JM; Asociación Española de Gastroenterología. Risk of upper gastrointestinal ulcer bleeding associated with selective cyclo-oxygenase-2 inhibitors, traditional non-aspirin non-steroidal anti-inflammatory drugs, aspirin and combinations. *Gut* 2006; **55**: 1731-1738 [PMID: 16687434 DOI: 10.1136/gut.2005.080754]

21 **Delaney JA**, Opatrny L, Brophy JM, Suissa S. Drug drug interactions between antithrombotic medications and the risk of gastrointestinal bleeding. *CMAJ* 2007; **177**: 347-351 [PMID: 17698822 DOI: 10.1503/cmaj.070186]

22 **Lanas Á**, Carrera-Lasfuentes P, Arguedas Y, García S, Bujanda L, Calvet X, Ponce J, Perez-Aísa Á, Castro M, Muñoz M, Sostres C, García-Rodríguez LA. Risk of upper and lower gastrointestinal bleeding in patients taking nonsteroidal anti-inflammatory drugs, antiplatelet agents, or anticoagulants. *Clin Gastroenterol Hepatol* 2015; **13**: 906-12.e2 [PMID: 25460554 DOI: 10.1016/j.cgh.2014.11.007]

23 **Shimomura A**, Nagata N, Shimbo T, Sakurai T, Moriyasu S, Okubo H, Watanabe K, Yokoi C, Akiyama J, Uemura N. New predictive model for acute gastrointestinal bleeding in patients taking oral anticoagulants: A cohort study. *J Gastroenterol Hepatol* 2018; **33**: 164-171 [PMID: 28544091 DOI: 10.1111/jgh.13830]

24 **Senoo K**, Lau YC, Dzeshka M, Lane D, Okumura K, Lip GY. Efficacy and safety of non-vitamin K antagonist oral anticoagulants vs. warfarin in Japanese patients with atrial fibrillation – meta-

analysis. *Circ J* 2015; **79**: 339-345 [PMID: 25501801 DOI: 10.1253/circj.CJ-14-1042]
25 **Miller CS**, Dorreen A, Martel M, Huynh T, Barkun AN. Risk of Gastrointestinal Bleeding in Patients Taking Non-Vitamin K Antagonist Oral Anticoagulants: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2017; **15**: 1674-1683.e3 [PMID: 28458008 DOI: 10.1016/j.cgh.2017.04.031]
26 **Serebruany VL**, Steinhubl SR, Berger PB, Malinin AI, Baggish JS, Bhatt DL, Topol EJ. Analysis of risk of bleeding complications after different doses of aspirin in 192,036 patients enrolled in 31 randomized controlled trials. *Am J Cardiol* 2005; **95**: 1218-1222 [PMID: 15877994 DOI: 10.1016/j.amjcard.2005.01.049]
27 **García Rodríguez LA**, Lin KJ, Hernández-Díaz S, Johansson S. Risk of upper gastrointestinal bleeding with low-dose acetylsalicylic acid alone and in combination with clopidogrel and other medications. *Circulation*2011; **123**: 1108-1115 [PMID: 21357821 DOI: 10.1161/CIRCULATIONAHA.110.973008]
28 **Rockall TA**, Logan RF, Devlin HB, Northfield TC. Risk assessment after acute upper gastrointestinal haemorrhage. *Gut* 1996; **38**: 316-321 [PMID: 8675081 DOI: 10.1136/gut.38.3.316]
29 **Sung JJ**, Tsoi KK, Ma TK, Yung MY, Lau JY, Chiu PW. Causes of mortality in patients with peptic ulcer bleeding: a prospective cohort study of 10,428 cases. *Am J Gastroenterol* 2010; **105**: 84-89 [PMID: 19755976 DOI: 10.1038/ajg.2009.507]
30 **Blatchford O**, Davidson LA, Murray WR, Blatchford M, Pell J. Acute upper gastrointestinal haemorrhage in west of Scotland: case ascertainment study. *BMJ* 1997; **315**: 510-514 [PMID: 9329304 DOI: 10.1136/bmj.315.7107.510]
31 **Stanley AJ**, Laine L, Dalton HR, Ngu JH, Schultz M, Abazi R, Zakko L, Thornton S, Wilkinson K, Khor CJ, Murray IA, Laursen SB; International Gastrointestinal Bleeding Consortium. Comparison of risk scoring systems for patients presenting with upper gastrointestinal bleeding: international multicentre prospective study. *BMJ* 2017; **356**: i6432 [PMID: 28053181 DOI: 10.1136/bmj.i6432]
32 **Travis AC**, Wasan SK, Saltzman JR. Model to predict rebleeding following endoscopic therapy for non-variceal upper gastrointestinal hemorrhage. *J Gastroenterol Hepatol* 2008; **23**: 1505-1510 [PMID: 18823441 DOI: 10.1111/j.1440-1746.2008.05594.x]
33 **Witt DM**, Delate T, Garcia DA, Clark NP, Hylek EM, Ageno W, Dentali F, Crowther MA. Risk of thromboembolism, recurrent hemorrhage, and death after warfarin therapy interruption for gastrointestinal tract bleeding. *Arch Intern Med* 2012; **172**: 1484-1491 [PMID: 22987143 DOI: 10.1001/archinternmed.2012.4261]
34 **Maulaz AB**, Bezerra DC, Michel P, Bogousslavsky J. Effect of discontinuing aspirin therapy on the risk of brain ischemic stroke. *Arch Neurol* 2005; **62**: 1217-1220 [PMID: 16087761 DOI: 10.1001/archneur.62.8.1217]
35 **Kim SY**, Hyun JJ, Suh SJ, Jung SW, Jung YK, Koo JS, Yim HJ, Park JJ, Chun HJ, Lee SW. Risk of Vascular Thrombotic Events Following Discontinuation of Antithrombotics After Peptic Ulcer Bleeding. *J Clin Gastroenterol* 2016; **50**: e40-e44 [PMID: 26084008 DOI: 10.1097/MCG.0000000000000354]
36 **Sung JJ**, Lau JY, Ching JY, Wu JC, Lee YT, Chiu PW, Leung VK, Wong VW, Chan FK. Continuation of low-dose aspirin therapy in peptic ulcer bleeding: a randomized trial. *Ann Intern Med* 2010; **152**: 1-9 [PMID: 19949136 DOI: 10.7326/0003-4819-152-1-201001050-00179]
37 **Nagata N**, Sakurai T, Shimbo T, Moriyasu S, Okubo H, Watanabe K, Yokoi C, Yanase M, Akiyama J, Uemura N. Acute Severe Gastrointestinal Tract Bleeding Is Associated With an Increased Risk of Thromboembolism and Death. *Clin Gastroenterol Hepatol* 2017; **15**: 1882-1889.e1 [PMID: 28634133 DOI: 10.1016/j.cgh.2017.06.028]
38 **Sugano K**, Matsumoto Y, Itabashi T, Abe S, Sakaki N, Ashida K, Mizokami Y, Chiba T, Matsui S, Kanto T, Shimada K, Uchiyama S, Uemura N, Hiramatsu N. Lansoprazole for secondary prevention of gastric or duodenal ulcers associated with long-term low-dose aspirin therapy: results of a prospective, multicenter, double-blind, randomized, double-dummy, active-controlled trial. *J Gastroenterol* 2011; **46**: 724-735 [PMID: 21499703 DOI: 10.1007/s00535-011-0397-7]
39 **Sanuki T**, Fujita T, Kutsumi H, Hayakumo T, Yoshida S, Inokuchi H, Murakami M, Matsubara Y, Kuwayama H, Kawai T, Miyaji H, Fujisawa T, Terao S, Yamazaki Y, Azuma T; Care Study Group. Rabeprazole reduces the recurrence risk of peptic ulcers associated with low-dose aspirin in patients with cardiovascular or cerebrovascular disease: a prospective randomized active-controlled trial. *J Gastroenterol* 2012; **47**: 1186-1197 [PMID: 22526273 DOI: 10.1007/s00535-012-0588-x]
40 **Sugano K**, Choi MG, Lin JT, Goto S, Okada Y, Kinoshita Y, Miwa H, Chiang CE, Chiba T, Hori M, Fukushima Y, Kim HS, Chang CY, Date M; LAVENDER Study Group. Multinational, double-blind, randomised, placebo-controlled, prospective study of esomeprazole in the prevention of recurrent peptic ulcer in low-dose acetylsalicylic acid users: the LAVENDER study. *Gut* 2014; **63**: 1061-1068 [PMID: 24326741 DOI: 10.1136/gutjnl-2013-304722]
41 **Yeomans N**, Lanas A, Labenz J, van Zanten SV, van Rensburg C, Rácz I, Tchernev K, Karamanolis D, Roda E, Hawkey C, Nauclér E, Svedberg LE. Efficacy of esomeprazole (20 mg once daily) for reducing the risk of gastroduodenal ulcers associated with continuous use of low-dose aspirin. *Am J Gastroenterol* 2008; **103**: 2465-2473 [PMID: 18637091 DOI: 10.1111/j.1572-0241.2008.01995.x]
42 **Satoh K**, Yoshino J, Akamatsu T, Itoh T, Kato M, Kamada T, Takagi A, Chiba T, Nomura S, Mizokami Y, Murakami K, Sakamoto C, Hiraishi H, Ichinose M, Uemura N, Goto H, Joh T, Miwa H, Sugano K, Shimosegawa T. Evidence-based clinical practice guidelines for peptic ulcer disease 2015. *J Gastroenterol* 2016; **51**: 177-194 [PMID: 26879862 DOI: 10.1007/s00535-016-1166-4]

P- Reviewer: Caboclo JF, Rodrigo L, Tarnawski AS
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3919-3926

DOI: 10.3748/wjg.v24.i34.3919

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

ORIGINAL ARTICLE

Retrospective Study

Predicting the presence of adenomatous polyps during colonoscopy with National Cancer Institute Colorectal Cancer Risk-Assessment Tool

Hassan Tariq, Muhammad Umar Kamal, Harish Patel, Ravi Patel, Muhammad Ameen, Shehi Elona, Maram Khalifa, Sara Azam, Aiyi Zhang, Kishore Kumar, Ahmed Baiomi, Danial Shaikh, Jasbir Makker

Hassan Tariq, Harish Patel, Kishore Kumar, Jasbir Makker, Division of Gastroenterology, Department of Medicine, BronxCare Health system, Bronx, NY 10457, United States

Muhammad Umar Kamal, Ravi Patel, Muhammad Ameen, Shehi Elona, Maram Khalifa, Sara Azam, Aiyi Zhang, Ahmed Baiomi, Danial Shaikh, Department of Medicine, BronxCare Health system, Bronx, NY 10457, United States

ORCID number: Hassan Tariq (0000-0002-9178-03420); Muhammad Umar Kamal (0000-0002-4323-3440); Harish Patel (0000-0003-3638-9495); Ravi Patel (0000-0003-1694-8747); Muhammad Ameen (0000-0002-4754-6778); Shehi Elona (0000-0002-6262-1334); Maram Khalifa (0000-0003-3362-1404); Sara Azam (0000-0002-0962-6697); Aiyi Zhang (0000-0002-3784-582X); Kishore Kumar (0000-0002-9768-5354); Ahmed Baiomi (0000-0003-2203-4028); Danial Shaikh (0000-0002-5798-9046); Jasbir Makker (0000-0002-1673-8515).

Author contributions: Tariq H, Kamal MU, Patel H and Makker J contributed to concept and design; Patel R, Ameen M, Shehi E, Khalifa M and Azam S contributed to acquisition of data; Tariq H, Kamal MU, Patel H, Zhang A and Makker J contributed to analysis and interpretation of data; Tariq H, Kamal MU, Patel H, Baiomi A, Shaikh D and Makker J drafted the manuscript, Tariq H, Kamal MU, Patel H, Kumar K and Makker J contributed to critical revision of the manuscript for important intellectual content; Zhang A performed statistical analysis; Patel H, Baiomi A and Makker J contributed to study supervision.

Institutional review board statement: The study was performed in agreement with the ethical guidelines of the Declaration of Helsinki and the protocol was approved by the local Ethics Committee (IRN# 12 14 17 13).

Informed consent statement: All participants have given written informed consent before inclusion in the study.

Conflict-of-interest statement: All authors declare no financial support or conflict of interest.

Data sharing statement: The raw data used for all analyses are available from the corresponding author.

Manuscript source: Unsolicited manuscript

Correspondence to: Muhammad Umar Kamal, MD, Doctor, Resident Physician, Department of Medicine, BronxCare Health system, 1650 Selwyn Avenue, Apt 10C, Bronx, NY 10457, United States. muhammadumarkamal@gmail.com
Telephone: +1-718-9601234
Fax: +1-718-9602055

Received: July 2, 2018
Peer-review started: July 3, 2018
First decision: July 18, 2018
Revised: July 25, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 14, 2018

Abstract

AIM

To evaluate the National Cancer Institute (NCI) Colorectal Cancer (CRC) Risk Assessment Tool as a predictor for the presence of adenomatous polyps (AP)

found during screening or surveillance colonoscopy.

METHODS

This is a retrospective single center observational study. We collected data of adenomatous polyps in each colonoscopy and then evaluated the lifetime CRC risk. We calculated the AP prevalence across risk score quintiles, odds ratios of the prevalence of AP across risk score quintiles, area under curves (AUCs) and Youden' s indexes to assess the optimal risk score cut off value for AP prevalence status.

RESULTS

The prevalence of AP gradually increased throughout the five risk score quintiles: *i.e.*, 27.63% in the first and 51.35% in the fifth quintile. The odd ratios of AP prevalence in the fifth quintile compared to the first and second quintile were 2.76 [confidence interval (CI): 1.71-4.47] and 2.09 (CI: 1.32-3.30). The AUC for all patients was 0.62 (CI: 0.58-0.66). Youden' s Index indicated the optimal risk score cutoff value discriminating AP prevalence status was 3.60.

CONCLUSION

Patients with the higher NCI risk score have higher risk of AP and subsequent CRC; therefore, measures to increase the effectiveness of CRC detection in these patients include longer withdrawal time, early surveillance colonoscopy, and choosing flexible colonoscopy over other CRC screening modalities.

Key words: National Cancer Institute Colorectal Cancer Risk-Assessment Tool; Colorectal cancer; Predictors of colorectal cancer; Adenomatous polyps; Colonoscopy

Core tip: Due to health, financial and social burden of colorectal cancer (CRC), it is necessary to assess the risk of cancer development earlier. National Cancer Institute (NCI) CRC risk prediction model helps identifying people who are at increased risk of developing CRC. Our study demonstrated that NCI CRC risk prediction tool could also estimate the risk of having Adenomatous polyps (AP) in patients undergoing screening or surveillance colonoscopy. The results revealed that the odds ratios of AP prevalence increase progressively throughout the five quintiles of risk scores. Therefore, measures to increase the effectiveness of CRC screening in these patients should be implemented using longer withdrawal times, early surveillance colonoscopy, and choosing flexible colonoscopy over other CRC screening modalities.

Tariq H, Kamal MU, Patel H, Patel R, Ameen M, Shehi E, Khalifa M, Azam S, Zhang A, Kumar K, Baiomi B, Shaikh D, Makker J. Predicting the presence of adenomatous polyps during colonoscopy with National Cancer Institute Colorectal Cancer Risk-Assessment Tool. *World J Gastroenterol* 2018; 24(34): 3919-3926 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3919.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3919

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer diagnosed in men and women in the United States. The lifetime risk for developing CRC is 1 in 22 for men and 1 in 24 for women. Estimates for new cases of CRC amount to 140000 yearly, and approximately 50000 people will die of CRC in 2018 alone[1]. CRC arises from colonic polyps, specifically 'adenomas', which result from either a sporadic mutation or a DNA mismatch repair within the mucosal lining of the intestine. Adenomas may grow in size and progress from low-grade dysplasia to high-grade dysplasia, to carcinoma-in-situ and eventually invasive carcinoma[2].

Studies have identified both genetic and environmental factors for developing CRC[3]. Currently in clinical practice many strategies are available to screen for CRC. Screening colonoscopies among them are known to decrease the incidence and mortality of CRC by identifying adenomas in asymptomatic individuals and surveillance colonoscopies at predefined intervals are employed to monitor them[4].

Given the financial and social impact of CRC on society, it is imperative to quantitatively assess the risk of developing CRC in individuals. Many tools are widely available that help calculate future risk of CRC, however they tend to be limited to specific patient groups, or be based on selected populations of patients, and have relatively poor discrimination or are not validated and/or published[5].

The Colorectal Cancer Risk Assessment Tool by the National Cancer Institute (NCI) is a validated tool that was developed using cancer incidence data from 13 NCI Surveillance, Epidemiology, and End Results (SEER) registries, and from national mortality rates. The tool uses the respondent's answers about risk and preventive factors to calculate that person's absolute risk of colorectal cancer for a specific time period (5-year, 10-year and lifetime risk)[6].

We conducted this study with the aim of evaluating the NCI Colorectal Cancer Risk Assessment Tool as a predictor of the presence of adenomatous polyps found during screening or surveillance colonoscopy.

MATERIALS AND METHODS

This is a retrospective single center observational study. The period of study was 6 mo between January 1st, 2017 and June 30th, 2017. The study was performed according to the Declaration of Helsinki and was approved by the Institution Review Board (IRB) of Bronx Lebanon hospital center.

Patient selection

The data was collected from the electronic medical records of patients and tabulated in Microsoft Excel ® (Microsoft Corp, Redmond, WA, United States). Findings at colonoscopy were extracted from final procedure reports, and pathology information was extracted from final pathology reports. Asymptomatic patients between 50 and 80 years old, undergoing screening colonoscopy or surveillance colonoscopy who had either excellent or good preparation with complete examination were included in the study population. Symptomatic patients, patients with indications for therapeutic or diagnostic colonoscopy, like for example, rectal bleeding, Iron-deficiency Anemia, Inflammatory Bowel Disease, CRC, Chronic diarrhea, Abnormal Imaging were excluded from participation. Incomplete colonoscopy examinations and patients with missing information/data were excluded from the study. Patients who met the above criteria were interviewed over the phone, and their lifetime NCI CRC Risk-Assessment Tool score was calculated. Patients with missing NCI colorectal cancer risk score were excluded as well. We choose adenomatous polyps (AP) over other kind of polyps in our study because these are more commonly associated with colorectal cancer. Additionally, we did not have other polyps like serrated polyps reported in our study group and hence were not reported in the study. All Authors had access to the study data and had reviewed and approved the final manuscript.

NCI CRC Risk-Assessment Tool and AP

The predictors included in the NCI CRC Risk-Assessment Tool for men are number of relatives with CRC, body mass index, servings of vegetables per day, aspirin and nonsteroidal anti-inflammatory drug use, usual number of cigarettes smoked per day and years of smoking in current and former smokers, prior negative sigmoidoscopy and/or colonoscopy, polyp history and current vigorous leisure time activity[4].

The predictors included in the NCI CRC Risk-Assessment Tool for women are number of relatives with CRC, body mass index, servings of vegetables per day, aspirin and nonsteroidal anti-inflammatory drug use, an age indicator, estrogen status within the last 2 years, prior negative sigmoidoscopy and/or colonoscopy, polyp history and current vigorous leisure time activity[4].

In the original publication, estimated 10-year and 20-year CRC risks were presented. The tool is now available on the Internet (http//www.cancer.gov/colorectalcancerrisk/.). The tool provides 5-year, 10-year and lifetime estimates. We used the predicted lifetime CRC risk. The data on the presence of adenomatous polyps and the numbers in each colonoscopy were collected.

Statistical analysis

Demographic information including age, gender, race, and if the appointment was screening or surveillance were reported and were stratified across AP status. Frequencies and percentages were reported for categorical variables. Means and standard deviations were reported for continuous variables. The associations between categorical variables and AP status were tested by Pearson's chi square tests. The associations between continuous variables and AP status were assessed by ANOVA tests. The frequency and percentage of AP prevalence were stratified across risk score quintiles. The AP prevalence trend across risk score quintiles were assessed by asymptotic linear by linear association test. The odds ratios (ORs) of the prevalence of AP across risk score quintiles and their 95% confidence intervals were computed. Receiver operating characteristic (ROC) curves were plotted to assess the discriminatory accuracy of the risk score for all patients and by genders. Area under curves (AUCs) and their confidence intervals were reported. Youden's indexes were used to assess the optimal risk score cut off value for AP prevalence status. Various disease statuses (AP prevalence, three and more AP prevalence) were stratified across risk score categories based on the optimal risk score cutoff value. Frequencies and percentages were reported. Pearson's chi square tests were applied to assess the associations between outcome diseases and risk score categories. All analyses were conducted on all patients and on screening and follow up patients separately.

The values were considered statistically significant if *P*-value was < 0.05 and values were considered more significant if *P*-values were < 0.01. Analyses were performed in R 1.0.153.

RESULTS

The prevalence of AP increased progressively through the five quintiles of risk scores: 27.63% in the first and lowest quintile, 33.53% in the second quintiles, 46.31% in the third quintiles, 52.21% in the fourth quintile, and 51.35% in the fifth and highest quintile. The ORs of AP prevalence in the third quintile compared to the first and second quintile were 2.26 [confidence interval (CI): 1.40-3.65] and 1.71 (CI: 1.08-2.70). The ORs of AP prevalence in the fourth quintile compared to the first and second quintile were 2.86 (CI: 1.75-4.67) and 2.16 (CI:1.36-3.45). The ORs of AP prevalence in the fifth quintile compared to the first and second quintile were 2.76 (CI: 1.71-4.47) and 2.09 (CI: 1.32-3.30).

Sample population characteristics and the prevalence of AP

The demographic composition of the sample population was indicated in Table 1. There were 749 patients who met the inclusion criteria. The mean age of the study population was 59.00 ± 7.38. Most of the sample population was female (436, 58.2%), Hispanics (501, 67%), and screening colonoscopy patients (606,

Table 1 Demographic information stratified by adenomatous polyp prevalence *n* (%)

	Adenoma absent	Adenoma present	Total	*P* value
Total	436 (58.2)	313 (41.79)	749 (100)	
Age				0.043
Mean ± SD	58.656 ± 7.321	59.764 10 ± 7.417	59.119 ± 7.376	
Gender				0.798
Male	180 (41.3)	133 (42.5)	313 (41.8)	
Female	256 (58.7)	180 (57.5)	436 (58.2)	
Race				0.243
White	29 (6.7)	33 (10.6)	62 (8.3)	
African american	104 (23.9)	74 (23.7)	178 (23.8)	
Asian	5 (1.1)	2 (0.6)	7 (0.9)	
Hispanic	298 (68.3)	203 (65.1)	501 (67.0)	
Indication of colonoscopy				0.279
Surveillance	77 (17.7)	66 (21.1)	143 (19.1)	
Screening	359 (82.3)	247 (78.9)	606 (80.9)	

Table 2 Adenoma prevalence by risk-score quintile for all patients

NCI lifetime risk score quintile	Score range	Number of individuals with adenomas (%)	OR of an adenoma (95%CI): row quintile *vs* column quintile			
			Q1	Q2	Q3	Q4
Q1 lowest	(1.1, 1.9)	42 (27.63)	-	-	-	-
Q2	(1.9, 2.8)	55 (33.54)	1.32 (0.82, 2.14)	-	-	-
Q3	(2.8, 4.0)	69 (46.31)	2.26 (1.40, 3.65)	1.71 (1.08, 2.70)	-	-
Q4	(4.0, 5.4)	71 (52.21)	2.86 (1.75, 4.67)	2.16 (1.36, 3.45)	1.27 (0.79, 2.02)	-
Q5 highest	(5.4, 12.3)	76 (51.35)	2.76 (1.71, 4.47)	2.09 (1.32, 3.30)	1.22 (0.78, 1.93)	0.97 (0.61, 1.54)

Asymptotic Linear-by-Linear Association Test: $P < 0.001$. OR: Odds ratio; CI: Confidence interval; NCI: National Cancer Institute.

80.9%). The mean age of patients without AP was slightly lower than the mean age of patients with AP (58.66 *vs* 59.76, $P = 0.043$).

The prevalence of AP by risk score quintiles

Table 2 indicates the prevalence of AP by risk score quintiles and the ORs of AP prevalence compared across score quintiles. The prevalence of AP increased progressively through the five quintiles of risk scores: 27.63% in the first and lowest quintile, 33.53% in the second quintiles, 46.31% in the third quintiles, 52.21% in the fourth quintile, and 51.35% in the fifth and highest quintile. The ORs of AP prevalence in the third quintile compared to the first and second quintile were 2.26 (CI: 1.40-3.65) and 1.71 (CI: 1.08-2.70). The ORs of AP prevalence in the fourth quintile compared to the first and second quintile were 2.86 (CI: 1.75-4.67) and 2.16 (CI: 1.36-3.45). The ORs of AP prevalence in the fifth quintile compared to the first and second quintile were 2.76 (CI: 1.71-4.47) and 2.09 (CI: 1.32-3.30).

Discriminatory accuracy of the risk prediction tool and cutoff value with highest sensitivity and specificity

The ROC curves assessing the discriminatory accuracy of the risk score for all patients and by gender were presented in Figures 1 and 2. The AUC for all patients was 0.62 (CI: 0.58-0.66). For Female, the AUC is 0.60 (CI: 0.55-0.66). For male, the AUC is 0.63 (CI: 0.57-0.69). Youden's Index indicated the optimal risk score cutoff value discriminating AP prevalence status was 3.60 based on data on all patients.

Table 3 indicates diseases prevalence stratified by risk score optimal cutoff value. The percentage of AP prevalence patients was smaller for patients with risk score lower than 3.6 compared to the patients with risk score 3.6 and above (30.8% *vs* 52.7%, $P < 0.001$). The percentage of patients having three and more adenomas is smaller for patients with risk score less than 3.6 compared to patients with risk score 3.6 and above (4.8% *vs* 14.6%, $P < 0.001$).

Subgroup analyses on screening and surveillance patients

To strengthen the results, we studied screening and surveillance patients separately. The prevalence of AP increased progressively in screening patients among the 5 quintiles (Table 4): 28.69% in the first and lowest quintile, 33.58% in the second quintile, 43.70% in the third quintile, 51.67% in the fourth quintile, and 47.75% in the fifth and highest quintile. The OR of having AP in the third quintile compared to the first quintile was 1.93 (CI: 1.13-3.29). The OR of having AP in the fourth quintile compared to the first and second quintiles were 2.66 (CI: 1.56-4.52) and 2.11 (CI: 1.27-3.51). The OR of having AP in the fifth quintile compared to the first and second quintiles were 2.27 (CI: 1.32-3.90) and 1.81 (CI: 1.08-3.03). The AUC discriminating risk score and AP prevalence was 0.60 (CI: 0.55-0.64) (Supplementary Figure 1). The optimal risk score cutoff value indicated by Youden's Index was 3.60. The percentage of patients

Table 3 Disease prevalence stratified by risk score optimal cutoff value *n* (%)

	< 3.6 (*n* = 373)	⩾ 3.6 (*n* = 376)	Total (*n* = 749)	*P* value
Adenoma present	115 (30.8)	198 (52.7)	313 (41.8)	< 0.001
Adenoma (number)				< 0.001
< 3	355 (95.2)	321 (85.4)	676 (90.3)	
⩾ 3	18 (4.8)	55 (14.6)	73 (9.7)	
Serrated adenoma present	1 (0.3)	2 (0.5)	3 (0.4)	1.000
Hyperplastic polyp > 10 mm present	7 (1.9)	10 (2.7)	17 (2.3)	0.632

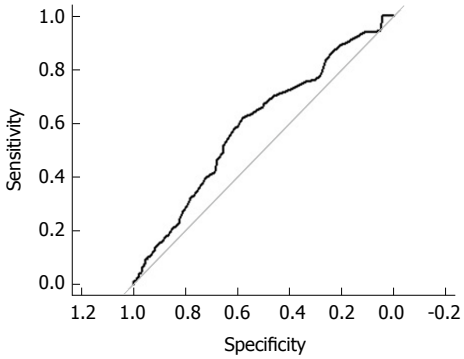


Figure 1 Receiver operating characteristic curve for National Cancer Institute Colorectal Cancer Risk-Assessment Tool for all patients.

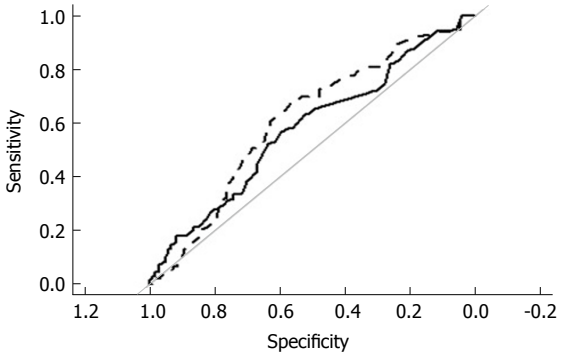


Figure 2 Receiver operating characteristic curves for National Cancer Institute Colorectal Cancer Risk-Assessment Tool for all patients by genders. Solid line indicates female, dashed line indicates male.

with AP prevalence is lower for patients with risk score lower than 3.6 compared to patients with risk score 3.6 and above (31.1% *vs* 50.3%, $P < 0.001$). The percentage of patients with three and more AP is lower for patients with risk score lower than 3.6 compared to patients with risk score 3.6 and above (4.6% *vs* 12.8%, $P < 0.001$) (Supplementary Table 1).

The prevalence of AP among surveillance patients mostly increased progressively among the five quintiles (Supplementary Table 2): 23.33% in the first and lowest quintile, 33.33% in the second quintile, 56.67% in the third quintile, 50.00% in the fourth quintile, and 68.97% in the fifth and highest quintile. The OR of having AP in the third quintile compared to the first quintile was 4.3 (CI: 1.41-13.07). The OR of having AP in the fourth quintile compared to the first quintile was 3.29 (CI: 1.03-10.53). The OR of having AP in the fifth quintile compared to the first and second quintiles were 7.3 (CI: 2.30-23.18) and 4.44 (CI: 1.49-13.26). The AUC discriminating risk score and AP prevalence was 0.70 (CI: 0.62-0.79) (Supplementary Figure 2). The optimal risk score cutoff value indicated by Youden's Index was 3.50. The percentage of AP prevalent patients is smaller for patients with risk score less than 3.5 compared to patients with risk score 3.5 and above (29.6% *vs* 62.5%, $P < 0.001$). The percentage of patients with three and more AP is smaller for patients with risk score less than 3.5 compared to patients with risk score 3.5 and above (5.6% *vs* 22.2%, $P = 0.009$) (Supplementary Table 3).

DISCUSSION

The results of our study suggest that the NCI's Risk Assessment Tool is a reasonable option for recognizing patients who are at a higher risk for the presence of adenomatous polyps, having a moderately good discriminatory accuracy for the presence of AP.

The primary endpoint of our study was the presence of any adenomatous polyp, whereas the secondary endpoint highlighted the presence of ⩾ 3 adenomatous polyps (high risk adenoma). In our study, a NIH risk score of 3.6, as calculated based on Youden's index, was the best cut off value as a predictor of AP. The NCI risk score of more than 3.6 as compared to NCI risk score of 3.6 or less had significantly higher chance of finding an adenomatous polyp (30.8% *vs* 52.7%, $P < 0.001$) or high-risk adenoma (4.8% *vs* 14.6%, $P < 0.001$).

The prevalence of AP increased progressively throughout the five quintiles of risk scores and plateaued on the fifth quintile in our study: 27.63% in the lowest quintile, and 51.35% in highest quintile. The AUC, which reflects the overall discriminatory accuracy of the risk-prediction tool, was 0.62 (0.60 for females, and 0.63 for males), similar to those observed in the validation study of the NCI CRC Risk-Assessment Tool using data from the NIH-AARP diet and health study with incident CRC as the outcome, in which the AUCs were 0.61 (95%CI: 0.59-0.62) for women and 0.61 (95%CI: 0.60-0.62) for men, thus demonstrating a moderately good range for the given AUCs (Figures 1 and 2, Supplementary Figures 1 and 2)[7].

These findings suggest that the NCI's Risk Assessment Tool has a moderate to good predictive value for estimating the risk for adenomatous polyps as well as future risk of CRC and can be utilized for such predictions. This dual risk estimation makes it a very effective tool for increasing the yield of colonoscopy.

Adenomas are well known precursors of CRC. The adenoma-carcinoma sequence has suggested genetic alterations and chromosomal instability as the underlying mechanism for colorectal tumorigenesis[8]. Genes such as the APC, K-Ras, p53 and others, have

Table 4 Adenoma prevalence by risk-score quintile for all screening patients

NCI lifetime risk score quintile	Score range	Number of individuals with adenomas (%)	OR of an adenoma (95%CI): row quintile *vs* column quintile			
			Q1	Q2	Q3	Q4
Q1 lowest	(1.1, 1.9)	35 (28.69)	-	-	-	-
Q2	(1.9, 2.8)	45 (33.58)	1.26 (0.74, 2.14)	-	-	-
Q3	(2.8, 4.0)	52 (43.70)	1.93 (1.13, 3.29)	1.53 (0.92, 2.56)	-	-
Q4	(4.0, 5.5)	62 (51.67)	2.66 (1.56, 4.52)	2.11 (1.27, 3.51)	1.38 (0.83, 2.29)	-
Q5 highest	(5.5, 12.3)	53 (47.75)	2.27 (1.32, 3.90)	1.81 (1.08, 3.03)	1.18 (0.70, 1.98)	0.85 (0.51, 1.43)

Asymptotic Linear-by-Linear Association Test: $P < 0.001$. OR: Odds ratio; CI: Confidence interval; NCI: National Cancer Institute.

been identified and implicated in the development of CRC[4]. It is often observed that CRC has a slower development in most cases *via* the adenoma-carcinoma sequence, which can take years. The estimated annual transition rates in both men and women from the advanced neoplasia to CRC was about 2.5%-3% among the groups of 55-64 years and about 5% to 5.5% in age groups of 70-79 years. This slower development gives physicians the potential for reducing the burden of the disease by early detection and subsequent removal of adenomas thereby haltering progression to CRC. Studies relate a 53% reduction in mortality due to CRC when adenomas are identified and removed during colonoscopy[9].

Adenomas that are greater than 1 cm, contain a substantial (> 25%) villous component, or have high-grade dysplasia are commonly referred to as advanced adenomas and carry an increased cancer risk[10]. However, a recent meta-analysis reported inconsistencies in detecting advanced adenomas based on size[11]. These discrepancies are due to a lack of standard methods for estimating adenoma size and the inter-observer variability and different pathology measurements[12-13]. It was seen that the utilization of an open biopsy forceps reported precise measurement only 37% of the time[14]. Sometimes the polyps are removed piecemeal and often break during collection[2]. Therefore, accuracy based on size in not reliable and should be used with caution for surveillance colonoscopies.

Calderwood *et al*[2] revealed that there in inconsistent association of villous histology of polyps and risk of advanced neoplasia in different studies**.** In contrast to European and United States guidelines, the British guidelines doesn't include advanced neoplastic features of polyps for recommendations of surveillance colonoscopies[2]. Based on this the British guidelines recommended one year follow up for 5 small adenomas or 3 adenomas if one is ⩾ 10 mm in size *vs* 3 years in European and United States guidelines for patients with ⩾ 3 adenomas or any adenoma ⩾ 10 mm size or with high grade dysplastic features or villous histology[15-17]. Due to these reasons and inconsistencies, we did not include advanced adenomas in our analysis.

In 2009, Freedman *et al*[4] developed the CRC risk assessment tool for white men and women without known susceptibility. This model was validated by Park *et al*[7] with recommendation that the model has a modest discriminatory power for the assessment of an individual's risk of CRC.

More than 50 proposed risk scores for colon cancer that have the potential to identify individuals at high risk[18]. A recent systematic review showed there is no clear improvement in discrimination as increasing numbers of variables are added to the risk assessment tools[19]. The two most commonly used and validated scores are the Cleveland Clinic test and the NCI test; which are both self-completed questionnaire. The Cleveland clinic test can only provide a suggested 10-year risk assessment, whereas the NCI test provides a 5-year and lifetime risk as well. Because the NCI tool has been subjected to external validation and because the predictors included in other risk scores are often a subset of the NCI tool's multiple predictors, we designed this study to explore the NCI tool.

We recommend the use of this score to help the patient in an informed decision-making; with patients having a higher NIH score should opt for the colonoscopy as compared to other available modalities. This is because colonoscopy has the additional advantage of clearing the colon of polyps and detecting cancerous lesions early in these patients. It also saves the time and cost of doing other tests which ultimately may require colonoscopy in such group of patients[20-22]. As the higher score is consistent with ⩾ 3 AP, it is reasonable to screen with colonoscopy to detect synchronous lesions throughout the entire colon.

It has been suggested by many studies that for adequate adenoma detection, the withdrawal time should be at least 6-9 min on average[10-11]. Preferably, an average withdrawal time for normal colonoscopies of 9 min is essential of adenoma detection. A higher adenoma (33.6%) detection rate was observed with withdrawal time of 9 min as compared to a lower adenoma (23.8%) detection rate with withdrawal time of 6 min[12]. We recommend that in patients having a higher NCI risk score, a higher withdrawal time should be used as it will increase the yield of adenomas and decrease the future risk of CRC in these patients.

Sanduleanu *et al*[23] defined interval CRC (iCRC) as "colorectal cancer diagnosed after a colorectal screening examination or test in which no cancer is detected, and before the date of the next recommended exam". Various factors contribute towards the development

of iCRC which include, missed lesions, incomplete polypectomy or rapid progression[24]. We suggest that the use of NCI score should be incorporated in clinical practice to make a decision about the duration before next colonoscopy to help decrease the incidence of interval CRC, though this has to be validated in prospective trials[25].

There are a few limitations of our study. It's a retrospective study mainly on minority group of patients. Majority of our study patient population included Hispanics and African Americans and a small number of Asians and white Americans. Therefore, it' s unclear whether generalizability to the more diverse United States population will be effective.

We didn't estimate the CRC risk based on the effect of location (proximal versus distal) of adenomas in this study. It is mentioned in previous studies that proximal lesions have a greater risk of adenoma detection as compared to the distal ones. These important clinical strategies need to be designed based on the location of the adenomas[26].

Conclusion and recommendations

In the last two decades, screening and surveillance colonoscopy guidelines implementation has remarkably decreased the CRC incidence and mortality. Our study intends to further decrease the morbidity and mortality associated with CRC.

Therefore, we recommended prospective trials to validate our hypothesis in a larger and heterogeneous population group with more diverse racial and ethnic backgrounds. This will help characterize the risk of future CRC risk and AP based on NCI score and assist the patients and provider decision-making about timing and mode of screening.

Patients with the higher NCI risk score have higher risk of AP and subsequent CRC; therefore, measures to increase the effectiveness of CRC detection in these patients include longer withdrawal, early surveillance, and choosing colonoscopy over other CRC screening modalities.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the third most common cancer diagnosed in men and women in the United States. The lifetime risk for developing CRC is 1 in 22 for men and 1 in 24 for women. Estimates for new cases of CRC amount to 140000 yearly, and approximately 50000 people will die of CRC in 2018 alone. CRC arises from colonic polyps, specifically 'adenomas', which result from either a sporadic mutation or a DNA mismatch repair within the mucosal lining of the intestine. Adenomas may grow and progress from low-grade dysplasia to high-grade dysplasia, to carcinoma-in-situ and eventually invasive carcinoma. It is there essential to diagnose the pre-cancerous and cancerous lesions are earlier stage.

Research motivation

Due to the financial and social impact of CRC on society, it is imperative to quantitatively assess the risk of developing CRC in individuals. Many tools are widely available that help calculate future risk of CRC, however they tend to be limited to specific patient groups, or be based on selected populations of patients, and have relatively poor discrimination or are not validated and/or published. Therefore, it is needed to developed tools which help predict future risk of CRC more specifically.

Research objectives

National Cancer Institute Colorectal Cancer Risk Assessment tool provides 5-year, 10-year and lifetime estimates and currently helps predict lifetime CRC risk. We conducted this study with the aim of evaluating the NCI Colorectal Cancer Risk Assessment Tool as a predictor of the presence of adenomatous polyps found during screening or surveillance colonoscopy.

Research methods

This is a retrospective single center observational study over a period of 6 mo duration. The data was collected from the electronic medical records of patients and tabulated in Microsoft Excel. Findings at colonoscopy were extracted from final procedure reports, and pathology information was extracted from final pathology reports. Asymptomatic patients between 50 and 80 years old, undergoing colonoscopy were included in the study population. Patients who met the above criteria were interviewed over the phone, and their lifetime NCI CRC Risk-Assessment Tool score was calculated. The predictors included in the NCI CRC Risk-Assessment Tool are number of relatives with CRC, body mass index, servings of vegetables per day, aspirin and nonsteroidal anti-inflammatory drug use, usual number of cigarettes smoked per day and years of smoking in current and former smokers, prior negative sigmoidoscopy and/or colonoscopy, polyp history and current vigorous leisure time activity *etc*.The authors in original paper estimated 10-year and 20-year CRC risks. This tool is available on the Internet and provides 5-year, 10-year and lifetime estimates. We used the predicted lifetime CRC risk. The data on the presence of adenomatous polyps and the numbers in each colonoscopy were collected.

Research results

After data analysis, it was noticed that the prevalence of AP increased progressively through the five quintiles of risk scores: 27.63% in the first and lowest quintile, 33.53% in the second quintiles, 46.31% in the third quintiles, 52.21% in the fourth quintile, and 51.35% in the fifth and highest quintile. The odd ratios (ORs) of AP prevalence in the third quintile compared to the first and second quintile were 2.26 (CI: 1.40-3.65) and 1.71 (CI: 1.08-2.70). The ORs of AP prevalence in the fourth quintile compared to the first and second quintile were 2.86 (CI: 1.75-4.67) and 2.16 (CI: 1.36-3.45). Youden's Index indicated the optimal risk score cutoff value discriminating AP prevalence status was 3.60 based on data on all patients. The percentage of AP prevalence patients was smaller for patients with risk score lower than 3.6 compared to the patients with risk score 3.6 and above (30.8% *vs* 52.7%, $P < 0.001$). The percentage of patients having three and more adenomas is smaller for patients with risk score less than 3.6 compared to patients with risk score 3.6 and above (4.8% *vs* 14.6%, $P < 0.001$).

Research conclusions

According to this study, patients with the higher NCI risk score have higher risk of AP and subsequent CRC. Our findings propose that patients who are categorized as high risk according to the NCI CRC risk assessment tool should undergo colonoscopy for the screening of CRC. Our study also revealed that the NCI CRC risk assessment tool can predict about the presence of AP, in addition to the lifetime risk of CRC. In these high risk patients, the measures to increase the effectiveness of CRC detection in these patients include longer withdrawal time, early surveillance colonoscopy, and choosing flexible colonoscopy over other CRC screening modalities. This study characterized the risk of future CRC risk and AP based on NCI score and will assist the patients and providers with informed decision-making about timing and mode of screening.

Research perspectives

In the last two decades, screening and surveillance colonoscopy guidelines implementation has remarkably decreased the CRC incidence and mortality. Our study was conducted to further decrease the morbidity and mortality associated with CRC. The tool can be used to predict the risk of future CRC and AP and therefor can assist the patients and providers with informed decision-making about timing and mode of screening. To further validate the

results of our study, there is a need to conduct prospective trials in a larger and heterogeneous population group with more diverse racial and ethnic backgrounds and involving multiple center.

REFERENCES

1 Key Statistics for Colorectal Cancer. Accessed March 23, 2018 Available from: URL: https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html
2 **Calderwood AH**, Lasser KE, Roy HK. Colon adenoma features and their impact on risk of future advanced adenomas and colorectal cancer. *World J Gastrointest Oncol* 2016; **8**: 826-834 [PMID: 28035253 DOI: 10.4251/wjgo.v8.i12.826]
3 **CDC**. What Are the Risk Factors for Colorectal Cancer? Accessed March 23, 2018 Available from: URL: https://www.cdc.gov/cancer/colorectal/basic_info/risk_factors.htm
4 **Freedman AN**, Slattery ML, Ballard-Barbash R, Willis G, Cann BJ, Pee D, Gail MH, Pfeiffer RM. Colorectal cancer risk prediction tool for white men and women without known susceptibility. *J Clin Oncol* 2009; **27**: 686-693 [PMID: 19114701 DOI: 10.1200/JCO.2008.17.4797]
5 **Hippisley-Cox J**, Coupland C. Development and validation of risk prediction equations to estimate survival in patients with colorectal cancer: cohort study. *BMJ* 2017; **357**: j2497 [PMID: 28620089 DOI: 10.1136/bmj.j2497]
6 Colorectal Cancer Risk Assessment Tool. Accessed March 23, 2018 Available from: URL: https://www.cancer.gov/colorectalcancerrisk/about-tool.aspx
7 **Park Y**, Freedman AN, Gail MH, Pee D, Hollenbeck A, Schatzkin A, Pfeiffer RM. Validation of a colorectal cancer risk prediction model among white patients age 50 years and older. *J Clin Oncol* 2009; **27**: 694-698 [PMID: 19114700 DOI: 10.1200/JCO.2008.17.4813]
8 **Leslie A**, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. *Br J Surg* 2002; **89**: 845-860 [PMID: 12081733 DOI: 10.1046/j.1365-2168.2002.02120.x]
9 **Zauber AG**, Winawer SJ, O'Brien MJ, Lansdorp-Vogelaar I, van Ballegooijen M, Hankey BF, Shi W, Bond JH, Schapiro M, Panish JF, Stewart ET, Waye JD. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. *N Engl J Med* 2012; **366**: 687-696 [PMID: 22356322 DOI: 10.1056/NEJMoa1100370]
10 **Brenner H**, Hoffmeister M, Stegmaier C, Brenner G, Altenhofen L, Haug U. Risk of progression of advanced adenomas to colorectal cancer by age and sex: estimates based on 840,149 screening colonoscopies. *Gut* 2007; **56**: 1585-1589 [PMID: 17591622 DOI: 10.1136/gut.2007.122739]
11 **Winawer SJ**, Zauber AG. The advanced adenoma as the primary target of screening. *Gastrointest Endosc Clin N Am* 2002; **12**: 1-9, v [PMID: 11916153 DOI: 10.1016/S1052-5157(03)00053-9]
12 **Moug SJ**, Vernall N, Saldanha J, McGregor JR, Balsitis M, Diament RH. Endoscopists' estimation of size should not determine surveillance of colonic polyps. *Colorectal Dis* 2010; **12**: 646-650 [PMID: 19486095 DOI: 10.1111/j.1463-1318.2009.01870.x]
13 **Schoen RE**, Gerber LD, Margulies C. The pathologic measurement of polyp size is preferable to the endoscopic estimate. *Gastrointest Endosc* 1997; **46**: 492-496 [PMID: 9434214 DOI: 10.1016/S0016-5107(97)70002-6]
14 **Rex DK**, Rabinovitz R. Variable interpretation of polyp size by using open forceps by experienced colonoscopists. *Gastrointest Endosc* 2014; **79**: 402-407 [PMID: 24119506 DOI: 10.1016/j.gie.2013.08.030]
15 **Lieberman DA**, Rex DK, Winawer SJ, Giardiello FM, Johnson DA, Levin TR. Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer. *Gastroenterology* 2012; **143**: 844-857 [PMID: 22763141 DOI: 10.1053/j.gastro.2012.06.001]
16 **Hassan C**, Quintero E, Dumonceau JM, Regula J, Brandão C, Chaussade S, Dekker E, Dinis-Ribeiro M, Ferlitsch M, Gimeno-García A, Hazewinkel Y, Jover R, Kalager M, Loberg M, Pox C, Rembacken B, Lieberman D; European Society of Gastrointestinal Endoscopy. Post-polypectomy colonoscopy surveillance: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy* 2013; **45**: 842-851 [PMID: 24030244 DOI: 10.1055/s-0033-1344548]
17 **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; British Society of Gastroenterology; Association of Coloproctology for Great Britain and Ireland. 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]
18 **Issa IA**, Noureddine M. Colorectal cancer screening: An updated review of the available options. *World J Gastroenterol* 2017; **23**: 5086-5096 [PMID: 28811705 DOI: 10.3748/wjg.v23.i28.5086]
19 **Usher-Smith JA**, Walter FM, Emery JD, Win AK, Griffin SJ. Risk Prediction Models for Colorectal Cancer: A Systematic Review. *Cancer Prev Res (Phila)* 2016; **9**: 13-26 [PMID: 26464100 DOI: 10.1158/1940-6207.CAPR-15-0274]
20 **Wong MC**, Ching JY, Chan VC, Sung JJ. The comparative cost-effectiveness of colorectal cancer screening using faecal immunochemical test vs. colonoscopy. *Sci Rep* 2015; **5**: 13568 [PMID: 26338314 DOI: 10.1038/srep13568]
21 **Janz T**, Lu K, Povlow MR, Urso B. A Review of Colorectal Cancer Detection Modalities, Stool DNA, and Fecal Immunochemistry Testing in Adults Over the Age of 50. *Cureus* 2016; **8**: e931 [PMID: 28097082 DOI: 10.7759/cureus.931]
22 **Wheeler SB**, Kuo TM, Meyer AM, Martens CE, Hassmiller Lich KM, Tangka FK, Richardson LC, Hall IJ, Smith JL, Mayorga ME, Brown P, Crutchfield TM, Pignone MP. Multilevel predictors of colorectal cancer testing modality among publicly and privately insured people turning 50. *Prev Med Rep* 2016; **6**: 9-16 [PMID: 28210537 DOI: 10.1016/j.pmedr.2016.11.019]
23 **Sanduleanu S**, le Clercq CM, Dekker E, Meijer GA, Rabeneck L, Rutter MD, Valori R, Young GP, Schoen RE; Expert Working Group on 'Right-sided lesions and interval cancers', Colorectal Cancer Screening Committee, World Endoscopy Organization. Definition and taxonomy of interval colorectal cancers: a proposal for standardising nomenclature. *Gut* 2015; **64**: 1257-1267 [PMID: 25193802 DOI: 10.1136/gutjnl-2014-307992]
24 **Kaminski MF**, Regula J, Kraszewska E, Polkowski M, Wojciechowska U, Didkowska J, Zwierko M, Rupinski M, Nowacki MP, Butruk E. Quality indicators for colonoscopy and the risk of interval cancer. *N Engl J Med* 2010; **362**: 1795-1803 [PMID: 20463339 DOI: 10.1056/NEJMoa0907667]
25 **Shaukat A**, Rector TS, Church TR, Lederle FA, Kim AS, Rank JM, Allen JI. Longer Withdrawal Time Is Associated With a Reduced Incidence of Interval Cancer After Screening Colonoscopy. *Gastroenterology*2015; **149**: 952-957 [PMID: 26164494 DOI: 10.1053/j.gastro.2015.06.044]
26 **Elhanafi S**, Ortiz AM, Yarlagadda A, Tsai C, Eloliby M, Mallawaarachchi I, Dwivedi A, Zuckerman MJ, Othman MO. Estimation of the Adenoma Detection Rate From the Polyp Detection Rate by Using a Conversion Factor in a Predominantly Hispanic Population. *J Clin Gastroenterol* 2015; **49**: 589-593 [PMID: 26035518 DOI: 10.1097/MCG.0000000000000179]

P- Reviewer: Hoensch HP, Luo HS **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3927-3957

DOI: 10.3748/wjg.v24.i34.3927

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

META-ANALYSIS

Epidemiology of viral hepatitis in Somalia: Systematic review and meta-analysis study

Mohamed Abdulkadir Hassan-Kadle, Mugtaba Sulaiman Osman, Pavel Petrovich Ogurtsov

Mohamed Abdulkadir Hassan-Kadle, Pavel Petrovich Ogurtsov, Center For the study of the Liver, Department of Hospital Medicine, Peoples' Friendship University of Russia (RUDN University), Moscow 117198, Russia

Mohamed Abdulkadir Hassan-Kadle, Abrar Research and Training Center, Abrar University, Department of Internal Medicine, Division of Gastroenterology and Hepatology, Dufle Specialist Hospital, Mogadishu 25, Somalia

Mugtaba Sulaiman Osman, Department of Psychiatry, School of Medicine, Royal College of Surgeons in Ireland RCSI, Beaumont Hospital, Dublin 9, Ireland

ORCID number: Mohamed Abdulkadir Hassan-Kadle (0000-0003-2304-6135); Mugtaba Sulaiman Osman (0000-0002-5170-6199); Ogurtsov Pavel Petrovich (0000-0001-7939-891X).

Author contributions: Hassan-Kadle MA acquired, collected, extracted, and interpreted the data, drafted and revised the article and made the final approval; Mugtaba SO designed the study, analysis and interpreted the data, drafted the article and made the final approval; Ogurtsov PP revised the article and made the final approval.

Supported by RUDN University Program 5-100.

Conflict-of-interest statement: The authors deny any conflict of interest.

PRISMA 2009 Checklist statement: This systematic review and meta-analysis was conducted as PRISMA guidelines.

Manuscript source: Unsolicited Manuscript

Correspondence to: Mohamed Abdulkadir Hassan-Kadle, MBChB, MD, Doctor, Abrar Research and Training Center, Abrar University, Department of Internal Medicine, Division of Gastroenterology and Hepatology, Dufle Specialist Hospital, 25 Mogadishu, Somalia. dr.kadle@abrar.edu.so
Telephone: +252-6-15910409

Received: March 17, 2018
Peer-review started: March 17, 2018
First decision: April 11, 2018
Revised: May 25, 2018
Accepted: June 21, 2018
Article in press: June 21, 2018
Published online: September 14, 2018

Abstract

AIM

To provide a clear understanding of viral hepatitis epidemiology and their clinical burdens in Somalia.

METHODS

A systematic review and meta-analysis was conducted as Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. A comprehensive literature search of published studies on viral hepatitis was performed from 1977-2016 in PubMed, Google Scholar, Science Direct, World Health Organization African *Index Medicus* and the Africa Journals Online databases, as well as on the Ministry of Health website. We also captured unpublished articles that were not available on online systems.

RESULTS

Twenty-nine studies from Somalia and Somali immigrants (United Kingdom, United States, Italy, Libya) with a combined sample size for each type of viral hepatitis [hepatitis A virus (HAV): 1564, hepatitis B virus (HBV): 8756, hepatitis C virus (HCV): 6257, hepatitis D virus (HDV): 375 and hepatitis E virus (HEV): 278] were analyzed. The overall pooled prevalence rate of HAV was 90.2% (95%CI: 77.8% to 96%). The

HAV prevalence among different age groups was as follows: < 1 year old, 61.54% (95%CI: 40.14% to 79.24%); 1-10 years old, 91.91% (95%CI: 87.76% to 94.73%); 11-19 years old, 96.31% (95%CI: 92.84% to 98.14%); 20-39 years old, 91.3% (95%CI: 83.07% to 95.73%); and > 40 years old, 86.96% (95%CI: 75.68% to 93.47%). The overall pooled prevalence of HBV was 18.9% (95%CI: 14% to 29%). The overall pooled prevalence among subgroups of HBV was 20.5% (95%CI: 5.1% to 55.4%) in pregnant women; 5.7% (95%CI: 2.7% to 11.5%) in children; 39.2% (95%CI: 33.4% to 45.4%) in patients with chronic liver disease, including hepatocellular carcinoma (HCC); 7.7% (95%CI: 4.2% to 13.6%), 12.4% (95%CI: 6.3% to 23.0%) and 11.8% (95%CI: 5.3% to 24.5%) in age groups < 20 years old, 20-39 years old and > 40 years old, respectively. The HBV prevalence among risk groups was 20% (95%CI: 7.19% to 44.64%) in female prostitutes, 21.28% (95%CI: 7.15% to 48.69%) in hospitalized adults, 5.56% (95%CI: 0.99% to 25.62%) in hospitalized children, 60% (95%CI: 31.66% to 82.92%) in patients with acute hepatitis, 33.55% (95%CI: 14.44% to 60.16%) in patients with ancylostomiasis, 12.34% (95%CI: 7.24% to 20.26%) in patients with leprosy and 20.19% (95%CI: 11.28% to 33.49%) in schistosomiasis patients. The overall pooled prevalence of HCV was estimated as 4.84% (95%CI: 3.02% to 7.67%). The prevalence rates among blood donors, risk groups, children and patients chronic liver disease (including HCC) was 0.87% (95%CI: 0.33% to 2.30%), 2.43% (95%CI: 1.21% to 4.8%), 1.37% (95%CI: 0.76% to 2.46%) and 29.82% (95%CI: 15.84% to 48.98%), respectively. The prevalence among genotypes of HCV was 21.9% (95%CI: 15.36% to 30.23%) in genotype 1, 0.87% (95%CI: 0.12% to 5.9%) in genotype 2, 25.21% (95%CI: 18.23% to 33.77%) in genotype 3, 46.24% (95%CI: 37.48% to 55.25%) in genotype 4, 2.52% (95%CI: 0.82% to 7.53%) in genotype 5, and 1.19% (95%CI: 0.07% to 16.38%) in genotype 6. The overall pooled prevalence of HDV was 28.99% (95%CI: 16.38% to 45.96%). The HDV prevalence rate among patients with chronic liver disease, including HCC, was 43.77% (95%CI: 35.09% to 52.84%). The overall pooled prevalence of HEV was 46.86% (95%CI: 5.31% to 93.28%).

CONCLUSION

Our study demonstrates a high prevalence of all forms of viral hepatitis in Somalia and it also indicates that chronic HBV was the commonest cause of chronic liver disease. This highlights needs for urgent public health interventions and strategic policy directions to controlling the burden of the disease.

Key words: Viral hepatitis; Hepatitis A virus; Hepatitis B virus; Hepatitis C virus; Hepatitis D virus; Hepatitis E virus; Systematic review; Meta-analysis, Somalia

Core tip: This is the first article reviewing epidemiology of viral hepatitis in Somalia with systematic review and meta-analysis of the published and unpublished reports from 1977 to 2016 among prevalence of all types' viral hepatitis in Somalia.

Hassan-Kadle MA, Mugtaba SO, Ogurtsov PP. Epidemiology of viral hepatitis in Somalia: Systematic review and meta-analysis study. *World J Gastroenterol* 2018; 24(34): 3927-3957 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3927.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3927

INTRODUCTION

Viral hepatitis is a major public health problem affecting several hundred million people globally. The most common types of viral hepatitis are six distinct types that have been identified as hepatitis A, B, C, D, E, G viruses, and they may present in acute form or chronic form, which causes substantial morbidity and mortality, including chronic hepatitis, cirrhosis and hepatocellular carcinoma. The World Health Organization (WHO) estimates that 257 million people worldwide are infected with hepatitis B virus (HBV), which constitutes 3.5% of the population. Africa has the second-largest number of chronic HBV carriers after the Western Pacific regions. Both regions are considered of high endemicity, and the prevalence rate of each region was 6.1% and 6.2%, respectively. It is estimated that approximately 71 million people were living with hepatitis C virus (HCV) infection, which accounts for 1% of the world's population, in 2015. The regions with the highest prevalence of HCV were the eastern Mediterranean region (2.3%) and the European region (1.5%), while the prevalence in Africa was 1.0%. Hepatitis D virus (HDV) affects nearly 15 million people, and hepatitis E virus (HEV) annually infects 20 million people, with over 3.3 million symptomatic cases of hepatitis E and 44600 hepatitis E-related deaths being recorded[1] Hepatitis E is one of the leading causes of major outbreaks of acute viral hepatitis worldwide, especially in developing nations[2]. Hepatitis A virus (HAV) infection spans the entire world, with specifically high prevalence rates in older children and adults[3].The number of deaths from viral hepatitis has increased from 1.10 million deaths in 2000 to 1.34 million deaths in 2015 compared to deaths from tuberculosis (from 1.67 to 1.37 million deaths), malaria (from 0.86 to 0.44 million deaths) and human Immunodeficiency virus (from 1.46 to 1.06 million deaths) between 2000 and 2015. Approximately 96% of these deaths resulted from complications of chronic HBV (66%) and HCV (30%), although hepatitis E and HAV infections accounted for 3.3% and 0.8% of these deaths, respectively[1]. HBV (887000 deaths) accounts for more deaths than HCV infection (399000 deaths),

while complications of both viruses (HBV and HCV) and cirrhosis (720000 deaths) account for more deaths than hepatocellular carcinoma (470000 deaths)[1].

In Somalia, viral hepatitis, especially HBV, is of significant public health importance. Somalia is an area of the world with a high prevalence HBV infection of > 8. There are several studies of the prevalence of HAV, HBV, HCV, HDV, and HEV in Somalia; however, to the best of our knowledge, there is no meta-analysis to provide an overall estimation of the prevalence of all viral hepatitis infections in this country. A recent report explored the reasons for such a dearth of data[4]. In Somalia, largely due to the unsettling decades-long civil war, medical staffs are underqualified and undertrained, and limited access to modern laboratory facilities poses substantial diagnostic challenges[4]. Somalia is considered to be a country that has no national strategy for the surveillance, prevention and control of viral hepatitis[5]. The provision of high-quality epidemiological data for viral hepatitis in Somalia could help motivate the drafting of action at the policy level. To synthesize such high-quality epidemiological estimates, we decided to undertake this systematic review and meta-analysis of studies that reported the population-level prevalence of each type of viral hepatitis (A, B, C, D and E) in Somalia. We also aimed to understand the burden of viral hepatitis in Somalia, especially HBV and HCV, and to inform public health practitioners, researchers and policy makers.

MATERIALS AND METHODS

Study area

Somalia is located along the Gulf of Aden and Indian Ocean in the sub-region of East Africa, and it is bordered by Djibouti and the Gulf of Aden to the north, the Indian Ocean to the east, Kenya to the southwest and Ethiopia to the west. Somalia has the longest coastline in Africa. It has a land mass of 637657 km^2 and a population of approximately 12 million, 61% of whom live in rural areas. Life expectancy at birth in 2015 was 49 years for males and 54 years for females[6]. The country is divided into eighteen administrative regions and 92 districts. Seventy-one percent of the Somali labor force is in agriculture, and almost everyone living in rural areas is involved in livestock or in farming. The per-capita total health expenditure, as a percentage of gross domestic products, was not mentioned in World Bank estimations. The proportion of Somalia' s population living below the poverty line has not been published to date, but the country was the fifth-poorest country of the world according the World Bank in 2016[7]. According to the United Nation, the Human Development Index of Somalia stood at 0.285, and the country ranked 165th out of 170 countries in 2012[8]. The country entered into a civil war in 1991, and many major structures collapsed, including the health sector and especially the public sectors.

Data sources and search strategy

We conducted an electronic literature search in several biomedical databases, including PubMed, Google Scholar, African Journal Online, WHO African Index Medicus and Science Direct, using different combinations of key words. The search encompassed published and unpublished studies from 1977 to 2016 with epidemiological and/or clinical data on the seroprevalence of viral hepatitis in Somalia. The key words used were as follows: ["hepatitis A" AND (seroprevalence OR prevalence) AND "Somalia"], ["hepatitis B" AND (seroprevalence OR prevalence) AND "Somalia"], ["hepatitis C" AND seroprevalence OR prevalence) AND "Somalia"], ["hepatitis D" AND (seroprevalence OR prevalence) AND "Somalia"], ["hepatitis E" AND (seroprevalence OR prevalence) AND "Somalia"]. We also reconducted the search using full written phrases, such as "Viral hepatitis", "hepatitis A", "hepatitis B" or "hepatitis B surface antigen", "hepatitis C", "hepatitis D", "hepatitis E", "epidemiology", and "Somali immigrants." We searched unpublished studies from other sources, such as universities and the website of the Ministry of Health (http://www.moh.gov.so/en/) for non-indexed studies or reports on the topic. The statement was reviewed systemically following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[9]. If the full text of studies were not reachable, we contacted the authors by email.

Study selection, inclusion and exclusion criteria

In this systematic review, we considered all studies published in peer-reviewed journals between 1977 and 2016, and unpublished primary data of each type of viral hepatitis in Somalia qualified for inclusion in the review, according to the PRISMA flow diagram[9], which excludes case reports, systematic reviews, case series editorials, letters to the editor, commentaries, magazines, newspaper reports/articles and studies of other Somali ethnicities living in neighboring countries published in peer-reviewed journals. The titles and abstracts were screened for relevance, and full-text papers considered relevant for further screening were obtained wherever possible. The references of all identified full-text articles and reviews of the literature were also checked to identify whether there were any additional articles that were missed during screening. For the study selection of this systematic review, we sub-analyzed all articles according to population sub-groups, such as blood donors, pregnant women, children, and patients with chronic liver disease and in the general population. These subgroups were examined with no age restriction. For other populations, the age restrictions imposed were children, defined as those 12 years of age and below, and adults, defined those 18 years of age and above. The search language was restricted to English. The studies also included Somali people who immigrated to Italy, the United States, United Kingdom and Libya and were screened for hepatitis viruses.

Data extraction

For the data extraction, the two investigators, Hassan-Kadle MA and Mugtaba SO, independently applied the inclusion criteria selected the studies and extracted the data. The extracted data included the following descriptive information: author(s), publication year, country of study (because some studies were conducted among Somali populations outside of Somalia), total sample size, and cases of each type of viral hepatitis in each study.

Statistical analysis

Calculation of pooled prevalence: The prevalence of viral hepatitis was extracted from every individual observational study by dividing the number of patients who tested positive over the total number included in the study. The prevalence in each study was multiplied by a weight inversely proportional to the total size of the study sample. The pooled prevalence is the sum of these weighted prevalence estimates in each individual study. We used the R software statistical packages "meta" and "metaphor" to calculate the pooled prevalence (Viechtbauer *et al*[10]; Schwarzer *et al*[11]). We chose to use a random effects model in the meta-analysis because we expected considerable heterogeneity among studies, due largely to the different settings in which studies were conducted. To compare the results, we included the results for a fixed effects meta-analysis. We attempted a meta-regression model by adjusting for the potential effect of the country where the study was conducted[12].

Testing heterogeneity: Heterogeneity testing was performed using the I^2 and the Q statistic methods. We interpreted the I^2 statistic results as follows: 0%, no observed heterogeneity; 25%, low; 50%, moderate; and 75%, high heterogeneity. We choose the *P* value = 0.05 as a cutoff for significant heterogeneity in interpreting the results of the Q statistic.

Publication bias: We used the funnel plot method to visually assess the studies by plotting the sample size against the observed prevalence. We assumed that smaller studies would vary more considerably around the pooled estimate than the larger studies would. Objective measures of the funnel plot symmetry were performed using Duval and Tweedie's trim and fill procedure (Duval *et al*[13]) and Egger's test (Egger *et al*[14]).

RESULTS

Search results

Our search yielded 504 citations, which were retrieved in the literature review. After reviewing the abstracts and titles, we excluded duplicates and irrelevant studies after applying the exclusion criteria. A total of 29 articles were included in this study and were analyzed. The 29 studies were conducted in Somalia and outside of Somalia (among Somali immigrants, Figure 1).

Epidemiology of HAV

Hepatitis A is a liver disease caused by HAV, and it occurs worldwide. This virus thus creates a public health concern, primarily in developing countries, due to its persistent circulation in the environment. Among the studies presented in Somalia, more than 90% of children had the HAV antibody by the age of 4 years[15-19]. In 1992, Mohamud KB and his colleagues studied a Somali sample of 593 subjects who were healthy rural and urban volunteers and child outpatients ages 0-83 years in three villages in Somalia (Mogadishu area: Buur-Ful village; Jowhar District: Mooda Moode; and Bur-Hakaba District and Bajuni Islands: Kismaio District). This sample showed a very high rate of HAV exposure of approximately 90%[15]. Another study by Bile *et al*[16] conducted at two institutions for children in Somalia (Shebeli: 596 subjects and Societe Organization Sociale, SOS: 76 subjects) showed a very high rate of HAV in the two samples of 96% and 59%, respectively. One study indicated that HAV in Somalia occurs primarily between 4 mo to 4 years of age, because the child has passive immunity from maternal antibodies during the first 3 mo of life[17]. Sebastiani *et al*[18] presented a result of 90.6%. Another study conducted in Italy for immigrant communities, which included 213 subjects, mostly originating from Somalia (177 or 83%), Ethiopia (21 or 10%), and Djibouti, Egypt and Saudi Arabia, showed a very high prevalence of HAV of 96%, including children (87.5% of children were under 12 years)[19].These reports of anti-HAV prevalence rates across the 4 studies ranged from 59.2% to 96%[15-19], as shown in Table 1. Four studies met the inclusion criteria for the meta-analysis of the prevalence of HAV infection. The four studies included examined the prevalence of HAV infection in the total of 1564 Somali participants, and the quality of the included studies also varied. We used the extracted data of the 4 studies to quantify the overall pooled prevalence of HAV infection. The pooled effect size for the prevalence of HAV infection among Somali people was 90.2% (95%CI: 77.8% to 96%). The heterogeneity was high (I^2 = 95.6%, 95%CI: 92.2% to 97.5%). Another indication of high heterogeneity was the Q-statistic [Q (degrees of freedom = 4) = 90.31, *P* value < 0.001]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 2.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 3). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The

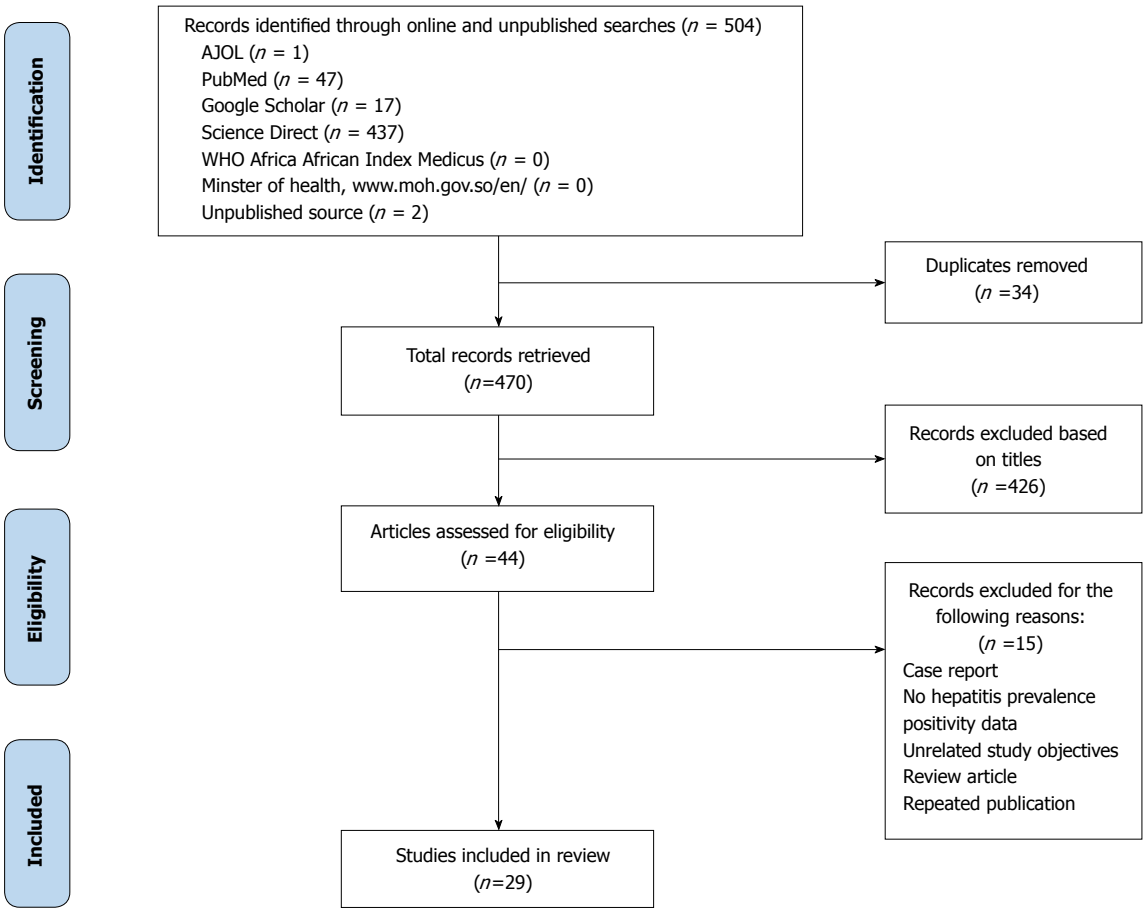


Figure 1 Schematic flow diagram of the studies reviewed for inclusion in analysis.

Table 1 Hepatitis A virus of overall prevalence at Somali population in Somalia and Somali immigrants *n* (%)

Author	Publication year	Total	Hepatitis A virus	Setting	Population
Faustini *et al*[19]	1994	213	204 (96)	Italy	Immigrants
Bile *et al*[16]	1992	596	572 (96)	Somalia	Local
Bile *et al*[16]	1992	76	45 (59.2)	Somalia	Local
Mohamud *et al*[15]	1992	593	534 (90)	Somalia	Local
Sebastiani *et al*[18]	1984	86	78 (90.6)	Somalia	Local

effect size imputed was 87.6%, which was close to the observed effect size. Notably, we could not perform Egger's test for the assessment of symmetry of the funnel plot due to the small number of included studies.

Sub-analysis according to country/setting

The majority of the studies (n = 3) were conducted in Somalia with a pooled prevalence of HAV of 88.1% (95%CI: 71.1% to 95.7%) and high heterogeneity (I^2 = 96.3%). These studies are followed by 1 study conducted in Italy, with a prevalence of HAV of 95.8% (95%CI: 92.1% to 97.8%, Figure 4).

Age pattern of HAV infection

Characteristics of the studies included: All studies met the inclusion criteria for the meta-analysis of the prevalence of HAV infection and examined the prevalence of HAV infection in a total of 1488 Somali participants belonging to 4 major age groups. The quality of the included studies varied (Table 2).

Pooled prevalence of HAV infection per age group: We used the extracted data of the 4 studies to quantify the overall pooled prevalence of HAV infection in each age group.

One study was conducted on patients younger than one year old. The pooled effect size for the prevalence of HAV infection among Somali people aged less than one year was 61.54% (95%CI: 40.14% to 79.24%).

The pooled effect size (out of 4 studies) for the prevalence of HAV infection among Somali people aged 1-10 years old was 91.91% (95%CI: 87.76% to 94.73%). The heterogeneity was moderate (I^2 = 59.9%). Another indication of moderate heterogeneity was the Q-statistic [Q (degrees of freedom = 3) = 7.49].

The pooled effect size (out of 3 studies) for the prevalence of HAV infection among Somali people aged 11-19 years old was 96.31% (95%CI: 92.84% to 98.14%). The heterogeneity was low (I^2 = 26.5%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 2) = 2.72].

The pooled effect size (out of 2 studies) for the prevalence of HAV infection among Somali people

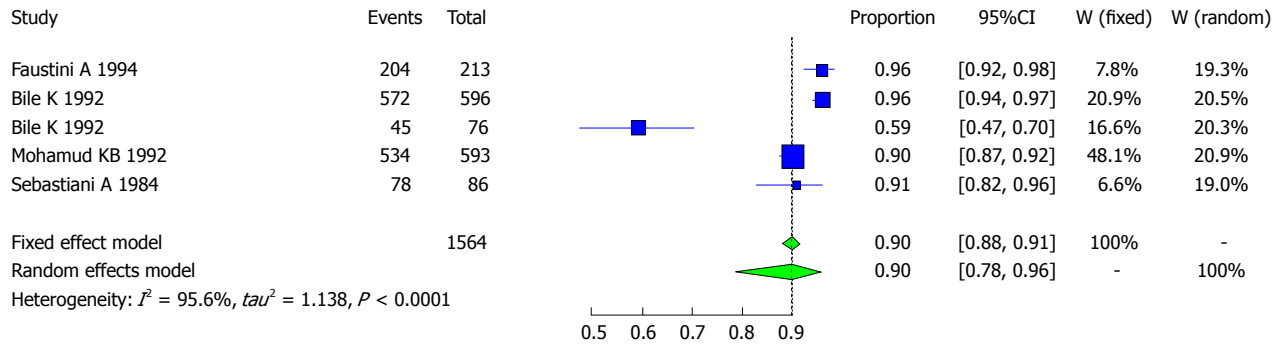


Figure 2 Meta-analysis and forest plot presentation of the anti-hepatitis A virus antibody from 1984 to 1994.

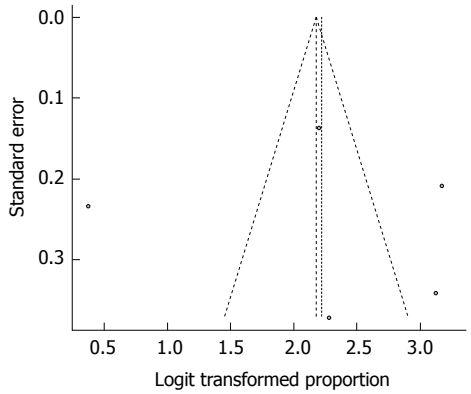


Figure 3 Bias assessment plot of studies reporting of hepatitis A virus prevalence in Somalia from 1984 to 1994.

aged 20-39 years old was 91.3% (95%CI: 83.07% to 95.73%). The heterogeneity was moderate (I^2 = 46.3%). Another indication of heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 1.86].

The pooled effect size (out of 2 studies) for the prevalence of HAV infection among Somali people aged over 40 years old was 86.96% (95%CI: 75.68% to 93.47%). The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.36]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CI, as shown in Figure 5.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 6). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, the Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 88.56%, which was not widely different from the observed effect size.

Epidemiology of HBV

Somalia is classified among countries as having a high hepatitis B surface antigen (HBsAg) endemicity of more than 8%[20,21]. The first study of HBV in the country was conducted in 1977, in which Delia S and his colleagues were presented with a higher frequency of HBsAg among patients with ancylostomiasis (33.33%) and with urinary schistosomiasis (25.92%) than among leprosy patients (9.67% in the L type and 6.89% in the T type), and the overall prevalence among these patients was shown to be high, with 76.1% (118/155) being observed among patients who were HBsAg positive and 11.11% of the controls. In the leprosy patients with schistosomiasis, the frequency was 40.0%[22]. Another study conducted in 1978 showed that HBsAg was found in 14.8%of these patients (54 cases), while the frequency was 34.0% among controls (47 cases). However, the overall prevalence in this study was 23.7% (77/101)[23].

In 1979, Nuti *et al*[24] studied 222 Somalian patients with the lepromatous form of leprosy (LL; *n* = 135 patients) and the tuberculoid form of the disease (TT; *n* = 87 patients) for HBV markers. The results showed that the proportion of leprosy and tuberculoid patients presenting with HBsAg was 24.4% (54/222) and 11.5% (26/222), respectively, but the overall prevalence of HBsAg-positive patients in this article was 36% (80/222)[24]. Another study published in this year revealed that among patients with acute viral hepatitis who were tested for the presence of HBsAg and the e-antigen and its corresponding antibodies, HBsAg was found in 60% of patients with hepatitis and 34% of controls, and the overall prevalence of HBsAg-positive patients was 48% (49/102)[25]. Nuti M and his colleagues also found that 14% (22/157) of patients were HBsAg-positive for the overall prevalence of their study, but in patients with bladder schistosomiasis and in controls, the prevalence was 19.4% (13/67) and 10% (9/90), respectively[26].

In 1985, a study conducted in three different villages of Somalia found that 12.08% (40/331) of subjects aged 1-83 years were HBsAg-positive, 29.9% were anti-HBs-positive, 43.8% were anti-HBc-positive, 21.4% were anti-HBe-positive, and the overall prevalence in this study was 12% (46/383) of subjects, including those under one year of age. Among the HBsAg-positive subjects, 34.7% were HBeAg-positive and 21.7% had anti-HBcAg-IgM[27]. A survey study in 1987 of HBV

Table 2 Age pattern of hepatitis A infection

Age group	Author/Publication year	Total	Cases	Total	Healthy	Serology
0-11 mo	Mohamud *et al* 1992[15]	52	32	52	20	Anti-HAV
1-11 yr	Mohamud *et al* 1992[15]	189	176	189	13	Anti-HAV
	Sebastiani *et al* 1984[18]	35	33	35	2	Anti-HAV
	Faustini *et al* 1994[19]	213	186	213	27	Anti-HAV
	Bile *et al* 1992[16]	234	220	234	14	Anti-HAV
	Total	723	647	723	76	Anti-HAV
11-19 yr	Mohamud *et al* 1992[15]	62	58	62	4	Anti-HAV
	Sebastiani *et al* 1984[18]	21	20	21	1	Anti-HAV
	Bile *et al* 1992[16]	362	353	362	9	Anti-HAV
	Total	445	431	445	14	Anti-HAV
20-39 yr	Mohamud *et al* 1992[15]	164	153	164	11	Anti-HAV
	Sebastiani *et al* 1984[18]	19	16	19	3	Anti-HAV
	Total	183	169	183	14	Anti-HAV
40+ yr	Mohamud *et al* 1992[15]	126	111	126	15	Anti-HAV
	Sebastiani *et al* 1984[18]	11	9	11	2	Anti-HAV
	Total	137	120	137	17	Anti-HAV

HAV: Hepatitis A virus.

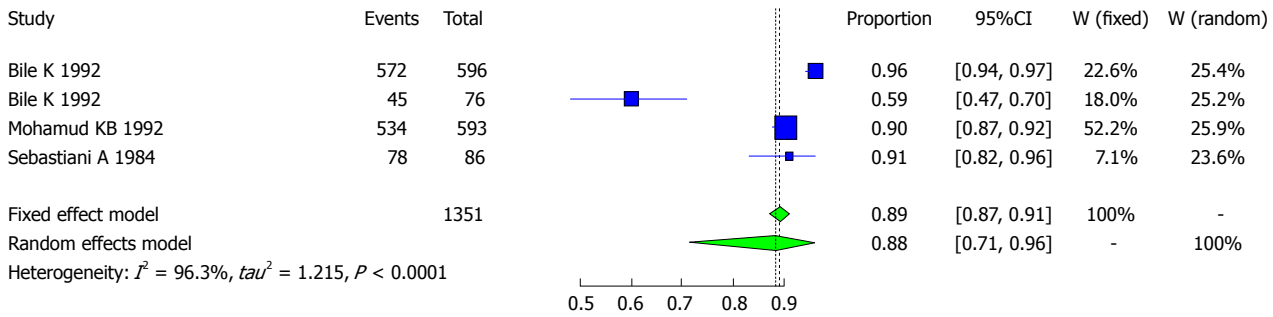


Figure 4 Forest plot of hepatitis A virus prevalence rates for studies conducted in Somalia from 1984 to 1992.

epidemiology was carried out among 383 adults from different areas of Somalia and in 135 pregnant women and 428 children from Mogadishu. The study showed a high incidence of HBsAg among nomadic males 20/85; (23%) and a lower incidence among males from agricultural and coastal areas, *i.e.*, 16/93 (17%) and 14/98 (14%), respectively. Meanwhile, the lowest frequency of HBsAg was observed among women from coastal areas (6/72; 8%) and among pregnant women (14/135; 10.4%), none of whom had HBeAg. However, a low number of children were HBsAg-positive, both under 4 years old (3/94; 3%) and 4-13 years of age (5/128; 4%). In the 15-19 age group, 50% of subjects showed seroconversion from HBeAg to anti-HBe. A total of 7 out of 41 HBsAg carriers aged over 20 had HBeAg, while the overall prevalence of 8.2% (78/946) was HBsAg-positive[28].

In 1987, Jama H and his colleagues conducted a study of sexual transmitted diseases among varied population groups, and these diseases were detected in 22.4% (49/218) of subjects in the overall populations, including 37% of pregnant women, 4% of neonates, 22% of educated women and 20% of prostitutes[29]. Another study in the country showed that 50% (52/104) of subjects was HBsAg-positive[30]. In 1989, a total of 1138 subjects with HBsAg were examined from different regions of Somalia; the results showed that 19.3% (220/1138) of subjects were HBsAg-reactive[31]. Bile KM and his colleagues conducted a case-control study that detected that 28.8% (67/232) of the overall prevalence was HBsAg subjects, including cases and control groups[32].

A total of 256 serum samples collected from blood donors (157 subjects) and hospitalized children (42 subjects) and adults (57 subjects) in Mogadishu were examined. The results showed that among 198 samples tested, the HBsAg carrier rate was 19.1% (22/115), 5.6%(2/36) and 21.3% (10/47) among blood donors, hospitalized children and hospitalized adults, respectively, but in the overall prevalence,17.1% (34/198) were positive for HBsAg[33].

The prevalence calculation carried out by Mohamud KB and his colleagues indicated that 10.5% (134/1272) of subjects were positive for HBsAg[15]. Another study

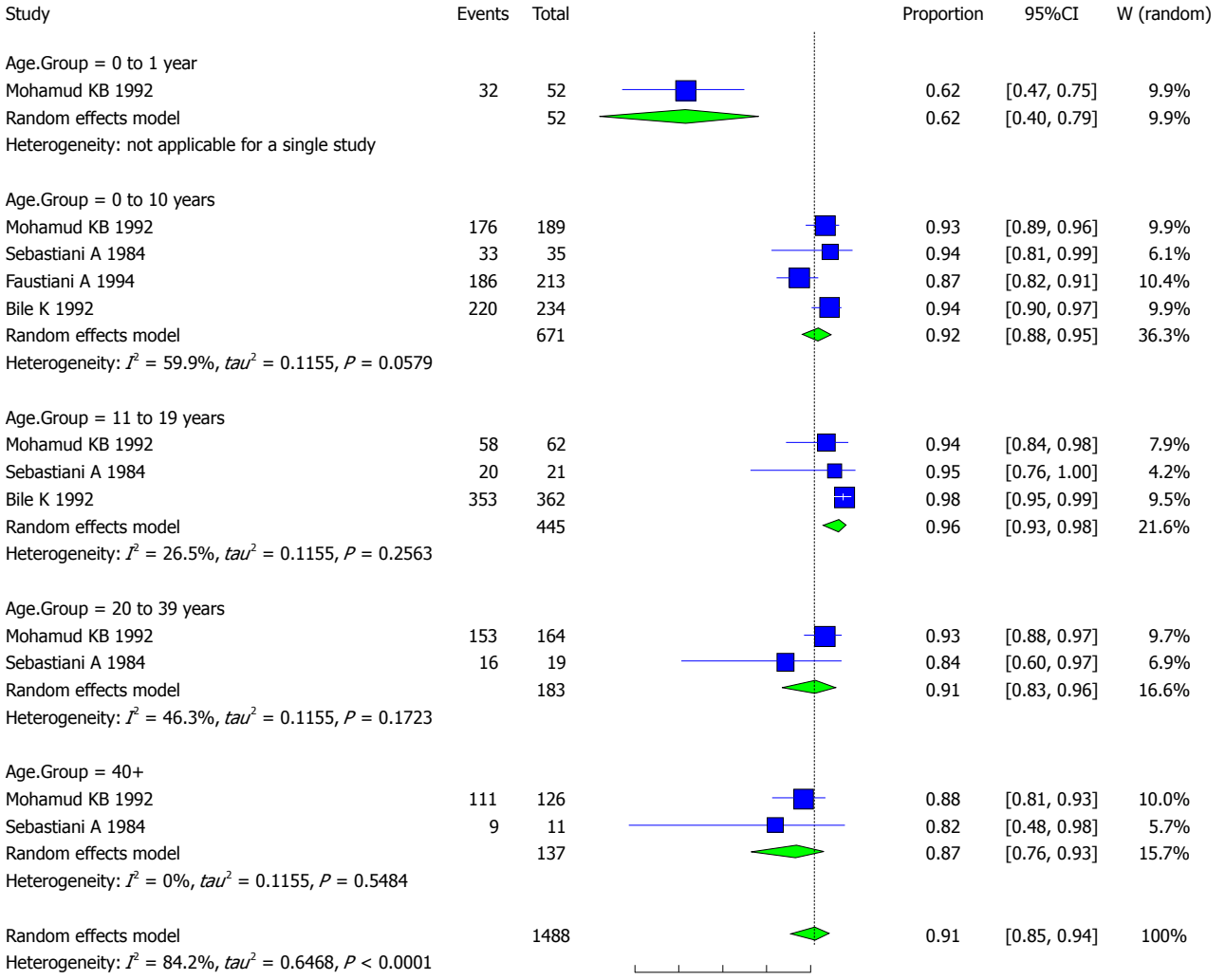


Figure 5 Forest plot of hepatitis A virus infection prevalence rates according to age groups from 1984 to 1994.

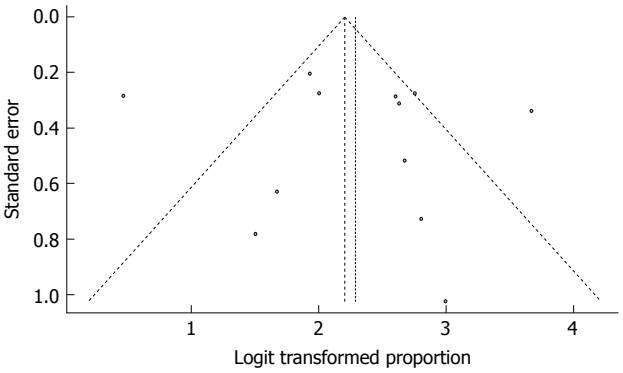


Figure 6 Bias assessment plot of studies reporting hepatitis A virus and age groups.

published in 1992 showed that 15.9% (95/596) of subjects were HBsAg-positive in one institution at Shabeli residence[16]. In a case-control study published in 1993, it was detected that 13.5% (29/124) of subjects in the overall prevalence of both groups were HBsAg-positive[34]. In 1994, a study conducted in Italy among immigrant communities detected HBsAg among 3.2% (7/213) of subjects[19]. Another study carried out in the United Kingdom among Somali immigrant communities showed that 5.6% (25/439) of subjects were positive for HBsAg[35]. Shire AM and his colleagues conducted a study in the United States and found that 13.6% (151/1109) of their sample were positive for HBsAg[36]. Unpublished studies performed in the country in 2011 and 2012 showed that 40.1% (59/147)[37] and 39.1% (61/156)[38] of subjects, respectively, were HBsAg reactive. A study in an immigrant community mostly originating from Somalia showed that 6.2% (31/500) of subjects had been detected with HBsAg[39].

Pooled prevalence of HBV infection

The reported HBsAg prevalence rates across the 23 studies ranged from 1.9% to 76%, as shown in Table 3. Additionally, the percentage of studies that reported prevalence rates that exceeded 8% was 82.6% (19/23), so we define this endemicity level of an area as high. A total of 17.3% (4/23) of studies reported prevalence rates of at least 8%. The 23 studies met the inclusion criteria for the meta-analysis of the prevalence of hepatitis virus infection and examined the prevalence

Table 3 Summary of studies of included studies on the prevalence of hepatitis B viral infection in a Somali population in Somalia and Somali immigrants (1977-2014) *n* (%)

No.	Author	Publication year	Total	HBsAg	Healthy	Setting	Population
1	Padovese *et al*[39]	2014	500	31 (6.2)	469	Italy	Immigrants
2	Kadle *et al*[38]	2012	156	61 (39.1)	95	Somalia	Local
3	Shire *et al*[36]	2012	1109	151 (13.6)	958	United States	Immigrants
4	Khadjio *et al*[37]	2011	147	59 (40.1)	88	Somalia	Local
5	Aweis *et al*[35]	2001	439	25 (5.6)	414	United Kingdom	Immigrants
6	Nur et al[33]	2000	198	34 (17.1)	164	Somalia	Local
7	Faustini *et al*[19]	1994	213	7 (3.2)	206	Italy	Immigrants
8	Bile *et al*[34]	1993	124	29 (13.5)	95	Somalia	Local
9	Bile *et al*[16]	1992	596	95 (15.9)	501	Somalia	Local
10	Mohamud *et al*[15]	1992	1272	134 (10.5)	1138	Somalia	Local
11	Bile *et al*[32]	1991	232	67 (28.8)	165	Somalia	Local
12	Aceti *et al*[31]	1989	1138	220 (19.3)	918	Somalia	Local
13	Bile *et al*[40]	1991	158	3 (1.9)	155	Somalia	Local
14	Aceti *et al*[30]	1991	104	52 (50)	52	Somalia	Local
15	Jama *et al*[29]	1987	218	49 (22.4)	169	Somalia	Local
16	Bile *et al*[28]	1987	946	78 (8.2)	868	Somalia	Local
17	Sebastiani *et al*[27]	1985	383	46 (12)	337	Somalia	Local
18	Nuti *et al*[26]	1979	102	49 (48)	58	Somalia	Local
19	Nuti *et al*[25]	1979	157	22 (14)	135	Somalia	Local
20	Nuti *et al*[24]	1978	101	24 (23.7)	77	Somalia	Local
21	Nuti *et al*[23]	1979	222	80 (36)	142	Somalia	Local
22	Delia *et al*[22]	1977	155	118 (76.1)	37	Somalia	Local
23	Sebastiani *et al*[18]	1984	86	11 (12.7)	75	Somalia	Local

HBsAg: Hepatitis B surface antigen.

of HBV infection in a total of 8756 Somali participants. The quality of the included studies also varied. We used the extracted data of the 23 studies to quantify the overall pooled prevalence of HBV infection. The pooled effect size for the prevalence of HBV infection among Somali people was 18.9% (95%CI: 14% to 29%). The heterogeneity was high (I^2 = 97.6%, 95%CI: 96.2% to 97.6%). Another indication of high heterogeneity was the Q-statistic [Q (degrees of freedom = 22) = 726.17, *P* value < 0.001]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CI, as shown in Figure 7.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 8). Notably, the total number of studies was reasonable, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 18.9%, which was notably similar to the observed effect size. Furthermore, Egger's test for assessment of symmetry of the funnel plot was not statistically significant (*t* = 0.6158, degrees of freedom = 21, *P*-value = 0.5447), providing further support for the absence of publication bias.

Sub-analysis according to country/setting

The majority of the studies (*n* = 19) were conducted in Somalia, with a pooled prevalence of HBV of 23% (95%CI: 16.9% to 30.6%) and high heterogeneity (I^2 = 96.9%). These findings are followed by 2 studies conducted in Italy, with a pooled prevalence of HBV of 4.6% (95%CI: 1.4% to 14.2%) and moderate heterogeneity (I^2 = 58.9%). One study was conducted in the United Kingdom, with a prevalence of HBV of 5.7% (95%CI: 1.1% to24.6%), and another study was conducted in the United States, with a prevalence of HBV of 13.6% (95%CI: 3.0% to 45.0%) (Figure 9).

Meta-regression analysis

Examining the effect of setting, significant variability could be explained by meta-regressing the meta-analysis over the four settings: Italy, Somalia, the United Kingdom, and the United States. Tests of moderators indicated the following: coefficient(s) 2, 3, 4: QM (df = 3) = 10.5687, *P*-value = 0.0143. For the prevalence results in Somalia compared to those of Italy, the *P* value was 0.0056, and the estimate was 1.8193. For the United Kingdom and the United States, the variability was not significantly different from that of Italy (*P* value=0.8373 and 0.2608, respectively).

Sub-analysis according to the population

The majority of the studies (*n* = 19) were conducted on the local population in Somalia with a pooled prevalence of HBV of 23% (95%CI: 16.9% to 30.6%) and high heterogeneity (I^2 = 96.9%). The remaining 4 studies were conducted on immigrants with a pooled prevalence of HBV of 23.1% (95%CI: 17.0% to 30.5%) and high heterogeneity (I^2 = 92.8%) (Figure 10).

Meta-regression analysis

Examining the effect of population, significant variability

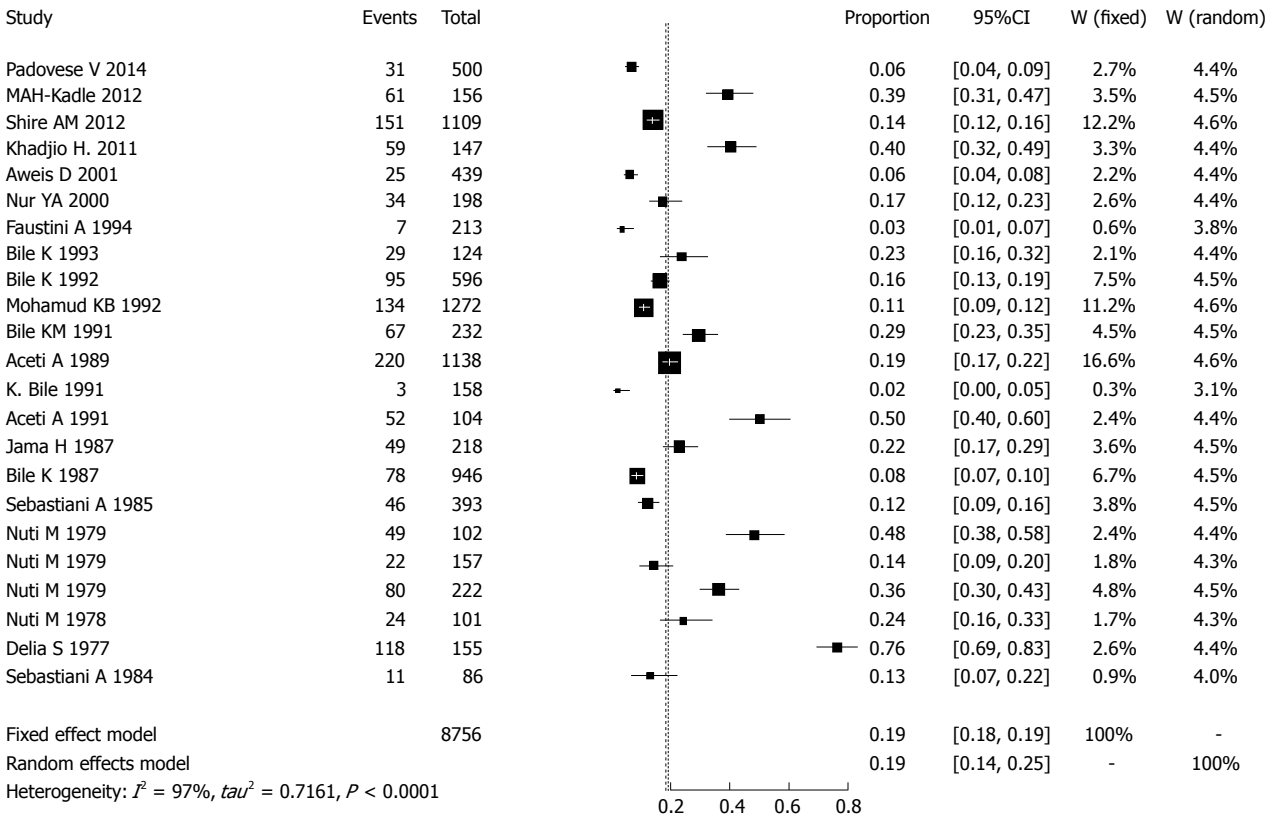


Figure 7 Forest plot of hepatitis B virus infection prevalence rates in Somalia in published and unpublished studies from 1977 to 2014.

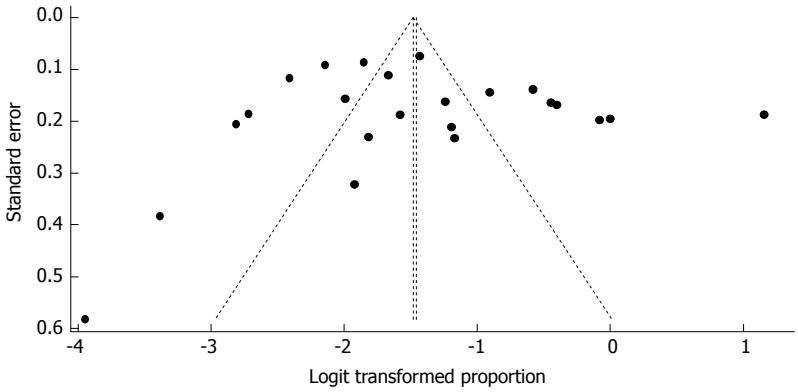


Figure 8 Bias assessment plot of studies reporting hepatitis B virus prevalence rate in Somalia from 1977 to 2014.

could be explained by meta-regressing the meta-analysis over the two population types: immigrants and locals. Tests of moderators indicated the following: coefficient 2: QM (df = 1) = 9.5448, *P*-val = 0.0020. For the prevalence results of the local Somali population compared to the immigrant population, the *P* value was 0.002, and the estimate was 1.4498.

Pregnant women and HBV infection

A total of 2 studies presented HBV prevalence rates among pregnant women, and these studies showed that 10.4% (14/135) of subjects were HBsAg-positive[28], while the other study presented 37% (19/52) of subjects as positive for HBsAg[29], as shown in Table 4. The two studies met the inclusion criteria for the meta-analysis of the prevalence of HBV infection during pregnancy. The pooled effect size for the prevalence of HBV infection during pregnancy among Somali people was 20.5% (95%CI: 5.1% to 55.4%). The heterogeneity was high (I^2 = 93.7%). See Figure 11.

HBV infection in children

A total of six studies showed HBV prevalence rates between 0% to 16% among children[16,27-30,40]. See Table 5. Six studies met the inclusion criteria for the meta-analysis of the prevalence of HBV infection among children. The pooled effect size for the prevalence of HBV infection in Somali children was 5.7% (95%CI:

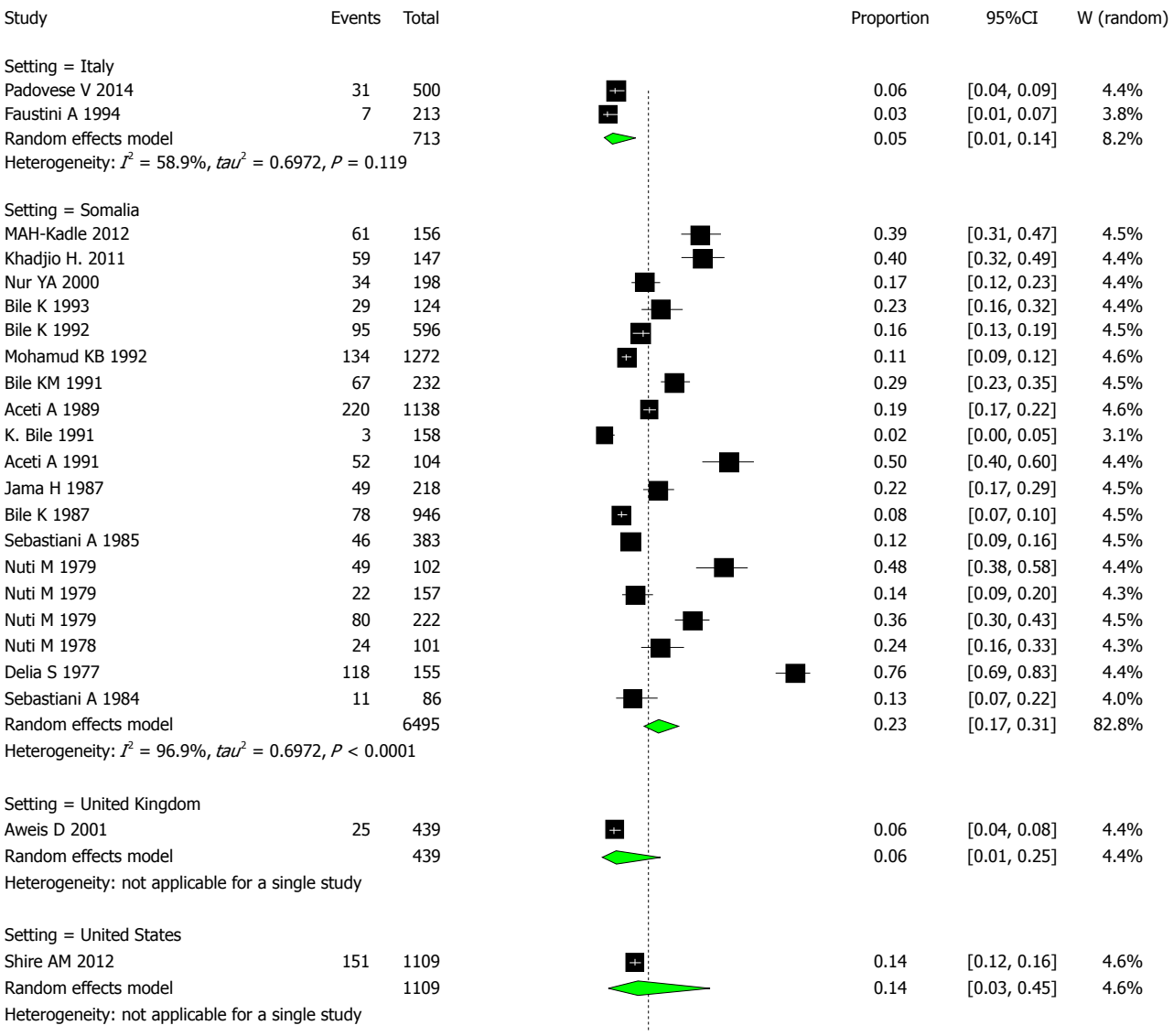


Figure 9 Forest plot of hepatitis B virus prevalence rates for studies conducted according to setting from 1977 to 2014.

2.7% to 11.5%). The heterogeneity was high (I^2 = 88.1%, 95%CI: 77.8% to 93.6%, Figure 12).

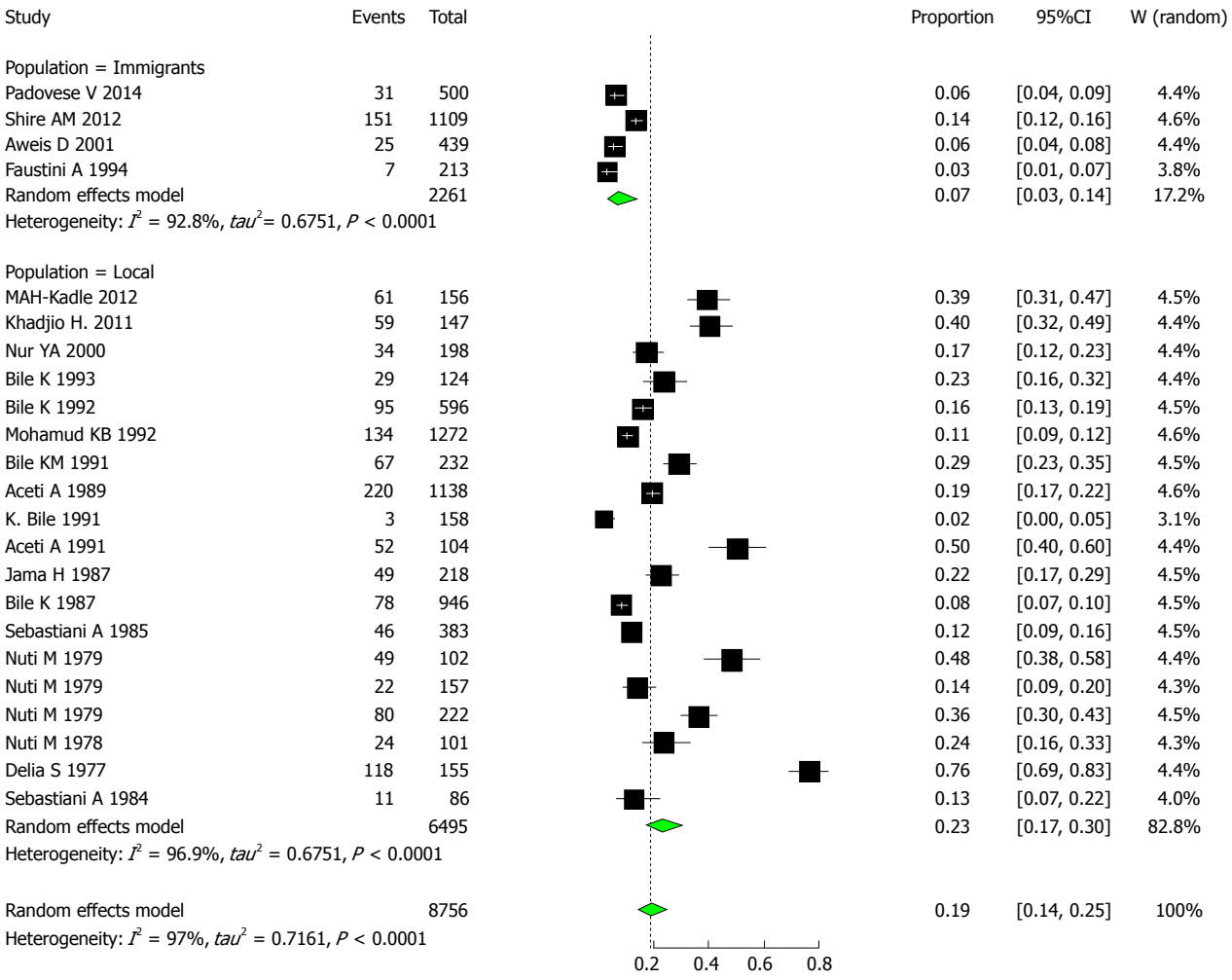
Hepatitis B in chronic liver disease (including hepatocellular carcinoma)

Six studies met the inclusion criteria for the meta-analysis of the prevalence of HBV infection among patients with chronic liver disease, including patients with hepatocellular carcinoma, in Somali people. Their prevalence ranges from 17.9% to 50%[30,32,34,36-38] (Table 6). The pooled effect size for the prevalence of hepatitis B among chronic liver disease patients in Somali people was 39.2% (95%CI: 33.4% to 45.4%). The heterogeneity was moderate (I^2 = 53.7%, 95%CI: 0% to 81.5%, Figure 13).

Despite the moderate heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 14). Moreover, the Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 41.5%, which was close to the observed effect size (*i.e.*, 39.2%). However, the heterogeneity, expectedly, was statistically significant (Q = 18.28, df = 7, P = 0.0108).

Sub-analysis according to the age group

The available studies (n = 5) contained data on the prevalence of HBV infection among three age groups



Results for subgroups (random effects model):

	K	Proportion	95%CI	Q	tau^2	I^2
Immigrants	4	0.0657	[0.0295, 0.1397]	41.71	0.6751	92.8%
Local	19	0.2306	[0.1699, 0.3049]	579.28	0.6751	96.9%

Figure 10 Forest plot of hepatitis B virus prevalence rates for studies conducted according to population from 1977 to 2014.

Table 4 Hepatitis B virus infection prevalence rates among pregnant women

Author	Year	Total	HBsAg	Healthy	Setting	Population
Bile *et al*[28]	1987	135	14 (10.3%)	121	Mogadishu	Pregnant women
Jama *et al*[29]	1987	52	19 (37%)	33	Mogadishu	Pregnant women

HBsAg: Hepatitis B surface antigen.

(below 20 years, 20-40 years, and above 40 years) that were available for extraction. The reported prevalence rate of these studies according to age group was as follows: below 20 (1% to 16%)[16,18,28,40]; 20-39 (6% to 16%)[18,28,40]; and above 40 (0% to 13%)[18,28,40] (Table 7). The HBV infection pooled effect was highest among the 20-39 age group at 12.4% (95%CI: 6.3% to 23.0%) followed by the above-40 age group at 11.8% (95%CI: 5.3% to 24.5%). The lowest pooled prevalence was in the below-20 age group at 7.7% (95%CI: 4.2% to 13.6%). The heterogeneity was very low for the 20-39 and the above-40 age groups (I^2 = 0%) but was very high for the below-20 age group (I^2 = 91.1%) (Figure 15).

The funnel plot indicated little evidence to support publication bias. Furthermore, Egger's test for the assessment of symmetry of the funnel plot was not statistically significant (t = -1.8748, degrees of freedom = 11, P-value = 0.0876), providing some support, at the 5% significance level, for the absence of publication bias (Figure 16).

Risk groups and HBV

Characteristics of the studies included: Seven studies met the inclusion criteria for the meta-analysis of the prevalence of HBV infection and their risk groups, as shown in Table 8. The 7 included studies examined the prevalence of HBV infection in a total of 1488 Somali participants belonging to 7 major risk groups.

Table 5 Hepatitis B infection prevalence rates among Somali children

Author	Year	Total	Cases	HBsAg	Healthy	Setting	Population
Sebastiani *et al*[27]	1985	219	25	11%	194	Jowhar, Bur-Hakaba, Kismaio and Afgoy	Children living in four villages
Bile *et al*[16]	1992	596	95	16%	501	Mogadishu area	Children in government-operated residence for abandoned children in Shebeli
Bile *et al*[16]	1992	76	3	4%	73	Mogadishu	Children in government-operated residences for abandoned children in SOS institution
Bile *et al*[40]	1991	106	0	0%	106	Lower Shabelle	Children living in Mukay Dumis
Nur *et al*[30]	2000	36	2	6%	34	Mogadishu	Hospitalized children
Bile *et al*[28]	1987	428	11	3%	417	Mogadishu	Children living in Mogadishu
Jama *et al*[29]	1987	26	1	4%	25	Mogadishu	Newborn babies

HBsAg: Hepatitis B surface antigen.

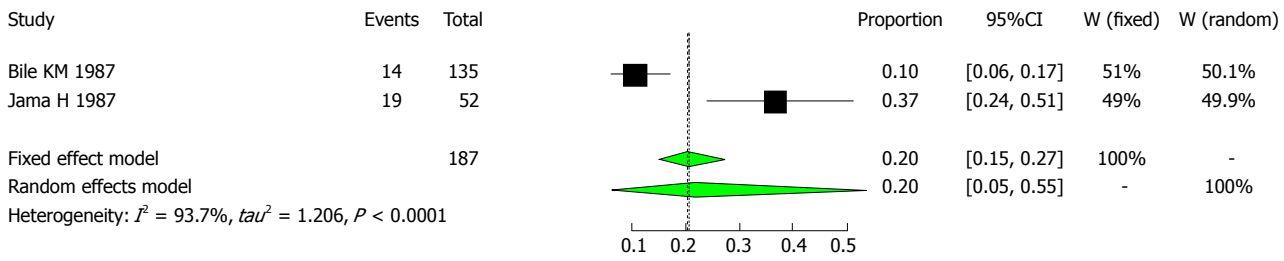


Figure 11 Forest plot of hepatitis B virus infection prevalence rates among pregnant women.

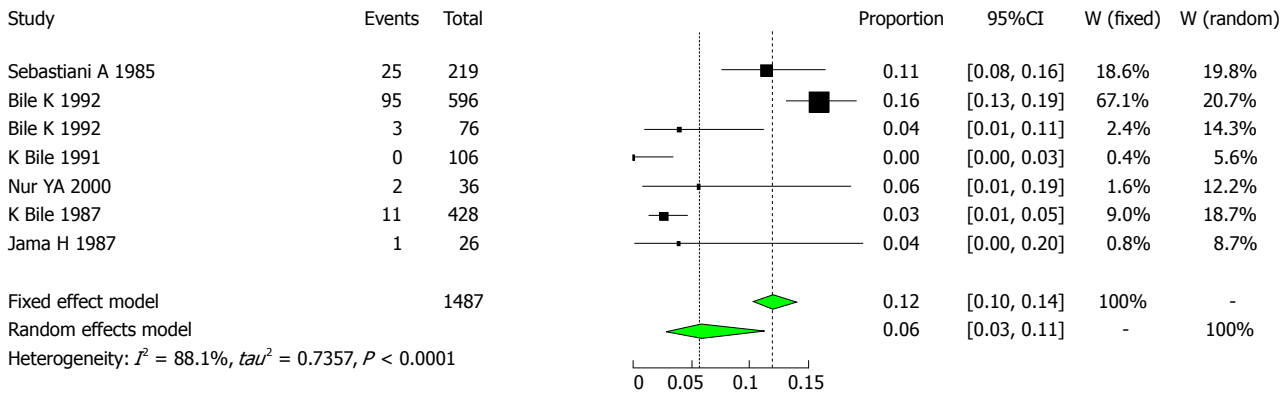


Figure 12 Forest plot of hepatitis B virus infection prevalence rates among Somali children.

Table 6 Studies among patients with chronic liver disease (including hepatocellular carcinoma) in Somalia *n* (%)

Author	Year	Total	HBsAg	Healthy	Setting	Population
Shire *et al*[36]	2012	30	5 (17.9)	25	United States	Immigrants
Kadle *et al*[38]	2012	156	61 (39.1)	95	Somalia	Local
Khadija *et al*[37]	2011	147	59 (40.1)	88	Somalia	Local
Bile *et al*[34]	1993	62	23 (37.1)	39	Somalia	Local
Bile *et al*[32]	1991	116	44 (37.9)	72	Somalia	Local
Aceti *et al*[30]	1991	104	52 (50)	52	Somalia	Local

HBsAg: Hepatitis B surface antigen.

The quality of the included studies also varied.

Pooled prevalence of HBV infection of Risk groups: We used the extracted data of the 12 studies to quantify the overall pooled prevalence of HAV infection in each group.

One study was conducted on female prostitutes. The pooled effect size for the prevalence of HBV infection among Somali prostitutes was 20% (95%CI: 7.19% to 44.64%).

One study was conducted on hospitalized adults. The pooled effect size for the prevalence of HBV infection among Somali hospitalized adults was 21.28% (95%CI: 7.15% to 48.69%).

One study was conducted on hospitalized children. The pooled effect size for the prevalence of HBV infection among Somali hospitalized children was 5.56% (95%CI: 0.99% to 25.62%).

One study was conducted on patients with acute hepatitis. The pooled effect size for the prevalence of HBV infection among Somali acute hepatitis patients was 60% (95%CI: 31.66% to 82.92%).

One study was conducted on patients with

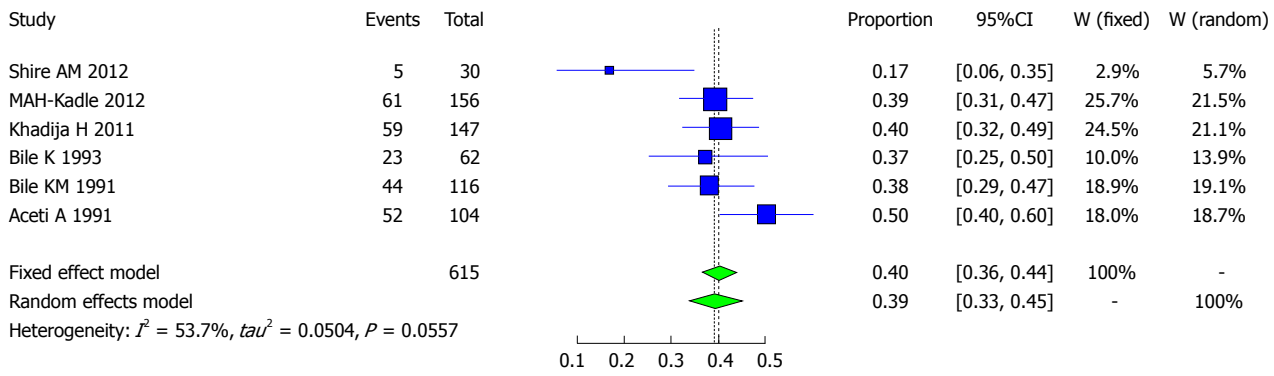


Figure 13 Forest plot of hepatitis B virus infection prevalence rates among patients with chronic liver disease, including hepatocellular carcinoma.

Table 7 Age pattern of hepatitis B virus infection *n* (%)

Age group	Author	Publication year	Total	HBsAg	Healthy
0-20	Sebastiani *et al*[40]	1985	219	25 (11)	194
	Bile *et al*[28]	1987	428	11 (6)	417
	Bile *et al*[40]	1991	119	1 (1)	118
	Bile *et al*[16]	1992	596	95 (16)	501
	Sebastiani *et al*[18]	1984	56	7 (13)	49
		Total	1418	139 (9.8)	1279
20-39	Sebastiani *et al*[40]	1985	94	13 (14)	81
	Bile *et al*[28]	1987	442	62 (14)	380
	Bile *et al*[40]	1991	36	2 (6)	34
	Sebastiani *et al*[18]	1984	19	3 (16)	16
		Total	591	80 (14)	511
40+	Sebastiani *et al*[40]	1985	70	8 (11)	62
	Bile *et al*[28]	1987	76	10 (13)	66
	Bile *et al*[40]	1991	3	0 (0)	3
	Sebastiani *et al*[18]	1984	11	1 (9.1)	10
		Total	160	19 (11.8)	141

HBsAg: Hepatitis B surface antigen.

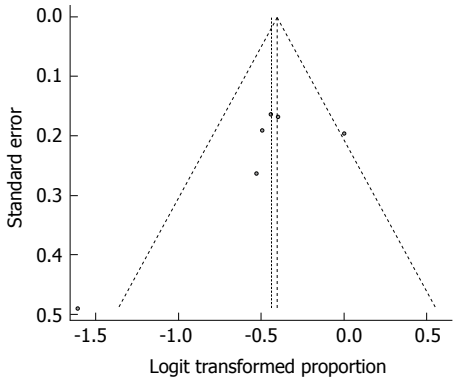


Figure 14 Bias assessment plot of studies reporting among patients with chronic liver disease, including hepatocellular carcinoma.

ancylostomiasis. The pooled effect size for the prevalence of HBV infection among Somali ancylostomiasis patients was 33.55% (95%CI: 14.44% to 60.16%).

Four studies were conducted on leprosy patients. The pooled effect size for the prevalence of HBV infection among Somali leprosy patients was 12.34% (95%CI: 7.24% to 20.26%). The heterogeneity was high (I^2 = 85.3%). Another indication of high heterogeneity was the Q-statistic [Q (degrees of freedom = 3) = 20.38].

Three studies were conducted on schistosomiasis patients. The pooled effect size for the prevalence of HBV infection among Somali schistosomiasis patients was 20.19% (95%CI: 11.28% to 33.49%). The heterogeneity was low (I^2 = 36%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 2) = 3.13].

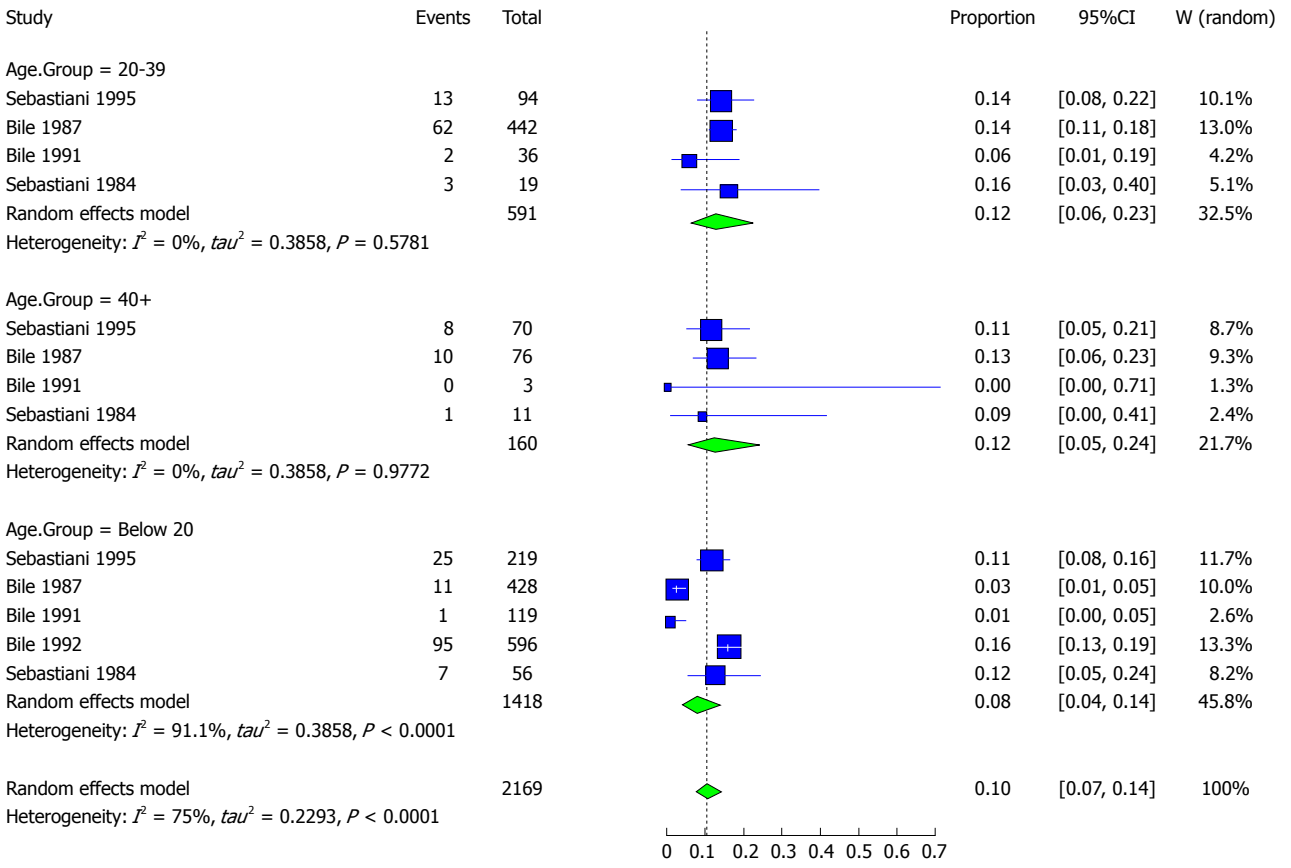
The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 17.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 18). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 28.5% which was not widely different from the observed effect size.

Epidemiology of HCV

There are few studies on HCV infection in Somalia. Watts DM and his colleagues studied 438 subjects, including female prostitutes (1.7% or 4/236), patients from a sexually transmitted disease clinic (2% or 2/80), male soldiers (1.3% or 1/79), and tuberculosis patients (2.3% or 1/43), while the overall prevalence showed that 1.8% (8/438) were anti-HCV positive[41].Another study investigated children from two institutions for children in Somalia, showing that 1.5% of children were positive for anti-HCV in one residence of 596 children, including boys (1.9% or 6/309) and girls (1% or 3/287). However, in corresponding individuals, 76 children (boys and girls) showed a result of 0%[16]. A study conducted among blood donors revealed that (0.6% or 1/157) were anti-HCV-positive, while the overall presence of anti-HCV in this study was (2.4% or 6/256)[33]. A case-control study of HCV in chronic liver disease patients showed an estimate of 4% (35/885) in overall subjects[42]. Bile K and his colleagues found that 23.3% (29/124) of subjects had antibodies for HCV in the overall prevalence of cases and controls[34]. In 2014, a study conducted in Malta



Results for subgroups (random effects model):

	K	Proportion	95%CI	Q	tau^2	I^2
Age.Group = 20-39	4	0.1242	[0.0632, 0.2298]	1.97	0.3858	0%
Age.Group = 40+	4	0.1182	[0.0526, 0.2445]	0.02	0.3858	0%
Age.Group = Below 20	5	0.0771	[0.0423, 0.1364]	45.13	0.3858	91.1%

Figure 15 Forest plot of hepatitis B virus infection prevalence rates among age groups.

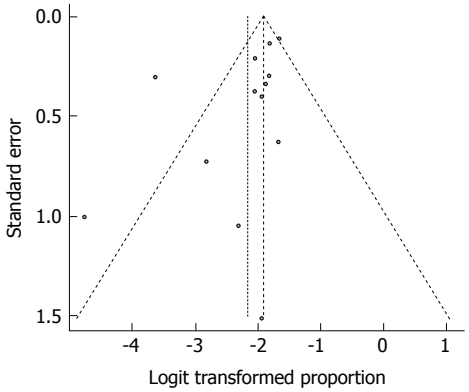


Figure 16 Bias assessment plot of studies reporting among age groups.

among asylum seekers showed that 0.6 %(3/500) of subjects were anti-HCV-positive[39]. Another study in Italy among immigrant communities that mostly came from Somalia indicated that 2.3% (5/213) had HCV-positive antibodies[19], and a study conducted in immigrant Somali communities in the United States showed that 9.1% (78/854) of subjects were anti-HCV-positive[36] (Table 9). Among population groups at risk for HCV in Somalia, studies have shown a result of 0% to 7%[33,41,42], shown in Table 10. Eleven studies met the inclusion criteria for the meta-analysis of the prevalence of HCV infection (Table 9).

The 11 included studies examined the prevalence of HCV infection in a total of 6257 Somali participants. The quality of the included studies also varied.

Pooled prevalence of HCV infection

We used the extracted data of the 12 studies to quantify the overall pooled prevalence of HCV infection. The pooled effect size for the prevalence of HCV infection among Somali people was 4.84% (95%CI: 3.02% to 7.67%). The heterogeneity was high (I^2 = 93.5%, 95%CI: 90.4% to 95.6%). Another indication of high heterogeneity was the Q-statistic [Q (degrees of freedom = 10) = 168.5, P value < 0.001]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CI, as shown in Figure 19.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little

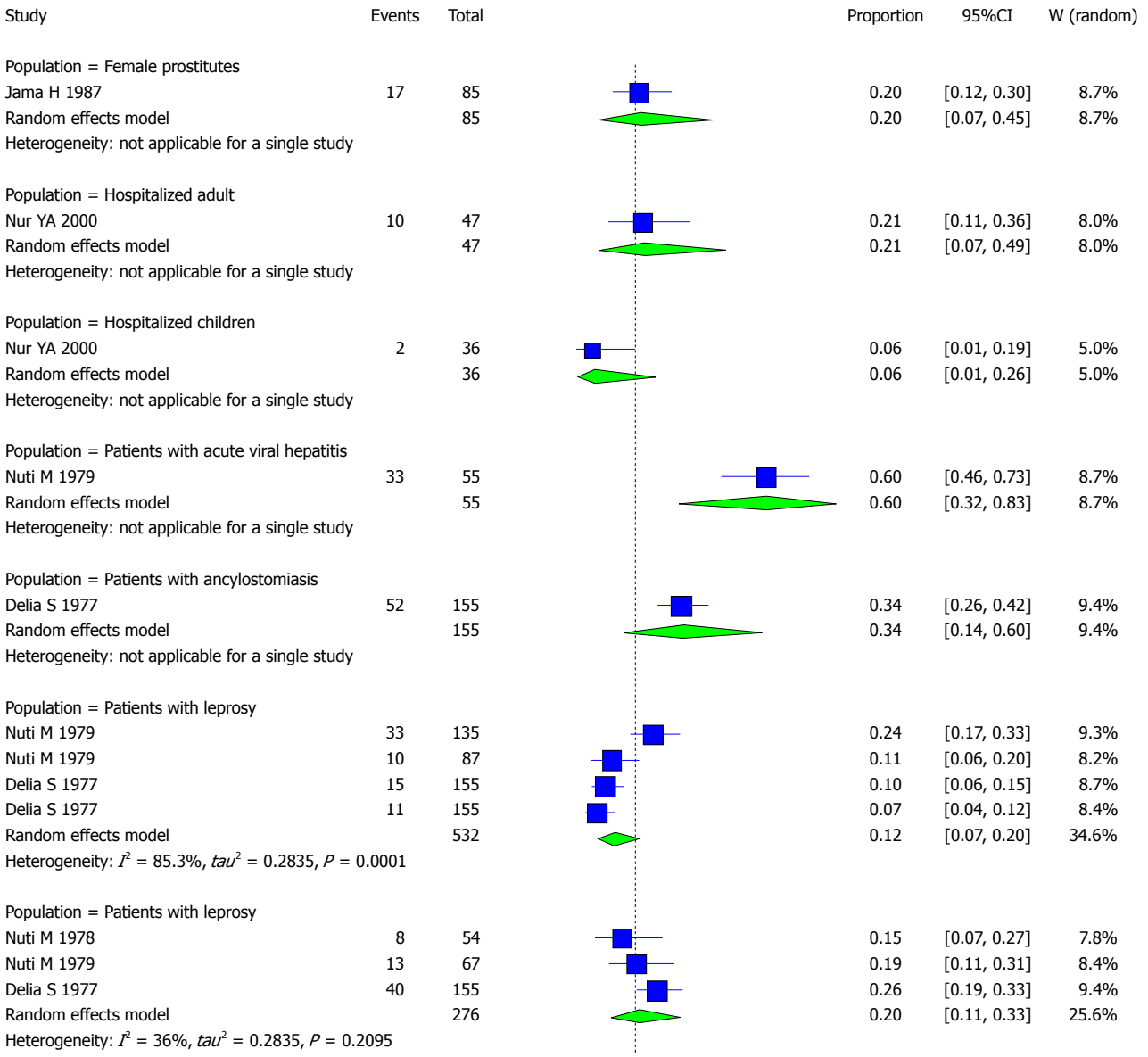


Figure 17 Forest plot of hepatitis B virus infection prevalence rates among risk groups.

Table 8 Risk groups and hepatitis B virus

Author	Year	Total	Cases	Healthy	Population
Nuti *et al*[24]	1979	135	33	102	Patients with leprosy
Nuti *et al*[24]	1979	87	10	77	Patients with leprosy
Nuti *et al*[23]	1978	54	8	46	Patients with schistosomiasis
Nuti *et al*[26]	1979	67	13	54	Patients with schistosomiasis
Delia *et al*[22]	1977	155	52	103	Patients with ancylostomiasis
Delia *et al*[22]	1977	155	40	115	Patients with schistosomiasis
Delia *et al*[22]	1977	155	15	140	Patients with leprosy
Delia *et al*[22]	1977	155	11	144	Patients with leprosy
Nuti *et al*[25]	1979	55	33	22	Patients with acute viral hepatitis
Jama *et al*[29]	1987	85	17	68	Female Prostitutes
Nur *et al*[33]	2000	36	2	34	Hospitalized children
Nur *et al*[33]	2000	47	10	37	Hospitalized adult

evidence of publication bias (Figure 20). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 8.14%, which was not widely different from the observed effect size. Furthermore, Egger's test for the assessment of symmetry of the funnel plot was not statistically significant (t = -1.6334, degrees of freedom = 10, P-value = 0.1334), providing further support for the absence of publication bias.

Sub-analysis according to country/setting

The majority of the studies (n = 8) were conducted in Somalia, with a pooled prevalence of HCV of 5.02% (95%CI: 2.18% to 11.13%) and high heterogeneity (I^2 = 94.9%). These findings are followed by 2 studies conducted in Italy, with a pooled prevalence of HCV of 1.22% (95%CI: 0.21% to 6.74%) and moderate heterogeneity (I^2 = 71.7%). There was 1 study from the United States with a prevalence of HCV of 9.13%

Table 9 Summary of studies on the overall prevalence of hepatitis C viral infection in a Somali population in Somalia and Somali immigrants (1970-2016)

	Year	Total	Cases	Total	Healthy	Setting	Population
Daw *et al*[43]	2016	2012	164	2012	1848	Libiya	Immigrants
Padovese *et al*[39]	2014	500	3	500	497	Italy	Immigrants
Kadle *et al*[38]	2012	156	30	156	126	Somalia	Local
Shire *et al*[36]	2012	854	78	854	776	United States	Immigrants
Khadjio *et al*[37]	2011	147	15	147	132	Somalia	Local
Nur *et al*[33]	2000	256	6	256	250	Somalia	Local
Faustini *et al*[19]	1994	213	5	213	208	Italy	Immigrants
Watts *et al*[41]	1994	438	8	430	90	Somalia	Local
Aceti *et al*[42]	1993	885	35	885	850	Somalia	Local
Bile *et al*[34]	1993	124	29	124	95	Somalia	Local
Bile *et al*[16]	1992	596	9	596	587	Somalia	Local
Bile *et al*[16]	1992	76	0	76	76	Somalia	Local

Table 10 Studies on hepatitis C virus among risk groups in Somalia

Author	Year	Total	Cases	Total	Healthy	Setting	Population
Aceti *et al*[42]	1993	287	0	287	278	Mogadishu	Hospitalized children with diseases other than hepatitis
Watts *et al*[41]	1994	236	4	236	232	Mogadishu, Marka, Kismayo	Female prostitutes
Nur *et al*[33]	2000	42	1	42	41	Mogadishu	Hospitalized children with measles, tuberculosis, anemia and other febrile illnesses
Watts *et al*[41]	1994	80	2	80	78	Mogadishu, Marka, Kismayo	Sexually transmitted disease patients
Nur *et al*[33]	2000	57	4	57	53	Mogadishu	Hospitalized adults with tuberculosis, malaria, acute respiratory infections, and unknown diagnosis (no clinically evident case of hepatitis)
Aceti *et al*[42]	1993	179	4	179	175	Mogadishu	Mixed populations (98 prisoners and 81 psychiatric patients)

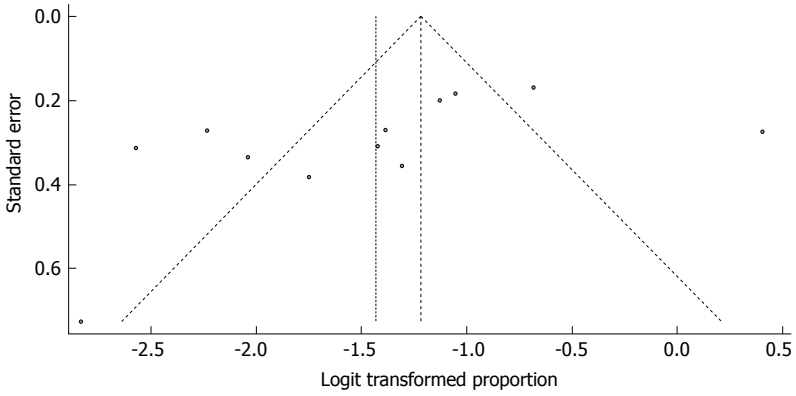


Figure 18 Bias assessment plot of studies reporting among risk groups.

(95%CI: 1.01% to 49.87%). There was 1 study from Libya with a prevalence of HCV of 8.15% (95%CI: 0.89% to 46.60%) (Figure 21).

Meta-regression analysis

Examining the effect of setting, insignificant variability could be explained by meta-regressing the meta-analysis over the three settings: Italy, Somalia, and the United States. Tests of moderators indicated the following: coefficient(s) 2,3: QM (df = 2) = 2.6557, *P* = 0.2651. For the prevalence results in Somalia compared to Italy, the *P* value was 0.1476, and the estimate was 1.4512. For the United States, the variability was not significantly different from that of Italy (*P* value=0.1560 and estimate = 2.0938).

Sub-analysis according to refugee status

The majority of the studies (*n* = 8) were conducted in Somalia among non-immigrant populations, with a pooled prevalence of HCV of 4.99% (95%CI: 2.11% to 11.36%) and high heterogeneity (I^2 = 94.9%). The remaining 4 studies were conducted in Italy, Libya and the United States among refugees, showing a pooled prevalence of HCV of 3.81% (95%CI: 1.54% to 9.13%) and high heterogeneity (I^2 = 90.3%) (Figure 22).

Meta-regression analysis

When examining the effect of refugee status type,

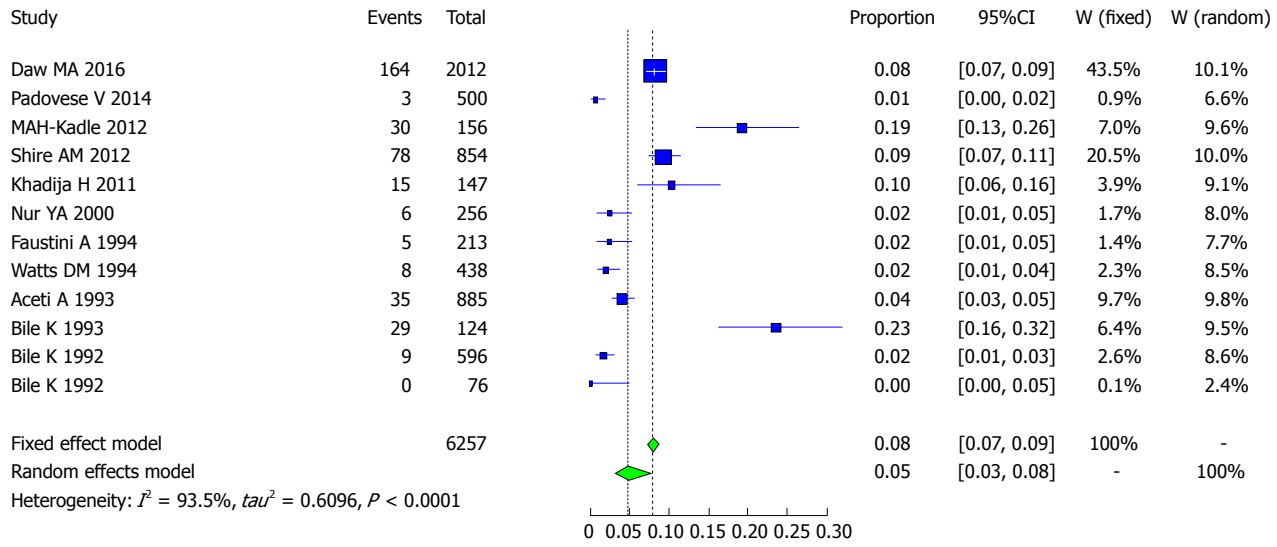


Figure 19 Forest plot of studies reporting chronic hepatitis C virus infection prevalence in Somalia.

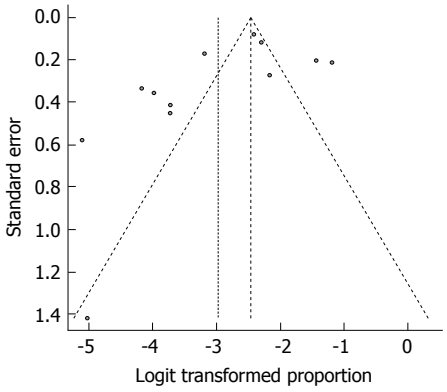


Figure 20 Funnel plot of studies reporting chronic hepatitis C virus infection prevalence in Somalia.

insignificant variability could be explained by meta-regressing the meta-analysis over the two statuses: immigrants *vs* the local population. Tests of moderators indicated the following: coefficient(s) 2: QM (df = 1) = 0.3335, P = 0.5636. For the prevalence in the local population compared to that in immigrants, the P value was 0.5636, and the estimate was 0.3383.

HCV infection in blood donors

Characteristics of the studies included: There are 2 studies that met the inclusion criteria for the meta-analysis of the prevalence of HCV infection among blood donors (Table 11). The included 2 studies examined the prevalence of HCV infection in a total of 466 Somali blood donors. The quality of the included studies also varied.

Pooled prevalence of HCV infection in blood donors

We used the extracted data of the 2 studies to quantify the overall pooled prevalence of HCV infection in Somali blood donors. The pooled effect size for the prevalence of HCV infection among Somali blood donors was 0.87% (95%CI: 0.33% to 2.30%). The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.13, P = 0.7139]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 23.

HCV infection in different genotypes

Characteristics of the studies included: Two studies met the inclusion criteria for the meta-analysis of the prevalence of HCV infection among different genotypes (Table 12)[36,43].

Pooled prevalence of HCV infection per genotype group: We used the extracted data of the 12 studies to quantify the overall pooled prevalence of HCV infection in each genotype group.

The pooled effect size (out of 2 studies) for the prevalence of HCV infection among Somali people of genotype 1 was 21.9% (95%CI: 15.36% to 30.23%). The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.20].

The pooled effect size (out of 2 studies) for the prevalence of HCV infection among Somali people of genotype 2 was 0.87% (95%CI: 0.12% to 5.9%). The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.10].

The pooled effect size (out of 2 studies) for the prevalence of HCV infection among Somali people of genotype 3 was 25.21% (95%CI: 18.23% to 33.77%). The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.02].

The pooled effect size (out of 2 studies) for the prevalence of HCV infection among Somali people of genotype 4 was 46.24% (95%CI: 37.48% to 55.25%).

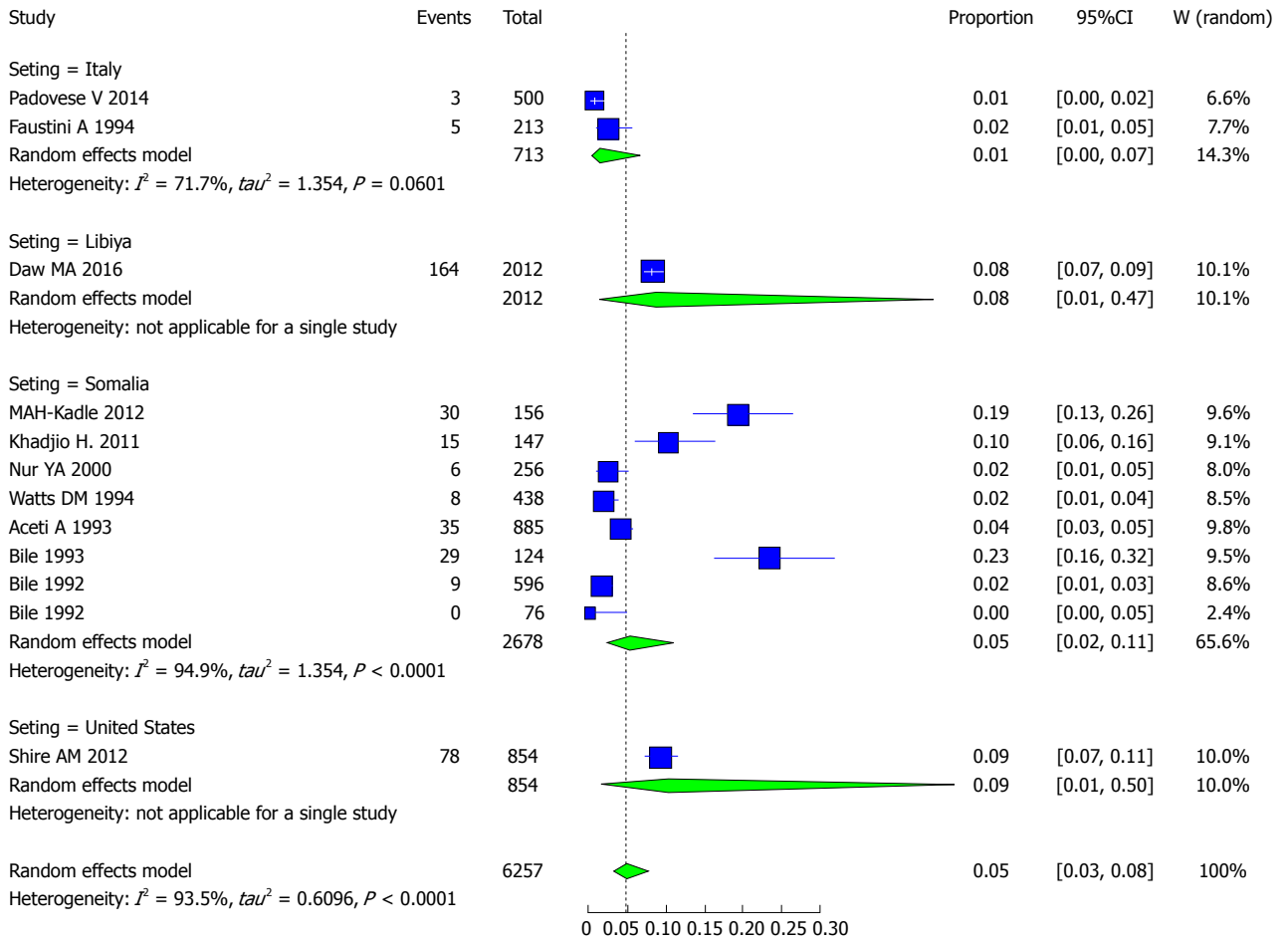


Figure 21 Forest plot of hepatitis C virus infection for studies conducted in Somalia and Outside of Somalia.

The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.57].

The pooled effect size (out of 2 studies) for the prevalence of HCV infection among Somali people of genotype 5 was 2.52% (95%CI: 0.82% to 7.53%). The heterogeneity was low (I^2 = 0%). Another indication of low heterogeneity was the Q-statistic [Q (degrees of freedom = 1) = 0.00].

The pooled effect size (out of 1 studies) for the prevalence of HCV infection among Somali people of genotype 6 was 1.19% (95%CI: 0.07% to 16.38%).

The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 24.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 25). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 25.84%, which was not widely different from the observed effect size.

Risk groups and HCV

Characteristics of the studies included: Three studies met the inclusion criteria for the meta-analysis of prevalence of HCV infection (Table 10). The 3 included studies examined the prevalence of HCV infection in the total of 881 Somali participants. The quality of the included studies also varied.

Pooled prevalence of HCV infection in risk groups: We used the extracted data of the 6 studies to quantify the overall pooled prevalence of HCV infection in Somali risk groups. The pooled effect size for the prevalence of HCV infection among Somali risk groups was 2.43% (95%CI: 1.21% to 4.8%). The heterogeneity was low (I^2 = 41.8%, 95%CI: 0% to 77%). Another indicator of low heterogeneity was the Q-statistic [Q (degrees of freedom = 5) = 8.6, P value = 0.1263]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 26.

As expected from the low heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 27). Notably, the total number of studies was small, and the individual studies were of variable sample size.

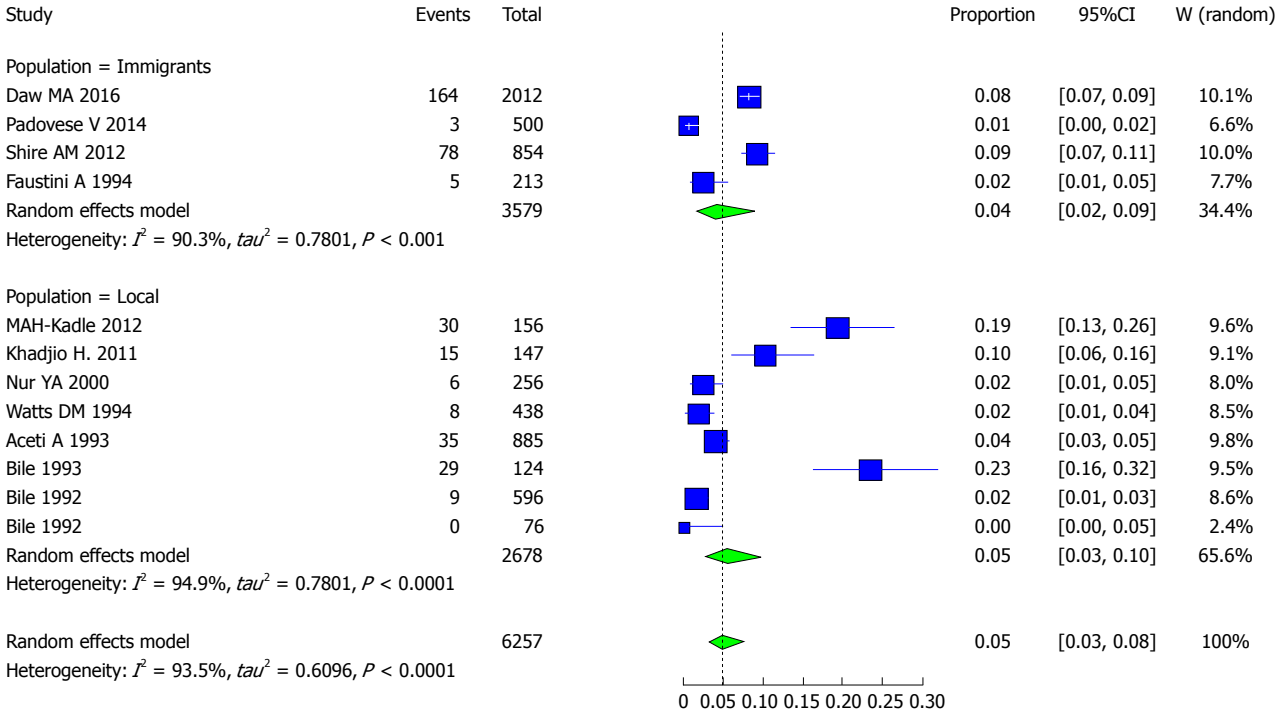


Figure 22 Forest plot of studies reporting chronic hepatitis C virus prevalence amongst the local population and Somali immigrants.

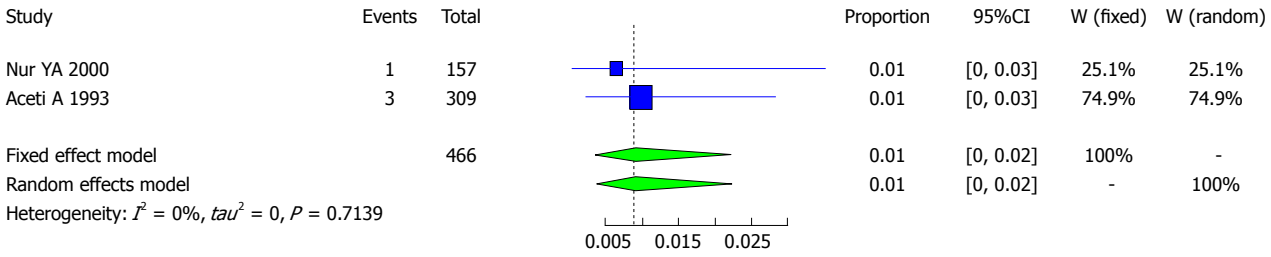


Figure 23 Forest plot of studies reporting chronic hepatitis C virus prevalence among blood donors in Somalia.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 3.36%, which was close to the observed effect size.

HCV infection in Somali children

Characteristics of the studies included: Three studies met the inclusion criteria for the meta-analysis of the prevalence of HCV infection (Table 13). The 3 included studies examined the prevalence of HCV infection in a total of 1001 Somali participants. The quality of the included studies also varied.

Pooled prevalence of HCV infection in children: We used the extracted data of the 4 studies to quantify the overall pooled prevalence of HCV infection in Somali children. The pooled effect size for the prevalence of HCV infection among Somali children was 1.37% (95%CI: 0.76% to 2.46%). The heterogeneity was low (I^2 = 0%, 95%CI: 0% to 83.8%). Another indicator of low heterogeneity was the Q-statistic [Q (degrees of freedom = 3) = 2.83, P value = 0.4181]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 28.

As expected from the low heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 29). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 1.63%, which was close to the observed effect size.

HCV infection in Somali chronic liver disease patients

Characteristics of the studies included: Five studies met the inclusion criteria for the meta-analysis of the prevalence of HCV infection (Table 14). The 5 included studies examined the prevalence of HCV infection in a total of 505 Somali participants. The quality of the

Table 11 Studies on hepatitis C among blood donors

Author	Year	Total	Cases	Healthy	Town	Group
Nur *et al*[33]	2000	157	1	156	Mogadishu	Blood donors
Aceti *et al*[42]	1993	309	3	306	Mogadishu	Blood donors

Table 12 Studies on distribution genotypes of hepatitis C virus infection in Somalia

Type of genotype	Author/Publication Year	Total	Cases	Healthy	Serology
Genotype 1	Daw *et al*[43] /2016	78	18	60	Anti-HCV
Genotype 1	Shire *et al*[36] / 2012	41	8	33	Anti-HCV
Genotype 2	Daw *et al*[43] /2016	78	0	78	Anti-HCV
Genotype 2	Shire *et al*[36] / 2012	41	0	41	Anti-HCV
Genotype 3	Daw *et al*[43] /2016	78	20	58	Anti-HCV
Genotype 3	Shire *et al*[36] / 2012	41	10	31	Anti-HCV
Genotype 4	Daw *et al*[43] /2016	78	38	40	Anti-HCV
Genotype 4	Shire *et al*[36] / 2012	41	17	24	Anti-HCV
Genotype 5	Daw *et al*[43] /2016	78	2	76	Anti-HCV
Genotype 5	Shire *et al*[36] / 2012	41	1	10	Anti-HCV
Genotype 6	Shire *et al*[36] / 2012	41	0	40	Anti-HCV

included studies also varied.

Pooled prevalence of HCV infection in chronic liver disease groups: We used the extracted data of the 6 studies to quantify the overall pooled prevalence of HCV infection in Somali chronic liver disease groups. The pooled effect size for the prevalence of HCV infection among Somali chronic liver disease groups was 29.82% (95%CI: 15.84% to 48.98%). The heterogeneity was high (I^2 = 92.3%, 95%CI: 85% to 96%). Another indicator of high heterogeneity was the Q-statistic [Q (degrees of freedom = 4) = 51.92, *P* value < 0.0001]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 30.

Despite the high heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 31). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 22.2%, which was close to the observed effect size.

Epidemiology of HDV infection

Hepatitis D, or delta hepatitis, is caused by the HDV, a unique RNA pathogen. It requires the HBV for its own replication and to infect as co-infection. HDV is transmitted by percutaneous or sexual contact with infected blood or blood products. The main vulnerable group consists of patients with chronic HBsAg infection who become super-infected with the virus. It is estimated that 5% of HBV-infected persons are also co-infected with HDV[1]. Aceti A and his colleagues conducted a study in 1989 showing that among 220 asymptomatic HBsAg-positive carriers, 16.80% (37) had the antibody for HDV[31]. In a study of the prevalence of HDV infection in patients with chronic liver disease in Somalia, 52 of the 104 patients studied were positive for HBsAg, and 26 (50%) had anti-delta antibodies[30]. In a 1993 study by Khalif Bile and his colleagues with 62 Somali patients with chronic liver disease, including hepatocellular carcinoma, 37.1% (23) of cases with chronic liver disease were positive for HBsAg, and 34.6% (8) of chronic liver disease patients had anti-HDV. Meanwhile, in the control group, 14.3% of those who were positive for HBsAg had anti-HDV[32]. Another study conducted among 67 HBsAg-positive patients showed that 30% (20) of these patients were anti-HDV-positive. Additionally, this study showed that 38.6% (17) of the 44 cases of chronic liver disease patients were HBsAg-positive[32]. One study examining the prevalence of HDV showed that 34.4% (10/29) of those who were HBsAg-positive and 39.1% (9/23) of chronic liver disease patients who were HBsAg-positive had antibodies for HDV[34]. Additionally, 0% of community immigrants from Somalia and neighboring countries who were positive for HBsAg were shown to have anti-HDV[19]. Five studies met the inclusion criteria for the meta-analysis of the prevalence of HDV infection, and the five included studies examined the prevalence of HDV infection in a total of 375 Somali participants. The quality of the included studies also varied (Table 15).

Pooled prevalence of HDV infection

We used the extracted data of the 5 studies to quantify the overall pooled prevalence of HDV infection. The pooled effect size for the prevalence of HDV infection among Somali people was 28.99% (95%CI: 16.38% to 45.96%). The heterogeneity was high (I^2 = 84.9%, 95%CI: 66% to 93%). Another indicator of high heterogeneity was the Q-statistic [Q (degrees of freedom = 4) = 26.4, *P* value < 0.001]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 32.

Despite the significant heterogeneity, the funnel plot displayed a symmetric spread of studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 33). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 28.99%, which was identical to the observed effect size.

Epidemiology of chronic liver disease and HDV infection

Three studies met the inclusion criteria for the meta-analysis of prevalence of chronic liver disease and HDV

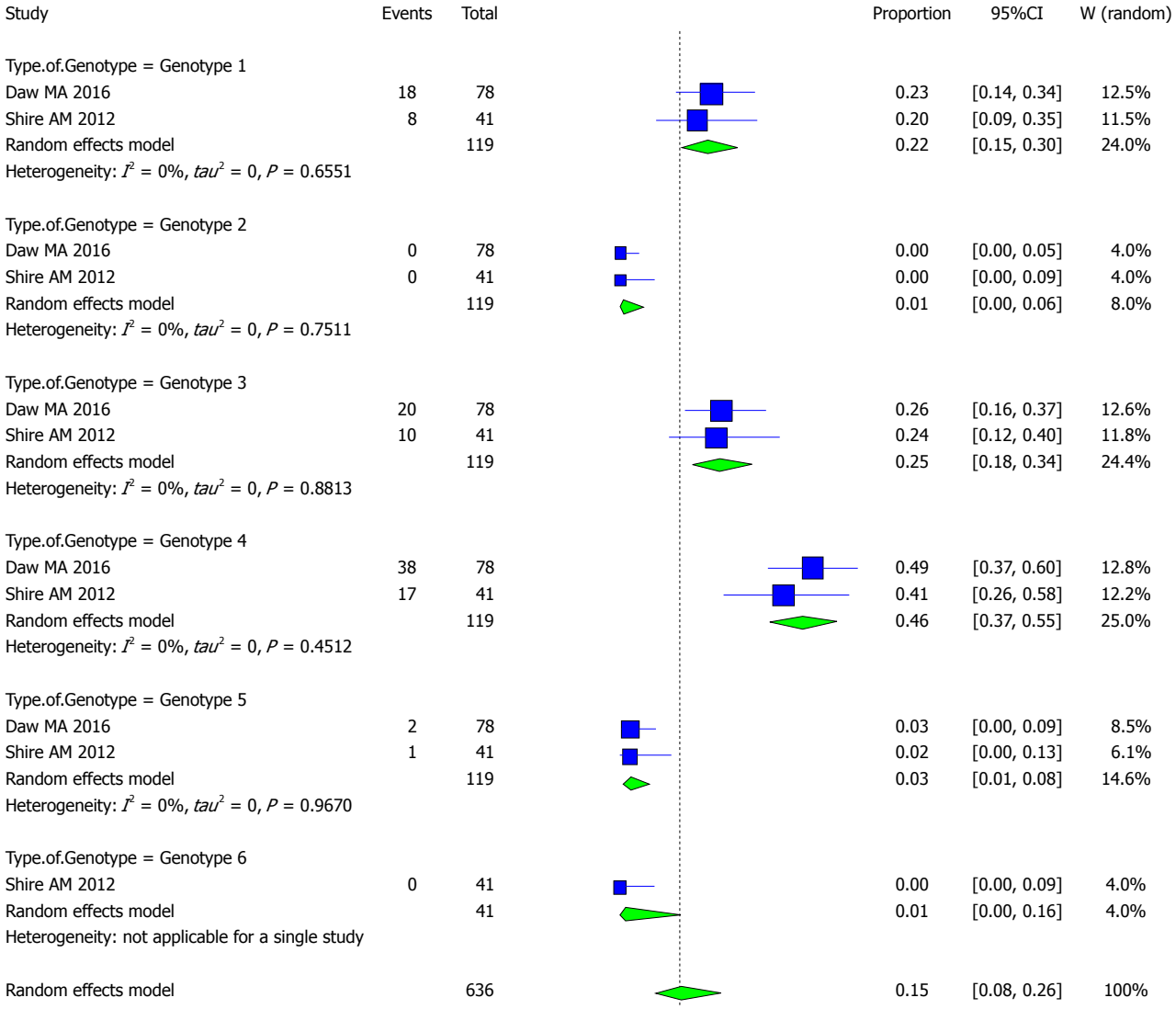


Figure 24 Forest plot of studies reporting on distribution genotypes of hepatitis C virus infection in Somalia.

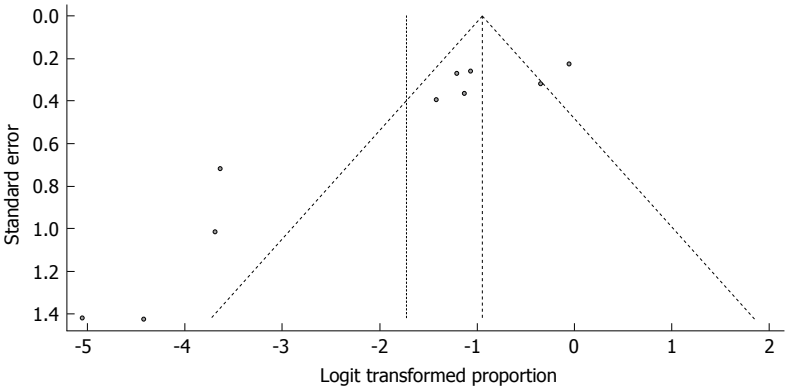


Figure 25 Bias assessment plot of studies reporting among the distribution of genotypes of hepatitis C virus.

infection. These three included studies had a total of 119 Somali participants, and the quality of the included studies varied (Table 16).

Pooled prevalence of chronic liver disease and HDV infection

We used the extracted data of the 3 studies to quantify the overall pooled prevalence of chronic liver disease and HDV infection. The pooled effect size for the prevalence of chronic liver disease and HDV infection among Somali people was 43.77% (95%CI: 35.09% to 52.84%). The heterogeneity was low but had a wide confidence interval (I^2 = 0%, 95%CI: 0% to 86%). Another indicator of low heterogeneity was the

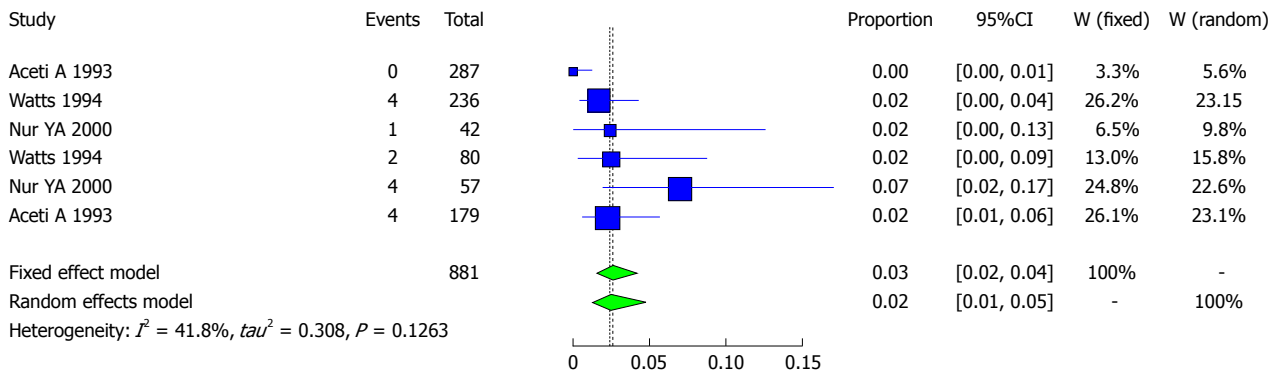


Figure 26 Forest plot of studies reporting chronic hepatitis C virus prevalence among risk groups in Somalia.

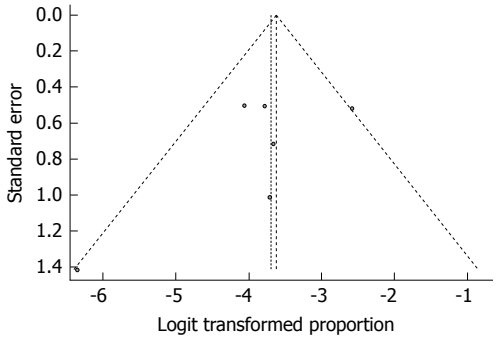


Figure 27 Bias assessment plot of studies reporting among risk groups in Somalia.

Q-statistic [Q (degrees of freedom = 2) = 1.49, *P* value = 0.4758]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 34.

The funnel plot displayed a symmetric spread of the three studies in terms of relative weight and effect size, thereby indicating little evidence of publication bias (Figure 35). Notably, the total number of studies was small, and the individual studies were of variable sample size.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did not support the possibility of missing studies from the analysis. The effect size imputed was 43.77%, which was identical to the observed effect size.

Epidemiology of HEV infection

It is believed that more than 50% of hepatitis E cases in developing countries are unrelated to HAV or HBV infection, and a high proportion of these cases seems to be enterically transmitted[44]. Studies on the outbreak of an acute viral hepatitis occurring in three villages in the lower Shabeli region of southern Somalia examined the presence of antibodies to the hepatitis E antigen, and it was found that the prevalence of anti-HEV ranged from 77.8% to 94.0% among the three villages and was widely distributed among all age groups, without a preponderance in any specific age. During the onset of the outbreak, 111 samples were collected in one of the three villages, which showed that a very low prevalence (4.5% or 5/111) was positive for IgG anti-HEV[45]. In another survey of 142 villages with a population of 245312 individuals, 11413 icteric cases were recorded, 346 of whom had died, corresponding to an attack rate and a case fatality rate of 4.6% and 3.0%, respectively. The role of HEV in this epidemic was proven by documenting anti-HEV in 88.2% (128 of 154) of the sampled cases, which served as a sign of recent infection with HEV. Further, the attack rate was found to be higher with increasing age, from 5% in the 1- to 4-year-old age group to 13% in the 5- to 15-year-old age group, and to 20% for persons older than 15 years of age. A high fatality rate of 13.8% was estimated among pregnant females. Therefore, the attack rate was higher (6.0%) in villages supplied with river water, while fewer cases were recorded in those relying on wells (1.7%) or ponds (1.2%) for their water supply[46]. Burans and his colleagues conducted a study during the operation of Restore of Hobe in Somalia in 1994, finding that among 31 Somalians with acute hepatitis, 20 (65%) had IgM anti-HEV[47]. Three studies met the inclusion criteria for the meta-analysis of the prevalence of HEV infection, and a total of 287 Somali participants were examined. The quality of included studies also varied (Table 17).

Pooled prevalence of HEV infection

We used the extracted data of the 3 studies to quantify the overall pooled prevalence of HEV infection. The pooled effect size for the prevalence of HEV infection among Somali people was 46.86% (95%CI: 5.31% to 93.28%). The heterogeneity was high (I^2 = 97.9%, 95%CI: 96% to 98.9%). Another indicator of high heterogeneity was the Q-statistic [Q (degrees of freedom = 2) = 93.4, *P* value < 0.001]. The result of this analysis is presented in the forest plot showing the effect sizes for the individual original studies and their 95%CIs, as shown in Figure 36.

Because the significant heterogeneity, the funnel plot displayed a somewhat asymmetric spread of studies in terms of relative weight and effect size, thereby indicating evidence of publication bias (Figure 37). Notably, the total number of studies was small, and

Table 13 Studies on hepatitis C virus infection among Somali children

Author	Year	Total	Cases	Total	Healthy	Setting	Population
Bile *et al*[16]	1992	596	9	596	587	Mogadishu area	Children in government-operated residences for abandoned children in Shebeli
Bile *et al*[16]	1992	76	0	76	76	Mogadishu area	Children in government-operated residence for abandoned children in SOS institution
Nur *et al*[33]	2000	42	1	42	41	Mogadishu	Hospitalized children
Aceti *et al*[42]	1993	287	0	287	278	Mogadishu	Hospitalized children with diseases other than hepatitis

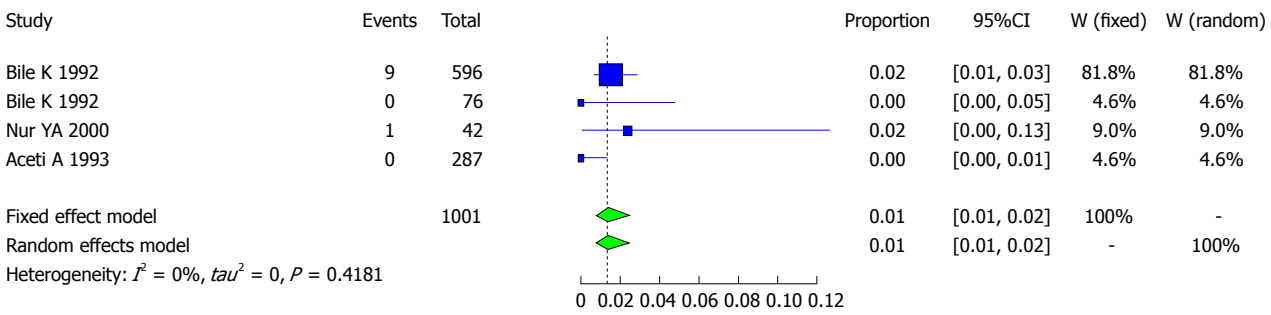


Figure 28 Forest plot of studies reporting chronic hepatitis C virus prevalence among Somali children.

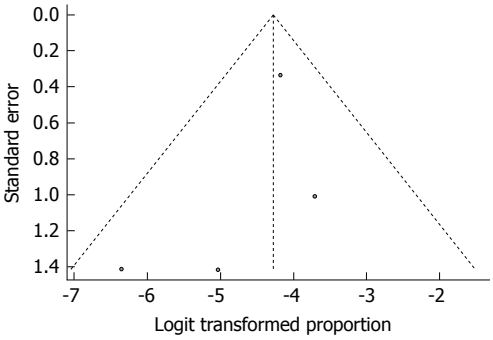


Figure 29 Bias assessment plot of studies reporting among children in Somalia.

the individual studies were of variable sample size. The study by Mushahwar *et al*[45] is clearly an outlier.

Moreover, Duval and Tweedie's trim and fill procedure for the detection of publication bias did support the possibility of missing studies from the analysis. The effect size imputed was 88.28%, which was well above the observed effect size. The funnel plot generated *via* Duval and Tweedie's trim and fill procedure indicated the absence of studies with a high prevalence if the balance was to be restored (Figure 38).

DISCUSSION

Viral hepatitis is major public health problem in the world, especially in developing countries. To our knowledge, this review is the first systematic review and meta-analysis study that has attempted to thoroughly summarize evidence on the different types of viral hepatitis infection in Somalia. The aim of this review was to understand the burden of viral hepatitis, especially HBV and HCV, in Somalia and to inform public health practitioners, researchers and policy makers in the development of national strategies for the prevention and control of viral hepatitis. The findings of this review clearly show the burden of the viral hepatitis in the country, particularly HBV. The estimation prevalence of HBV in most African countries was 5%-20%[48], and the WHO HBV endemic definition of HBsAg prevalence is 5%-7%[5]. In our review, we identified the prevalence of HBV in Somalia, as detected by HBsAg, to be high (18.9%). Somalia was categorized among countries with a high endemicity of HBV (prevalence $\geqslant$ 8%)[20,21]. The rate of global prevalence (3.61%) reported by Sweitzer and his colleagues, as well as the rate of 8.83% of the WHO African region, was significantly less than our study result[49]. In countries such the United States, Iran and Kosovo, the HBV prevalence rate has been estimated to be approximately < 0.27%, 2.14%, and 4.2%, respectively[50-52]. Therefore, this comparative information highlighted the extent of the HBV burden in Somalia. The pooled prevalence in this study was higher than that of a recent report from African countries, such as Ethiopia, Cameroon, Ghana, and Nigeria, the pooled prevalence of which was 7.4%, 11.2%, 12.3%, and 13.6%, respectively[48,53-55].

Additionally, HBV infection among blood donors (19.1%)[33] and the pooled prevalence of pregnant women (20.5%) also remain high in Somalia, which justifies the requirements of a national HBV screening program for all pregnant women in antenatal clinics and a national policy to vaccinate all pregnant women and adults who test negative for HBV, so as to prevent and reduce the risk of HBV of mother-to-child transmission. We also need to establish a national blood policy throughout Somalia for the prevention or reduction of receiving contaminated blood, the occurrence of which remains high within this HBV population. According to studies of HBV infection among children, the prevalence (5.7%) is lower than that of other groups, while that

Table 14 Studies on hepatitis C virus infection among patients with chronic liver disease, including hepatocellular carcinoma, in Somalia

Author	Year	Total	Cases	Total	Healthy	Setting	Population
Shire *et al*[36]	2012	30	22	30	8	United States	Immigrants
Kadle *et al*[38]	2012	156	30	156	126	Somalia	Local
Khadija *et al*[37]	2011	147	15	147	132	Somalia	Local
Bile *et al*[34]	1993	62	25	62	37	Somalia	Local
Aceti *et al*[42]	1993	110	28	110	82	Somalia	Local

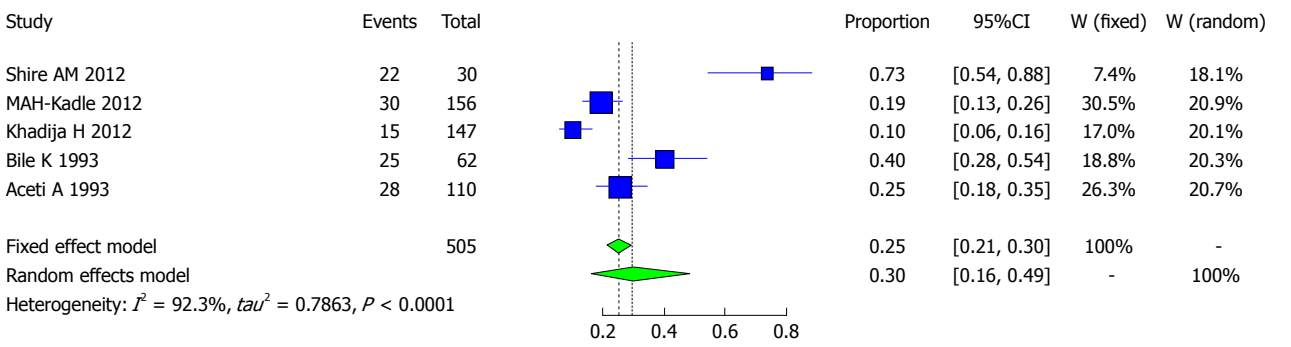


Figure 30 Forest plot of studies reporting chronic hepatitis C virus prevalence among patients with chronic liver disease, including hepatocellular carcinoma, in Somalia.

Table 15 Summary of studies on overall prevalence of hepatitis D viral infection of HBsAg-positive carriers in the Somali population in Somalia and Somali immigrants (1970-2016) *n* (%)

Author	Year	Total	Hepatitis D virus	Healthy	Setting	Population
Aceti *et al*[31]	1989	220	37 (16.8)	138	Somalia	Local
Bile *et al*[32]	1991	67	20 (30)	47	Somalia	Local
Aceti *et al*[30]	1991	52	26 (50)	26	Somalia	Local
Bile *et al*[34]	1993	29	10 (34.4)	19	Somalia	Local
Faustini *et al*[19]	1994	7	0 (0)	7	Italy	Immigrants
Total		375	93 (24.8)	237		

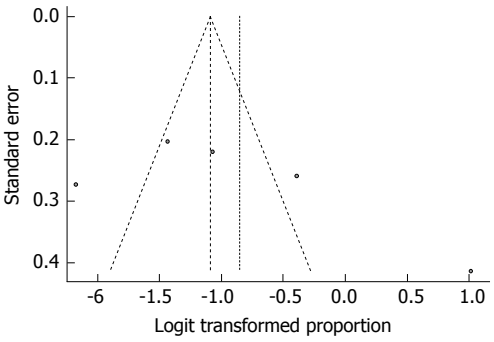


Figure 31 Bias assessment plot of studies reporting chronic liver disease.

of Somali immigrants around the world and of chronic liver disease patients was 23% and 39.2%, respectively. These findings therefore demonstrate the true epidemiological burden of HBV in Somalia.

Regarding HCV, we found a high pooled prevalence of 4.84% in Somalia. This prevalence rate is considerably higher than the reported pooled prevalence in Somalia of 0.9% by Chaabna *et al*[56] and 1.5% by Karoney *et al*[57]. Meanwhile, in Djibouti and Sudan, the pooled prevalence rate was 0.3% and 1.0%, respectively[56]. We also found close prevalence rates in our study and found that the pooled prevalence rate in Ethiopia, Ghana, and the Democratic Republic of the Congo was 3.1%[48], 3.0%[58] and 2.9%[59], respectively, but the overall prevalence rate is 3.0% in all sub-Saharan African countries[60]. However, in Cameroon, the estimated prevalence of anti-HCV is 11.6%[61] and the reported prevalence rate for Egypt is 14.7%[61,62]; thus, the estimated prevalence of anti-HCV is much lower in Somalia than in these two countries. The pooled prevalence rate of sub-groups examined in this study, such as blood donors, Somali immigrants, risk groups, children and patients with chronic liver disease, was 0.8%, 3.81%, 2.43%, 1.37%,and 29.82%, respectively. These findings may emphasize that the level of chronic HCV in Somalia may be significantly high. Genotypes commonly found in Africa are 1, 4 and 5[57]. Therefore, in this review, we also present the most frequent genotype among the Somali population, which is genotype 4. Most African countries, such as Uganda, Sudan, Rwanda, Burundi, Cameroon and Egypt, also show a predominance of HCV genotype 4[57]. The next most prevalent genotype in our country is genotype 3, which is also similar to our neighboring countries, such as Ethiopia, Kenya, and Eritrea[57].Meanwhile, the next most prevalent genotype in Somalia is genotype 1.

The number of studies of HDV in Somalia among Somali patients and chronic liver disease patients was very low, but the data available thus far demonstrated a considerable prevalence of HDV infection. Our results

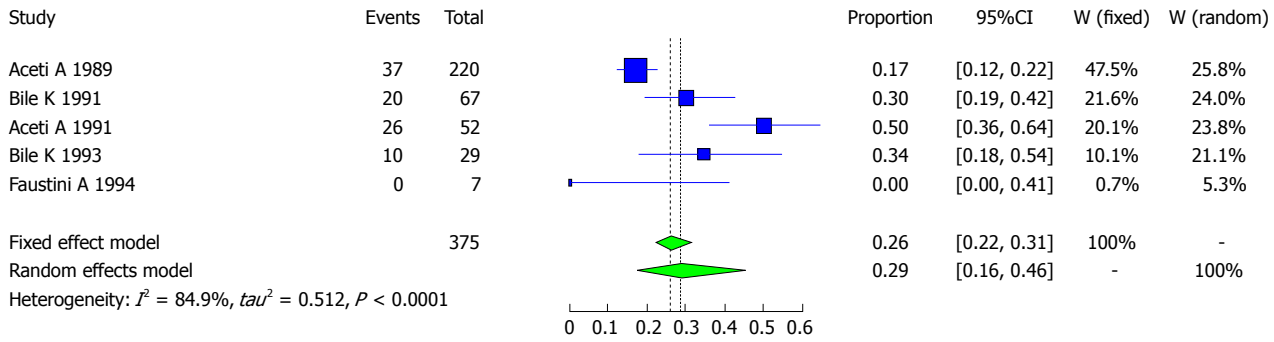


Figure 32 Forest plot of studies reporting hepatitis D virus infection prevalence in Somalia.

Table 16 Studies among patients with chronic liver disease (including hepatocellular carcinoma) in Somalia *n* (%)

Author	Year	Total	Hepatitis D virus	Total	Healthy	Setting	Population
Bile *et al*[34]	1993	23	9 (39.1)	23	14	Somalia	Local
Bile *et al*[32]	1991	44	17 (38.6)	44	27	Somalia	Local
Aceti *et al*[30]	1991	52	26 (50)	52	26	Somalia	Local
Total		119	52 (43.6)	119	67		

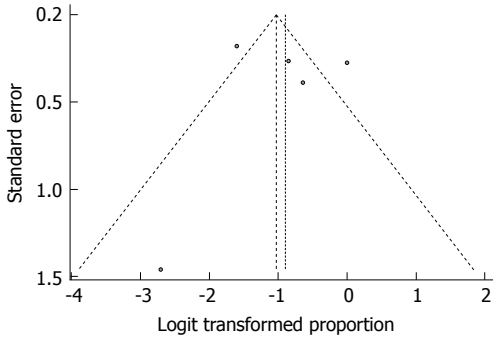


Figure 33 Bias assessment plot of studies reporting of hepatitis D virus infection in Somalia.

showed that the pooled prevalence rate of HDV in Somalia was 28.99%. The pooled prevalence of Sudan and Uganda, which are other sub-Saharan African countries, was 27.8%[63] and 30.6%[63], respectively, and the pooled prevalence rate of the EMRO region is 14.74%[64]. Therefore, our result is similar to that of other countries in our region. The worldwide estimation rate also indicated that 5% of HBsAg carriers were infected with HDV[1]. The pooled prevalence rate in this review for patients with chronic liver disease, including hepatocellular carcinoma and positivity for anti-HDV, was shown to be 43.77%, and this result is similar to the prevalence rates among patients in the EMRO region with chronic liver disease (chronic hepatitis disease 27.8% and cirrhosis/hepatocellular carcinoma 36.57%)[64]. However, the previous pooled estimation in Somalia showed a similar prevalence rate as chronic hepatitis disease (47.36%) and cirrhosis/hepatocellular carcinoma (33.20%)[64]. Other countries in the EMRO region also showed results similar to our findings. For example, the prevalence of chronic hepatitis disease and cirrhosis/hepatocellular carcinoma in Pakistan was 37.38% and 53.77%, respectively, and the prevalence of the same conditions in Egypt was 24.37% and 29.6%, respectively[64]. These results indicate that HDV infection is endemic in Somalia.

Although the number of prevalence studies of hepatitis A and E virus infections among Somali patients were also very low in Somalia due to the lack of government support in the country in the last three decades, the available data for HAV and HEV still illustrated a high prevalence rate in the country. These viruses were transmitted through the fecal-oral route, and many environmental and socio-economic factors promoted the transmission routes. This systematic review showed a high pooled prevalence rate of HAV, which is 90.2%, and this prevalence rate was close to the individual estimation rates of old reports in Somalia[15,16,18,19]. We also indicate that the estimates of HEV infection in previous reports in Somalia may quite high, ranging between 4.5% and 88.2%[45-47], which is similar to estimates for the Ghanaian population of anti-HEV, with their range of 5.8% and 71.55%[65]. However, this review showed a pooled prevalence rate of HEV of 46.86%, which is also high among Somali patients. HEV infection in Africa is widespread and is predicted to be a hazard to numerous lives, in particular for pregnant women and their fetuses[2]. Serological studies from Egypt have shown that the seroprevalence of anti-HEV can reach close to 100% in the general population[2]. Another serological study from Ghana showed that anti-HEV can also reach close to 50%, and a study in Nigeria showed nearly100% of anti-HEV in the general population[2]. Meanwhile, a number of large outbreaks occurred and were reported at different times in many countries in Africa, such as Kenya, Sudan, Ethiopia, Somalia, Uganda and Chad[2,45]. The outbreak that occurred in Somalia showed a range of 77.8% to 94.0% among three villages in the country[45]. A survey from 142 villages showed 11413 jaundice patients in a population of 245312 individuals, of whom 346 persons died, with a corresponding attack rate of 4.6% and a case fatality rate of 3.0%[46]. An older report demonstrated that the attack rate was higher with increasing age, from 5%, 13% and 20% for the

Table 17 Studies on hepatitis E virus in Somalia *n* (%)

Author	Year	Total	Hepatitis E virus	Healthy	Setting	Population
Burans *et al*[47]	1994	31	20 (64.5)	11	Somalia	Local
Mushahwar *et al*[45]	1993	111	5 (4.5)	106	Somalia	Local
Bile *et al*[46]	1994	145	128 (88.2)	17	Somalia	Local
Total		287	153 (53.3)	134		

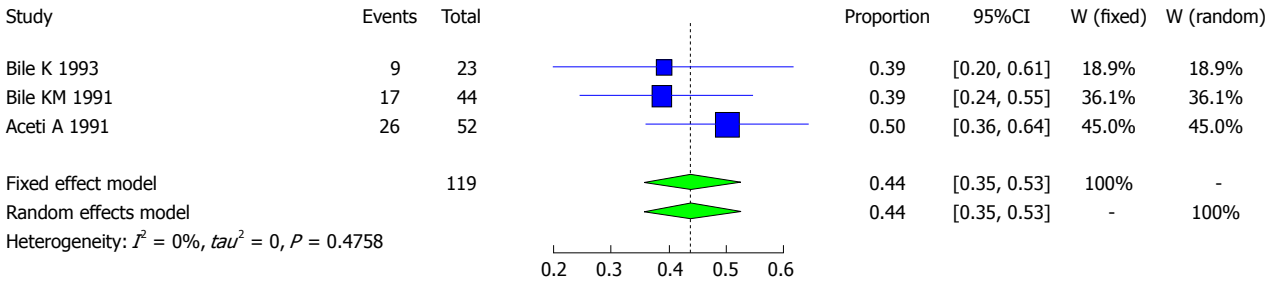


Figure 34 Forest plot of hepatitis D virus infection prevalence rates among patients with chronic liver disease in Somalia.

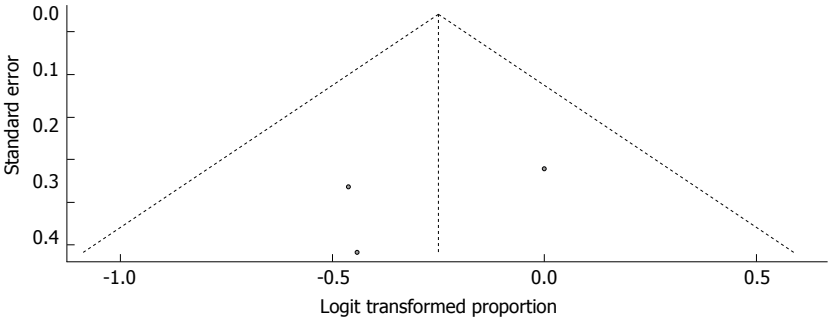


Figure 35 Bias assessment plot of studies reporting of hepatitis D virus infection among chronic liver disease in Somalia.

age groups of 1-4 years, 5-15 years and above 15 years old, respectively. Meanwhile, pregnant women had a high fatality rate, estimated to be 13.8%[46], in comparison to the rates in other African countries, such Ghana, (66.7%)[65], Sudan (31.1%)[66] and the Central African Republic (14.3%)[67]. However, the fatality rate of pregnant women was higher than that of our older report in the country[46]. This systematic review and meta-analysis study indicates that the two viruses of HAV and HEV are endemic in the country.

This systematic review and meta-analysis study had several limitations. The main limitation is that to the best of our knowledge, this review is the first that focuses on all types of viral hepatitis infection in Somalia. The second limitation of this review is the relatively small number of studies identified on all types of viral hepatitis in the country before and after the civil war. This lack of studies highlights the fact that research and knowledge of the viral hepatitis infection burden in Somalia are less developed than in other African and Arab countries. The third limitation is that the majority of our studies involved samples from only four or five regions of Somalia, especially in South Somalia, and samples of Somali immigrants around the world. Subsequently, we did not have samples from the southwestern, central, and northern regions of the country, such as Gedo, Bakol, Hiran, Galgadud, Mudug, Nugal, East, Sol, Sanag, Togder, Northwest and Awdal regions, which were included in the studies reviewed, while most studies were completed before the government collapsed, and few studies were done during and after the civil war.

In conclusions, although this systematic review and meta-analysis study is based on a limited number of studies and a small sample size, the study effects were a potential limitation of the review. However, the results of this review indicate that all types of viral hepatitis infection are common among Somalis, particularly HBV, which is more prevalent and endemic. This study has also documented a high prevalence rate of HCV infection in Somalia. The prevalence of these viruses could be understood as one of the nation's public health problems, and urgent public health interventions are needed to reduce the infection rate of the country, because these preventable diseases, particularly HBV and HAV, can be eradicated by vaccination. Preventive measures include increasing awareness and knowledge about the transmission of all forms of viral hepatitis, screening all donated blood, targeting high-risk groups, providing treatment for affected persons, and generally improving

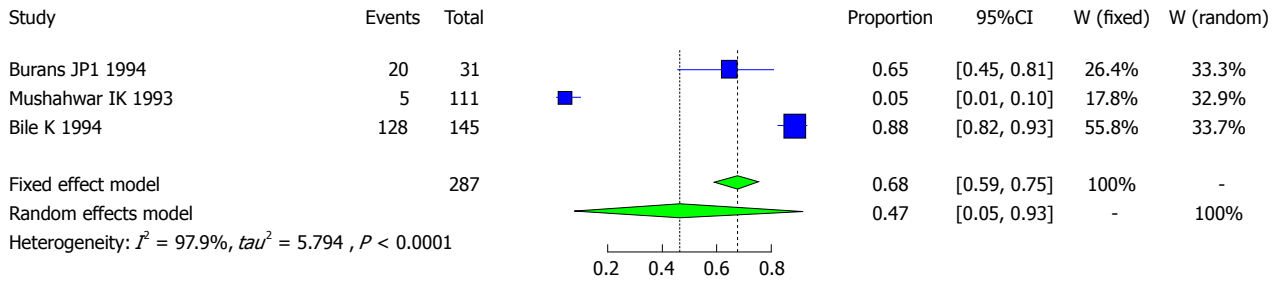


Figure 36 Forest plot of studies reporting hepatitis E virus infection prevalence in Somalia.

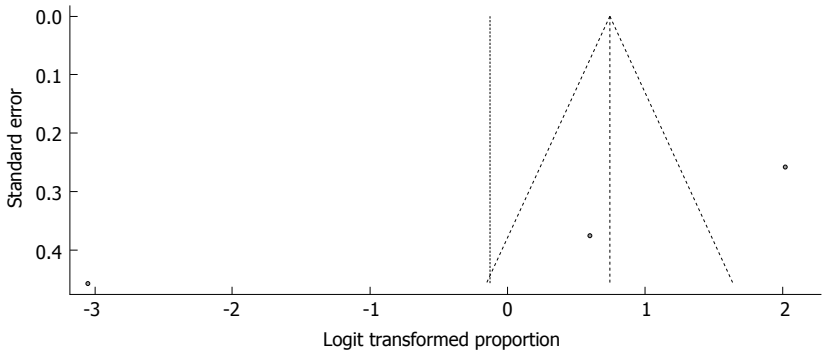


Figure 37 Funnel plot of studies reporting hepatitis E virus infection prevalence in Somalia.

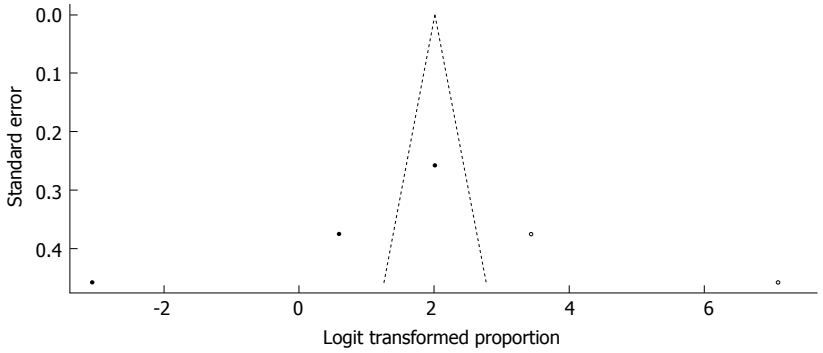


Figure 38 Bias assessment plot of studies reporting hepatitis E virus prevalence in Somalia.

the sanitary and living conditions. Other efforts needed to reduce the burden of the disease and strengthen the Somali health system include the establishment of a national blood policy and prevention and control viral hepatitis policy. Additionally, further studies are needed to fully understand the population factors underlying the high prevalence of all forms viral hepatitis, particularly in underrepresented regions and among adults, children, blood donors and high-risk groups, to offer better perspectives on all forms of the viral hepatitis burden in Somalia. The generation of up-to-date data is recommended, especially regarding the magnitude of HAV, HCV, and their genotypes, HDV and HEV.

ARTICLE HIGHLIGHTS

Research background

Viral hepatitis is a major public health problem affecting several hundred million people globally. The most common types of viral hepatitis are six distinct types (hepatitis A, B, C, D, E, G viruses), and they may present in acute form or chronic form causes substantial morbidity and mortality (including chronic hepatitis, cirrhosis and hepatocellular carcinoma).

Research motivation

In the field of viral hepatitis, there is a lack of researches last three decades in country and all articles about viral hepatitis published and unpublished are scattered. In this systematic review and meta-analysis the authors aim to provide a clear understanding of viral hepatitis epidemiology and their clinical burdens in Somalia according to the related documents published and unpublished articles during the last decades.

Research objectives

The main objective of this study is to determine the prevalence of all viral hepatitis in Somalia especially hepatitis B virus (HBV) and hepatitis C virus (HCV), and to inform public health practitioners, researchers and policy makers, and to be a baseline data for future Hepatology researches in the country.

Research methods

A systematic review and meta-analysis was conducted as Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. A comprehensive

literature search of published studies on viral hepatitis was performed from 1977-2016 in PubMed, Google Scholar, Science Direct, World Health Organization African Index Medicus and the Africa Journals Online databases, as well as on the Ministry of Health website. We also captured unpublished articles that were not available on online systems.

Research results

Twenty-nine studies from Somalia and Somali immigrants (United Kingdom, United States, Italy, Libya) with a combined sample size for each type of viral hepatitis were analyzed. The overall pooled prevalence rate of hepatitis A virus was 90.2%. The overall pooled prevalence of HBV was 18.9%. The overall pooled prevalence of HCV was estimated as 4.84%. The overall pooled prevalence of hepatitis D virus was 28.99%. The overall pooled prevalence of hepatitis E virus was 46.86%.

Research conclusions

This study demonstrates a high prevalence of all forms of viral hepatitis in Somalia and it also indicates that chronic HBV was the commonest cause of chronic liver disease.

Research perspectives

Viral hepatitis in Somalia demonstrated a high rate in its all types of hepatitis especially hepatitis B virus and C, while hepatitis B determined the common cause of chronic liver disease in Somalia. According to this systematic review and meta-analysis, further studies are needed in order to search out data about viral hepatitis in different regions of the country, risk factors and its complications.

REFERENCES

1 **World Health Organization.** Global Hepatitis Report 2017 Accessed March 16, 2018. Available from: URL: apps.who.int/iris/bitstream/10665/255016/1/9789241565455-eng.pdf
2 **Kim JH**, Nelson KE, Panzner U, Kasture Y, Labrique AB, Wierzba TF. A systematic review of the epidemiology of hepatitis E virus in Africa. *BMC Infect Dis* 2014; **14**: 308 [PMID: 24902967 DOI: 10.1186/1471-2334-14-308]
3 **Jacobsen KH**, Wiersma ST. Hepatitis A virus seroprevalence by age and world region, 1990 and 2005. *Vaccine* 2010; **28**: 6653-6657 [PMID: 20723630 DOI: 10.1016/j.vaccine.2010.08.037]
4 **Hassan-Kadle MA.** The Diagnostic Challenges, Possible Etiologies and Lack of Researches of Hepatocellular Carcinoma in Somalia. *Open J Gastroenterol* 2017; **7**: 115 [DOI: 10.4236/ojgas.2017.73013]
5 **World Health Organization**. Global policy report on the prevention and control of viral hepatitis, 2013. Accessed March 16, 2018. Available from: URL: apps.who.int/iris/bitstream/10665/85397/1/9789241564632_eng.pdf
6 **Central Intelligence Agency.** The world fact book. Accessed March 16, 2018. Available from: URL: https://www.cia.gov/library/publications/the-world-factbook/geos/so.html
7 **World Bank.** Somalia overview. Accessed March 16, 2018. Available from: URL: http://www.worldbank.org/en/country/somalia/overview
8 **African Development Bank Group.** Somalia Country Brief 2013-2015. Accessed March 16, 2018. Available from: URL: http://www.afdb.org/fileadmin/uploads/afdb/Documents/Project-and-Operations/2013-2015%20-%20Somalia%20-%20Country%20Brief.pdf
9 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Reprint--preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Phys Ther* 2009; **89**: 873-880 [PMID: 19723669 DOI: 10.1016/j.jclinepi.2009.06.005]
10 **Viechtbauer W**. Package 'metafor'. Meta-Analysis Package for R. Accessed March 16, 2018. Available from: URL: https://cran.r-project.org/web/packages/metafor/metafor.pdf
11 **Schwarzer G.** Package 'meta'. General Package for Meta-Analysis. Accessed March 16, 2018. Available from: URL: https://cran.r-project.org/web/packages/meta/meta.pdf
12 **Normand SL**. Meta-analysis: formulating, evaluating, combining, and reporting. *Stat Med* 1999; **18**: 321-359 [PMID: 10070677 DOI: 10.1002/(SICI)1097-0258(19990215)18:3<321::AID-SIM28>3.0.CO;2-P]
13 **Duval S**, Tweedie R. Trim and fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* 2000; **56**: 455-463 [PMID: 10877304 DOI: 10.1111/j.0006-341X.2000.00455.x]
14 **Egger M**, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997; **315**: 629-634 [PMID: 9310563 DOI: 10.1136/bmj.315.7109.629]
15 **Mohamud KB**, Aceti A, Mohamed OM, Pennica A, Maalin KA, Biondi R, Hagi MA, Quaranta G, Paparo SB, Celestino D. [The circulation of the hepatitis A and B viruses in the Somali population]. *Ann Ital Med Int* 1992; **7**: 78-83 [PMID: 1334688]
16 **Bile K**, Mohamud O, Aden C, Isse A, Norder H, Nilsson L, Magnius L. The risk for hepatitis A, B, and C at two institutions for children in Somalia with different socioeconomic conditions. *Am J Trop Med Hyg* 1992; **47**: 357-364 [PMID: 1524149 DOI: 10.4269/ajtmh.1992.47.357]
17 **Bile K**, Aceti A, Hagi MA, Paparo BS, Pennica A, Celestino D, Sebastiani A. Primary hepatitis A virus infection in developing countries: decline of maternal antibodies and time of infection. *Boll Ist Sieroter Milan* 1986; **65**: 464-466 [PMID: 3034305]
18 **Sebastiani A,** Aceti A, Russo V, Paparo BS, Pennica A, Bile K, Farah AA, Farah AH. Age-specific prevalence of hepatitis A and hepatitis B virus infection in fluvial Somalian village. *Estratto dal* 1984; **30**: 17-23
19 **Faustini A**, Franco E, Saitto C, Cauletti M, Zaratti L, Papini P, Ali' Ahmed S, Zampieri F, Ierussi A, Panà A. Hepatitis A, B, C and D in a community in Italy of immigrants from NE Africa. *J Public Health Med* 1994; **16**: 71-78 [PMID: 8037956 DOI: 10.1093/oxfordjournals.pubmed.a042938]
20 **Averhoff F**. Hepatitis B. Accessed March 16, 2018. Available from: URL: http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/hepatitis-b
21 **Howell J,** Ladep NG, Lemoin M, Thursz MR, Taylor-Robinson SD. Hepatitis B in Sub-Saharan Africa. *South Sudan Medical J* 2014; **7**: 59-61
22 **Delia S**, Nuti M, Soro S. [Relations between hepatitis B surface antigen and parasitic diseases: observations in patients with ancylostomiasis and schistosomiasis]. *Quad Sclavo Diagn* 1977; **13**: 238-243 [PMID: 594309]
23 **Nuti M**, Abdullhai SE, Thamer G. [E antigen (HBeAG) and surface antigen (HBsAg) in bladder schistosomiasis]. *Parassitologia* 1978; **20**: 153-159 [PMID: 553266]
24 **Nuti M**, Tarabini G, Palermo P, Tarabini GL, Thamer G. Leprosy and hepatitis B virus markers: incidence of HBsAg and HBeAg in Somalian patients. *Int J Lepr Other Mycobact Dis* 1979; **47**: 580-584 [PMID: 122628]
25 **Nuti M**, Harare O, Thamer G. The surface antigen (HBsAg) and the e-antigen (HBeAg) in Somalian patients with acute viral hepatitis. *Trans R Soc Trop Med Hyg* 1979; **73**: 185-187 [PMID: 473307 DOI: 10.1016/0035-9203(79)90207-4]
26 **Nuti M**, Abdullahi Elmi S, Alario C. [Diffusion of hepatitis B surface antigens in subjects with bladder schistosomiasis]. *Boll Ist Sieroter Milan* 1979; **58**: 220-223 [PMID: 518754]
27 **Sebastiani A**, Aceti A, Paparo BS, Pennica A, Ilardi I, Bile K, Mohamud OM. Hepatitis B virus circulation in three different villages of Somalia. *Trans R Soc Trop Med Hyg* 1985; **79**: 162-164 [PMID: 4002285 DOI: 10.1016/0035-9203(85)90323-2]
28 **Bile KM**, Aden A, Lindberg G, Nilsson L, Lidman K, Norder H, Magnius L. Epidemiology of hepatitis B in Somalia: inference from a cross-sectional survey of serological markers. *Trans R Soc Trop Med Hyg* 1987; **81**: 824-828 [PMID: 3502433 DOI: 10.1016/0035-9203(87)90043-5]
29 **Kolmos HJ**, Zimakoff J. [DASPID--an interdisciplinary Danish study group for the elucidation of peritonitis among peritoneal dialysis patients]. *Ugeskr Laeger* 1991; **153**: 1068 [PMID: 2024336]

30 **Aceti A**, Mohamed OM, Paparo BS, Mohamud OM, Quaranta G, Maalin KA, Sebastiani A. High prevalence of anti-hepatitis delta virus antibody in chronic liver disease in Somalia. *Trans R Soc Trop Med Hyg* 1991; **85**: 541-542 [PMID: 1755067 DOI: 10.1016/0035-9203(91)90249-X]

31 **Aceti A**, Paparo BS, Celestino D, Pennica A, Caferro M, Grilli A, Sebastiani A, Mohamud OM, Abdirahman M, Bile K. Sero-epidemiology of hepatitis delta virus infection in Somalia. *Trans R Soc Trop Med Hyg* 1989; **83**: 399-400 [PMID: 2617589 DOI: 10.1016/0035-9203(89)90516-6]

32 **Bile KM**, Abdirahman M, Aden C, Norder H, Magnius L, Lindberg G, Nilsson LH. Minor role of hepatitis B virus in the causation of chronic liver disease in Somalia indicated by a case-control study. *Trans R Soc Trop Med Hyg* 1991; **85**: 104-108 [PMID: 1712517 DOI: 10.1016/0035-9203(91)90177-Z]

33 **Nur YA**, Groen J, Elmi AM, Ott A, Osterhaus AD. Prevalence of serum antibodies against bloodborne and sexually transmitted agents in selected groups in Somalia. *Epidemiol Infect* 2000; **124**: 137-141 [PMID: 10722141 DOI: 10.1017/S0950268899003441]

34 **Bile K**, Aden C, Norder H, Magnius L, Lindberg G, Nilsson L. Important role of hepatitis C virus infection as a cause of chronic liver disease in Somalia. *Scand J Infect Dis* 1993; **25**: 559-564 [PMID: 7506842 DOI: 10.3109/00365549309008543]

35 **Aweis D**, Brabin BJ, Beeching NJ, Bunn JE, Cooper C, Gardner K, Iriyagolle C, Hart CA. Hepatitis B prevalence and risk factors for HBsAg carriage amongst Somali households in Liverpool. *Commun Dis Public Health* 2001; **4**: 247-252 [PMID: 12109390]

36 **Shire AM**, Sandhu DS, Kaiya JK, Oseini AM, Yang JD, Chaiteerakij R, Mettler TA, Giama NH, Roberts RO, Therneau TM, Petersen GM, Knutson KL, Roberts LR. Viral hepatitis among Somali immigrants in Minnesota: association of hepatitis C with hepatocellular carcinoma. *Mayo Clin Proc* 2012; **87**: 17-24 [PMID: 22212964 DOI: 10.1016/j.mayocp.2011.08.001]

37 **Olow KH,** Warsame FS. Prevalence of Liver Cirrhosis Post - Hepatitis B and C Among Adults in Mogadishu Hospitals. 2011

38 **Kadle** M, Hassan AM, Yasin AM, Sheikh AH, Omar MS, Sayid AM. Frequency of Hepatocellular Carcinoma and Its Associated of Hepatitis B and C in Patients Attending Mogadishu Hospitals. Accessed March 16, 2018. Available from: URL: https://www.researchgate.net/publication/ 309564485_Frequency_of_Hepatocellular_Carcinoma_and_its_associated_Hepatitis_B_and_C_in_Patients_attending_Mogadishu_hospitals

39 **Padovese V**, Egidi AM, Melillo TF, Farrugia B, Carabot P, Didero D, Costanzo G, Mirisola C. Prevalence of latent tuberculosis, syphilis, hepatitis B and C among asylum seekers in Malta. *J Public Health (Oxf)* 2014; **36**: 22-27 [PMID: 23559596 DOI: 10.1093/pubmed/fdt036]

40 **Bile K**, Abdirahman M, Mohamud O, Aden C, Isse A, Nilsson L, Norder H, Magnius L. Late seroconversion to hepatitis B in a Somali village indicates the important role of venereal transmission. *J Trop Med Hyg* 1991; **94**: 367-373 [PMID: 1758006]

41 **Watts DM**, Corwin AL, Omar MA, Hyams KC. Low risk of sexual transmission of hepatitis C virus in Somalia. *Trans R Soc Trop Med Hyg* 1994; **88**: 55-56 [PMID: 8154002 DOI: 10.1016/0035-9203(94)90495-2]

42 **Aceti A**, Taliani G, Bruni R, Sharif OS, Moallin KA, Celestino D, Quaranta G, Sebastiani A. Hepatitis C virus infection in chronic liver disease in Somalia. *Am J Trop Med Hyg* 1993; **48**: 581-584 [PMID: 7683179 DOI: 10.4269/ajtmh.1993.48.581]

43 **Daw MA**, El-Bouzedi A, Ahmed MO, Dau AA, Agnan MM; In association with the Libyan Study Group of Hepatitis & amp; HIV. Epidemiology of hepatitis C virus and genotype distribution in immigrants crossing to Europe from North and sub-Saharan Africa. *Travel Med Infect Dis* 2016; **14**: 517-526 [PMID: 27502972 DOI: 10.1016/j.tmaid.2016.05.020]

44 **Bradley DW**. Enterically-transmitted non-A, non-B hepatitis. *Br Med Bull* 1990; **46**: 442-461 [PMID: 2116213 DOI: 10.1093/oxfordjournals.bmb.a072409]

45 **Mushahwar IK**, Dawson GJ, Bile KM, Magnius LO. Serological studies of an enterically transmitted non-A, non-B hepatitis in Somalia. *J Med Virol* 1993; **40**: 218-221 [PMID: 8355020 DOI: 10.1002/jmv.1890400309]

46 **Bile K**, Isse A, Mohamud O, Allebeck P, Nilsson L, Norder H, Mushahwar IK, Magnius LO. Contrasting roles of rivers and wells as sources of drinking water on attack and fatality rates in a hepatitis E epidemic in Somalia. *Am J Trop Med Hyg* 1994; **51**: 466-474 [PMID: 7943574 DOI: 10.4269/ajtmh.1994.51.466]

47 **Burans JP**, Sharp T, Wallace M, Longer C, Thornton S, Batchelor R, Clemens V, Hyams KC. Threat of hepatitis E virus infection in Somalia during Operation Restore Hope. *Clin Infect Dis* 1994; **18**: 100-102 [PMID: 8054416 DOI: 10.1093/clinids/18.1.100]

48 **Belyhun Y**, Maier M, Mulu A, Diro E, Liebert UG. Hepatitis viruses in Ethiopia: a systematic review and meta-analysis. *BMC Infect Dis* 2016; **16**: 761 [PMID: 27993129 DOI: 10.1186/s12879-016-2090-1]

49 **Schweitzer A**, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet* 2015; **386**: 1546-1555 [PMID: 26231459 DOI: 10.1016/S0140-6736(15)61412-X]

50 **Wasley A**, Kruszon-Moran D, Kuhnert W, Simard EP, Finelli L, McQuillan G, Bell B. The prevalence of hepatitis B virus infection in the United States in the era of vaccination. *J Infect Dis* 2010; **202**: 192-201 [PMID: 20533878 DOI: 10.1086/653622]

51 **Alavian SM,** Hajarizadeh B, Ahmadzad-Asl M, Kabir A, Bagheri-Lankarani K. Hepatitis B Virus infection in Iran: A systematic review. *Hepatitis monthly* 2008; **8**: 281-294

52 **Fejza H**, Telaku S. Prevalence of HBV and HCV among blood donors in Kosovo. *Virol J* 2009; **6**: 21 [PMID: 19216773 DOI: 10.1186/1743-422X-6-21]

53 **Bigna JJ**, Amougou MA, Asangbeh SL, Kenne AM, Noumegni SRN, Ngo-Malabo ET, Noubiap JJ. Seroprevalence of hepatitis B virus infection in Cameroon: a systematic review and meta-analysis. *BMJ Open* 2017; **7**: e015298 [PMID: 28667212 DOI: 10.1136/bmjopen-2016-015298]

54 **Ofori-Asenso R**, Agyeman AA. Hepatitis B in Ghana: a systematic review & amp;amp; meta-analysis of prevalence studies (1995-2015). *BMC Infect Dis* 2016; **16**: 130 [PMID: 26987556 DOI: 10.1186/s12879-016-1467-5]

55 **Musa BM**, Bussell S, Borodo MM, Samaila AA, Femi OL. Prevalence of hepatitis B virus infection in Nigeria, 2000-2013: a systematic review and meta-analysis. *Niger J Clin Pract* 2015; **18**: 163-172 [PMID: 25665986 DOI: 10.4103/1119-3077.151035]

56 **Chaabna K**, Kouyoumjian SP, Abu-Raddad LJ. Hepatitis C Virus Epidemiology in Djibouti, Somalia, Sudan, and Yemen: Systematic Review and Meta-Analysis. *PLoS One* 2016; **11**: e0149966 [PMID: 26900839 DOI: 10.1371/journal.pone.0149966]

57 **Karoney MJ**, Siika AM. Hepatitis C virus (HCV) infection in Africa: a review. *Pan Afr Med J* 2013; **14**: 44 [PMID: 23560127 DOI: 10.11604/pamj.2013.14.44.2199]

58 **Agyeman AA**, Ofori-Asenso R, Mprah A, Ashiagbor G. Epidemiology of hepatitis C virus in Ghana: a systematic review and meta-analysis. *BMC Infect Dis* 2016; **16**: 391 [PMID: 27507267 DOI: 10.1186/s12879-016-1708-7]

59 **Muzembo BA**, Akita T, Matsuoka T, Tanaka J. Systematic review and meta-analysis of hepatitis C virus infection in the Democratic Republic of Congo. *Public Health* 2016; **139**: 13-21 [PMID: 27450441 DOI: 10.1016/j.puhe.2016.06.017]

60 **Madhava V**, Burgess C, Drucker E. Epidemiology of chronic hepatitis C virus infection in sub-Saharan Africa. *Lancet Infect Dis* 2002; **2**: 293-302 [PMID: 12062995 DOI: 10.1016/S1473-3099(02)00264-5]

61 **Gower E**, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol* 2014; **61**: S45-S57 [PMID: 25086286 DOI: 10.1016/j.jhep.2014.07.027]

62 **Mohamoud YA**, Mumtaz GR, Riome S, Miller D, Abu-Raddad LJ. The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis. *BMC Infect Dis* 2013; **13**: 288 [PMID: 23799878 DOI: 10.1186/1471-2334-13-288]

63 **Mudawi HM**. Epidemiology of viral hepatitis in Sudan. *Clin Exp Gastroenterol* 2008; **1**: 9-13 [PMID: 21677820 DOI: 10.2147/CEG.S3887]
64 **Amini N**, Alavian SM, Kabir A, Aalaei-Andabili SH, Saiedi Hosseini SY, Rizzetto M. Prevalence of hepatitis d in the eastern mediterranean region: systematic review and meta analysis. *Hepat Mon* 2013; **13**: e8210 [PMID: 23554822 DOI: 10.5812/hepatmon.8210]
65 **Ofori-Asenso R**, Agyeman AA. Hepatitis E infection among Ghanaians: a systematic review. *Infect Dis Poverty* 2017; **6**: 29 [PMID: 28162095 DOI: 10.1186/s40249-017-0239-9]
66 **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]
67 **Goumba CM**, Yandoko-Nakouné ER, Komas NP. A fatal case of acute hepatitis E among pregnant women, Central African Republic. *BMC Res Notes* 2010; **3**: 103 [PMID: 20398305 DOI: 10.1186/1756-0500-3-103]

P- Reviewer: Esmat SM, Pokorska-Spiewak M, Wang K
S- Editor: Wang JL **L- Editor**: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3958-3964

DOI: 10.3748/wjg.v24.i34.3958

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

CASE REPORT

Unicentric Castleman disease presenting as a retroperitoneal peripancreatic mass: A report of two cases and review of literature

Jia-Lin Cheng, Jing Cui, Yi Wang, Zong-Zhen Xu, Feng Liu, Shu-Bin Liang, Hu Tian

Jia-Lin Cheng, Shu-Bin Liang, Taishan Medical University, Tai'an 271016, Shandong Province, China

Jia-Lin Cheng, Zong-Zhen Xu, Feng Liu, Shu-Bin Liang, Hu Tian, Department of General Surgery, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan 250014, Shandong Province, China

Jing Cui, Department of Pathology, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan 250014, Shandong Province, China

Yi Wang, Department of Medical Imaging, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan 250014, Shandong Province, China

ORCID number: Jia-Lin Cheng (0000-0002-4306-4894); Jing Cui (0000-0002-8266-9984); Yi Wang (0000-0002-1832-0876); Zong-Zhen Xu (0000-0003-2693-8884); Feng Liu (0000-0002-7271-8658); Shu-Bin Liang (0000-0001-8839-8049); Hu Tian (0000-0002-8015-9083).

Author contributions: Cheng JL designed the report and wrote the paper; Cheng JL, Xu ZZ, Liu F and Tian H performed the surgery; Cheng JL, Cui J, Wang Y and Liang SB collected the patient's clinical data; Cheng JL and Tian H analyzed the data and revised the paper; all authors have read and approved the final version of this manuscript.

Supported by the Key Research and Development Plan of Shandong Province, No. 2016GSF201108.

Informed consent statement: The patients included in this study gave written informed consent for the use and disclosure of their protected health information.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

CARE Checklist (2013) statement: The authors have read the CARE Checklist (2013), and the manuscript was prepared and revised according to the CARE Checklist (2013).

Manuscript source: Unsolicited manuscript

Correspondence to: Hu Tian, MD, PhD, Chief Doctor, Professor, Surgeon, Department of General Surgery, Shandong Provincial Qianfoshan Hospital, Shandong University, No. 16766, Jingshi Road, Jinan 250014, Shandong Province, China. tianhu6585@163.com
Telephone: +86-531-89269817
Fax: +86-531-82963647

Received: April 23, 2018
Peer-review started: April 27, 2018
First decision: July 4, 2018
Revised: July 10, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: September 14, 2018

Abstract

Castleman disease (CD) is a rare disorder of lymph nodes and related tissues. CD generally occurs in the mediastinum, as well as in cervical, retroperitoneal and axillary regions. The disease is classified into two major types: unicentric CD (UCD) and multicentric CD. The occurrence of UCD in the retroperitoneal peripancreatic region is quite rare. We encountered two cases of retroperitoneal peripancreatic UCD in our hospital during the past three years. Following a series of medical examinations, including magnetic resonance imaging, computed tomography, ultrasonography and postoperative histopathological examination, these two

patients were diagnosed with UCD, which presented as a retroperitoneal peripancreatic mass. The mass in each patient was completely excised, and no postoperative radiochemotherapy was administered. Both patients recovered well without recurrence during a follow-up period of 30 mo and 8 mo.

Key words: Unicentric Castleman disease; Peripancreatic; Retroperitoneal; Tumor

Core tip: We report two typical cases of unicentric Castleman disease (UCD) presenting as a retroperitoneal peripancreatic mass. The clinical manifestations and imaging findings of these masses were nonspecific. UCD is difficult to diagnose prior to surgery, and the diagnosis is mainly by postoperative pathological examination. The occurrence of UCD in the abdominal cavity is very rare, especially in the retroperitoneal peripancreatic region. Importantly, both patients recovered well without postoperative radiochemotherapy. These case reports provide a valuable reference for the diagnosis and treatment of this disease.

Cheng JL, Cui J, Wang Y, Xu ZZ, Liu F, Liang SB, Tian H. Unicentric Castleman disease presenting as a retroperitoneal peripancreatic mass: A report of two cases and review of literature. *World J Gastroenterol* 2018; 24(34): 3958-3964 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3958.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3958

INTRODUCTION

Castleman disease (CD) is generally identified as a polyclonal lymphoid proliferation of unknown etiology, and is also known as angiofollicular lymph node hyperplasia. It was first described by Benjamin Castleman in 1954 as "localized mediastinal lymph node hyperplasia resembling thymoma", and was named after him[1]. This disease can occur in any age group, but is mostly seen in patients aged 30-50 years, with no significant gender differences[2]. Clinically, the disease is divided into two major types: unicentric CD (UCD) and multicentric CD, based on its locations at a single lymph node and a series of lymph nodes, respectively[3]. CD, which develops in all lymph nodes, especially in the mediastinum, can also occur in the cervical, retroperitoneal, and axillary regions. However, invasion into the retroperitoneal peripancreatic region is quite rare[4]. We herein report two rare cases of retroperitoneal peripancreatic UCD over a three-year period from 2014 to 2017.

CASE REPORT

Case 1

A 48 year old woman was referred to our department on April 28, 2014 for a detailed physical examination due to retroperitoneal peripancreatic lymph node enlargement over seven days. The patient initially had no apparent discomfort or noteworthy positive signs. Magnetic resonance imaging (MRI) of the epigastrium showed an elliptical hyperintense signal below the pancreas on the T1 weighted image (T1WI) (Figure 1B). The size of the lesion was 3.0 cm × 1.9 cm with a clear boundary, with slight hyperintensity on the fat-suppression T2 weighted image (T2WI) (Figure 1C). In addition, marked enhancement on the arterial phase (Figure 1D and E) and reduced enhancement on the delayed phase (Figure 1F) were observed, which were probably due to lymph node enlargement. Laboratory examinations revealed that the patient was negative for the human immunodeficiency virus (HIV) antibody. Tumor markers and other laboratory test results were also within the normal range.

Based on the above results, a retroperitoneal peripancreatic mass was diagnosed preoperatively. Laparotomy was performed through an oblique incision along the left lower rib, and no ascites were found. When the gastrocolic ligament was incised, a tumor was discovered closely adhered to the lower edge of the pancreas (Figure 2A). The tumor volume was 3.0 cm × 2.0 cm × 2.0 cm, had a soft flexible texture and a clear boundary (Figure 2B). The tumor was completely removed with blunt and sharp separation for further testing (Figure 2C). Lymphoid tissue hyperplasia was identified in a frozen biopsy. Postoperative pathology indicated that the retroperitoneal peripancreatic single lymph node was compatible with CD (Figure 3B-D). Immunohistochemical staining revealed that cells were slightly positive for CD20, CD3, CD21, bcl-6, and bcl-2, a small number of cells were positive for CD138, and 20% of cells were positive for Ki-67. During the follow-up at 30 mo after surgery, the patient was recovering well with no signs of recurrence.

Case 2

A 57 year old woman was admitted to our hospital on July 18, 2017 because of tiredness and fever. The patient had a body temperature of 39 degrees without other discomfort. Physical examination indicated no positive symptoms. MRI of the epigastrium showed that there was an elliptical hyperintense signal below the pancreas on the T1WI (Figure 4A) and slight hyperintensity on the fat-suppression T2WI (Figure 4B). The internal signal was heterogeneous and a clearly bounded tumor 3.4 cm × 2.8 cm in size was observed. Marked enhancement on the arterial phase was inhomogeneous (Figure 4C). In addition, the venous phase and delayed phase continued to intensify (Figure 4D-F). On account of the hypervascular mass located under the pancreas, it was highly possible that the mass was a nerve incretory tumor.

Laboratory examinations showed that the HIV antibody was negative and the white blood cell count was 14.6×10^9/L. Tumor markers and other laboratory test results were within the normal range. The preoperative

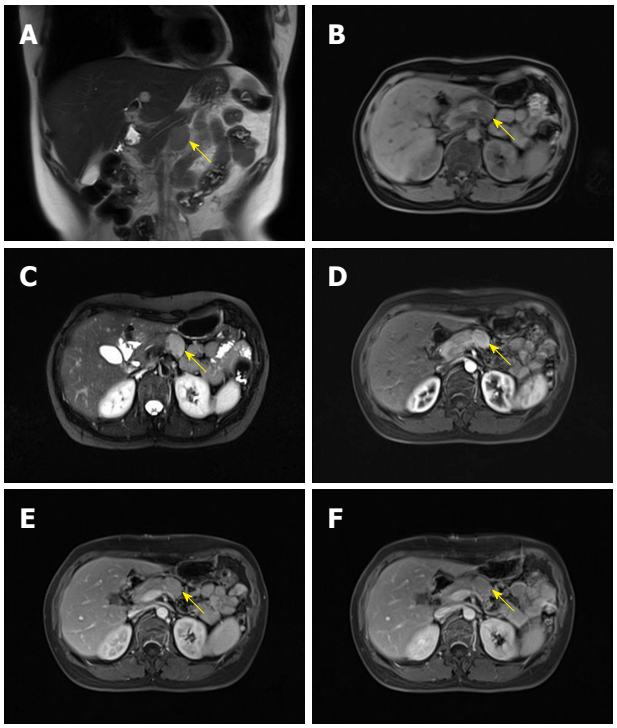


Figure 1 Magnetic resonance imaging of the lesion (yellow arrow) in Case 1. A: Coronal T2-weighted image sequence; B: Transverse T1-weighted fat-suppression sequence; C: Transverse T2-weighted fat-suppression sequence; D: Transverse T1-weighted fat-suppression sequence (early arterial phase); E: Transverse T1-weighted fat-suppression sequence (late arterial phase); F: Transverse T1-weighted fat-suppression sequence (delayed phase).

diagnosis was an retroperitoneal neuroendocrine tumor. Laparoscopic surgery was performed to remove the retroperitoneal tumor. No ascitic fluid or obvious conglutination were found. When the gastrocolic ligament was incised, the lesion was located behind and below the transverse mesocolon. Intraoperative ultrasonography indicated that the tumor had an abundant blood supply, and its right side was close to the inferior mesenteric artery and vein (Figure 5A). The flexible tumor was 3.0 cm × 3.0 cm in size with a clear border (Figure 5B). The tumor was completely removed by blunt and sharp separation for further analysis (Figure 5C). Rapid histopathological examination revealed that the mass was possibly retroperitoneal CD together with a follicular dendritic cell tumor, which was confirmed by subsequent postoperative pathology (Figure 6B-F). Immunohistochemical staining showed that a portion of cells were positive for CD21 and negative for CK, a small number of cells were positive for S-100 and slightly positive for CD1a and CD23, and about five percent of cells were positive for Ki67. The patient recovered well after surgery without chemotherapy, and no relapse was found during the follow-up period.

DISCUSSION

CD is a rare lymphoproliferative disease with a favorable prognosis; however, the pathogenesis of this disease is unclear. Munshi *et al*[5] showed that the incidence of CD is approximately 21/million. This disease can occur in all lymph nodes, especially in the mediastinum, neck, retroperitoneum and axillary regions; however, its occurrence in the retroperitoneal peripancreatic region is more rare[4]. The etiology of CD is thought to be related to herpes simplex virus 8 and HIV[6-7]. Another report confirmed that the pathogenesis of CD was closely related to the proliferation of T cells and B cells stimulated by interleukin 6[8]. CD mainly manifests as a painless enlargement of deep or superficial lymph nodes, and may be accompanied by fever, fatigue, anemia, night sweats, and an accelerated erythrocyte sedimentation rate[9]. The two cases in this report showed some differences in clinical manifestation. The tumor in Case 1 was found by physical examination without apparent patient discomfort, and the other was diagnosed by the symptoms of fever and fatigue. In addition, there was no obvious abdominal discomfort in either patient.

Retroperitoneal peripancreatic UCD is usually concealed and difficult to diagnose. In addition, there is no specific manifestation to distinguish it from a neuroendocrine tumor, paraganglioma, or lymphoma[10]. Imaging manifestations of the disease are very difficult to distinguish from other diseases, and the preoperative imaging diagnosis does not usually agree with the postoperative histopathological diagnosis. These difficulties can lead to misdiagnosis. Computed tomography (CT), MRI, and ultrasound can provide reference values for the diagnosis of retroperitoneal UCD. On CT imaging, retroperitoneal UCD is generally a well-defined mass with different morphologies, such as elliptical, kidney-shaped, and dumbbell-shaped, which can easily be distinguished from a spherical paraganglioma. In these two cases, both masses were oval in shape. The plain CT scan images of this disease show low or equal density lesions. The majority of lesions exhibit inhomogeneous enhancement on enhanced CT images. In addition, some tumors have a rich vascular supply, accompanied by microcalcifications[11]. On the MRI, most retroperitoneal UCD lesions show an isointense or hypo-intense signal on T1WI, and a slight hyperintense or hyperintense signal on T2WI. Although the enhanced scan characteristics are similar in both CT and MRI, MRI is superior to CT in terms of identifying the relationship between the mass and neighboring vessels or tissues[12]. In addition, ultrasound also has some advantages in estimating the location of the mass and its adjacent feeding vessels. In Case 2 in our report, we quickly clarified the location of the mass and its relationship with surrounding blood vessels (such as the inferior mesenteric artery and vein) using intraoperative ultrasonography, effectively reducing the risk of bleeding during resection of the mass and significantly improving the safety of surgery.

Due to the unclear specificity in clinical manifestations and imaging findings, the diagnosis of retroperitoneal peripancreatic UCD is still dependent on histopathology.

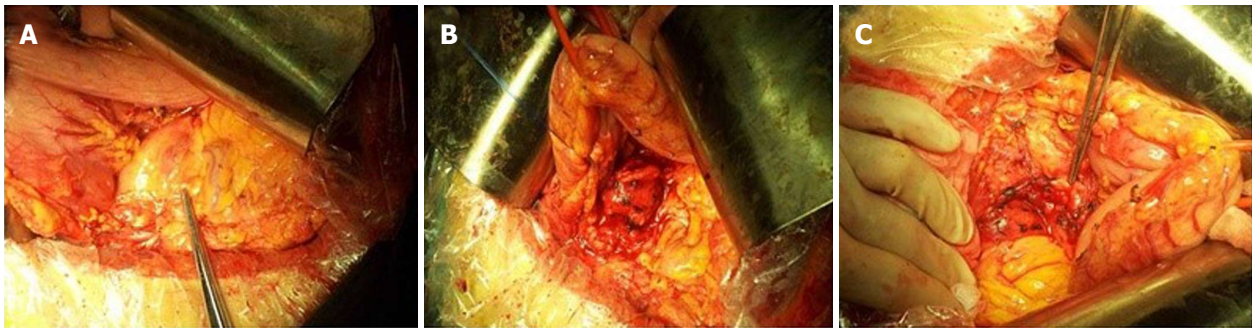


Figure 2 Surgery of the retroperitoneal peripancreatic unicentric Castleman disease in Case 1. A: The tumor was close to the lower side of the pancreas with a clear margin and a volume of 3 cm × 2 cm × 2 cm; B: Image of the free tumor; C: The tumor was completely excised.

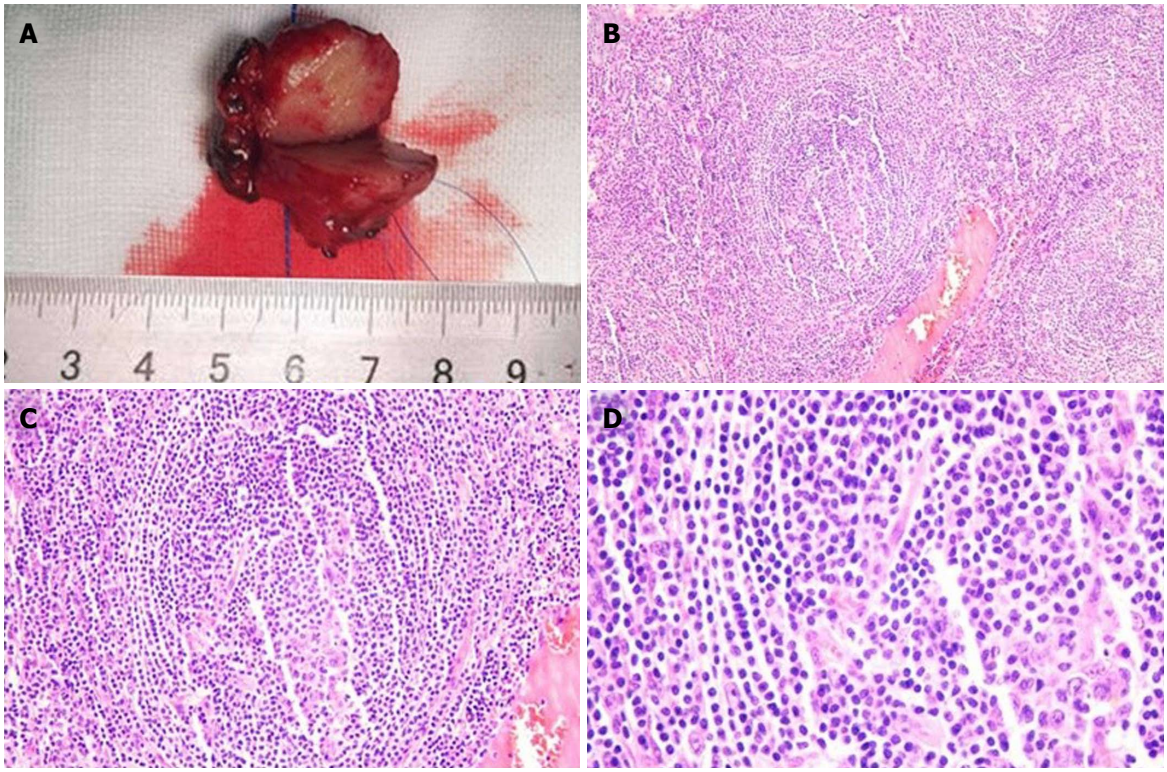


Figure 3 Pathological examinations of the tumor in Case 1. A: The tumor was 3 cm × 2 cm × 2 cm in size. It had a smooth surface, complete envelope, and was white to tan in appearance; B: Pathological section analysis of UCD (HE, × 100); C: Pathological section analysis of UCD (HE, × 200); D: Pathological section analysis of UCD (HE, × 400). HE: Hematoxylin and eosin; UCD: Unicentric Castleman disease.

In addition, different pathological types also have a direct impact on the treatment and prognosis of the disease. In general, CD can be classified into three types based on pathological characteristics: hyaline vascular type (HV), plasma cell type (PC) and mixed type. The percentage of HV type and PC type is approximately 90% and 10%, respectively. Mixed type cases are rarely reported. Most HV type cases manifest as UCD, while PC type cases usually occur in the form of multicentric CD[13]. In microscopic images of HV type, the centers of the huge folliculus lymphaticus are atrophied, blood vessels degenerate with hyaline vascular changes during this period, and a portion have an onion-like skin appearance[2,14-15]. In microscopic images of PC type CD, the plasma cells are mainly spread throughout the interfollicular areas[16]. Pathologically, the two cases in this report were HV type UCD. In Case 1, mantle cell hyperplasia appeared as an onion-like skin surrounding a neurodegenerative vascularized germinal center. In Case 2, the single penetrated vessel appeared as a lollipop shape with hyaline degeneration.

At present, complete resection of the tumor is still the most effective treatment for retroperitoneal UCD with low recurrence and high cure rates[17]. However, recurrence has been reported in some cases, and both radiotherapy and chemotherapy were unsatisfactory for recurrent UCD[18]. Fortunately, no recurrence of retroperitoneal UCD was found in either of our two cases after surgery. During the resection of retroperitoneal UCD, special attention should be paid to

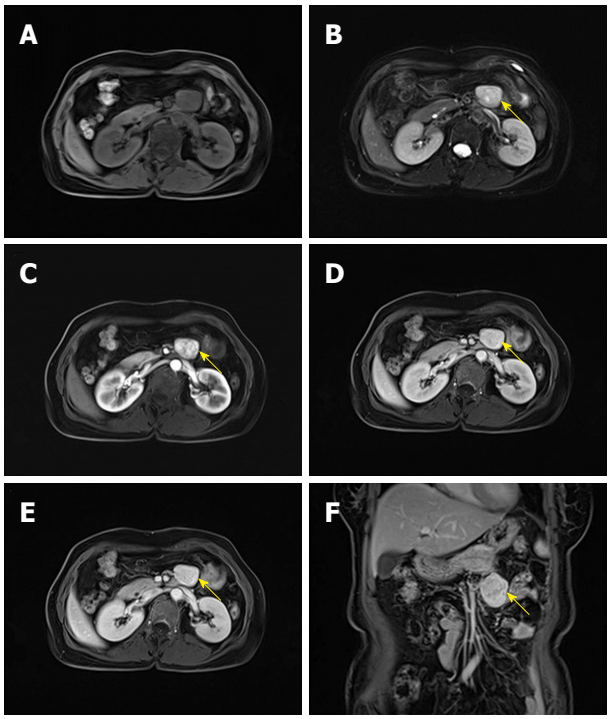


Figure 4 Magnetic resonance imaging of the lesion (yellow arrow) in Case 2. A: Transverse T1-weighted fat-suppression sequence; B: Transverse T2-weighted fat-suppression sequence; C: Transverse T1-weighted fat-suppression sequence (arterial phase); D: Transverse T1-weighted fat-suppression sequence (venous phase); E: Transverse T1-weighted fat-suppression sequence (delayed phase); F: Coronal T1-weighted fat-suppression sequence (delayed phase).

the affected area between the tumor and surrounding vessels, tissues and organs, which is conducive to the complete resection of tumor and reducing blood loss during surgery. In Case 2, we performed laparoscopic resection of the tumor, which has the advantages of reduced surgical trauma, clear intraoperative vision, reduced intraoperative bleeding, high safety, and faster postoperative recovery. In Case 2, we found UCD together with follicular dendritic cell tumor (FDCT), which is a rare low-grade malignant tumor and may arise from CD[19]. However, it was difficult to determine whether the FDCT in Case 2 was secondary to CD. Surgical resection is the first choice in the treatment of FDCT, and recurrence has been observed after surgery. Adjuvant radiotherapy and chemotherapy can be followed after surgery, especially in patients with unresectable FDCT[20]. Case 2 did not undergo radiochemotherapy after surgery. We believe that the treatment of this patient was reasonable. In this report, we described two rare cases of retroperitoneal peripancreatic UCD, which will contribute to an improvement in the understanding of UCD and provide a significant reference for future cases.

ARTICLE HIGHLIGHTS

Case characteristics

Case 1 is a 48 year old female patient who presented with lymph node enlargement; and Case 2 is a 57 year old female patient who presented with tiredness and fever.

Clinical diagnosis

In Case 1, the patient initially had no apparent discomfort or noteworthy positive physical signs. In Case 2, the patient felt tired and had a fever with no other discomfort.

Differential diagnosis

Neuroendocrine tumor, paraganglioma and lymphoma.

Laboratory diagnosis

In Case 1, laboratory examinations revealed that the HIV antibody was negative. Carbohydrate antigen 19-9, carcinoembryonic antigen and other laboratory test results were also within the normal range. In Case 2, laboratory examinations showed that the HIV antibody was negative and the white blood cell count was 14.6 $\times$ 10^9/L. Carbohydrate antigen 19-9, carcinoembryonic antigen and other laboratory test results were also within the normal range.

Imaging diagnosis

In Case 1, MRI of the epigastrium showed that there was an elliptical hyperintense signal below the pancreas on the T1WI, and slight hyperintensity on the T2WI. In addition, marked enhancement on the arterial phase and reduced enhancement on the delayed phase were observed. In Case 2, MRI of the epigastrium showed that there was an elliptical hyperintense signal below the pancreas on the T1WI and slight hyperintensity on the T2WI. Marked enhancement on the arterial phase was inhomogeneous. In addition, the venous phase and delayed phase continued to intensify.

Pathological diagnosis

In Case 1, the retroperitoneal single lymph node was compatible with Castleman disease. In addition, immunohistochemical staining revealed that cells were slightly positive for CD20, CD3, CD21, bcl-6, and bcl-2, a small number of cells were positive for CD138, and 20% of cells were positive for Ki-67. In Case 2, the diagnosis was retroperitoneal Castleman disease together with a follicular dendritic cell tumor. Immunohistochemical staining showed that a portion of cells were positive for CD21 and cells were negative for CK, a small number of cells were positive for S-100 and slightly positive for CD 1a and CD23, and about five percent of cells were positive for Ki-67.

Treatment

In Case 1, laparotomy was performed to remove the retroperitoneal peripancreatic tumor without subsequent radiochemotherapy. In Case 2, laparoscopic surgery was performed to remove the retroperitoneal peripancreatic tumor without subsequent radiochemotherapy.

Related reports

Unicentric Castleman disease (UCD) is a rare disease of lymph nodes and related tissues, and occurrence in the retroperitoneal peripancreatic region is quite rare. Only a few cases of UCD together with follicular dendritic cell tumor have been identified. At present, complete resection of the tumor is still the most effective treatment for retroperitoneal peripancreatic UCD with low recurrence and high cure rates.

Term explanation

Follicular dendritic cell tumor is an uncommon neoplasm that has features of follicular dendritic cell differentiation, and typically presents as a slow-growing, painless mass without systemic symptoms. The tumor can occur on various parts of the body such as lymph nodes, tonsils, armpits and mediastinum, but is most common in the neck lymph nodes.

Experiences and lessons

At present, complete resection of the tumor is still the most effective treatment for retroperitoneal peripancreatic UCD with low recurrence and high cure rates. The diagnosis of retroperitoneal peripancreatic UCD is still dependent on histopathology. The two rare cases of retroperitoneal peripancreatic UCD

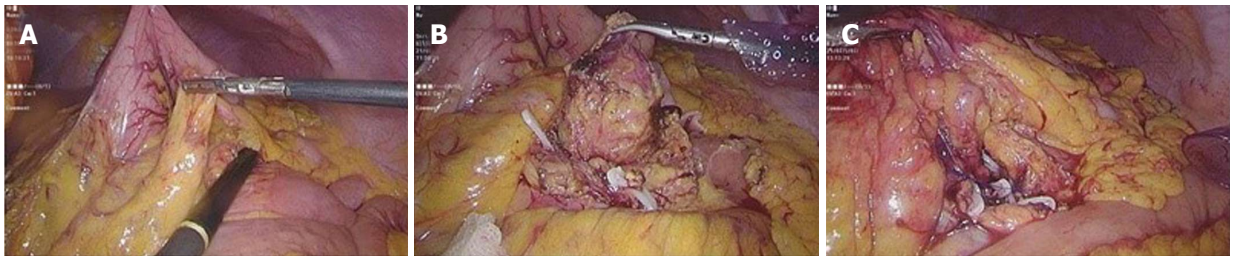


Figure 5 Surgery of the retroperitoneal peripancreatic unicentric Castleman disease in Case 2. A: The lesion was located below and behind the transverse mesocolon with a volume of 3.5 cm × 3 cm × 3 cm. Intraoperative ultrasonography indicated that the tumor had an abundant blood supply, and its right side was close to the inferior mesenteric artery and vein; B: Image of the free tumor; C: The tumor was completely excised.

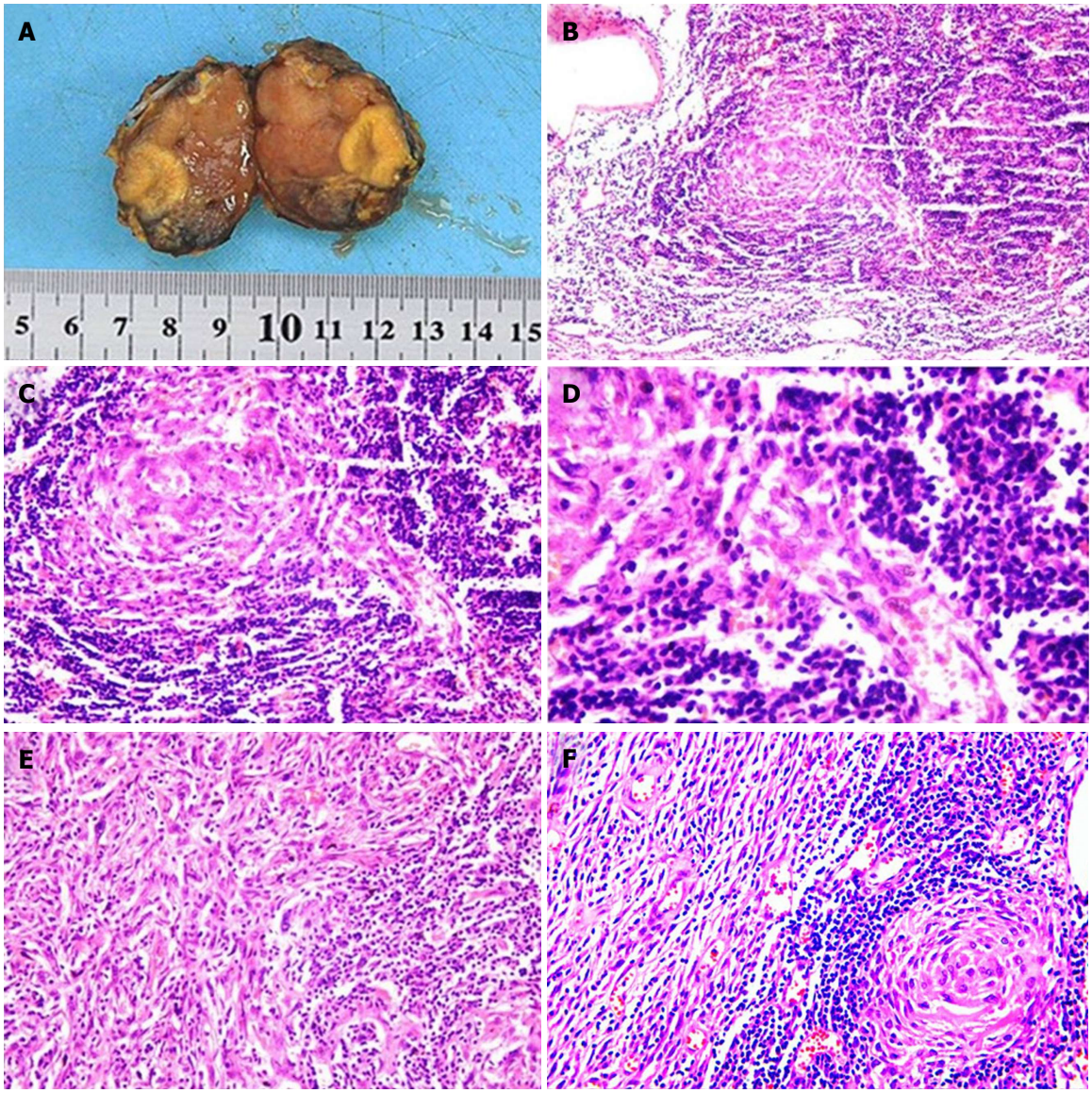


Figure 6 Pathological examinations of the tumor in Case 2. A: The tumor was 3.5 cm × 3 cm × 3 cm in size. It had a smooth surface, complete envelope, and was white to tan in appearance; B: Pathological section analysis of UCD (HE, × 100); C: Pathological section analysis of UCD (HE, × 200); D: Pathological section analysis of UCD (HE, × 400); E: Pathological section analysis of FDCT (HE, × 200); F: Pathological section analysis of UCD together with FDCT (HE, × 200). HE: Hematoxylin and eosin; UCD: Unicentric Castleman disease; FDCT: Follicular dendritic cell tumor.

included in this report will improve our understanding of this disease and provide a significant reference for future cases.

REFERENCES

1 **Castleman B**, Iverson L, Menendez VP. Localized mediastinal lymphnode hyperplasia resembling thymoma. *Cancer* 1956; **9**: 822-830 [PMID: 13356266 DOI: 10.1002/1097-0142(195607/08)9:4<822::AID-CNCR2820090430>3.0.CO;2-4]

2 **Talat N**, Belgaumkar AP, Schulte KM. Surgery in Castleman's disease: a systematic review of 404 published cases. *Ann Surg* 2012; **255**: 677-684 [PMID: 22367441 DOI: 10.1097/SLA.0b013e318249dcdc]

3 **Chan KL**, Lade S, Prince HM, Harrison SJ. Update and new approaches in the treatment of Castleman disease. *J Blood Med* 2016; **7**: 145-158 [PMID: 27536166 DOI: 10.2147/JBM.S60514]

4 **Charalabopoulos A**, Misiakos EP, Foukas P, Tsapralis D, Charalampopoulos A, Liakakos T, Macheras A. Localized peripancreatic plasma cell Castleman disease. *Am J Surg* 2010; **199**: e51-e53 [PMID: 20079482 DOI: 10.1016/j.amjsurg.2009.05.020]

5 **Munshi N**, Mehra M, van de Velde H, Desai A, Potluri R, Vermeulen J. Use of a claims database to characterize and estimate the incidence rate for Castleman disease. *Leuk Lymphoma* 2015; **56**: 1252-1260 [PMID: 25120049 DOI: 10.3109/10428194.2014.953145]

6 **Pria AD**, Pinato D, Roe J, Naresh K, Nelson M, Bower M. Relapse of HHV8-positive multicentric Castleman disease following rituximab-based therapy in HIV-positive patients. *Blood* 2017; **129**: 2143-2147 [PMID: 28143881 DOI: 10.1182/blood-2016-10-747477]

7 **Siegel MO**, Ghafouri S, Ajmera R, Simon GL. Immune reconstitution inflammatory syndrome, human herpesvirus 8 viremia, and HIV-associated multicentric Castleman disease. *Int J Infect Dis* 2016; **48**: 49-51 [PMID: 27173076 DOI: 10.1016/j.ijid.2016.05.005]

8 **Musters A**, Assaf A, Gerlag DM, Tak PP, Tas SW. Discovery of Innovative Therapies for Rare Immune-Mediated Inflammatory Diseases via Off-Label Prescription of Biologics: The Case of IL-6 Receptor Blockade in Castleman's Disease. *Front Immunol* 2015; **6**: 625 [PMID: 26697019 DOI: 10.3389/fimmu.2015.00625]

9 **Talarico F**, Negri L, Iusco D, Corazza GG. Unicentric Castleman's disease in peripancreatic tissue: case report and review of the literature. *G Chir* 2008; **29**: 141-144 [PMID: 18419976]

10 **Stevens EA**, Strowd RE, Mott RT, Oaks TE, Wilson JA. Angiofollicular lymph node hyperplasia resembling a spinal nerve sheath tumor: a rare case of Castleman's disease. *Spine J* 2009; **9**: e18-e22 [PMID: 19482516 DOI: 10.1016/j.spinee.2009.04.011]

11 **Shah D**, Darji P, Lodha S, Bolla S. Unicentric Castleman's disease of abdomen. *J Radiol Case Rep* 2013; **7**: 26-33 [PMID: 23705043 DOI: 10.3941/jrcr.v7i3.1031]

12 **Dong A**, Dong H, Zuo C. Castleman disease of the porta hepatis mimicking exophytic hepatocellular carcinoma on CT, MRI, and FDG PET/CT. *Clin Nucl Med* 2014; **39**: e69-e72 [PMID: 23486333 DOI: 10.1097/RLU.0b013e31827a25fd]

13 **Ghosh A**, Pradhan SV, Talwar OP. Castleman's disease - hyaline vascular type - clinical, cytological and histological features with review of literature. *Indian J Pathol Microbiol* 2010; **53**: 244-247 [PMID: 20551525 DOI: 10.4103/0377-4929.64335]

14 **Hölzle F**, Landers A, Platz-Baudin C, Basrei D, Wolff KD. [Castleman's disease]. *Mund Kiefer Gesichtschir* 2005; **9**: 121-125 [PMID: 15647952 DOI: 10.1007/s10006-004-0592-2]

15 **Schaefer IM**, Günnel H, Schweyer S, Korenkov M. Unicentric castleman's disease located in the lower extremity: a case report. *BMC Cancer* 2011; **11**: 352 [PMID: 21838880 DOI: 10.1186/1471-2407-11-352]

16 **Wang HW**, Pittaluga S, Jaffe ES. Multicentric Castleman disease: Where are we now? *Semin Diagn Pathol* 2016; **33**: 294-306 [PMID: 27296355 DOI: 10.1053/j.semdp.2016.05.006]

17 **Xu J**, Zhou BO, Cao HL, Wang BO, Yan S, Zheng SS. Surgical management of isolated retroperitoneal Castleman's disease: A case report. *Oncol Lett* 2016; **11**: 2123-2126 [PMID: 26998133 DOI: 10.3892/ol.2016.4177]

18 **Baek HJ**, Kook H, Han DK, Shin MG, Kim HS, Hwang TJ. Unicentric Castleman disease relapsed after rituximab-CHOP chemotherapy or radiation therapy in an adolescent. *J Pediatr Hematol Oncol* 2012; **34**: e206-e208 [PMID: 22258344 DOI: 10.1097/MPH.0b013e3182352dc7]

19 **Jain P**, Prieto VG, Manning JT, Fowler N, Medeiros LJ, Kanagal-Shamanna R. Synchronous presentation of intra-nodal follicular dendritic cell sarcoma and Castleman disease. *Am J Hematol* 2017; **92**: 478-479 [PMID: 27862216 DOI: 10.1002/ajh.24603]

20 **Jain P**, Milgrom SA, Patel KP, Nastoupil L, Fayad L, Wang M, Pinnix CC, Dabaja BS, Smith GL, Yu J, Hu S, Bueso Ramos CE, Kanagal-Shamanna R, Medeiros LJ, Oki Y, Fowler N. Characteristics, management, and outcomes of patients with follicular dendritic cell sarcoma. *Br J Haematol* 2017; **178**: 403-412 [PMID: 28382648 DOI: 10.1111/bjh.14672]

P- Reviewer: Govindarajan GK, Lee WJ **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 September 21; 24(35): 3965-4092

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 35 September 21, 2018

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 35 September 21, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Amedeo Lonardo, MD, Doctor, Senior Lecturer, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena 41126, Italy

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
August 28, 2018

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 3965-3973

DOI: 10.3748/wjg.v24.i35.3965

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

EDITORIAL

Role of endoscopic therapy in early esophageal cancer

Sonika Malik, Gautam Sharma, Madhusudhan R Sanaka, Prashanthi N Thota

Sonika Malik, Department of Internal Medicine, Cleveland Clinic Akron General Medical Center, Akron, OH 44307, United States

Gautam Sharma, Department of Anesthesiology, University Hospitals, Cleveland, OH 44106, United States

Madhusudhan R Sanaka, Prashanthi N Thota, Department of Gastroenterology, Digestive Disease Surgery Institute, Cleveland Clinic, OH 44195, United States

ORCID number: Sonika Malik (0000-0001-5332-4680); Gautam Sharma (0000-0003-1512-640X); Madhusudhan R Sanaka (0000-0003-2506-8602); Prashanthi N Thota (0000-0001-7179-4774).

Author contributions: All authors contributed to the conception and design, acquisition of data and drafting of manuscript; all authors approved the final version of the article, including the authorship list.

Conflict-of-interest statement: Authors deny any conflict-of-interest.

Manuscript source: Invited manuscript

Correspondence to: Prashanthi N Thota, MD, FACG, Medical Director, Esophageal Center, Digestive Disease and Surgery Institute, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH 44195, United States. thotap@ccf.org
Telephone: +1-216-4440780
Fax: +1-216-4454222

Received: July 9, 2018
Peer-review started: July 9, 2018
First decision: July 17, 2018
Revised: July 23, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 21, 2018

Abstract

Esophageal carcinoma is a highly lethal cancer associated with high morbidity and mortality. Esophageal squamous cell carcinoma and esophageal adenocarcinoma are the two distinct histological types. There has been significant progress in endoscopic diagnosis and treatment of early stages of cancer using resection and ablation techniques, as shown in several trials in the recent past. Earlier detection of esophageal cancer and advances in treatment modalities have lead to improvement in the 5-year survival from 5% to about 20% in the past decade. Endoscopic eradication therapy is the preferred modality of treatment in cancer limited to mucosal layer of the esophagus as there is very low risk of lymph node metastasis, leading to high cure rates, low risk of recurrence and with few adverse effects. The most common adverse events seen are strictures, bleeding and rarely perforation which can be endoscopically managed. In patients with recurrent advanced disease or invasive tumor, esophagectomy with lymph node dissection remains the mainstay of treatment. There is debate on post-endoscopic surveillance with some studies suggesting closer follow up with upper endoscopy every 6 mo for the first 1-2 years and then annually for the 3 years while others recommending the appropriate action only if symptoms or other abnormalities develop. Overall, the field of endoscopic therapy is still evolving and focus should be placed on careful patient selection using a multidisciplinary approach.

Key words: Endoscopic mucosal resection; Endoscopic submucosal dissection; Radiofrequency ablation; Argon plasma coagulation; Esophageal cancer; Photodynamic therapy; Cryotherapy

Core tip: Endoscopic eradication therapy (EET) plays a pivotal role in the management of patients with early esophageal cancer who are at very low risk for lymph node metastases. The main advantage of EET over surgery is the lower morbidity and mortality rates with similar cure rates, five-year survival rates and better quality of life. These excellent outcomes are tempered by the need for multiple treatment sessions for complete eradication and risk of post eradication recurrences. Careful patient selection by a multidisciplinary approach and patient compliance are crucial for treatment success.

Malik S, Sharma G, Sanaka MR, Thota PN. Role of endoscopic therapy in early esophageal cancer. *World J Gastroenterol* 2018; 24(35): 3965-3973 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/3965.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.3965

INTRODUCTION

Esophageal carcinoma is the sixth most common cause of cancer death globally[1]. Two histological forms exist: esophageal squamous cell cancer (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the most common histological subtype globally, especially in areas of eastern Asia and Africa[2] and is associated with heavy tobacco and alcohol use. The other histological form is EAC, seen more commonly in the Western world and associated with chronic gastroesophageal reflux disease[3]. Esophageal carcinoma is a lethal cancer with a five-year survival rate of 19%[4] due to the fact that more than 70% of cancers have spread beyond the esophagus by the time of diagnosis. In patients with cancer confined to the esophagus, the curative treatment is usually esophagectomy with or without chemoradiation therapy[5]. These intensive treatment regimens lead to poor quality of life even after therapy and can be associated with recurrences. However, with the advent of organ sparing endoscopic interventions in the past two decades, cure rates of early cancer similar to those with surgery are reported with much better quality of life. There has been significant progress in endoscopic diagnosis and treatment of dysplastic tissue and early stages of cancer using resection and ablation techniques, as shown in several studies in the recent past. Detection of esophageal cancer at an earlier stage and better utilization of treatment modalities, mainly surgery, has lead to improvement in the 5-year survival from 5% to about 20% in the past decade[4,6]. This editorial provides an understanding of the role of endotherapy in the treatment of early esophageal cancer, patient selection and the clinical outcomes of different modalities.

EARLY ESOPHAGEAL CANCER

Early esophageal cancer is defined as disease confined to the mucosa or submucosa of the esophagus with no evidence of spread. About 20% of all the cases of esophageal cancers diagnosed constitute early cancer[7]. Surgery with esophagectomy is considered the standard treatment with an estimated 5-year survival rates up to 90% in tumors which are confined to the mucosal layer[8]. However, esophagectomy, being a surgical procedure, has its own pitfalls with an operative mortality rate of about 2% and morbidity rate of 10%[8,9].

Endoscopic eradication therapy (EET) has emerged as a popular method for treatment of early esophageal cancer with favorable outcomes. Studies have found comparable outcomes between EET versus surgery in terms of long term survival[10,11]. A recent population based study utilizing the Surveillance, Epidemiology and End Results Program (SEER) database compared 2-year and 5-year overall survival and esophageal cancer specific mortality in patients undergoing EET and esophagectomy. They found no difference in cancer related mortality at 2-year (EET: 10.5% *vs* esophagectomy: 12.7%, $P = 0.27$) and 5-year (EET: 36.7% *vs* esophagectomy: 42.8%, $P = 0.16$) follow up[7]. These results were found in patients with T0 (carcinoma *in situ*/high grade dysplasia) and T1a (limited to lamina propria or muscularis mucosa). It is imperative to understand that patients with cancer limited to the mucosa have a very low risk of lymph node metastasis (0% to 2%)[11] and hence are candidates for EET. However, there are no randomized controlled trials that can provide definitive evidence comparing EET with surgery in terms of superiority.

PATIENT SELECTION

EET, which entails endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), is increasingly used in the management of early esophageal cancer with expanding indications. It is not only used for treating precancerous lesions such as Barrett's esophagus (BE) with dysplasia, squamous dysplasia and intramucosal cancer (IMC) but also in selected cases of submucosal cancers (T1b)[12]. Submucosal tumors are further classified into sm1 (involving one third of submucosa), sm2 (involving two thirds of submucosa and sm3 (involving lower third of the submucosa). Since EET will address only disease confined to esophagus, the key factor is to exclude those patients who may have local or distant metastases. Therefore, patients should be evaluated with endoscopic ultrasound (EUS) and positron emission tomography (PET) scan for the spread of disease and have a multidisciplinary evaluation with oncologists, thoracic surgeons, pathologists and radiation oncologists before embarking on EET. Patients are to be counselled on the need for multiple treatment sessions and post eradication surveillance.

Candidates for EET in ESSC

According to the 2011 National Comprehensive Cancer Network (NCCN) guidelines, patients with squamous cell dysplasia (low-grade and high grade intra-epithelial neoplasm/carcinoma *in situ*) and T1a where the tumor

invades the lamina propria and muscularis mucosa without lymph node or vascular invasion are candidates for EET[13]. In ESSC, the risk of lymph node metastases is 0% to 6% for IMC, 8% to 30% for sm1, 30% for sm2 and 60% to 70% for sm3 cancers[11,14-16]. Therefore treatment differs between T1a and T1b with the latter being treated with esophagectomy. As per Japanese esophageal society guidelines for early SCC, indications for EET are IMC involving the epithelium and lamina propria occupying less than 2/3 of the lumen of the esophagus with relative indications being involvement of the muscularis mucosa or < 200 μm invasion of the submucosa[17]. For lesions larger than 15 mm, lesion with poor lifting and for better assessment of the depth of invasion in case of suspicion for submucosal invasion, ESD may be considered[18].

Candidates for EET in EAC

In EAC, the rate of lymph node metastasis in T1a is 0% to 2% and for sm1, sm2 and sm3 cancers is 0% to 22%, 0% to 30% and 20% to 70% respectively[11]. Therefore, EET is considered in high grade dysplasia, and IMC lesions with low risk of submucosal invasion. These include Type Ⅰ (polypoidal), Ⅱa (slightly elevated), Ⅱb (flat) and Ⅱc (slightly depressed) based on Paris classification and smaller lesions (less than 20 mm), histological grades G1 and G2, or high grade dysplasia on biopsy[19]. Lesions which are excavated, poorly/undifferentiated on biopsy or with lymphadenopathy detected on EUS are better managed by surgery.

Overall, the rate of lymph node metastasis is higher in submucosal invasion by SCC as compared to EAC[20]. Therefore, stage T1b in ESCC is usually treated surgically with esophagectomy.

EET TECHNIQUES

EET involves endoscopic resection of the cancer followed by ablation of surrounding precancerous tissue to prevent recurrences. The widely used resection techniques are EMR and ESD.

EMR

EMR was initially started in Japan for management of gastric cancer[21], however, it is now being increasingly used for carcinomas of gastrointestinal tract confined to mucosa and submucosa not only for therapeutic benefits but also for its diagnostic value in staging and ascertaining the depth of invasion as well as degree of differentiation and lymphovascular invasion[22-25].

EMR technique: The commonly used techniques can be categorized as cap- and ligation-assisted. The simplest technique available is based on snare resection after lifting the submucosa with a saline-epinephrine injection. This is rarely performed in the esophagus now as this is restricted to pedunculated lesions only.

The cap-assisted technique, first described by Inoue and Endo, involves attaching a specially developed transparent plastic cap to the end of the endoscope[26]. After submucosal injection under the target lesion, the lesion is sucked into the cap and resected using a diathermy loop that has previously been loaded onto a specially designed groove on the lower edge of the cap. This is often referred to as the "suck and cut" technique. Preloading of the loop is easily done in the gastric antrum by applying slight suction to the mucosa and carefully advancing the snare until it is placed exactly in the rim at the distal margin of the cap. The borders of the lesion are marked with electrocautery, before the submucosal injection as it is difficult to identify the borders following the injection.

Another alternative to cap-assisted technique is using a ligation device, based on the same principle of "suck and cut"[27]. This is the commonest technique performed in the United States. After the target lesion is sucked into the cylinder of the ligation device, there is creation of pseudo-polyp using a rubber band which is released simultaneously at the base of this polyp. The band is subsequently released in a few seconds following the prolapse of the mucosa and submucosa into the cylinder. Following this, the ligation device is removed after withdrawing and re-introducing the endoscope and introducing the loop. The main advantage of this method in comparison with cap resection, is that previous submucosal injection is not necessary. The ligation devices come in both single use and reusable forms[28]. Recently, newer ligation cylinders have been developed with multiple rubber bands and a facility for advancing a snare through the working channel of a regular endoscope enabling the endoscopist to perform multiple resections without having to withdraw and reintroduce the endoscope[29].

Both the above described techniques have shown similar efficacy and safety in terms of resection of early esophageal cancers. In a prospective randomized study, 100 consecutive endoscopic resections were performed in 72 patients with early stage esophageal cancer[30]. There were no significant differences in terms of the maximum diameter of the resection specimen, resection area and the complication rate. Stepwise endoscopic resection technique (SRER) entails resection of BE in multiple sessions with an eventual complete removal of the segment[24]. Eligible candidates are those with BE length of 5 cm or less, a histological diagnosis of high grade intraepithelial neoplasia or invasive cancer without poor differentiation, lymphovascular spread or positive resection margins[24].

Outcomes of EMR: EMR, as an effective treatment modality for early EAC was first reported by Ell *et al*[27] in 2000, where complete remission was seen in 97% of the patients with low risk EAC versus 59% in high risk EAC. However, recurrent carcinomas developed in 14% and 17% in high risk and low risk groups respectively at a mean follow up of 1 year[27]. To reduce the risk of recurrences, SRER was described by Pouw *et al*[24], which led to sustained eradication of neoplasia in 95.3% but

Table 1 Comparison of various modalities of endoscopic resection techniques for esophageal cancer

Technique	Focal EMR with ablation	SRER	ESD
Efficacy in ESCC	Curative resection rate 58.6%[34]	Curative resection rate 92.3%[36]	Curative resection rate 99% to 100%[46]
Efficacy in EAC	Eradication of neoplasia 93.4% and eradication of metaplasia 73.1%[75]	Eradication of neoplasia 94.9% and eradication of metaplasia 79.6%[75]	95% to 97% curative resection rate[44,45]
Recurrence	11% for ESCC[34], 1.4% for EAC[75]	0.7% for EAC[75] 6.9 % at one year for ESCC[36]	0.9% to 9.8% for ESCC[58], 0.17% for EAC[56]
Adverse events	Stricture (5% to 14%)[25,61,62], bleeding (1.1%)[75] and perforation (0.2%)[75]	Strictures (33% to 88%)[24,37], bleeding (2.4% to 25%)[24] and perforation (1.3%)[75]	Stricture (15% to 60%)[52], bleeding (0.9% to 6.7%)[52,53] and perforations (3%)[55]

ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma; EMR: Endoscopic mucosal resection; SRER: Stepwise radical endoscopic resection; ESD: Endoscopic submucosal dissection.

found to be associated with strictures requiring repeated dilations in 49% of patients. Therefore focal EMR with ablation of residual mucosa has been described with equivalent outcomes but low rate of strictures compared to SRER[25,31]. A recent study of 1000 patients with IMC undergoing EMR, showed excellent long term outcomes with 96% of the patients achieving complete response with 10-year survival rate of 75%[32]. Other studies have also shown 5-year survival in upto 95% to 98% patients with both early SCC and EAC[27,33]. The long-term outcomes of EMR for SCC are less reported. In a Japanese study of 204 patients with early SCC treated with EMR followed by ablation in the presence of positive margins, 11% of patients experienced recurrence during a median follow of 36 mo[34]. All patients were able to be treated with subsequent endoscopic therapy. Another study of patients undergoing EMR for ESCC involving muscularis propria or submucosa showed no significant difference in 5-year survival when compared to the surgical group (95.0% and 93.5% respectively)[35].

Adverse events after EMR: EMR is generally a safe procedure with a low risk of complications. With focal EMR, strictures and bleeding are seen in about 2% respectively[36]. SRER or extensive EMR can cause bleeding in 2.4% to 25% cases[24]. The rate of stricture formation is also higher as compared to focal EMR, with rates as high as 33%-88% as shown in some studies[24,37]. Perforations are rare with EMR and seen in 0-5% of patients[38]. Focal EMR is associated with high recurrence rate up to 25% to 33% in BE during follow up if the residual BE is left untreated[39-41]. Therefore, focal EMR should always be followed by ablation of residual BE.

ESD

Similar to EMR, the technique of ESD was first attempted in 1988 in Japan for early gastric cancers[42] before being applied for treatment of early esophageal cancers. The procedure involves three steps. After marking the resection borders of the lesion using argon plasma coagulation (APC) or ESD knife, a submucosal cushion is created by injecting normal saline or diluted sodium hyaluronate solution. Finally, dissection of the submucosal layer is achieved after a circumferential incision around the lesion with stripping of the lesion from the muscularis propria. This allows *en bloc* resection of the lesion, irrespective of the size and shape. A carbon dioxide insufflation system is usually used in order to prevent mediastinal emphysema[43].

Outcomes of ESD: Multiple studies have been published in the literature to look at long term outcomes of ESD. Curative *en bloc* resection rates have been reported to be as high as 100% in ESCC and 95% to 97% in EAC[44,45]. Resection with tumor free lateral and basal margins (R0 resection) rate was 90% in lesions involving muscularis layer (M3) and about 70% if lesion extends beyond M3 in BE[44]. More recently, a meta-analysis of 21 studies on ESD for ESCC, the pooled *en bloc* resection rate was 99% to 100% for the tumors and R0 resection rate 90% to 92%. Larger tumors with diameter greater than 2.5 cm achieved lower curative resection rates as compared to the smaller tumors[46].

Overall, ESD has led to excellent *en bloc* resection rates and curative resection rates in Asian countries[47,48]. Studies conducted in Europe have shown high *en bloc* resection rates up to 95% and curative resection rates up to 72% with a recurrence rate of 2.5%. Disease specific survival was 97% with an overall survival of about 96%[44]. Ishihara *et al*[49] in their retrospective study on 136 patients in Japan, showed that ESD was associated with high curative resection rates irrespective of the size of the lesion.

ESD has been studied in BE related neoplasia in studies described from western world, with lesions with high risk for submucosal invasion, size more than 15 mm and poorly lifting in nature. The resection rates were not sufficiently high enough to warrant its use over piecemeal EMR. When performed with ablation, it achieved complete remission in 96% of patients[50]. A European study showed comparable resection rates in BE neoplasia with both EMR and ESD with complete remission, however, ESD was associated with more adverse events and was more time consuming[51].

Adverse events after ESD: ESD is a technically demanding procedure with a learning curve and has

adverse events similar to, but more frequent, than in EMR. The most common complications are bleeding and stricture formation. In patients with BE undergoing ESD, bleeding rates are 0.9% to 6.7%[52] and strictures are reported in 15% to 60% of patients[52,53]. In patients undergoing ESD for ESCC, the rates of strictures may be higher if the entire circumferential esophagus is dissected and > 5 cm of longitudinal mucosal defect length is present[54]. Perforation rates of 3.3% have been reported after esophageal ESD in Japan of which, over half the patients required open thoracotomy for repair[55]. A lower hospital volume and female sex were factors associated with a higher occurrence of complications. In a meta-analysis of ESD in BE related neoplasia, the pooled estimates for perforation and bleeding were 1.5% and 1.7% respectively with a stricture rate of 11.6%[56]. Incidence of recurrence after curative resection was 0.17% (95%CI: 0%-0.3%) at a mean follow-up 22.9 mo (95%CI: 17.5-28.3)[56].

Comparison between EMR and ESD

In patients with BE-related neoplastic lesions larger than 15 mm, lesions with poor lifting and to better assess depth of invasion if submucosal invasion is suspected, ESD is considered superior to EMR[18,57]. However, EMR is preferred in BE associated neoplasia as ESD has not been proven to be superior to EMR in terms of clinical outcomes in a randomized controlled trial[51]. Ishihara *et al*[49] in their retrospective study, showed that in lesions smaller than 15 mm, both the techniques are comparable in achieving complete resection. In patients with ESCC, higher R0 resection rates (100% *vs* 53%) and lower recurrence (0.9% *vs* 9.8%) has been associated with ESD as compared to EMR as shown in a large retrospective study[58]. Similar results were seen in a more recent meta-analysis in Asian population undergoing ESD versus EMR for early esophageal cancer[38]. Strictures are frequently encountered in both EMR and ESD. In a meta-analysis of eight studies, the rates of stricture formation were comparable in both groups[38]. A comparison of both techniques are presented in Table 1.

ABLATION TECHNIQUES

Ablative treatment mainly implies destruction of the neoplastic tissue by thermal injury in the form of heat through burning, coagulation and necrosis or freezing. Following EMR or ESD, radiofrequency ablation (RFA) or argon plasma coagulation (APC) are performed typically after 8-12 wk to eradicate the remaining BE or squamous dysplasia[59]. It is important to confirm the absence of any residual cancer by pathological staging of the specimen. Other ablative techniques such as cryotherapy and photodynamic therapy (PDT) have been used for in patients with early stage cancer independent of EMR.

RFA

This is the most widely used ablation technique, primarily for ablation of dysplastic tissue after resection of cancer. It should not be used as the sole treatment modality for early esophageal cancer. This technique uses a radiofrequency generator and balloon/focal ablation catheters which deliver radiofrequency energy waveform to the epithelium, leading to water vaporization, protein coagulation and subsequent tissue necrosis[60].

Combination of focal resection of cancer followed by ablation of residual BE with RFA showed a complete eradication of dysplasia and intestinal metaplasia of 91% and 78%, respectively at one year follow up[31]. Recurrence of metaplasia is usually seen in the distal part of esophagus or at the gastro-esophageal junction with rates between 13% and 33%, necessitating the need for lifelong surveillance[31]. Complications seen with RFA are strictures and rarely, bleeding. Strictures can develop in 5% to 14% patients with comparable rates in patients with prior EMR[25,61,62].

In contrast to EAC, there is sparse literature on the use of RFA in early ESCC. In a study of 13 patients with ESCC who underwent EMR and subsequent circumferential RFA, complete eradication was seen after a median of two RFA sessions[63]. Three patients developed stenosis and none had recurrence at 17 mo follow up. Similarly, in a bigger cohort study on 29 patients undergoing EMR and RFA for either moderate grade, high grade or flat type early SCC, 97% patients had complete response at 12 mo with no further progression[64]. Results from a United Kingdom registry study on early SCC, showed some contrasting results with 50% patients achieving complete eradication at 1 year follow up and 30% progressing to invasive cancer eventually[65]. Flat ESCC may be under-staged as it is based on endoscopic appearance only, hence endoscopic resection therapies should be preferred over ablation as there might be a higher risk for tumor progression with failure to completely eradicate them.

APC

This is a focal noncontact method of thermal ablation which uses argon gas to conduct electrical current to the target tissue and causes tissue necrosis. Its utility in the management of ESCC is mainly limited to T1a and T1b lesions which are not amenable to endoscopic therapy due to underlying comorbidities. It has shown to be somewhat successful in achieving curative rates with less recurrences. Min *et al*[66] in their study on 19 patients with ESCC who underwent APC, showed that 95% patients achieved complete response at 1 year follow up. However, it is less effective in improving long term survival in patients with early esophageal cancer[67]. APC has shown promising results in the treatment of flat BE lesions which recur or are residual after EMR, ESD or RFA[68]. However, its use is fairly limited to precancerous lesions of the esophagus and indications should be carefully considered in management of early cancer. There are limited studies on the role of APC in the treatment of BE lesions. No studies have reported the

use of APC in EAC.

PDT

This involves the use of laser therapy which is used to activate a photosensitizer, administered either orally or intravenously, to the target lesion leading to ischemia and cell necrosis. Main indication of PDT is palliative treatment in advanced cancers and after failure of chemotherapy and high grade dysplasia in BE. However, some studies supported the use of this technique in treatment of early esophageal cancer. Overholt *et al*[69] reported use of PDT in 13 patients with T1-T2 EAC with eradication of 77% of cancers. In a case series of 14 patients with T1 cancers, 13 patients had complete eradication of cancer[70]. Tanaka *et al*[71] in their retrospective study of 38 patients with early SCC lesions which were too large for endoscopic treatment, showed complete remission in 87% patients after undergoing PDT. Similar rates have been observed in patients with carcinoma *in situ* or T1a ESSC lesions[72]. Main complications of the procedure are stricture formation and photosensitivity. They can develop in about 30% and 70% of patients respectively[69,70].

Cryotherapy

This method involves the use of liquid nitrogen to deliver thermal energy at -196 degrees Celsius causing repeated cycles of freezing and thawing target tissue which leads to immediate as well as delayed necrosis. In a multicenter study of 88 patients with EAC (39 with T1a, 25 with T1b, 9 with unspecified T1, and 15 with T2), complete response of intraluminal disease was seen in 55.8% of the patients[73]. The complete response rates were 76.3% for T1a, 45.8% for T1b, 66.2% for all T1, and 6.7% for T2 during a mean follow-up was 18.4 mo. There were no deaths or perforations related to spray cryotherapy. Strictures developed in 12 of 88 patients (13.6%) but were present before spray cryotherapy in 3 out of 12 patients. Use of a new cryoballoon system using nitrous oxide has been developed and its use was reported in 9 patients with squamous dysplasia and one with ESCC. All patients were disease free at last visit, with a median follow-up time of 10.7 mo[74].

CONCLUSION

EET plays a pivotal role in the management of patients with early esophageal cancer who are at a very low risk for lymph node metastases. The main advantage of EET over surgery is the lower morbidity and mortality rates with similar cure rates, five year survival rates and better quality of life. These excellent outcomes are tempered by the need for multiple treatment sessions for complete eradication and risk of post eradication recurrences. Careful patient selection by a multidisciplinary approach and patient compliance are crucial for treatment success.

REFERENCES

1 **Global Burden of Disease Cancer Collaboration**, Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, Allen C, Hansen G, Woodbrook R, Wolfe C, Hamadeh RR, Moore A, Werdecker A, Gessner BD, Te Ao B, McMahon B, Karimkhani C, Yu C, Cooke GS, Schwebel DC, Carpenter DO, Pereira DM, Nash D, Kazi DS, De Leo D, Plass D, Ukwaja KN, Thurston GD, Yun Jin K, Simard EP, Mills E, Park EK, Catalá-López F, deVeber G, Gotay C, Khan G, Hosgood HD 3rd, Santos IS, Leasher JL, Singh J, Leigh J, Jonas JB, Sanabria J, Beardsley J, Jacobsen KH, Takahashi K, Franklin RC, Ronfani L, Montico M, Naldi L, Tonelli M, Geleijnse J, Petzold M, Shrime MG, Younis M, Yonemoto N, Breitborde N, Yip P, Pourmalek F, Lotufo PA, Esteghamati A, Hankey GJ, Ali R, Lunevicius R, Malekzadeh R, Dellavalle R, Weintraub R, Lucas R, Hay R, Rojas-Rueda D, Westerman R, Sepanlou SG, Nolte S, Patten S, Weichenthal S, Abera SF, Fereshtehnejad SM, Shiue I, Driscoll T, Vasankari T, Alsharif U, Rahimi-Movaghar V, Vlassov VV, Marcenes WS, Mekonnen W, Melaku YA, Yano Y, Artaman A, Campos I, MacLachlan J, Mueller U, Kim D, Trillini M, Eshrati B, Williams HC, Shibuya K, Dandona R, Murthy K, Cowie B, Amare AT, Antonio CA, Castañeda-Orjuela C, van Gool CH, Violante F, Oh IH, Deribe K, Soreide K, Knibbs L, Kereselidze M, Green M, Cardenas R, Roy N, Tillmann T, Li Y, Krueger H, Monasta L, Dey S, Sheikhbahaei S, Hafezi-Nejad N, Kumar GA, Sreeramareddy CT, Dandona L, Wang H, Vollset SE, Mokdad A, Salomon JA, Lozano R, Vos T, Forouzanfar M, Lopez A, Murray C, Naghavi M. The Global Burden of Cancer 2013. *JAMA Oncol* 2015; **1**: 505-527 [PMID: 26181261 DOI: 10.1001/jamaoncol.2015.0735]

2 **Arnold M**, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut* 2015; **64**: 381-387 [PMID: 25320104 DOI: 10.1136/gutjnl-2014-308124]

3 **Edgren G**, Adami HO, Weiderpass E, Nyrén O. A global assessment of the oesophageal adenocarcinoma epidemic. *Gut* 2013; **62**: 1406-1414 [PMID: 22917659 DOI: 10.1136/gutjnl-2012-302412]

4 **Njei B**, McCarty TR, Birk JW. Trends in esophageal cancer survival in United States adults from 1973 to 2009: A SEER database analysis. *J Gastroenterol Hepatol* 2016; **31**: 1141-1146 [PMID: 26749521 DOI: 10.1111/jgh.13289]

5 **Jacobs M**, Macefield RC, Elbers RG, Sitnikova K, Korfage IJ, Smets EM, Henselmans I, van Berge Henegouwen MI, de Haes JC, Blazeby JM, Sprangers MA. Meta-analysis shows clinically relevant and long-lasting deterioration in health-related quality of life after esophageal cancer surgery. *Qual Life Res* 2014; **23**: 1097-1115 [PMID: 24129668 DOI: 10.1007/s11136-013-0545-z]

6 **Gavin AT**, Francisci S, Foschi R, Donnelly DW, Lemmens V, Brenner H, Anderson LA; EUROCARE-4 Working Group. Oesophageal cancer survival in Europe: a EUROCARE-4 study. *Cancer Epidemiol* 2012; **36**: 505-512 [PMID: 22910036 DOI: 10.1016/j.canep.2012.07.009]

7 **Wani S**, Drahos J, Cook MB, Rastogi A, Bansal A, Yen R, Sharma P, Das A. Comparison of endoscopic therapies and surgical resection in patients with early esophageal cancer: a population-based study. *Gastrointest Endosc* 2014; **79**: 224-232.e1 [PMID: 24060519 DOI: 10.1016/j.gie.2013.08.002]

8 **Wani S**, Early D, Edmundowicz S, Sharma P. Management of high-grade dysplasia and intramucosal adenocarcinoma in Barrett's esophagus. *Clin Gastroenterol Hepatol* 2012; **10**: 704-711 [PMID: 22507878 DOI: 10.1016/j.cgh.2012.03.030]

9 **Bennett C**, Vakil N, Bergman J, Harrison R, Odze R, Vieth M, Sanders S, Gay L, Pech O, Longcroft-Wheaton G, Romero Y, Inadomi J, Tack J, Corley DA, Manner H, Green S, Al Dulaimi D, Ali H, Allum B, Anderson M, Curtis H, Falk G, Fennerty MB, Fullarton G, Krishnadath K, Meltzer SJ, Armstrong D, Ganz R, Cengia G, Going JJ, Goldblum J, Gordon C, Grabsch H, Haigh C, Hongo M, Johnston D, Forbes-Young R, Kay E, Kaye P, Lerut T, Lovat LB, Lundell L, Mairs P, Shimoda T, Spechler S, Sontag S, Malfertheiner P, Murray I, Nanji M, Poller D, Ragunath K, Regula J, Cestari R, Shepherd N, Singh R, Stein HJ, Talley NJ, Galmiche JP, Tham TC, Watson P, Yerian L, Rugge M, Rice TW, Hart J, Gittens S, Hewin D, Hochberger J, Kahrilas P, Preston S, Sampliner R, Sharma P, Stuart R, Wang K, Waxman I, Abley C, Loft D, Penman I, Shaheen NJ, Chak A, Davies G, Dunn L, Falck-Ytter Y, Decaestecker J, Bhandari P, Ell C, Griffin SM, Attwood S, Barr H, Allen J, Ferguson MK, Moayyedi P, Jankowski

JA. Consensus statements for management of Barrett's dysplasia and early-stage esophageal adenocarcinoma, based on a Delphi process. *Gastroenterology* 2012; **143**: 336-346 [PMID: 22537613 DOI: 10.1053/j.gastro.2012.04.032]

10 **Prasad GA**, Wu TT, Wigle DA, Buttar NS, Wongkeesong LM, Dunagan KT, Lutzke LS, Borkenhagen LS, Wang KK. Endoscopic and surgical treatment of mucosal (T1a) esophageal adenocarcinoma in Barrett's esophagus. *Gastroenterology* 2009; **137**: 815-823 [PMID: 19524578 DOI: 10.1053/j.gastro.2009.05.059]

11 **Cho JW**, Choi SC, Jang JY, Shin SK, Choi KD, Lee JH, Kim SG, Sung JK, Jeon SW, Choi IJ, Kim GH, Jee SR, Lee WS, Jung HY; Korean ESD Study Group. Lymph Node Metastases in Esophageal Carcinoma: An Endoscopist's View. *Clin Endosc* 2014; **47**: 523-529 [PMID: 25505718 DOI: 10.5946/ce.2014.47.6.523]

12 **Fitzgerald RC**, di Pietro M, Ragunath K, Ang Y, Kang JY, Watson P, Trudgill N, Patel P, Kaye PV, Sanders S, O'Donovan M, Bird-Lieberman E, Bhandari P, Jankowski JA, Attwood S, Parsons SL, Loft D, Lagergren J, Moayyedi P, Lyratzopoulos G, de Caestecker J; British Society of Gastroenterology. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. *Gut* 2014; **63**: 7-42 [PMID: 24165758 DOI: 10.1136/gutjnl-2013-305372]

13 **Raghu Subramanian C,** Triadafilopoulos G. Diagnosis and therapy of esophageal squamous cell dysplasia and early esophageal squamous cell cancer. *Gastroenterol Rep* 2017; **5**: 247-57 [DOI: 10.1093/gastro/gox022]

14 **Kandiah K**, Chedgy FJ, Subramaniam S, Thayalasekaran S, Kurup A, Bhandari P. Early squamous neoplasia of the esophagus: The endoscopic approach to diagnosis and management. *Saudi J Gastroenterol* 2017; **23**: 75-81 [PMID: 28361837]

15 **Ancona E**, Rampado S, Cassaro M, Battaglia G, Ruol A, Castoro C, Portale G, Cavallin F, Rugge M. Prediction of lymph node status in superficial esophageal carcinoma. *Ann Surg Oncol* 2008; **15**: 3278-3288 [PMID: 18726651 DOI: 10.1245/s10434-008-0065-1]

16 **He LJ**, Shan HB, Luo GY, Li Y, Zhang R, Gao XY, Wang GB, Lin SY, Xu GL, Li JJ. Endoscopic ultrasonography for staging of T1a and T1b esophageal squamous cell carcinoma. *World J Gastroenterol* 2014; **20**: 1340-1347 [PMID: 24574809 DOI: 10.3748/wjg.v20.i5.1340]

17 **Ono S**, Fujishiro M, Koike K. Endoscopic submucosal dissection for superficial esophageal neoplasms. *World J Gastrointest Endosc* 2012; **4**: 162-166 [PMID: 22624067 DOI: 10.4253/wjge.v4.i5.162]

18 **Pimentel-Nunes P**, Dinis-Ribeiro M, Ponchon T, Repici A, Vieth M, De Ceglie A, Amato A, Berr F, Bhandari P, Bialek A, Conio M, Haringsma J, Langner C, Meisner S, Messmann H, Morino M, Neuhaus H, Piessevaux H, Rugge M, Saunders BP, Robaszkiewicz M, Seewald S, Kashin S, Dumonceau JM, Hassan C, Deprez PH. Endoscopic submucosal dissection: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy*2015; **47**: 829-854 [PMID: 26317585 DOI: 10.1055/s-0034-1392882]

19 **Higuchi K**, Tanabe S, Koizumi W, Sasaki T, Nakatani K, Saigenji K, Kobayashi N, Mitomi H. Expansion of the indications for endoscopic mucosal resection in patients with superficial esophageal carcinoma. *Endoscopy*2007; **39**: 36-40 [PMID: 17252458 DOI: 10.1055/s-2006-945148]

20 **Hölscher AH**, Bollschweiler E, Schneider PM, Siewert JR. Prognosis of early esophageal cancer. Comparison between adeno- and squamous cell carcinoma. *Cancer* 1995; **76**: 178-186 [PMID: 8625089 DOI: 10.1002/1097-0142(19950715)76:2<178::AID-CNCR2820760204>3.0.CO;2-D]

21 **Soetikno RM**, Gotoda T, Nakanishi Y, Soehendra N. Endoscopic mucosal resection. *Gastrointest Endosc* 2003; **57**: 567-579 [PMID: 12665775 DOI: 10.1067/mge.2003.130]

22 **Hull MJ**, Mino-Kenudson M, Nishioka NS, Ban S, Sepehr A, Puricelli W, Nakatsuka L, Ota S, Shimizu M, Brugge WR, Lauwers GY. Endoscopic mucosal resection: an improved diagnostic procedure for early gastroesophageal epithelial neoplasms. *Am J Surg Pathol* 2006; **30**: 114-118 [PMID: 16330950 DOI: 10.1097/01.pas.0000180438.56528.a0]

23 **Larghi A**, Lightdale CJ, Memeo L, Bhagat G, Okpara N, Rotterdam H. EUS followed by EMR for staging of high-grade dysplasia and early cancer in Barrett's esophagus. *Gastrointest Endosc* 2005; **62**: 16-23 [PMID: 15990814 DOI: 10.1016/S0016-5107(05)00319-6]

24 **Pouw RE**, Seewald S, Gondrie JJ, Deprez PH, Piessevaux H, Pohl H, Rösch T, Soehendra N, Bergman JJ. Stepwise radical endoscopic resection for eradication of Barrett's oesophagus with early neoplasia in a cohort of 169 patients. *Gut* 2010; **59**: 1169-1177 [PMID: 20525701 DOI: 10.1136/gut.2010.210229]

25 **van Vilsteren FG**, Pouw RE, Seewald S, Alvarez Herrero L, Sondermeijer CM, Visser M, Ten Kate FJ, Yu Kim Teng KC, Soehendra N, Rösch T, Weusten BL, Bergman JJ. Stepwise radical endoscopic resection versus radiofrequency ablation for Barrett's oesophagus with high-grade dysplasia or early cancer: a multicentre randomised trial. *Gut* 2011; **60**: 765-773 [PMID: 21209124 DOI: 10.1136/gut.2010.229310]

26 **Inoue H**, Endo M, Takeshita K, Yoshino K, Muraoka Y, Yoneshima H. A new simplified technique of endoscopic esophageal mucosal resection using a cap-fitted panendoscope (EMRC) *Surg Endosc* 1992; **6**: 264-265 [PMID: 1465738 DOI: 10.1007/BF02498820]

27 **Ell C**, May A, Gossner L, Pech O, Günter E, Mayer G, Henrich R, Vieth M, Müller H, Seitz G, Stolte M. Endoscopic mucosal resection of early cancer and high-grade dysplasia in Barrett's esophagus. *Gastroenterology*2000; **118**: 670-677 [PMID: 10734018 DOI: 10.1016/S0016-5085(00)70136-3]

28 **Ell C**, May A, Wurster H. The first reusable multiple-band ligator for endoscopic hemostasis of variceal bleeding, nonvariceal bleeding and mucosal resection. *Endoscopy* 1999; **31**: 738-740 [PMID: 10604616 DOI: 10.1055/s-1999-81]

29 **Soehendra N**, Seewald S, Groth S, Omar S, Seitz U, Zhong Y, de Weerth A, Thonke F, Schroeder S. Use of modified multiband ligator facilitates circumferential EMR in Barrett's esophagus (with video). *Gastrointest Endosc* 2006; **63**: 847-852 [PMID: 16650552 DOI: 10.1016/j.gie.2005.06.052]

30 **May A**, Gossner L, Behrens A, Kohnen R, Vieth M, Stolte M, Ell C. A prospective randomized trial of two different endoscopic resection techniques for early stage cancer of the esophagus. *Gastrointest Endosc* 2003; **58**: 167-175 [PMID: 12872081 DOI: 10.1067/mge.2003.339]

31 **Orman ES**, Li N, Shaheen NJ. Efficacy and durability of radiofrequency ablation for Barrett's Esophagus: systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2013; **11**: 1245-1255 [PMID: 23644385 DOI: 10.1016/j.cgh.2013.03.039]

32 **Pech O**, May A, Manner H, Behrens A, Pohl J, Weferling M, Hartmann U, Manner N, Huijsmans J, Gossner L, Rabenstein T, Vieth M, Stolte M, Ell C. Long-term efficacy and safety of endoscopic resection for patients with mucosal adenocarcinoma of the esophagus. *Gastroenterology* 2014; **146**: 652-660.e1 [PMID: 24269290 DOI: 10.1053/j.gastro.2013.11.006]

33 **Inoue H**, Fukami N, Yoshida T, Kudo SE. Endoscopic mucosal resection for esophageal and gastric cancers. *J Gastroenterol Hepatol* 2002; **17**: 382-388 [PMID: 11982716 DOI: 10.1046/j.1440-1746.2002.02732.x]

34 **Nakagawa K**, Koike T, Iijima K, Shinkai H, Hatta W, Endo H, Ara N, Uno K, Asano N, Imatani A, Shimosegawa T. Comparison of the long-term outcomes of endoscopic resection for superficial squamous cell carcinoma and adenocarcinoma of the esophagus in Japan. *Am J Gastroenterol* 2014; **109**: 348-356 [PMID: 24394751 DOI: 10.1038/ajg.2013.450]

35 **Shimizu Y**, Tsukagoshi H, Fujita M, Hosokawa M, Kato M, Asaka M. Long-term outcome after endoscopic mucosal resection in patients with esophageal squamous cell carcinoma invading the muscularis mucosae or deeper. *Gastrointest Endosc* 2002; **56**: 387-390 [PMID: 12196777 DOI: 10.1016/S0016-5107(02)70043-6]

36 **Jin XF**, Sun QY, Chai TH, Li SH, Guo YL. Clinical value of multiband mucosectomy for the treatment of squamous intraepithelial neoplasia of the esophagus. *J Gastroenterol Hepatol* 2013; **28**: 650-655 [PMID: 23301863 DOI: 10.1111/jgh.12111]

37 **Conio M**, Fisher DA, Blanchi S, Ruggeri C, Filiberti R, Siersema PD. One-step circumferential endoscopic mucosal cap resection of Barrett's esophagus with early neoplasia. *Clin Res Hepatol Gastroenterol* 2014; **38**: 81-91 [PMID: 23856637 DOI: 10.1016/j.clinre.2013.05.015]

38 **Guo HM**, Zhang XQ, Chen M, Huang SL, Zou XP. Endoscopic submucosal dissection vs endoscopic mucosal resection for superficial esophageal cancer. *World J Gastroenterol* 2014; **20**: 5540-5547 [PMID: 24833885 DOI: 10.3748/wjg.v20.i18.5540]

39 **Pech O**, Behrens A, May A, Nachbar L, Gossner L, Rabenstein T, Manner H, Guenter E, Huijsmans J, Vieth M, Stolte M, Ell C. Long-term results and risk factor analysis for recurrence after curative endoscopic therapy in 349 patients with high-grade intraepithelial neoplasia and mucosal adenocarcinoma in Barrett's oesophagus. *Gut* 2008; **57**: 1200-1206 [PMID: 18460553 DOI: 10.1136/gut.2007.142539]

40 **May A**, Gossner L, Pech O, Fritz A, Günter E, Mayer G, Müller H, Seitz G, Vieth M, Stolte M, Ell C. Local endoscopic therapy for intraepithelial high-grade neoplasia and early adenocarcinoma in Barrett's oesophagus: acute-phase and intermediate results of a new treatment approach. *Eur J Gastroenterol Hepatol* 2002; **14**: 1085-1091 [PMID: 12362099 DOI: 10.1097/00042737-200210000-00009]

41 **Peters FP**, Kara MA, Rosmolen WD, Aalders MC, Ten Kate FJ, Bultje BC, Krishnadath KK, Fockens P, van Lanschot JJ, van Deventer SJ, Bergman JJ. Endoscopic treatment of high-grade dysplasia and early stage cancer in Barrett's esophagus. *Gastrointest Endosc* 2005; **61**: 506-514 [PMID: 15812401 DOI: 10.1016/S0016-5107(05)00063-5]

42 **Hirao M**, Masuda K, Asanuma T, Naka H, Noda K, Matsuura K, Yamaguchi O, Ueda N. Endoscopic resection of early gastric cancer and other tumors with local injection of hypertonic saline-epinephrine. *Gastrointest Endosc* 1988; **34**: 264-269 [PMID: 3391382 DOI: 10.1016/S0016-5107(88)71327-9]

43 **Ning B**, Abdelfatah MM, Othman MO. Endoscopic submucosal dissection and endoscopic mucosal resection for early stage esophageal cancer. *Ann Cardiothorac Surg* 2017; **6**: 88-98 [PMID: 28446997 DOI: 10.21037/acs.2017.03.15]

44 **Probst A**, Aust D, Märkl B, Anthuber M, Messmann H. Early esophageal cancer in Europe: endoscopic treatment by endoscopic submucosal dissection. *Endoscopy* 2015; **47**: 113-121 [PMID: 25479563 DOI: 10.1055/s-0034-1391086]

45 **Isomoto H**, Yamaguchi N, Minami H, Nakao K. Management of complications associated with endoscopic submucosal dissection/endoscopic mucosal resection for esophageal cancer. *Dig Endosc* 2013; **25** Suppl 1: 29-38 [PMID: 23368404 DOI: 10.1111/j.1443-1661.2012.01388.x]

46 **Sun F**, Yuan P, Chen T, Hu J. Efficacy and complication of endoscopic submucosal dissection for superficial esophageal carcinoma: a systematic review and meta-analysis. *J Cardiothorac Surg* 2014; **9**: 78 [PMID: 24885614 DOI: 10.1186/1749-8090-9-78]

47 **Park HC**, Kim DH, Gong EJ, Na HK, Ahn JY, Lee JH, Jung KW, Choi KD, Song HJ, Lee GH, Jung HY, Kim JH. Ten-year experience of esophageal endoscopic submucosal dissection of superficial esophageal neoplasms in a single center. *Korean J Intern Med* 2016; **31**: 1064-1072 [PMID: 27618866 DOI: 10.3904/kjim.2015.210]

48 **Nagami Y**, Ominami M, Shiba M, Minamino H, Fukunaga S, Kameda N, Sugimori S, Machida H, Tanigawa T, Yamagami H, Watanabe T, Tominaga K, Fujiwara Y, Arakawa T. The five-year survival rate after endoscopic submucosal dissection for superficial esophageal squamous cell neoplasia. *Dig Liver Dis* 2017; **49**: 427-433 [PMID: 28096057 DOI: 10.1016/j.dld.2016.12.009]

49 **Ishihara R**, Iishi H, Uedo N, Takeuchi Y, Yamamoto S, Yamada T, Masuda E, Higashino K, Kato M, Narahara H, Tatsuta M. Comparison of EMR and endoscopic submucosal dissection for en bloc resection of early esophageal cancers in Japan. *Gastrointest Endosc* 2008; **68**: 1066-1072 [PMID: 18620345 DOI: 10.1016/j.gie.2008.03.1114]

50 **Neuhaus H**, Terheggen G, Rutz EM, Vieth M, Schumacher B. Endoscopic submucosal dissection plus radiofrequency ablation of neoplastic Barrett's esophagus. *Endoscopy* 2012; **44**: 1105-1113 [PMID: 22968641 DOI: 10.1055/s-0032-1310155]

51 **Terheggen G**, Horn EM, Vieth M, Gabbert H, Enderle M, Neugebauer A, Schumacher B, Neuhaus H. A randomised trial of endoscopic submucosal dissection versus endoscopic mucosal resection for early Barrett's neoplasia. *Gut* 2017; **66**: 783-793 [PMID: 26801885 DOI: 10.1136/gutjnl-2015-310126]

52 **Kim JS**, Kim BW, Shin IS. Efficacy and safety of endoscopic submucosal dissection for superficial squamous esophageal neoplasia: a meta-analysis. *Dig Dis Sci* 2014; **59**: 1862-1869 [PMID: 24619279 DOI: 10.1007/s10620-014-3098-2]

53 **Chevaux JB**, Piessevaux H, Jouret-Mourin A, Yeung R, Danse E, Deprez PH. Clinical outcome in patients treated with endoscopic submucosal dissection for superficial Barrett's neoplasia. *Endoscopy* 2015; **47**: 103-112 [PMID: 25412090 DOI: 10.1055/s-0034-1390982]

54 **Miwata T**, Oka S, Tanaka S, Kagemoto K, Sanomura Y, Urabe Y, Hiyama T, Chayama K. Risk factors for esophageal stenosis after entire circumferential endoscopic submucosal dissection for superficial esophageal squamous cell carcinoma. *Surg Endosc* 2016; **30**: 4049-4056 [PMID: 26703127 DOI: 10.1007/s00464-015-4719-3]

55 **Odagiri H**, Yasunaga H. Complications following endoscopic submucosal dissection for gastric, esophageal, and colorectal cancer: a review of studies based on nationwide large-scale databases. *Ann Transl Med* 2017; **5**: 189 [PMID: 28616404 DOI: 10.21037/atm.2017.02.12]

56 **Yang D**, Zou F, Xiong S, Forde JJ, Wang Y, Draganov PV. Endoscopic submucosal dissection for early Barrett's neoplasia: a meta-analysis. *Gastrointest Endosc* 2018; **87**: 1383-1393 [PMID: 28993137 DOI: 10.1016/j.gie.2017.09.038]

57 **Yang D**, Coman RM, Kahaleh M, Waxman I, Wang AY, Sethi A, Shah AR, Draganov PV. Endoscopic submucosal dissection for Barrett's early neoplasia: a multicenter study in the United States. *Gastrointest Endosc* 2017; **86**: 600-607 [PMID: 27688205 DOI: 10.1016/j.gie.2016.09.023]

58 **Takahashi H**, Arimura Y, Masao H, Okahara S, Tanuma T, Kodaira J, Kagaya H, Shimizu Y, Hokari K, Tsukagoshi H, Shinomura Y, Fujita M. Endoscopic submucosal dissection is superior to conventional endoscopic resection as a curative treatment for early squamous cell carcinoma of the esophagus (with video). *Gastrointest Endosc* 2010; **72**: 255-264, 264.e1-264.e2 [PMID: 20541198 DOI: 10.1016/j.gie.2010.02.040]

59 **di Pietro M**, Canto MI, Fitzgerald RC. Endoscopic Management of Early Adenocarcinoma and Squamous Cell Carcinoma of the Esophagus: Screening, Diagnosis, and Therapy. *Gastroenterology* 2018; **154**: 421-436 [PMID: 28778650 DOI: 10.1053/j.gastro.2017.07.041]

60 **Shaheen NJ**, Overholt BF, Sampliner RE, Wolfsen HC, Wang KK, Fleischer DE, Sharma VK, Eisen GM, Fennerty MB, Hunter JG, Bronner MP, Goldblum JR, Bennett AE, Mashimo H, Rothstein RI, Gordon SR, Edmundowicz SA, Madanick RD, Peery AF, Muthusamy VR, Chang KJ, Kimmey MB, Spechler SJ, Siddiqui AA, Souza RF, Infantolino A, Dumot JA, Falk GW, Galanko JA, Jobe BA, Hawes RH, Hoffman BJ, Sharma P, Chak A, Lightdale CJ. Durability of radiofrequency ablation in Barrett's esophagus with dysplasia. *Gastroenterology* 2011; **141**: 460-468 [PMID: 21679712 DOI: 10.1053/j.gastro.2011.04.061]

61 **Okoro NI**, Tomizawa Y, Dunagan KT, Lutzke LS, Wang KK, Prasad GA. Safety of prior endoscopic mucosal resection in patients receiving radiofrequency ablation of Barrett's esophagus. *Clin Gastroenterol Hepatol*2012; **10**: 150-154 [PMID: 22056303 DOI: 10.1016/j.cgh.2011.10.030]

62 **Li N**, Pasricha S, Bulsiewicz WJ, Pruitt RE, Komanduri S, Wolfsen HC, Chmielewski GW, Corbett FS, Chang KJ, Shaheen NJ. Effects of preceding endoscopic mucosal resection on the efficacy and safety of radiofrequency ablation for treatment of Barrett's esophagus: results from the United States Radiofrequency Ablation Registry. *Dis Esophagus* 2016; **29**: 537-543 [PMID: 26121935 DOI: 10.1111/dote.12386]

63 **van Vilsteren FG**, Alvarez Herrero L, Pouw RE, ten Kate FJ, Visser M, Seldenrijk CA, van Berge Henegouwen MI, Weusten BL, Bergman JJ. Radiofrequency ablation for the endoscopic eradication of esophageal squamous high grade intraepithelial neoplasia and mucosal squamous cell carcinoma. *Endoscopy* 2011; **43**: 282-290 [PMID: 21455869 DOI: 10.1055/s-0030-1256309]

64 **Bergman JJ**, Zhang YM, He S, Weusten B, Xue L, Fleischer DE, Lu N, Dawsey SM, Wang GQ. Outcomes from a prospective trial of endoscopic radiofrequency ablation of early squamous cell neoplasia of the esophagus. *Gastrointest Endosc* 2011; **74**: 1181-1190 [PMID:

21839994 DOI: 10.1016/j.gie.2011.05.024]
65 **Haidry RJ**, Butt MA, Dunn J, Banks M, Gupta A, Smart H, Bhandari P, Smith LA, Willert R, Fullarton G, John M, Di Pietro M, Penman I, Novelli M, Lovat LB. Radiofrequency ablation for early oesophageal squamous neoplasia: outcomes form United Kingdom registry. *World J Gastroenterol* 2013; **19**: 6011-6019 [PMID: 24106401 DOI: 10.3748/wjg.v19.i36.6011]
66 **Min BH**, Kim ER, Lee JH, Kang KJ, Rhee PL, Kim JJ, Rhee JC. Feasibility and efficacy of argon plasma coagulation for early esophageal squamous cell neoplasia. *Endoscopy* 2013; **45**: 575-578 [PMID: 23801315 DOI: 10.1055/s-0033-1344025]
67 **Wang GQ**, Hao CQ, Wei WQ, Zhao L. Long-term outcomes of endoscopic argon plasma coagulation (APC) therapy for early esophageal cancer and precancerous lesions. *Zhonghua Zhong Liu Za Zhi* 2013; **35**: 456-458 [PMID: 24119907]
68 **Manner H**, May A, Kouti I, Pech O, Vieth M, Ell C. Efficacy and safety of Hybrid-APC for the ablation of Barrett's esophagus. *Surg Endosc* 2016; **30**: 1364-1370 [PMID: 26104794 DOI: 10.1007/s00464-015-4336-1]
69 **Overholt BF**, Panjehpour M, Haydek JM. Photodynamic therapy for Barrett's esophagus: follow-up in 100 patients. *Gastrointest Endosc* 1999; **49**: 1-7 [PMID: 9869715 DOI: 10.1016/S0016-5107(99)70437-2]
70 **Wolfsen HC**, Woodward TA, Raimondo M. Photodynamic therapy for dysplastic Barrett esophagus and early esophageal adenocarcinoma. *Mayo Clin Proc* 2002; **77**: 1176-1181 [PMID: 12440553 DOI: 10.4065/77.11.1176]
71 **Tanaka T**, Matono S, Nagano T, Murata K, Sueyoshi S, Yamana H, Shirouzu K, Fujita H. Photodynamic therapy for large superficial squamous cell carcinoma of the esophagus. *Gastrointest Endosc* 2011; **73**: 1-6 [PMID: 21074765 DOI: 10.1016/j.gie.2010.08.049]
72 **Savary JF**, Grosjean P, Monnier P, Fontolliet C, Wagnieres G, Braichotte D, van den Bergh H. Photodynamic therapy of early squamous cell carcinomas of the esophagus: a review of 31 cases. *Endoscopy* 1998; **30**: 258-265 [PMID: 9615874 DOI: 10.1055/s-2007-1001252]
73 **Tsai FC**, Ghorbani S, Greenwald BD, Jang S, Dumot JA, McKinley MJ, Shaheen NJ, Habr F, Wolfsen HC, Abrams JA, Lightdale CJ, Nishioka NS, Johnston MH, Zfass A, Coyle WJ. Safety and efficacy of endoscopic spray cryotherapy for esophageal cancer. *Dis Esophagus* 2017; **30**: 1-7 [PMID: 28881903 DOI: 10.1093/dote/dox087]
74 **Canto MI**, Abrams JA, Künzli HT, Weusten B, Komatsu Y, Jobe BA, Lightdale CJ. Nitrous oxide cryotherapy for treatment of esophageal squamous cell neoplasia: initial multicenter international experience with a novel portable cryoballoon ablation system (with video). *Gastrointest Endosc* 2018; **87**: 574-581 [PMID: 28720474 DOI: 10.1016/j.gie.2017.07.013]
75 **Desai M**, Saligram S, Gupta N, Vennalaganti P, Bansal A, Choudhary A, Vennelaganti S, He J, Titi M, Maselli R, Qumseya B, Olyaee M, Waxman I, Repici A, Hassan C, Sharma P. Efficacy and safety outcomes of multimodal endoscopic eradication therapy in Barrett's esophagus-related neoplasia: a systematic review and pooled analysis. *Gastrointest Endosc* 2017; **85**: 482-495.e4 [PMID: 27670227 DOI: 10.1016/j.gie.2016.09.022]

P- Reviewer: Yamamoto K, Kim GHH **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 3974-3979

DOI: 10.3748/wjg.v24.i35.3974

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

EDITORIAL

Biomarkers for hepatocellular carcinoma: What's new on the horizon?

Matthias Ocker

Matthias Ocker, Department of Translational Medicine Oncology, Bayer AG, Berlin 13353, Germany

Matthias Ocker, Charité University Medicine Berlin, Berlin 10117, Germany

ORCID number: Matthias Ocker (0000-0001-8263-6288).

Author contributions: Ocker M solely contributed to the manuscript.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Matthias Ocker, MD, Professor, Department of Translational Medicine Oncology, Bayer AG, Muellerstrasse 178, Berlin 13353, Germany. matthias.ocker@bayer.com
Telephone: +49-30-468194799
Fax: +49-30-468994799

Received: June 11, 2018
Peer-review started: June 12, 2018
First decision: July 6, 2018
Revised: July 29, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 21, 2018

Abstract

Treatment of advanced hepatocellular carcinoma remains unsatisfying and so far only prognostic biomarkers like α-fetoprotein have been established. No clear predictive biomarker is currently available for standard of care therapies, including targeted therapies like sorafenib. Novel therapeutic options like immune checkpoint inhibitors may pose new challenges to identification and validation of such markers. Currently, PD-L1 expression *via* immunohistochemistry and tumor mutational burden *via* next-generation sequencing are explored as predictive biomarkers for these novel treatments. Limited tissue availability due to lack of biopsies still restricts the use of tissue based approaches. Novel methods exploring circulating or cell free nucleic acids (DNA, RNA or miRNA-containing exosomes) could provide a new opportunity to establish predictive biomarkers. Epigenetic profiling and next-generation sequencing approaches from liquid biopsies are under development. Sample size, etiologic and geographical background need to be carefully addressed in such studies to achieve meaningful results that could be translated into clinical practice. Proteomics, metabolomics and molecular imaging are further emerging technologies.

Key words: Hepatocellular carcinoma; Biomarker; Next-generation sequencing; Liquid Biopsy; Functional imaging; Molecular imaging; Circulating free DNA; Circulating tumor cells; Immune checkpoint inhibitors

Core tip: Hepatocellular carcinoma (HCC) is a heterogeneous disease with various underlying etiologies and an overall still poor prognosis. Biomarkers to identify optimal treatment for distinct patients are still lacking for HCC due to limited availability of biopsies. Novel treatment options, esp. immune checkpoint inhibitors, may need novel biomarker approaches and non-tissue based technologies might provide a solution to identify those biomarkers. In this article, the current status of biomarker identification for HCC is discussed.

Ocker M. Biomarkers for hepatocellular carcinoma: What's new on the horizon? *World J Gastroenterol* 2018; 24(35): 3974-3979 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/3974.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.3974

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver and ranks 3rd in cancer-related deaths worldwide[1,2]. While its incidence continues to be high in Africa and Asia, Western countries also showed increasing incidences rates in the past decades due to chronic hepatitis C virus (HCV) infection, alcohol consumption and high rates of obesity linked to non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and subsequent development of chronic liver disease with cirrhosis[3,4]. Although, a slight improvement in global HCC mortality was recently reported, certain sub-populations and regions esp. in Western countries still have an unfavourable prognosis and risk assessment with continuous high incidence and mortality[5,6]. Curative treatment options like surgical resection or orthotopic liver transplantation are only feasible in a minority of patients at early disease stages and with preserved liver function. Thus, the overall prognosis for patients with HCC remains unsatisfying, its 5 year survival being a dismal 6.9%, an incidence to mortality ratio of 0.95 and a median overall survival of only 11 mo[7]. For advanced stages, treatment is based on multi-kinase inhibitors like sorafenib or regorafenib, while recent data indicate that immune checkpoint inhibitors will lead to increased response rates also in this setting[8,9].

Biomarkers are defined as characteristics that are measured as indicators of physiologic or pathologic processes or in response to various diagnostic or therapeutic procedures. The reader is referred to the recent definitions of the FDA-NIH Biomarker Working group for full definition of different biomarker types[10]. Various prognostic biomarkers, *e.g.*, α-fetoprotein (AFP), AFP-L3 or Des-γ-carboxyprothrombin (DCP), are currently used or under investigation for the early diagnosis and surveillance of HCC patients. Here, newer biomarkers like osteopontin, glypican-3 or high c-met expression have shown additional value, esp. when combining these parameters as was shown for osteopontin and AFP[11-13]. Yet, little progress was achieved in developing predictive biomarkers for targeted and other novel treatment options[14,15]. In this article, the current status of predictive biomarkers for identification and selection of patients for novel therapies will be discussed.

BIOMARKERS FOR TARGETED THERAPIES

Sorafenib is the current standard of care for advanced HCC. Initial phase 2 data indicated that pretreatment levels of phosphorylated ERK (p-ERK) correlated with time to progression (TTP)[16], which was later confirmed by several preclinical and *in vitro* studies[17,18]. Due to limited availability of tissue samples, this finding could not be confirmed in the registrational phase 3 study (SHARP trial)[19]. Instead, an extensive program investigating different biomarkers from plasma samples was initiated. Surprisingly, none of the investigated biomarkers predicted the response to sorafenib while biomarkers related to clinical performance, *e.g.*, vascular invasion or AFP, as well as markers of angiogenesis like Ang2 or VEGF were shown to be independent predictors of survival in patients with advanced HCC[20] and thus need to be seen rather as prognostic biomarkers. In other smaller studies, elevated p-ERK and VEGF2 tissue levels were shown to be predictive for poor response to sorafenib treatment in a cohort of 77 advanced HCC patients[21]. Similarly, high p-ERK correlated to poor overall survival, but not time to progression, in another study with 44 patients[22]. Interestingly, also the multi-kinase inhibitor regorafenib, which was recently approved for second-line therapy of HCC, stratified patients only on clinical parameters and thus does not have a predictive biomarker available so far for HCC patients[23], while promising results on plasma circulating cell free DNA were obtained for patients with colorectal cancer[24].

BIOMARKERS FOR IMMUNE CHECKPOINT INHIBITORS

The recent success of immune checkpoint inhibitors in other cancer diseases also triggered various approaches in HCC. As HCC development is commonly based on chronic inflammatory liver diseases (viral hepatitis, NASH), a clear rationale to investigate this new treatment paradigm is clearly given. Initial results using anti-CTLA-4 or anti-PD-1 antibodies are encouraging and lead to accelerated approval of the anti-PD-1 antibody nivolumab for the treatment of advanced HCC[25,26]. Combination of checkpoint inhibitors seem feasible and the use of such drugs together with locoregional procedures in early disease settings might even further improve outcome of patients[27].

Still, a significant number of patients (> 60%) do not respond to these novel therapies. Biomarker-based enrichment was initially based on immunohistochemical expression of the respective checkpoint targets, but recent data from various indications suggest that this is not the strongest predictor for treatment response[28]. This could be due to the still tissue based scoring of target expression with an intrinsic risk for sampling error in heterogenous solid tumors[29,30] or due to the still not completely understood biology of checkpoint inhibition[31] as evidenced by approx. 10% of patients who do not express PD-L1 but respond to treatment[32].

LIQUID BIOPSIES

It is intriguing that many promising and potent drugs,

e.g., sunitinib, everolimus, brivanib or tivantinib, failed in HCC clinical trials. Besides careful selection of patients based on clinical parameters like liver function or vessel invasion, all-comer trials are nowadays not considered appropriate and identification of specific patient subgroups based on distinct molecular subtypes is therefore urgently requested[33-35].

In HCC, lack of biopsies and different etiologic backgrounds hampered the identification and validation of such markers for the currently available treatments. Even today, practice guidelines do not recommend taking biopsies of every patient although risk of bleeding and needle track seeding are infrequent and should not be seen as a reason against taking a diagnostic biopsy[36,37]. The latest EASL practice guidelines strongly recommend liver biopsy and blood sampling from patients participating in clinical and diagnostic trials[37].

Genomic profiling established distinct molecular subclasses of HCC that were also linked to specific gene mutations and clinical and histological features. Two major groups, the proliferative (chromosomal instable) and the non-proliferative (chromosomal stable) group, were defined which comprise approx. 50% of HCC cases each. Further analyses defined a stem cell/hepatoblast like and a TGFβ related subgroup in the proliferative group, as well as a hepatocyte like and a Wnt/β-catenin related subgroup in the non-proliferative group[34,38]. While the overall impact of this classification is still under debate, additional common mutations and genetic alterations were described. Overall, mutations in telomerase signaling, the p53 and cell cycle control pathway as well as in Wnt/β-catenin signaling are commonly observed while rarer events include mutations in the Ras/PI3K/mTOR pathway, JAK/STAT signaling and other pathways[38,39]. Interestingly, no clear individual oncogenic driver has been identified in HCC so far and HCC is considered a cancer with medium to low mutational burden.

Analyzing tumor nucleic acids from other sources than tissue, *i.e.*, from circulating tumor cells, cell free DNA/RNA or exosomes, could help to overcome the above mentioned limitations. Liquid biopsies usually detect expression levels, methylation status or mutations of distinct tumor related nucleic acids, including DNA, RNA and miRNAs originating from circulating tumor cells or being shed into the blood directly from living or dying tumor cells. While this approach is considered to reduce sampling error compared to solid tumor biopsies[40,41], there are still technical limitations to this technology. The success rate of detecting circulating tumor cells is depending on the size of the tumor and results seem highly variable, ranging from approx. 25% to 100% success rate within different populations and with different technical approaches[42]. Similar results were obtained for genetic analyses of circulating DNAs[43,44]. Surprisingly, also these studies seem to be underpowered when considering different etiologic and geographical background. A clear advantage of liquid biopsies is also the option of taking serial samples from a patient to detect changes during disease history and imposed by treatment[45].

PROTEOMICS, GLYCOMICS AND IMAGING

As the liver is the primary secretory and metabolizing organ of the body, the use of proteomics and glycomics (usually by liquid chromatography-mass spectrometry (LC-MS) or matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis) could provide an option to identify novel biomarkers, too, without the current limitations of tissue based analyses. Several proteomic factors, *e.g.*, CD44v9[46] or Hippocalcin-like 1 (HPCAL1)[47], including various multimarker approaches were used as prognostic or diagnostic biomarkers for HCC or to predict recurrence[48-52]. Similarly, glycomics-based tests like GlycoCirrhoTest or analysis of N-glycans were developed as further diagnostic tools for better surveillance of patients at risk of HCC development[14,53-55]. So far, these approaches were not used in a predictive setting in advanced HCC.

Beyond LC-MS or MALDI-TOF analysis, functional and molecular imaging represents a further technology to identify potential biomarkers for HCC. Functional imaging is using dynamic computed tomography, dynamic magnetic resonance imaging and diffusion weighted magnetic resonance imaging approaches and is now well established to detect changes in *e.g.,* fibrosis grade or angiogenesis and for early diagnosis of HCC[56]. The development of novel radiotracers (beyond ^{18}F-FDG) for PET imaging could bridge the findings from proteomics and metabolomics analyses to imaging and thus add useful and important information on tissue distribution to these data. Today, molecular imaging for primary liver tumors is still limited by *e.g.,* lack of specific tracer uptake into malignant cells[57].

CONCLUSION

Predictive biomarkers are considered key for the success of developing new drugs. The further development of emerging technologies that are not dependent on tissue will also increase our knowledge for the better treatment of patients but more homogeneous study design regarding technologies and patient characteristics need to be done to achieve meaningful sample sizes with results that can be robustly translated into clinical applications.

REFERENCES

1 **Njei B**, Rotman Y, Ditah I, Lim JK. Emerging trends in hepatocellular carcinoma incidence and mortality. *Hepatology* 2015; **61**: 191-199 [PMID: 25142309 DOI: 10.1002/hep.27388]

2 **Global Burden of Disease Liver Cancer Collaboration**, Akinyemiju T, Abera S, Ahmed M, Alam N, Alemayohu MA, Allen C, Al-Raddadi R, Alvis-Guzman N, Amoako Y, Artaman A, Ayele TA, Barac A, Bensenor I, Berhane A, Bhutta Z, Castillo-Rivas J, Chitheer A, Choi

JY, Cowie B, Dandona L, Dandona R, Dey S, Dicker D, Phuc H, Ekwueme DU, Zaki MS, Fischer F, Fürst T, Hancock J, Hay SI, Hotez P, Jee SH, Kasaeian A, Khader Y, Khang YH, Kumar A, Kutz M, Larson H, Lopez A, Lunevicius R, Malekzadeh R, McAlinden C, Meier T, Mendoza W, Mokdad A, Moradi-Lakeh M, Nagel G, Nguyen Q, Nguyen G, Ogbo F, Patton G, Pereira DM, Pourmalek F, Qorbani M, Radfar A, Roshandel G, Salomon JA, Sanabria J, Sartorius B, Satpathy M, Sawhney M, Sepanlou S, Shackelford K, Shore H, Sun J, Mengistu DT, Topór-Mądry R, Tran B, Ukwaja KN, Vlassov V, Vollset SE, Vos T, Wakayo T, Weiderpass E, Werdecker A, Yonemoto N, Younis M, Yu C, Zaidi Z, Zhu L, Murray CJL, Naghavi M, Fitzmaurice C. The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. *JAMA Oncol* 2017; **3**: 1683-1691 [PMID: 28983565 DOI: 10.1001/jamaoncol.2017.3055]

3 **Ozakyol A**. Global Epidemiology of Hepatocellular Carcinoma (HCC Epidemiology). *J Gastrointest Cancer* 2017; Epub ahead of print [PMID: 28626852 DOI: 10.1007/s12029-017-9959-0]

4 **Younossi Z**, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 11-20 [PMID: 28930295 DOI: 10.1038/nrgastro.2017.109]

5 **Bertuccio P**, Turati F, Carioli G, Rodriguez T, La Vecchia C, Malvezzi M, Negri E. Global trends and predictions in hepatocellular carcinoma mortality. *J Hepatol* 2017; **67**: 302-309 [PMID: 28336466 DOI: 10.1016/j.jhep.2017.03.011]

6 **White DL**, Thrift AP, Kanwal F, Davila J, El-Serag HB. Incidence of Hepatocellular Carcinoma in All 50 United States, From 2000 Through 2012. *Gastroenterology* 2017; **152**: 812-820.e5 [PMID: 27889576 DOI: 10.1053/j.gastro.2016.11.020]

7 **Greten TF**, Papendorf F, Bleck JS, Kirchhoff T, Wohlberedt T, Kubicka S, Klempnauer J, Galanski M, Manns MP. Survival rate in patients with hepatocellular carcinoma: a retrospective analysis of 389 patients. *Br J Cancer* 2005; **92**: 1862-1868 [PMID: 15870713 DOI: 10.1038/sj.bjc.6602590]

8 **Kudo M**. Systemic Therapy for Hepatocellular Carcinoma: 2017 Update. *Oncology* 2017; **93** Suppl 1: 135-146 [PMID: 29258077 DOI: 10.1159/000481244]

9 **Kudo M**. Immuno-Oncology in Hepatocellular Carcinoma: 2017 Update. *Oncology* 2017; **93** Suppl 1: 147-159 [PMID: 29258079 DOI: 10.1159/000481245]

10 **FDA-NIH Biomarker Working Group**. BEST (Biomarkers, EndpointS, and other Tools) Resource [Internet]. 2016 [PMID: 27010052]

11 **Duarte-Salles T**, Misra S, Stepien M, Plymoth A, Muller D, Overvad K, Olsen A, Tjønneland A, Baglietto L, Severi G, Boutron-Ruault MC, Turzanski-Fortner R, Kaaks R, Boeing H, Aleksandrova K, Trichopoulou A, Lagiou P, Bamia C, Pala V, Palli D, Mattiello A, Tumino R, Naccarati A, Bueno-de-Mesquita HB, Peeters PH, Weiderpass E, Quirós JR, Agudo A, Sánchez-Cantalejo E, Ardanaz E, Gavrila D, Dorronsoro M, Werner M, Hemmingsson O, Ohlsson B, Sjöberg K, Wareham NJ, Khaw KT, Bradbury KE, Gunter MJ, Cross AJ, Riboli E, Jenab M, Hainaut P, Beretta L. Circulating Osteopontin and Prediction of Hepatocellular Carcinoma Development in a Large European Population. *Cancer Prev Res* (Phila) 2016; **9**: 758-765 [PMID: 27339170 DOI: 10.1158/1940-6207.CAPR-15-0434]

12 **Xiao WK**, Qi CY, Chen D, Li SQ, Fu SJ, Peng BG, Liang LJ. Prognostic significance of glypican-3 in hepatocellular carcinoma: a meta-analysis. *BMC Cancer* 2014; **14**: 104 [PMID: 24548704 DOI: 10.1186/1471-2407-14-104]

13 **Kondo S**, Ojima H, Tsuda H, Hashimoto J, Morizane C, Ikeda M, Ueno H, Tamura K, Shimada K, Kanai Y, Okusaka T. Clinical impact of c-Met expression and its gene amplification in hepatocellular carcinoma. *Int J Clin Oncol* 2013; **18**: 207-213 [PMID: 22218908 DOI: 10.1007/s10147-011-0361-9]

14 **Black AP**, Mehta AS. The search for biomarkers of hepatocellular carcinoma and the impact on patient outcome. *Curr Opin Pharmacol* 2018; **41**: 74-78 [PMID: 29772420 DOI: 10.1016/j.coph.2018.04.002]

15 **Lou J**, Zhang L, Lv S, Zhang C, Jiang S. Biomarkers for Hepatocellular Carcinoma. *Biomark Cancer* 2017; **9**: 1-9 [PMID: 28469485 DOI: 10.1177/1179299X16684640]

16 **Abou-Alfa GK**, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, De Greve J, Douillard JY, Lathia C, Schwartz B, Taylor I, Moscovici M, Saltz LB. Phase Ⅱ study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2006; **24**: 4293-4300 [PMID: 16908937 DOI: 10.1200/JCO.2005.01.3441]

17 **Liang Y**, Chen J, Yu Q, Ji T, Zhang B, Xu J, Dai Y, Xie Y, Lin H, Liang X, Cai X. Phosphorylated ERK is a potential prognostic biomarker for Sorafenib response in hepatocellular carcinoma. *Cancer Med* 2017; **6**: 2787-2795 [PMID: 29030911 DOI: 10.1002/cam4.1228]

18 **Zhang Z**, Zhou X, Shen H, Wang D, Wang Y. Phosphorylated ERK is a potential predictor of sensitivity to sorafenib when treating hepatocellular carcinoma: evidence from an in vitro study. *BMC Med* 2009; **7**: 41 [PMID: 19698189 DOI: 10.1186/1741-7015-7-41]

19 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

20 **Llovet JM**, Peña CE, Lathia CD, Shan M, Meinhardt G, Bruix J; SHARP Investigators Study Group. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012; **18**: 2290-2300 [PMID: 22374331 DOI: 10.1158/1078-0432.CCR-11-2175]

21 **Negri FV**, Dal Bello B, Porta C, Campanini N, Rossi S, Tinelli C, Poggi G, Missale G, Fanello S, Salvagni S, Ardizzoni A, Maria SE. Expression of pERK and VEGFR-2 in advanced hepatocellular carcinoma and resistance to sorafenib treatment. *Liver Int* 2015; **35**: 2001-2008 [PMID: 25559745 DOI: 10.1111/liv.12778]

22 **Personeni N**, Rimassa L, Pressiani T, Destro A, Ligorio C, Tronconi MC, Bozzarelli S, Carnaghi C, Di Tommaso L, Giordano L, Roncalli M, Santoro A. Molecular determinants of outcome in sorafenib-treated patients with hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2013; **139**: 1179-1187 [PMID: 23568548 DOI: 10.1007/s00432-013-1429-x]

23 **Bruix J**, Qin S, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Breder V, Gerolami R, Masi G, Ross PJ, Song T, Bronowicki JP, Ollivier-Hourmand I, Kudo M, Cheng AL, Llovet JM, Finn RS, LeBerre MA, Baumhauer A, Meinhardt G, Han G; RESORCE Investigators. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **389**: 56-66 [PMID: 27932229 DOI: 10.1016/S0140-6736(16)32453-9]

24 **Wong AL**, Lim JS, Sinha A, Gopinathan A, Lim R, Tan CS, Soh T, Venkatesh S, Titin C, Sapari NS, Lee SC, Yong WP, Tan DS, Pang B, Wang TT, Zee YK, Soong R, Trnkova Z, Lathia C, Thiery JP, Wilhelm S, Jeffers M, Goh BC. Tumour pharmacodynamics and circulating cell free DNA in patients with refractory colorectal carcinoma treated with regorafenib. *J Transl Med* 2015; **13**: 57 [PMID: 25889309 DOI: 10.1186/s12967-015-0405-4]

25 **Sangro B**, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, Riezu-Boj JI, Larrea E, Alfaro C, Sarobe P, Lasarte JJ, Pérez-Gracia JL, Melero I, Prieto J. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; **59**: 81-88 [PMID: 23466307 DOI: 10.1016/j.jhep.2013.02.022]

26 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

27 **Duffy AG**, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, Davis JL, Hughes MS, Heller T, ElGindi M,

Uppala A, Korangy F, Kleiner DE, Figg WD, Venzon D, Steinberg SM, Venkatesan AM, Krishnasamy V, Abi-Jaoudeh N, Levy E, Wood BJ, Greten TF. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J Hepatol* 2017; **66**: 545-551 [PMID: 27816492 DOI: 10.1016/j.jhep.2016.10.029]

28 **Carbone DP**, Reck M, Paz-Ares L, Creelan B, Horn L, Steins M, Felip E, van den Heuvel MM, Ciuleanu TE, Badin F, Ready N, Hiltermann TJN, Nair S, Juergens R, Peters S, Minenza E, Wrangle JM, Rodriguez-Abreu D, Borghaei H, Blumenschein GR Jr, Villaruz LC, Havel L, Krejci J, Corral Jaime J, Chang H, Geese WJ, Bhagavatheeswaran P, Chen AC, Socinski MA; CheckMate 026 Investigators. First-Line Nivolumab in Stage IV or Recurrent Non-Small-Cell Lung Cancer. *N Engl J Med* 2017; **376**: 2415-2426 [PMID: 28636851 DOI: 10.1056/NEJMoa1613493]

29 **McLaughlin J**, Han G, Schalper KA, Carvajal-Hausdorf D, Pelekanou V, Rehman J, Velcheti V, Herbst R, LoRusso P, Rimm DL. Quantitative Assessment of the Heterogeneity of PD-L1 Expression in Non-Small-Cell Lung Cancer. *JAMA Oncol* 2016; **2**: 46-54 [PMID: 26562159 DOI: 10.1001/jamaoncol.2015.3638]

30 **Casadevall D**, Clavé S, Taus Á, Hardy-Werbin M, Rocha P, Lorenzo M, Menéndez S, Salido M, Albanell J, Pijuan L, Arriola E. Heterogeneity of Tumor and Immune Cell PD-L1 Expression and Lymphocyte Counts in Surgical NSCLC Samples. *Clin Lung Cancer* 2017; **18**: 682-691.e5 [PMID: 28549836 DOI: 10.1016/j.cllc.2017.04.014]

31 **Zerdes I**, Matikas A, Bergh J, Rassidakis GZ, Foukakis T. Genetic, transcriptional and post-translational regulation of the programmed death protein ligand 1 in cancer: biology and clinical correlations. *Oncogene* 2018; **37**: 4639-4661 [PMID: 29765155 DOI: 10.1038/s41388-018-0303-3]

32 **Cyriac G**, Gandhi L. Emerging biomarkers for immune checkpoint inhibition in lung cancer. *Semin Cancer Biol* 2018 [PMID: 29782924 DOI: 10.1016/j.semcancer.2018.05.006]

33 **Gerbes A**, Zoulim F, Tilg H, Dufour JF, Bruix J, Paradis V, Salem R, Peck-Radosavljevic M, Galle PR, Greten TF, Nault JC, Avila MA. Gut roundtable meeting paper: selected recent advances in hepatocellular carcinoma. *Gut* 2018; **67**: 380-388 [PMID: 29150490 DOI: 10.1136/gutjnl-2017-315068]

34 **Nault JC**, Galle PR, Marquardt JU. The role of molecular enrichment on future therapies in hepatocellular carcinoma. *J Hepatol* 2018; **69**: 237-247 [PMID: 29505843 DOI: 10.1016/j.jhep.2018.02.016]

35 **Zhang B**, Finn RS. Personalized Clinical Trials in Hepatocellular Carcinoma Based on Biomarker Selection. *Liver Cancer* 2016; **5**: 221-232 [PMID: 27493897 DOI: 10.1159/000367763]

36 **Heimbach JK**, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, Zhu AX, Murad MH, Marrero JA. AASLD guidelines for the treatment of hepatocellular carcinoma. *Hepatology* 2018; **67**: 358-380 [PMID: 28130846 DOI: 10.1002/hep.29086]

37 **European Association for the Study of the Liver**. European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

38 **Llovet JM**, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015; **12**: 408-424 [PMID: 26054909 DOI: 10.1038/nrclinonc.2015.103]

39 **Khemlina G**, Ikeda S, Kurzrock R. The biology of Hepatocellular carcinoma: implications for genomic and immune therapies. *Mol Cancer* 2017; **16**: 149 [PMID: 28854942 DOI: 10.1186/s12943-017-0712-x]

40 **Huang A**, Zhao X, Yang XR, Li FQ, Zhou XL, Wu K, Zhang X, Sun QM, Cao Y, Zhu HM, Wang XD, Yang HM, Wang J, Tang ZY, Hou Y, Fan J, Zhou J. Circumventing intratumoral heterogeneity to identify potential therapeutic targets in hepatocellular carcinoma. *J Hepatol* 2017; **67**: 293-301 [PMID: 28323123 DOI: 10.1016/j.jhep.2017.03.005]

41 **Zhai W**, Lim TK, Zhang T, Phang ST, Tiang Z, Guan P, Ng MH, Lim JQ, Yao F, Li Z, Ng PY, Yan J, Goh BK, Chung AY, Choo SP, Khor CC, Soon WW, Sung KW, Foo RS, Chow PK. The spatial organization of intra-tumour heterogeneity and evolutionary trajectories of metastases in hepatocellular carcinoma. *Nat Commun* 2017; **8**: 4565 [PMID: 28240289 DOI: 10.1038/ncomms14565]

42 **Okajima W**, Komatsu S, Ichikawa D, Miyamae M, Ohashi T, Imamura T, Kiuchi J, Nishibeppu K, Arita T, Konishi H, Shiozaki A, Morimura R, Ikoma H, Okamoto K, Otsuji E. Liquid biopsy in patients with hepatocellular carcinoma: Circulating tumor cells and cell-free nucleic acids. *World J Gastroenterol* 2017; **23**: 5650-5668 [PMID: 28883691 DOI: 10.3748/wjg.v23.i31.5650]

43 **Bettegowda C**, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih lM, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA Jr. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014; **6**: 224ra24 [PMID: 24553385 DOI: 10.1126/scitranslmed.3007094]

44 **Ng CKY**, Di Costanzo GG, Terracciano LM, Piscuoglio S. Circulating Cell-Free DNA in Hepatocellular Carcinoma: Current Insights and Outlook. *Front Med* (Lausanne) 2018; **5**: 78 [PMID: 29632864 DOI: 10.3389/fmed.2018.00078]

45 **Wan JCM**, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* 2017; **17**: 223-238 [PMID: 28233803 DOI: 10.1038/nrc.2017.7]

46 **Kakehashi A**, Ishii N, Sugihara E, Gi M, Saya H, Wanibuchi H. CD44 variant 9 is a potential biomarker of tumor initiating cells predicting survival outcome in hepatitis C virus-positive patients with resected hepatocellular carcinoma. *Cancer Sci* 2016; **107**: 609-618 [PMID: 26882440 DOI: 10.1111/cas.12908]

47 **Zhang Y**, Liu Y, Duan J, Yan H, Zhang J, Zhang H, Fan Q, Luo F, Yan G, Qiao K, Liu J. Hippocalcin-like 1 suppresses hepatocellular carcinoma progression by promoting p21(Waf/Cip1) stabilization by activating the ERK1/2-MAPK pathway. *Hepatology* 2016; **63**: 880-897 [PMID: 26659654 DOI: 10.1002/hep.28395]

48 **Tan GS**, Lim KH, Tan HT, Khoo ML, Tan SH, Toh HC, Ching Ming Chung M. Novel proteomic biomarker panel for prediction of aggressive metastatic hepatocellular carcinoma relapse in surgically resectable patients. *J Proteome Res* 2014; **13**: 4833-4846 [PMID: 24946162 DOI: 10.1021/pr500229n]

49 **Lee SC**, Tan HT, Chung MC. Prognostic biomarkers for prediction of recurrence of hepatocellular carcinoma: current status and future prospects. *World J Gastroenterol* 2014; **20**: 3112-3124 [PMID: 24696598 DOI: 10.3748/wjg.v20.i12.3112]

50 **Liu CH**, Chen TC, Chau GY, Jan YH, Chen CH, Hsu CN, Lin KT, Juang YL, Lu PJ, Cheng HC, Chen MH, Chang CF, Ting YS, Kao CY, Hsiao M, Huang CY. Analysis of protein-protein interactions in cross-talk pathways reveals CRKL protein as a novel prognostic marker in hepatocellular carcinoma. *Mol Cell Proteomics* 2013; **12**: 1335-1349 [PMID: 23397142 DOI: 10.1074/mcp.O112.020404]

51 **Junrong T**, Huancheng Z, Feng H, Yi G, Xiaoqin Y, Zhengmao L, Hong Z, Jianying Z, Yin W, Yuanhang H, Jianlin Z, Longhua S, Guolin H. Proteomic identification of CIB1 as a potential diagnostic factor in hepatocellular carcinoma. *J Biosci* 2011; **36**: 659-668 [PMID: 21857112 DOI: 10.1007/s12038-011-9101-6]

52 **Cheng J**, Xie HY, Xu X, Wu J, Wei X, Su R, Zhang W, Lv Z, Zheng S, Zhou L. NDRG1 as a biomarker for metastasis, recurrence and of poor prognosis in hepatocellular carcinoma. *Cancer Lett* 2011; **310**: 35-45 [PMID: 21763068 DOI: 10.1016/j.canlet.2011.06.001]

53 **Verhelst X**, Vanderschaeghe D, Castéra L, Raes T, Geerts A, Francoz C, Colman R, Durand F, Callewaert N, Van Vlierberghe H. A Glycomics-Based Test Predicts the Development of Hepatocellular Carcinoma in Cirrhosis. *Clin Cancer Res* 2017; **23**: 2750-2758 [PMID: 27986746 DOI: 10.1158/1078-0432.CCR-16-1500]

54 **Kamiyama T**, Yokoo H, Furukawa J, Kurogochi M, Togashi T, Miura N, Nakanishi K, Kamachi H, Kakisaka T, Tsuruga Y, Fujiyoshi M, Taketomi A, Nishimura S, Todo S. Identification of novel serum biomarkers of hepatocellular carcinoma using glycomic analysis. *Hepatology* 2013; **57**: 2314-2325 [PMID: 23322672 DOI: 10.1002/hep.26262]

55 **Goldman R**, Ressom HW, Varghese RS, Goldman L, Bascug G, Loffredo CA, Abdel-Hamid M, Gouda I, Ezzat S, Kyselova Z, Mechref Y, Novotny MV. Detection of hepatocellular carcinoma using glycomic analysis. *Clin Cancer Res* 2009; **15**: 1808-1813 [PMID: 19223512 DOI: 10.1158/1078-0432.CCR-07-5261]

56 **Ayuso C**, Rimola J, Vilana R, Burrel M, Darnell A, García-Criado Á, Bianchi L, Belmonte E, Caparroz C, Barrufet M, Bruix J, Brú C. Diagnosis and staging of hepatocellular carcinoma (HCC): current guidelines. *Eur J Radiol* 2018; **101**: 72-81 [PMID: 29571804 DOI: 10.1016/j.ejrad.2018.01.025]

57 **Ronot M**, Clift AK, Vilgrain V, Frilling A. Functional imaging in liver tumours. *J Hepatol* 2016; **65**: 1017-1030 [PMID: 27395013 DOI: 10.1016/j.jhep.2016.06.024]

P- Reviewer: Vradelis S, Yao DF **S- Editor**: Wang XJ **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 3980-3999

DOI: 10.3748/wjg.v24.i35.3980

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

REVIEW

Pediatric hepatocellular carcinoma

Rajeev Khanna, Sanjeev Kumar Verma

Rajeev Khanna, Department of Pediatric Hepatology, Institute of Liver and Biliary Sciences, New Delhi 110070, India

Sanjeev Kumar Verma, Department of Pediatrics, King George Medical University, Uttar Pradesh 226003, India

ORCID number: Rajeev Khanna (0000-0003-0800-4068); Sanjeev Kumar Verma (0000-0001-5309-4336).

Author contributions: Khanna R and Verma SK collectively did literature search and compilation of studies on pediatric hepatocellular carcinoma; the draft was prepared by both Khanna R and Verma SK, and a final editing was done by Khanna R before approval; Khanna R will serve as the guarantor of the manuscript.

Conflict-of-interest statement: There are no potential conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Rajeev Khanna, MD, Associate Professor, Department of Pediatric Hepatology, Institute of Liver and Biliary Sciences, D-1, Vasant Kunj, New Delhi 110070, India. drrajeev_khanna@rediffmail.com
Telephone: +91-9654246963

Received: May 13, 2018
Peer-review started: May 14, 2018
First decision: June 15, 2018
Revised: July 11, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 21, 2018

Abstract

Pediatric hepatocellular carcinoma (HCC) is the second common malignant liver tumor in children after hepatoblastoma. It differs from the adult HCC in the etiological predisposition, biological behavior and lower frequency of cirrhosis. Perinatally acquired hepatitis-B virus, hepatorenal tyrosinemia, progressive familial intrahepatic cholestasis, glycogen storage disease, Alagille's syndrome and congenital portosystemic shunts are important predisposing factors. Majority of children (87%) are older than 5 years of age. Following mass immunization against hepatitis-B, there has been a drastic fall in the incidence of new cases of pediatric HCC in the Asia-Pacific region. Management is targeted on complete surgical removal either by resection or liver transplantation. There is a trend towards improving survival of children transplanted for HCC beyond Milan criteria. Chemotherapeutic regimens do not offer good results but may be helpful for down-staging of advanced HCC. Surveillance of children with chronic liver diseases with ultrasound and alpha-fetoprotein may be helpful in timely detection, intervention and overall improvement in outcome of HCC.

Key words: Hepatocellular carcinoma; Children; Risk-factors; Outcome; Liver transplantation

Core tip: The review elaborately describes various risk factors, treatment options and outcome of children with hepatocellular carcinoma.

Khanna R, Verma SK. Pediatric hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(35): 3980-3999 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/3980.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.3980

INTRODUCTION

Hepatocellular carcinoma (HCC) is a rare malignancy of childhood affecting predominantly adolescent males with an overall poor survival. Both hepatoblastoma and HCC together account for 0.5%-1.5% of all childhood malignancies and 4% of all pediatric liver transplantations

(LT)[1-4]. Hepatoblastoma constitutes about 67%-80% of all pediatric liver cancers worldwide, remaining 20%-33% are HCC. The proportion of HCC among pediatric liver cancers varies and depends on the geographical locale and prevalence of Hepatitis-B virus (HBV) infection in the community[2,3,5]. There are two distinct subsets of HCC in the pediatric age group - first, in the setting of cirrhosis or underlying metabolic, infectious or vascular liver disease, and second, sporadic HCC without preceding liver disease. Tyrosinemia and perinatally acquired HBV infection are two major risk factors[1,2,6,7]. Cirrhosis is absent in 26%-62% of childhood HCC[6,7]. Fibrolamellar variant of HCC is seen in 24% with a relatively better prognosis[8]. In the present review, we tried to elaborate various risk factors, therapeutic and preventive strategies and outcome of pediatric HCC.

ADULT *VS* PEDIATRIC HCC - ARE THEY DIFFERENT DISEASES?

Pediatric HCC differs from its adult counterpart in various aspects[9,10]. Adult HCC usually develops in the setting of chronic necro-inflammation going on for several years to decades secondary to alcohol, viral hepatitis-B or C or non-alcoholic fatty liver disease (NAFLD)[11]. Contrastingly, pediatric HCC develops either in a cirrhotic or non-cirrhotic background and inciting agent is either a toxic metabolite (tyrosinemia) or a mutated virus (HBV)[1,2,6]. Gender predilection, although has been shown to be present in studies from East, is not as marked as in adults[6-8,12-18]. White children are equally predisposed to develop HCC as blacks[6-8,15]. The Surveillance, Epidemiology, and End Results (SEER) database has shown a stable incidence of pediatric HCC in United States over last 4 decades, whereas in adults the incidence has almost tripled between 1975 and 2005 primarily because of hepatitis-C, and also contributed by hepatitis-B, alcohol, obesity and NAFLD[5,11]. Fibrolamellar variant of HCC (FL-HCC) comprises 15%-25% of pediatric HCC, the corresponding figure in adult population is 5% with a relatively better prognosis[8,15,19]. There are diverse genetic pathways and mutations for carcinogenesis in adults with HCC - the common ones being Wnt/beta-catenin (*CTNNB1* and *AXIN1* genes), telomere maintenance (*TERT* promoter gene), cell-cycle regulator (*TP53* and *CDKN2A* genes), epigenetic modifier (*MLL*, *ARID1A* and *ARID2* genes), AKT/mTOR and Ras/raf/MAP kinase (*FGF 19*, *PIK3CA* and *RPS6KA3* genes), stress oxidative (*NFE2L2* and *KEAP1* genes) and JAK/STAT (*IL6ST* and *JAK1* genes) pathways[20]. Kim *et al*[21] compared expression of G1 phase regulatory proteins and loss of heterozygosity (LOH) on chromosomal arms 8p, 13q and 17p in children and adults with HBV-HCC. The expression of cyclin D1 was lower and LOH higher at 13q in childhood HCC. The different staging systems used in adult HCC, like Barcelona Clinics on Liver Cancer (BCLC) staging, Milan criteria, University College of San Francisco (UCSF) criteria, Japan Integrated staging score (JIS), Hong Kong Liver Cancer Classification (HKLC) or under-7 criteria, for purpose of staging and stratifying patients who may or may not benefit from LT have not been prospectively applied in children[11]. Small case series have tested the applicability of Milan criteria in pediatric HCC and found that in contrast to adults LT offers better cure in children falling beyond Milan criteria[14,18,22,23]. The reasons may be related to different biological behavior of the tumor and less aggressive nature of the disease. The survival of children with HCC has improved dramatically over last 3 decades with comparable or even better survival rates than adults with similar severity of disease[13,18,22-26]. This is primarily due to early diagnosis, improvement in surveillance and most importantly, advancement in surgical techniques. Lastly, in contrast to adult HCC which is chemo-resistant and radio-resistant, a proportion (39%) of pediatric HCC respond to chemotherapy[27].

EPIDEMIOLOGY OF PEDIATRIC HCC

Geographical variation in the etiological predisposition for pediatric HCC is largely determined by the prevalence of HBV infection. In regions with high endemicity of HBV, most of the pediatric HCC cases are secondary to perinatally acquired HBV, while in the low endemic regions, tyrosinemia and other metabolic disorders are common causes[7,10,12,18,22,27-31] (Tables 1 and 2).

Age and gender distribution

As per the SEER database conducted over a period of 1973 to 1997 in United States, pediatric HCC constituted 0.3% of all pediatric malignancies and 31% of all primary hepatic malignancies. The overall age-adjusted incidence rate of pediatric HCC was estimated to be 0.41 (0.24-0.65) per 1000000. The rates were slightly higher for males than females (0.45 *vs* 0.37 per 1000000). Of these, 12.9% were among children below 5 years of age and 34% were between 15 to 19 years of age. Incidence is highest (0.8 per million) among adolescents. This is in contrast to hepatoblastoma where a majority (91.3%) of cases belong to under-5 year age group[5]. In most of the studies, males outnumbered females with a factor ranging from 1.27 to 2.45[6-8,12-18].

Epidemiological studies from the East have shown higher incidence of pediatric HCC as compared to West and this is primarily related to the high prevalence of perinatally acquired HBV infection. In certain areas it was reported as the commonest childhood liver malignancy[6,7]. There is bimodal age distribution of cases with first peak at around 1 year of age, then a decline to trough at 4 years and a second peak at around 12 to 15 years of age[6]. Male preponderance is more marked in the East with a male to female ratio of 2.1-13.3:1[6,7,12]. A South-African study has found a gender ratio-difference of 2.5 between Hepatitis-B surface antigen (HBsAg) positive *vs* negative cases of pediatric HCC[7].

Racial differences

As per SEER database, whites and non-Hispanics com-

Table 1 Clinical characteristic of children with hepatocellular carcinoma in series from East

Study → Ref. (yr)	Moore *et al*[7] (2004)	Wang *et al*[38] (2017)	Chen *et al*[10,29] (1988, 1998)	Hsu *et al*[28] (1987)	Zhang *et al*[12] (2013)	Arikan *et al*[13] (2006)	Palaniappan *et al*[26] (2016)
Region	South Africa	China	Taiwan	Taiwan	China	Turkey	India
No. of subjects	68	65	44, 55	51	45	13 (Incidental diagnosis in 3)	12 (8 diagnosed in explant livers)
Males (%)	68 (78 in HBV subgroup)	80	Males dominated	77	93	46	75
Age (yr)	Mean 10.5	Median 17 (8-20)	-	Mean 11 (4-15)	Mean 13.5 (3-18)	6.4 ± 4.8	Median 5.9 (1.6-15.4)
Age-group (*n* or %)	91% > 6 yr of age	-	-	39% < 9 yr of age	-	-	< 5 yr→3 5-10 yr→7 > 10 yr→2
Size (cm)	-	10.2 ± 4.1	-	-	> 5 cm in 73% > 10 cm in 67%	Median 3.4 (0.5-8)	22.5 (2-40)
Multifocality	-	62%	62%	-	56% Satellite lesions in 18%	92% Median number of nodules 3 (1-10)	42% Median number of nodules 1 (1-5)
Etiology	67% HBV	82% HBV	100% HBV	100% HBV	71% HBV	6 Tyr 6 HBV 1 GSD1	5 Tyr 2 BA 1 BSEP 1 MDR3 1 NSPILBD 1 PSC 1 Caroli's
Histology (*n* or %)	HCC 87% FLHCC 13%	-	-	-	-	Well diff 2 Mod diff 4 Poorly diff 6	Well diff 8 Mod diff 4 (High grade dysplasia or nodules in adjoining liver in 50%)
Cirrhosis	-	-	71%, 68%	74% (95%) in < 9 yr of age	47% Child A/B/C 32/8/5	100% Mean CTP 11.1 ± 3.1 and PELD 19.7 ± 2.4 (1-44)	-
AFP	High in 69%	> 25 ng/mL in 91%	-	-	93999 ± 31228 ng/mL High in 87%	8000-250000 ng/dL High in 92%	Median AFP 7.3(1.2-28000) IU/L High AFP (> 100 IU/L) in 42%
Extent or stage (*n* or %)	-	TNM Stage Ⅲ /Ⅳ in 74%	-	Advance stage in 98%	-	TNM stage Ⅰ (20%), Ⅳ (80%) Advance disease in 92%	-
Vascular invasion	-	31%	42% PV 17% IVC	-	47%	54%	17%
Metastases	-	17%	29%	-	24%	0	-
Resectability	-	40%	< 10%, 18%	13%	27%	NA	NA
Outside Milan	NA	NA	NA	NA	NA	NA	NA

AFP: Alpha-fetoprotein; BA: Biliary atresia; BSEP: Bile salt export pump deficiency (PFIC2); diff: Differentiated; FLHCC: Fibrolamellar variant of HCC; GSD: Glycogen storage disease; HBV: Hepatitis-B virus; IVC: Inferior vena cava; LT: Liver transplantation; MDR3: Multidrug resistance protein3 deficiency (PFIC3); NA: Not assessed; NSPILBD: Non-syndromic paucity of bile ducts; PRETEXT: Pretreatment tumor extent evaluation; PSC: Primary sclerosing cholangitis; PV: Portal vein; TNM: Tumor node metastasis stage; Tyr: Tyrosinemia; Mod: Moderately.

prise 72% and 82% of pediatric HCC, respectively[8]. Hence, the racial differences are not as pronounced as in adults with HCC.

Time-trend of pediatric HCC

Epidemiological data from 2003 has shown that there is a declining trend in pediatric HCC from 1973 to 1997. This is in contrast to almost double the number of cases of hepatoblastoma[5]. In a subsequent analysis by SEER published in 2013, the overall incidence of pediatric HCC has been estimated to be 0.59 per million per year with a relatively stable incidence over last 4 decades (0.62 in 1970s, 0.43 in 1980s, 0.59 in 1990s and 0.62 in 2000s per million per year)[8]. Contrastingly, data from East has shown an 85% reduction in the incidence of pediatric HCC 20-years following introduction of HBV immunization[32].

Changing epidemiology in the East following hepatitis-B vaccination

There has been a dramatic change in the incidence of HBV related HCC in pediatric age-group following

Table 2 Clinical characteristics of children with hepatocellular carcinoma in series from West

Study→Ref. (yr)	SEER[8,15,30] (2013, 2013, 2014)	POG[16] (2002)	SIOPEL 1[27] (2002)	Lack *et al*[31] (1983)	Pham *et al*[17] (2007)	Ismail *et al*[22] (2009)	Romano *et al*[18] (2011)	Beaunoyer *et al*[23] (2007)
Region	United States	United States	Europe	United States	United States	Poland	Italy	United States
Number of subjects	238, 80, 218	46	39	32	22	21	10	10
Males (%)	58	57	72	63	50	-	40	60
Age (yr)	12.9 ± 5.2	-	Median 12 (4-15)	Mean 9.7 (0.5-21)	13.1 ± 1.1 (2-18)	Median 11.1 (2-18)	Median 1.8 (0.5-7.2)	Median 10.1 (4.4-16.3)
Age-group (%)	0-4 (8-9) 5-9 (14-16) 10-14 (27-29) 15-19 (48-49)	< 1 y (6.5) 1-9 y (37) ⩾ 10 y (56.5)	-	-	-	⩽ 2 (5) 2-10 (38) ⩾ 10 (57)	< 2 (50) 2-5 (30) ⩾ 5 (20)	3-12 (70) ⩾ 13 (30)
Size (cm)	0-5 (21%) 5.1-10 (24%) 10.1-15 (36%) > 15 (19%)	-	Median 14 (7-26) cm	-	11.8 ± 0.6 (5-25) cm	-	-	Median 5.8 (2-10.5) cm > 5 cm in 60%
Multifocality	Satellite lesions 28%	-	56% (4 diffuse)	-	-	-	50% (2-10 in No.)	70%
Etiology	-	-	13 HBV 1 Tyr 1 Biliary	-	-	4 Tyr 2 HBV 1 HCV 1 A1ATD 1 AIH 1 PFIC 11 no liver ds	3 BA 3 PFIC2 2 Tyr 1 CC 1 GSD4	4 Viral (3HBV) 1 Tyr 1 Alagille's 1 PFIC 3 No Liver ds
Histology (*n* or %)	HCC NOS 58%-74% FLHCC 24%-41% Clear cell 1%-2%	HCC 78% FLHCC 22%	Epithelial 75% FLHCC 15% Poorly diff or clear cell 10%	HCC 84% FLHCC 16%	-	High grade[2] (29%) Low grade (52%) FLHCC (19%)	G1 (1) G2 (6) G3 (3)[2] No FLHCC	HCC in 9 FLHCC in 1
Cirrhosis	-	-	38%	16%	-	52%	100%	50%
AFP	-	⩾ 20 ng/mL in 67%	Median 9677 (1-1400000) ng/mL > 10 ng/mL in 69%	-	-	1.7-713000 IU/mL	Median 2322 (3-35000) ng/mL	Median 446927 ng/mL
Extent or Stage (*n* or %)	Local 27%-28% Regional 35%-37% Distant 34%-35%	Stage Ⅰ (8), Ⅱ (0), Ⅲ (25), Ⅳ (13)[1]	PRETEXT Ⅰ (1), Ⅱ (14), Ⅲ (11), Ⅳ (13)	-	CCG/POG Stage Ⅰ (60%), Ⅱ (11.5%), Ⅲ (17%), Ⅳ (11.5%)[1]	PRETEXT Ⅰ (4), Ⅱ (7), Ⅲ (5), Ⅳ (5)	PRETEXT Ⅰ (4), Ⅱ (1), Ⅲ (1), Ⅳ (4)	TNM Stage Ⅰ (0), Ⅱ (3), Ⅲ (7), Ⅳ (0) T2 (3), T3 (6), T4 (1)
Vascular invasion	48%	-	21%	-	-	29%	20%	30%
Metastases	35%	-	Lung 31% Other 18%		-	0	-	-
Resectability	25%	22%	36%	22%	67%	48%	NA (All LT)	NA
Outside Milan	-	-	-	-	-	86%	60%	70%

[1]Staging done as Ⅰ, complete gross resection with pathologically negative margins; Ⅱ, gross total resection with microscopic residual disease at margins; Ⅲ, gross total resection with nodal involvement or tumor spill; and Ⅳ, metastatic disease with either complete or incomplete resection or biopsy; [2]Grade as per Edmondson and Steiner. A1ATD: Alpha-1 antitrypsin deficiency; AFP: Alpha-fetoprotein; AIH: Autoimmune hepatitis; BA: Biliary atresia; CC: Choledochal cyst; CCG: Children's cancer group; diff: Differentiated; FLHCC: Fibrolamellar variant of HCC; GSD: Glycogen storage disease; HBV: Hepatitis-B virus; HCV: Hepatitis-C virus; LT: Liver transplantation; NA: Not assessed; PFIC: Progressive familial intrahepatic cholestasis; PRETEXT: Pretreatment tumor extent evaluation; POG: Pediatric oncology group; SEER: Surveillance epidemiology and end-results; SIOPEL: Group for epithelial liver tumors of the international society of pediatric oncology; TNM: Tumor node metastasis stage; Tyr: Tyrosinemia.

introduction of mass immunization against HBV in the Asia-Pacific region. Chang *et al*[33] showed that following universal HBV vaccination, the average annual incidence of HCC in children 6-14 years of age declined from 0.70 to 0.36 over a decade. This translated in a corresponding (50%) reduction in mortality due to pediatric HCC in the same age group. Study by Taiwan Childhood Hepatoma Study Group further showed that the vaccination program also resulted in reducing boy-girl incidence ratio for the disease from 4.5 in 1981-1984 to 1.9 in 1990-1996. There was a dramatic reduction in incidence of disease in boys (relative risk 0.72) over this time

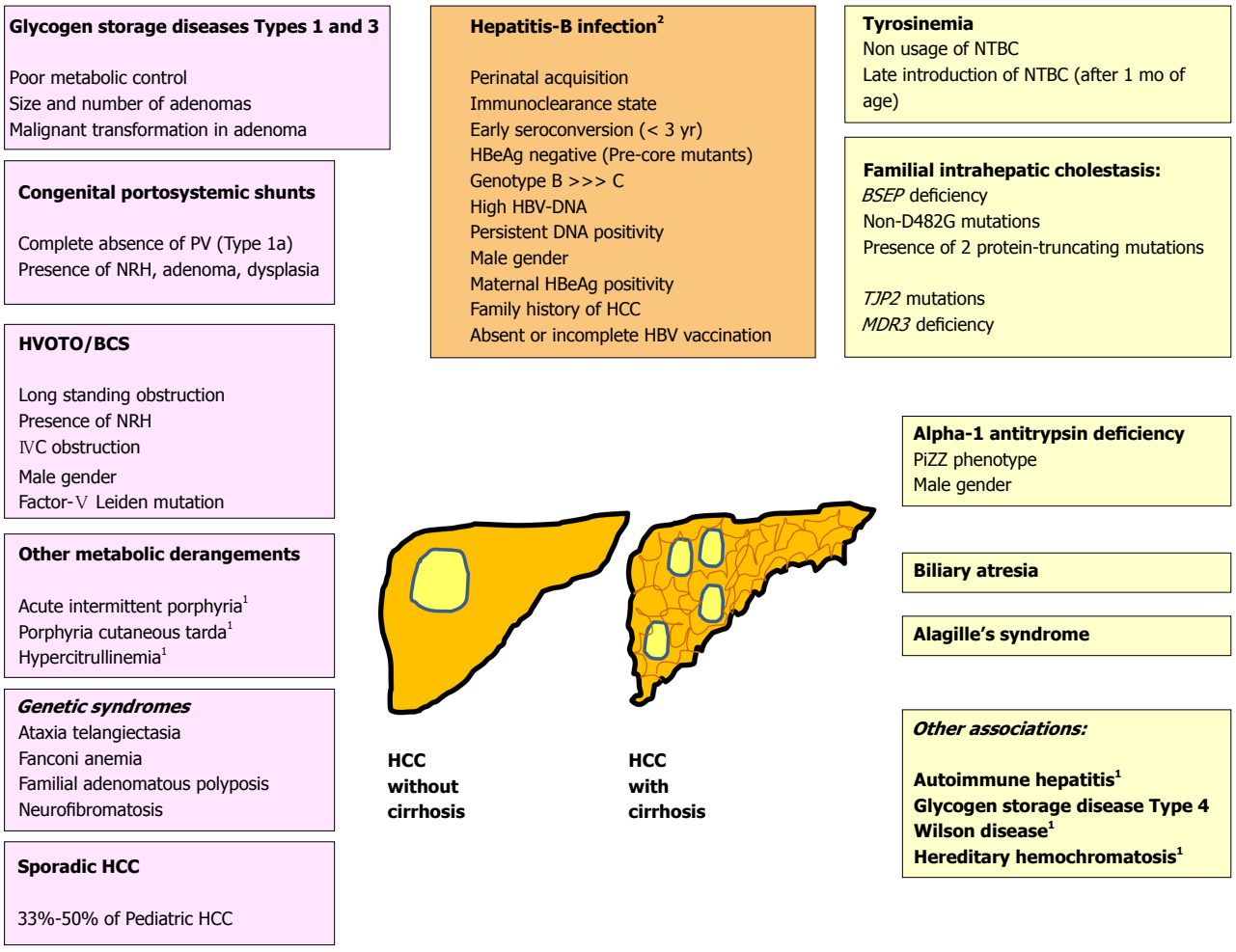


Figure 1 Risk factors for pediatric hepatocellular carcinoma. [1]Conditions cause HCC in adults, and very rarely in children; [2]HBV related HCC may occur in presence or absence of cirrhosis. BCS: Budd-Chiari syndrome; HCC: Hepatocellular carcinoma; HVOTO: Hepatic venous outflow tract obstruction; IVC: Inferior vena cava; NRH: Nodular regenerative hyperplasia; BSEP: Bile salt export pump; HCC: Hepatocellular carcinoma; MDR3: Multidrug resistance protein-3; NTBC: [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (Nitisinone); PiZZ: Homozygous PiZ phenotype of alpha-1 antitrypsin; PV: Portal vein, TJP: Tight junction protein.

period. However, for girls there was no decrease in the incidence and with increasing age a rising incidence was observed[34]. Subsequent analysis by the same group of workers showed that vaccination resulted in almost 70% reduction in the risk of developing HCC adjusted to age and gender. Moreover, in the vaccination cohort, the risk of developing HCC was found to be related to incomplete vaccination (odd's ratio, OR = 4.32), prenatal maternal HBsAg seropositivity (OR = 29.5), prenatal HBeAg seropositivity [with administration of HBV immunoglobulin (OR = 5.13) and without it (OR = 9.43)][32].

PATHOGENIC RISK FACTORS

Various pathogenic risk factors of pediatric HCC have been illustrated in Figure 1. Hepatocellular cancer may develop in a cirrhotic or a non-cirrhotic liver, and in the later scenario it may or may not be associated with underlying liver disease. Differentiation on the basis of these two points is important for treatment allocation and outcome as shown in Figure 2.

Hepatitis-B

Hepatitis-B infection is the commonest cause of HCC in adults who live in regions with high endemicity like Southeast Asia and sub-Saharan Africa. HBV constitutes 65% of HCC cases in adults from China and Far East as compared to < 20% from United States. Chronic carriers of HBV have upto 30-fold increased risk of development of HCC[11]. Pediatric HCC in the setting of HBV infection usually develops with perinatally acquired infection and is more common in males with genotype B[31,35]. Gender disparity is universal for all types of HCC and is related to the protection against the tumor by estrogen *via* a complex pathway involving hepatocyte nuclear factor-4[36]. Family studies from Far-East have confirmed that early infection with HBV, particularly through HBsAg positive mother, leads to persistent chronic infection in children, subsequently leading to development of chronic hepatitis, post-necrotic cirrhosis and HCC[36,37]. Majority of tumors are multi-centric, although cirrhosis may be

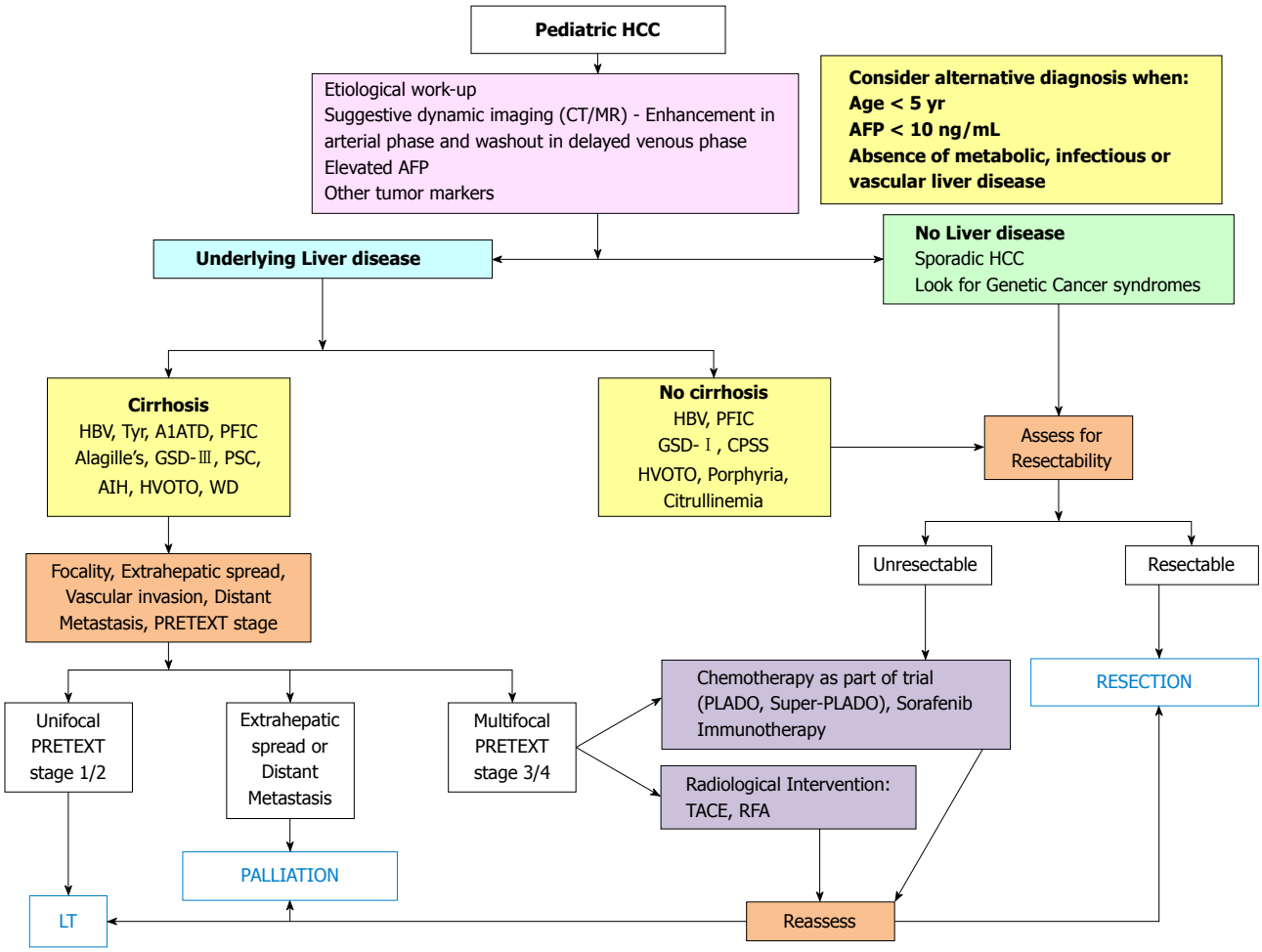


Figure 2 Algorithmic management of pediatric hepatocellular carcinoma. A1ATD: Alpha-1 antitrypsin deficiency; AFP: Alpha-fetoprotein; AIH: Autoimmune hepatitis; CPSS: Congenital portosystemic shunt; GSD: Glycogen storage disorder; HBV: Hepatitis-B virus; HVOTO: Hepatic venous outflow tract obstruction; LT: Liver transplantation; PFIC: Progressive familial intrahepatic cholestasis; PLADO: Cisplatin + Doxorubicin; PRETEXT: Pretreatment tumor extent evaluation; PSC: Primary sclerosing cholangitis; RFA: Radiofrequency ablation; Super-PLADO: Cisplatin, Carboplatin and Doxorubicin; TACE: Transarterial chemo-embolization; Tyr: Tyrosinemia; WD: Wilson disease.

absent in 26%-53% of cases[12,38] (Figures 3 and 4). Pediatric HCC secondary to HBV differs from non-HBV cases with regard to male predominance (68%-93% *vs* 50%), older age of presentation (14.5 years *vs* 10.9 years), presence of cirrhosis (56% *vs* 23%) and portal vein invasion (56% *vs* 23%), but there is no difference with respect to tumor size, number of lesions or metastases[6-8,12-16,30]. Thus, HBV related HCC represents more aggressive and advance disease, and complements its adult counterpart. An old study from Taiwan on HBV related pediatric HCC showed that the presence of cirrhosis is more common in younger children below 9 years of age (95% *vs* 58%). Hepatitis-B early antigen (HBeAg) in serum and hepatitis-B core antigen in the liver tissue were detected in 18% and 11% of cases, respectively. This coupled with high frequency of cirrhosis especially in the younger children indicated that early seroconversion from HBeAg to anti-HBe in association with severe liver injury plays an important role in development of HCC[28]. In a large cohort of chronic hepatitis-B children from India, HCC was detected in 4.3% at an age between 9 to 14 years with elevated transaminases, however 80% didn't have cirrhosis[39].

Numerous risk factors for development of HBV related HCC have been studied in adults. Concomitant liver insults like hepatitis-C, human immunodeficiency virus, alcohol intake, smoking, non-alcoholic fatty liver disease, diabetes, ingestion of aflatoxin from *Aspergillus flavum* are common in adults but have not been studied in children[11]. Similarly, numerous genetic associations are known in adults with HBV-HCC, namely loss of heterozygosity on the KIF1b locus, deletion or chromosomal loss of DLC1 (Deleted in Liver Cancer 1) locus, mutations in cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) gene, promoter region of the Minichromosome maintenance protein-7 (*MCM7*) gene and enhancer Ⅱ (EnhⅡ), basal core promoter (BCP) and precore regions of HBV[40]. It has been shown that Pre-S mutations, C1653T, T1753V, and A1762T/G1764A are associated with an increased risk of HCC[41]. A recent study from China on mother-cord-infant pairs has shown that the HCC-risk mutations were present in the mothers' peripheral blood and cord blood but mostly absent in peripheral blood of 7-mo-old infants. Further, in

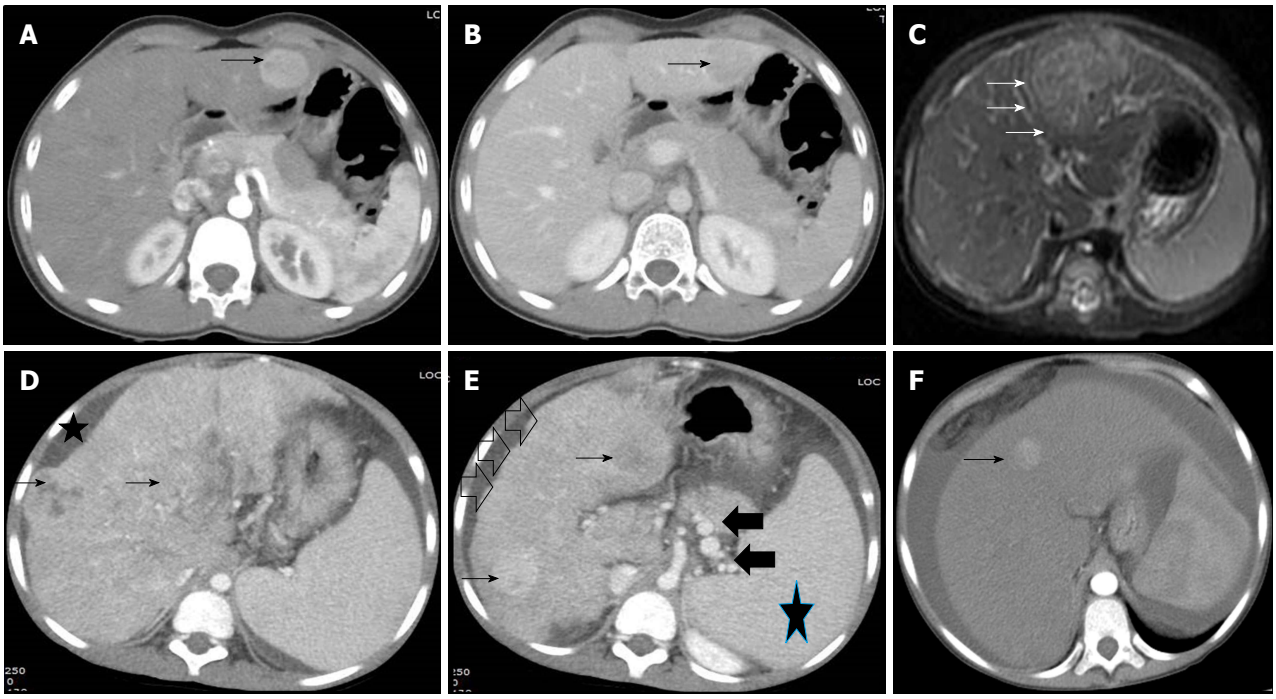


Figure 3 Radiological images in children with hepatocellular carcinoma. A and B: 14 and half years old boy with chronic hepatitis-B (Immunoclearance phase), HBeAg+, DNA 5 log, AFP = 3 ng/mL (normal), with a segment 3 lesion (2.5 cm × 2.4 cm), BCLC stage A. Contrast-enhanced CT (CECT) image showing enhancement of the lesion (arrow) in the arterial phase (A) and washout in delayed venous phase (B) in a background of non-cirrhotic liver; C: 10 mo old boy with infantile cholestasis, bile salt export pump (BSEP) deficiency on immunostaining, incidentally found to have lesion in segment Ⅱ and Ⅳa abutting the capsule, BCLC stage B. The lesion was T2 hyperintense with enhancement in the arterial phase (arrows) and washout in the delayed phase; D and E: 8-1/2 yr old boy with decompensated chronic liver disease, chronic hepatitis-B (Immunoclearance phase), HBeAg+, DNA 7 log, AFP = 250000 ng/mL, with multifocal HCC, BCLC stage D, Child-Pugh C. Contrast-enhanced CT (CECT) image showing heterogenous lesion (arrows, D and E) involving whole of the right lobe with enhancement in the arterial phase in few lesions (arrows, E) in a background of cirrhotic liver with nodular margins (open black arrows, E), ascites (star, D) and collaterals (black solid arrows, E) and splenomegaly (star, E); F: 14 yr old girl with hepatic venous outflow tract obstruction, ascites, portal hypertension, incidentally found to have a lesion in segment 4 (1 cm × 1 cm), BCLC stage D, Child-Pugh C. Contrast-enhanced CT (CECT) image showing enhancement of lesion in the arterial phase (arrow).

56 mother-child pairs with documented mother to child transmission of infection, it was shown that HCC mutations evolved over first 15 years of age - Pre-S deletion in genotype B2; C1653T, G2946A/C, C1T/A, A7C, and pre-S1 start codon mutation in genotype C2; and A1762T/G1764A and G1896A in both the genotypes, the mutations consecutively increased in frequencies with increasing age[42].

Tyrosinemia

Tyrosinemia type Ⅰ or hepatorenal tyrosinemia is caused by deficiency of enzyme fumaryl acetoacetate hydrolase (FAH). Due to blockage in the last step of the tyrosine degradation pathway, there is accumulation of toxic metabolites - maleylacetoacetate, fumarylacetoacetate (FAA), and succinylacetone (SA) - which lead to hepatic and renal manifestations of the disease and carcinogenesis. The incidence of HCC in tyrosinemia is reported to be 14%-75%, the prevalence increases with age of the child[43,44]. Nitisinone [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione, (NTBC)] blocks the enzyme parahydroxyphenylpyruvic acid dioxygenase, which is the second step in the tyrosine degradation pathway, and thus prevents accumulation of FAA and SA. Usage of NTBC along with low tyrosine diet leads to survival rates greater than 90% in these children. Further, if NTBC is started early (within 30 d of birth), there is reduction in incidence of HCC from 37% to 1%, and need of LT from 71% to 26%[43-45]. However, animal models have shown that NTBC treatment fails to normalize the tyrosinemia-induced alterations in expression of transcripts encoding proteins involved in protein turnover, signal transduction, and cell growth and proliferation[46]. One of the studies on 16 children who underwent LT at a median age of 16 mo has shown the presence of HCC in 12 (75%) liver explants - 7 were multifocal, 3 with microvascular invasion, and all were well differentiated. There was 86% tumor-free survival at a median of 6.6 years[47]. Contrastingly, report from Iran has shown that HCC was present in 5 (23%) of the 22 explants, the diagnosis was confirmed before LT in 2 children[48].

Progressive familial intrahepatic cholestasis

Among the list of Progressive familial intrahepatic cholestasis (PFIC) disorders, Bile salt export pump (BSEP) deficiency or PFIC type-2 children are especially predisposed at a young age to develop HCC (Figure 3). There is poor excretion of bile salts through the canalicular membrane leading to constant exposure of hepatocytes to bile salts with chronic inflammation and carcinogenesis. Studies from United States and Europe

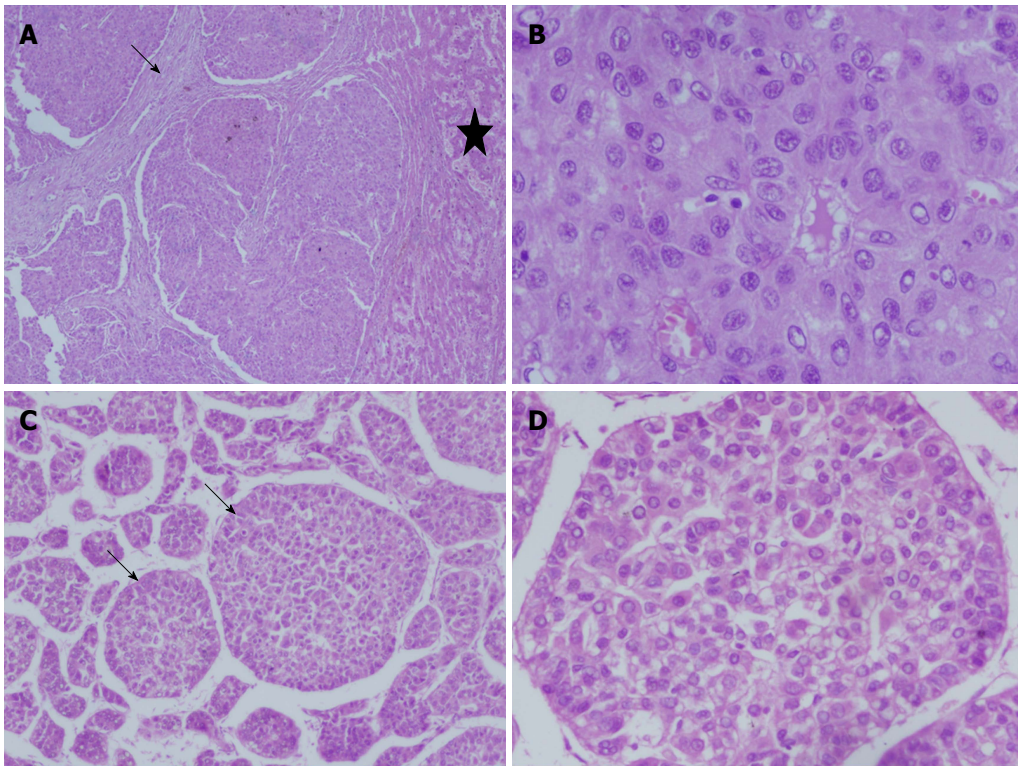


Figure 4 Liver histology images in children with hepatocellular carcinoma. A and B: 15 yr old boy with HBeAg negative chronic hepatitis-B, DNA 4 log, precore mutant, AFP = 150000 ng/mL, with a large HCC in right lobe, with portal vein thrombosis, BCLC stage C, Child Pugh A, underwent right hepatectomy. Low power view showing tumor nodules separated by fibrous bands (arrow), adjacent non-malignant parenchyma (star) (100 ×, HE stain) (A). High power view showing malignant nuclear details with prominent nucleoli and eoisnophilic cytoplasm (400 ×, HE stain) (B); C and D: 14 and half years old boy with chronic hepatitis-B (Immunoclearance phase), HBeAg+, DNA 5 log, AFP = 3 ng/mL (normal), with a segment 3 lesion (2.5 cm × 2.4 cm), BCLC stage A, underwent non-anatomic resection. Low power view of tumor showing broadened trabeculae (arrows) (100 ×, HE stain) (C). Trabeculae with malignant cytological features - nuclear atypia and anisocytosis (200 ×, HE stain) (D).

showed that HCC occurs in 5%-15% of children with BSEP deficiency at a young age (13 to 28 mo)[49-52]. Children with D482G mutations have less severe disease and portal hypertension, while HCC is common in those with non-D482G mutations[51]. From the largest cohort of 128 European children, single-strand conformation polymorphism analysis and sequencing of ABCB11 gene identified high risk of HCC (38% *vs* 10%) in children with presence of 2 protein-truncating mutations[52]. Exome sequencing of the genomes of humans affected by BSEP and of *Mdr2* knock-out mice revealed that a very few somatic mutations accumulated over time in the cancer genes. This stands in contrast to adults with HCC as well as other malignancies where a number of mutations accumulate over a period of time. Further, in BSEP individuals and animals, there is massive gene amplification that affected components of signal transduction pathways, such as the ErbB, the PI3K/Akt and the mitogen-activated protein kinase (MAPK) signalling pathways and in particular, activators of c-Jun-N terminal kinases (JNK)[53]. Another study has provided further pathophysiologic insights into BSEP mediated HCC. It showed that BSEP expression is severely diminished in HCC patients associated with alteration of farsenoid-X receptor (regulatory nuclear receptors) with increase in (FXR-α1/FXR-α2) ratio, the later is induced by inflammation and may be reversible[54].

HCC has also been described in a new variant of PFIC with severe cholestasis - TJP2 (tight junction protein 2) deficiency. Protein truncating mutations in TJP2 gene cause failure of protein localization and disruption of tight junction structure leading to severe cholestatic liver disease. In presence of these mutations, Claudin (CLDN1) fails to localise normally to cholangiocyte borders and biliary canaliculus margins, despite normal protein levels. In the unusually hostile environment of the canalicular and cholangiocytic membranes, exposure to high concentrations of detergent bile acids due to TJP2 deficiency lead to hepatobiliary disintegrity, producing severe liver injury and HCC[55,56]. Finally, in multidrug resistance protein-3 (MDR3) deficiency (PFIC type 3), HCC has been reported but is less common than BSEP deficiency[57].

Glycogen storage disorders

In Glycogen storage disorders, HCC develops on a soil of adenoma (adenoma-carcinoma sequence). Adenomas are common in Glycogen storage disorder (GSD) type Ⅰ (glucose-6-phosphatase deficiency) with a frequency of 16%-75%. Most of these are seen in the second and third decade with a mean age between 11 to 19 years. In comparison to adenomas in general

population, adenomas of GSD-Ⅰa children are more in number, bilobar in distribution, and without any gender predisposition. There is regression in size and number of adenomas with good metabolic control. Malignant transformation in such adenomas occurs rarely, and in view of rarity of the event as well as the disease, it is difficult to estimate the overall risk[58-61]. However, the risk is related to size, number and duration of adenomas, and their recurrence[60]. There is no effective biomarker as alpha-fetoprotein (AFP) and carcinoembryonic antigen levels are often normal in this setting. In a genetic study, it has been shown that chromosomal aberrations are present in 60% of GSD-Ⅰa adenomas which is almost comparable to general population adenomas, but simultaneous gain of chromosome 6p and loss of 6q were only seen in GSD-Ⅰa adenomas. Moreover, these adenomas also showed reduced expression of insulin-like growth factor-2 receptor (IGF2R) and large tumour suppressor kinase-1 (LATS1) candidate tumor suppressor genes at 6q in more than 50%. This indicates a possible role of chromosome 6q in malignant transformation of such adenomas[62].

Type Ⅲ GSD (debrancher enzyme deficiency, or Forbes disease, or limit dextrinosis) differs from type Ⅰ in that the liver enzymes are elevated with hepatocellular injury, and evidence of ballooning degeneration and fibrosis. Adenomas are seen in 4%-25% of patients. Malignant transformation takes place less often, but is almost always in the background of cirrhosis[63,64]. Liver cancer is rarely seen in Type 4 GSD (brancher enzyme deficiency, or Andersen's disease, or Amylopectinosis), but has never been reported in types 6 and 9 diseases[18].

Alpha-1 antitrypsin deficiency

Individuals with homozygous (PiZZ) alpha-1-antitrypsin deficiency (A1ATD) are at an increased risk for liver damage, cirrhosis and HCC. Liver cancer is rare in the pediatric age group but has been reported[22]. In a recent systemic review, the prevalence of HCC in adults with A1ATD is 1.3% with a low yearly cumulative rate of HCC than other causes of cirrhosis (0.88% per year *vs* 2.7% for hepatitis-C cirrhosis)[65,66]. However, autopsy data in adults has shown the prevalence of HCC of 16%[67]. Animal models of A1ATD have shown that approximately 69% of the PiZZ mice develop tumors by 16-19 mo of age and HCC was present without evidence of precancerous lesions or benign adenoma. There was upregulation of Cyclin-D gene, and elevation of c-Fos and melanoma cell adhesion molecule (MCAM), all three factors involved in cell proliferation, tumorigenesis and metastasis[68]. The liver injury is related to increased oxidative stress with an upregulation of redox- regulating genes and reduction in levels of antioxidant enzymes[69].

Alagille's syndrome

Alagille's syndrome is a multisystem disorder characterized by bile ductal paucity and chronic cholestasis. Mutations/deletions in two genes are known to cause Alagille's syndrome: *JAGGED1* (encoding the Notch signaling pathway ligand JAGGED1) in 89% and *NOTCH2* (encoding one of the Notch receptors) in a small minority. Carcinogenesis in Alagille's syndrome is rare but reported in children and adults[23,70,71]. HCC in Alagille's syndrome is seen in first or second decade in a background of cirrhosis with extensive pruritus[71]. Constitutive intracellular domain of Notch2 signalling in the liver leads to up-regulation of pro-proliferative genes and proliferation of hepatocytes and biliary epithelial cells, and stimulates diethylnitrosamine induced HCC formation in mice[72]. Recently, yes associated protein (YAP), which is a transcriptional co-activator and is responsible to make cancer cells proliferate in an anchorage independent fashion and become apoptosis resistant, has been shown to act by upregulating JAGGED-1 and activation of NOTCH pathway in mouse models and humans with HCC, colorectal and pancreatic cancers[73]. This may have putative role in Alagille's syndrome related HCC, and need to be studied.

Congenital portosystemic shunts

Nodular transformation in livers of children with congenital portosystemic shunts (CPSS) is related to differential vascular supply[74]. Such nodules may be benign or malignant. Two largest reviews on CPSS and largest series from France have shown that extra-hepatic CPSS (59%) is slightly more common than intra-hepatic CPSS (41%)[74-76]. The common communications are patent ductus venosus, portal vein to inferior vena cava (IVC), or left portal venous system to IVC[74]. Liver masses have been described in both extra-hepatic (35%) and intra-hepatic (13%) types at a median age of 8 years, however the malignant tumors (hepatoblastoma, HCC and sarcoma) are specifically described in extrahepatic variety. As per available literature, HCC is seen in 2.5% of CPSS cases[76]. Shunt occlusion leads to reduction in size of benign tumors like focal nodular hyperplasia and hepatic adenoma, however for malignant tumors, resection and shunt occlusion or LT is indicated[74].

Biliary atresia: Study from King's College London has shown that the prevalence of HCC in biliary atresia is 1.3% (5 out of 387). All except one were below 5 years of age (range 1.1-4.9 years)[77]. On the other hand, biliary atresia constitutes 0-30% among all causes of pediatric HCC, particularly from the West, however the figures may indicate a referral and surveillance bias[18,22,23,26,27]. Cancer in biliary atresia develops on a soil of biliary cirrhosis with ongoing chronic necroinflammation and destructive cholangitis[77,78].

Budd-Chiari syndrome: HCC is a known complication of Budd-Chiari syndrome in adults with a 5-year cumulative incidence of 4%. It commonly develops in a liver with nodular regenerative hyperplasia, and is more commonly associated with male gender, factor-Ⅴ Leiden mutation and inferior vena cava obstruction[79]. We have described HCC in a 14 years old girl with Budd-Chiari syndrome and Celiac disease[80] (Figure 3).

Other liver disorders: Autoimmune hepatitis (AIH) and Wilson disease (WD) are two commonest chronic liver diseases in older children[81]. From a systematic review and meta-analysis on adults with AIH, the risk of HCC is 3.06 per 1000 patient-years, slightly lower than that with other causes of cirrhosis in adults like hepatitis-B, C, or primary biliary cholangitis[82]. The risk of HCC is related to presence of cirrhosis at the time of diagnosis of AIH (OR = 4.08) and abnormal transaminases at final observation (OR = 3.66)[82,83]. Although incidence of HCC in children is rare, but as AIH is a common pediatric liver condition so it is important to be aware of the carcinogenesis risk in the long term. Copper accumulation in WD is said to be protective against tumorigenesis. The estimated annual risk of HCC from a Dutch study on 140 adults with WD followed up over 15 years was calculated to be 0.09%[84]. From another multicenter European study of 1186 WD patients, the prevalence of HCC was found to be 0.67%[85]. Hence, the risk is significantly low in WD to advocate regular HCC surveillance. Non-alcoholic fatty liver disease, type 2 diabetes, hereditary hemochromatosis and porphyria (acute intermittent and porphyria cutanea tarda) carry high risk of development of HCC in adults, but not in children[86].

Genetic cancer syndromes and sporadic HCC

Certain genetic cancer syndromes confer high risk of development of HCC in the absence of a metabolic, infectious or vascular disease of liver (Figure 1). Sporadic HCC developing in the absence of a known or unknown liver disease or genetic cancer predisposition is seen in 0-62% of children with HCC, the proportion is high in areas with low endemicity of HBV infection[10,12,13,18,22,23,27-29,31].

PATHOLOGY

Histologically, most of the pediatric HCCs resemble the adult type. The common proliferation patterns are solid, trabecular, acinar or scirrhous, and occasionally clear cell or steatotic change[47,87]. According to the SEER database, the most common histological subtype of pediatric HCC is non-fibrolamellar HCC constituting 73% of cases, followed by fibrolamellar HCC in 25% and clear cell carcinoma in 2% of cases. These histological subtypes do not differ with regard to gender, race, ethnicity and tumor stage at presentation[8]. Various grading systems including Edmondson and Steiner grading have been described[13,14,18,22,47]. Tumor grades Ⅰ and Ⅱ or low grade tumors (28%) refer to neoplastic cells with small nuclei which are slightly enlarged, hyperchromatic, and have single mitotic figure. While, high grade tumors (Ⅲ and Ⅳ) comprise cells with large, hyperchromatic pleomorphic nuclei with numerous mitotic figures and sometimes anaplastic foci[22] (Figure 4). An elegant study from London described pathological details of 26 HCC nodules in 12 children - morphology was solid in 62%, trabecular in 35% (two-thirds with thick trabeculae) and scirrhous in 4%. HCC in children with tyrosinemia are more often well differentiated, have grade Ⅰ morphology, and are solid or trabecular with diffuse clear cell change[87]. Premalignant dysplastic foci are commonly found in adjoining areas of tyrosinemia livers[47,87]. HCC in biliary atresia have moderately pleomorphic nuclei with eosinophilic cytoplasm arranged in a trabecular pattern. In BSEP deficiency, HCCs have varied morphology and proliferation pattern. On immunohistochemistry, epithelial cell adhesion molecule (EpCAM), cytokeratin (CK)-19 and Glypican-3 have been found to be more commonly expressed in pediatric HCC in contrast to adult HCC. In contrast to hepatoblastoma, pediatric HCC livers less frequently expressed beta-catenin and more frequently expressed p53. Further in contrast to fibrolamellar HCC, there was less expression of CK-7, which is a marker of biliary proliferation[87].

CLINICAL FEATURES

The common symptoms of pediatric HCC are abdominal mass and pain. Children with advance disease often have cachexia and jaundice. Other signs and symptoms of decompensated end-stage liver disease and portal hypertension (ascites, variceal bleed, encephalopathy, spider naevi, clubbing) are present in children with underlying cirrhosis. Upto one-third of pediatric HCCs are detected incidentally on imaging[13,14]. Liver is usually firm to hard in consistency with irregular margins. Nodular surface may be appreciable. Splenomegaly and ascites are related to the degree of portal hypertension (Tables 1 and 2).

DIAGNOSIS

The diagnosis of HCC in a child with cirrhosis or a predisposing liver disease is usually presumed on basis of elevated AFP levels and abnormal nodule (space occupying lesion) on ultrasound.

Imaging

Ultrasound typically shows presence of a heterogenous hyperechoic mass of variable size with increased vascularity[2]. For further characterization of the mass, a dynamic imaging, either contrast-enhanced computerized tomography (CECT) or magnetic resonance (CEMR) imaging is needed. Typical characteristics of HCC on dynamic imaging are hyper-enhancement in the arterial phase (10-20 s) and washout in the portal venous (60-80 s) and delayed venous (3-5 min) phases. The "enhancement" is because of the intranodular arterial supply of HCC, while "washout" is related to early venous drainage of the contrast, progressive enhancement of background cirrhotic liver, reduced intra-nodular portal venous drainage, tumoral hypercellularity with corresponding reduction in extracellular volume, and intrinsic hypoattenuation/hypointensity[88] (Figure 1). From the recent adult systematic review and meta-analysis, it was

found that CEMR has higher sensitivity (82% *vs* 66%) and lower negative likelihood ratio (0.20 *vs* 0.37) over CECT for the diagnosis of HCC[89]. It has been proposed that in adults with non-typical characteristics on imaging, combined interpretation of dynamic and hepatobiliary phase of Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MRI with diffusion-weighted imaging (DWI) can improve the diagnostic yield of HCC[90]. Although MR avoids radiation hazard to young children, in view of technical complexity, expertise, availability, susceptibility to motion artifacts, need for intubation and poor image quality in presence of ascites, CT is mostly preferred to look for tumor extent, vascular invasion, resectability and metastases[91]. Contrast-enhanced ultrasound (CEUS) has been shown to be promising in children with an advantage of minimizing the exposure to ionizing radiation. In a study on 44 children with focal liver lesions, agreement between CEUS and other imaging modality (CT or MR) was seen in 85%, with the specificity and negative predictive value of CEUS of 98% and 100%, respectively[92]. Sonovue® (sulfur hexafluoride with phospholipid shell), used as contrast for CEUS, has been tested for other indications in pediatric age-group and is found to be extremely safe[93].

Tumor markers

Serum AFP elevation, not very sensitive, but has a fairly good specificity for detection of HCC in at risk population with liver disease. In adults, it also has a role in prognostication and surveillance[91]. Because of the low incidence of HCC in pediatric age-group and the fact that AFP levels are elevated in presence of regeneration, the exact role of surveillance with AFP may be limited. Nevertheless, the levels are mostly high (67%-92%), as high up to 1400000 ng/mL[7,12-14,16,18,22,26,27] (Tables 1 and 2). Caution should be taken in children with tyrosinemia with very high AFP values, where a serial change from the baseline should be considered more informative. Other tumor markers like fucosylated AFP or Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), des-gamma-carboxy prothrombin (DCP) or protein adducts in absence of vitamin-K absence (PIVKA-Ⅱ), glypican-3, Golgi protein-73, hepatocyte growth factor, insulin growth factor 1 and transforming growth factor-β1 have not been prospectively studied in children[90].

Cytology/biopsy

As per the adult literature on HCC, American association for study of liver diseases (AASLD) does not suggest biopsy in patients with cirrhosis with a lesion radiologically suggestive of HCC. For lesions which are indeterminate on imaging, repeating a different imaging, or with another contrast, or biopsy is suggested to confirm the diagnosis[91]. APASL consensus suggests biopsy when the nodule is non-hypervascular, or hypervascular without washout with a size of $\geqslant$ 1 cm[90]. No such recommendations exist for pediatric age-group. However, we suggest that in children without cirrhosis, histological evaluation should be done. While in those without cirrhosis, diagnosis should be based on suggestive imaging and high AFP.

DIFFERENTIAL DIAGNOSIS

Hepatoblastoma is an important differential of HCC especially in children younger than 5 years of age. Predisposing factors like low birth weight, maternal pre-eclampsia, parental smoking, and certain syndromes like hemihypertrophy (Beckwith-Weidmann syndrome), familial adenomatous polyposis, Li-Fraumeni syndrome, Trisomy 18 and Simpson Golabi-Behmel syndrome favor hepatoblastoma. Imaging features and AFP may not help to differentiate from HCC. Presence of calcification, cystic areas or necrosis may suggest hepatoblastoma. Evidence of embryonal-type epithelial or mesenchymal elements on histopathology is almost diagnostic of hepatoblastoma. However in the presence of trabecular architecture or well differentiated fetal epithelial cells, a definite diagnosis may be difficult[1,2]. A term "hepatocellular neoplasm not otherwise specified" has been coined for such tumors[94]. In older children, other benign and malignant lesions like focal nodular hyperplasia, adenoma and undifferentiated embryonal sarcoma should be considered in the differential of HCC[1].

MANAGEMENT

Staging

Most of the groups working on pediatric liver tumors like Group for Epithelial liver tumors of the International Society of Pediatric Oncology (SIOPEL), Pediatric Oncology group (POG), Children's Cancer Group (CCG) and Japanese study group for Pediatric Liver tumor (JPLT) have been using pre-treatment extent of tumor (PRETEXT) staging system. This system has been recently updated by the Children's Hepatic tumors International Collaboration (CHIC), and has been found to be a powerful predictor of overall survival of children with hepatoblastoma and HCC. In contrast to adult HCC, where size and number based classifications are more popular for purpose of decision about resection, transplantation or palliation, the PRETEXT system is based on determining number of contiguous tumor-free liver sections - left lateral, left medial, right anterior and right posterior. These sections are divided from right to left by (1) right hepatic vein, (2) Cantlie's line, and (3) a plane extending along hepatic fissure and umbilical portion of left portal vein. The stage is determined by calculating the number of contiguous sections that have to be resected to completely remove the tumor. The stage further includes annotation factors like V (hepatic venous or inferior vena cava involvement), P (portal vein involvement), E (extrahepatic disease contiguous with main liver tumor), F (multifocality), R (tumor rupture), C (caudate lobe involvement), N (lymph node metastases) and M (distant metastases)[95]. Application

of Milan criteria and BCLC staging system in children is limited[14,18,22,23].

Treatment modalities and outcome

Table 3 summarizes various treatment modalities and outcome of children with HCC[6-8,10,12-18,23-31,38,96-101]. The main factors considered for decision making in adults with HCC are (1) Liver functional status - ascites, albumin, bilirubin, alkaline phosphatase, portal vein thrombosis, Child-Turcotte-Pugh (CTP) score; (2) tumor factors - size and number of nodules, extent, vascular invasion, presence of metastases, TNM staging, AFP levels; (3) portal hypertension - presence of varices, hepatic venous pressure gradient (HVPG); (4) general status of patient - performance status, symptoms[11,90,91,102,103]. Most of the clinical experience obtained from treatment of hepatoblastoma has been applied for treatment of HCC in children. The best option for non-metastatic HCC tumors is complete surgical removal either by resection or LT. Resection offers good cure rates, however only 27% (range 10%-67%) of tumors are resectable. Over a period of 4 decades there has been a dramatic improvement in the 5-year survival rates of children with HCC from 4%-10% to 56%-80% - the change is primarily related to increased surveillance and improvement in surgical techniques, particularly LT[7,8,10,12,28,29,35,96-98]. An algorithmic approach for management of these children is presented in Figure 2. All children with non-metastatic HCC should be assessed for resection or LT to achieve optimal outcomes. Systemic neoadjuvant chemotherapy can be used in children awaiting LT to serve as a "bridge" to LT and prevent disease progression. Interventional radiology techniques using transarterial locoregional chemotherapy can be tried in older children with large unresectable tumors and to control tumor burden as a "bridge" to resection or LT.

Resection: As per AASLD, resection is only recommended in those adults with a single nodule and CTP class A without evidence of portal hypertension[91]. However, the guidelines differ in the Asia-Pacific region and a more aggressive approach is followed[90]. As per Japanese guidelines, patients with CTP A or B are considered for resection and/or radiofrequency ablation even with 1-3 nodules, up to or more than 3 cm in diameter, but without portal vein invasion or extrahepatic spread[102]. Hong Kong consensus further extends this to suggest resection in intrahepatic portal vein or branch hepatic vein invasion, or with a single extrahepatic metastasis in selected patients, but not with main portal vein invasion[103]. In children, resection rates from the eastern part of the world, where HBV is the commonest cause of pediatric HCC, vary from < 10% to 27%[6,28,29,31,97,98]. With improvement in surgical expertise, the resection rates in pediatric HCC have improved to 40% in the latest series from China with a median survival of more than 30 mo[12,38]. From the SEER database of 60 children with HCC who underwent resection, the 5-year survival rate was 53%[30].

Chemotherapy: There are several trials in adults with HCC where neoadjuvant and adjuvant therapies have been used but none has been convincing enough to translate into a recommendation. The survival rates following chemotherapy in HCC in adults have been dismal. AASLD suggests against the usage of adjuvant therapy following successfully resected or ablated HCC in adults[91]. SIOPEL and POG/CCG have done trials in pediatric HCC on the lines of hepatoblastoma[16,27,100]. SIOPEL-1 study analyzed the outcome of 37 children, who received pre-operative chemotherapy of 1 to 6 PLADO courses (cisplatin and doxorubicin). Response was partial in 49%, while others had either no response or disease progression. Resection was possible in 17 (46%) of these children with successful tumor excision in 36%, while 51% never became resectable. At a median follow-up of 75 mo, 8 (28%) children survived, all with complete resection. There was a dismal outcome of 28% at 5 years. PRETEXT stage and metastases predicted survival in that group of children[27]. In the POG/CCG trial, children with HCC were randomly assigned to receive regimen A (cisplatin, vincristine and fluorouracil) or regimen B (PLADO). There was a poor 5-year event free survival of 19% ± 6% with no difference between the two regimens. Survival and outcome depended on the stage of disease - all 8 stage Ⅰ patients with complete resection survived post chemotherapy, while 18 (47%) of 38 patients with advanced (stage Ⅲ or Ⅳ) disease had an event (progression, death or new neoplasm) before surgery, and only 2 (10%) of 20 who received chemotherapy became resectable[16]. From the more recent SIOPEL 2 and 3 studies evaluating outcome in 85 children with super PLADO (cisplatin, carboplatin and doxorubicin), 13 patients had upfront surgery. Of the remaining 72, only 29 (40%) showed response to chemotherapy and 39 (46%) never became resectable. Complete resection (including LT) was achievable in 40%. Survival was more with resection or LT (59%) *vs* not (10%). Tumor-free margin at resection was predictive of good outcome[100]. The results were comparable to the older studies conducted by the German Society for Pediatric Oncology and Hematology (GPOH) where the chemotherapy used were ifosfamide, cisplatin and doxorubicin in HB-89 trial, with addition of carboplatin and ifosfamide in HB-94 trial. The overall survival rates were 33% and 32%, respectively[104]. The more recent trial from GPOH (HB-99) showed 3-year event free and overall survival of children with HCC after primary complete resection followed by 2 cycles of carboplatin/etoposide of 72% and 89%, respectively. However, these figures were 12% and 20% in those with non-resectable malignancy[105]. Cytopenias, vomitings, opportunistic infections, ototoxicity, nephrotoxicity, myocardial toxicity and elevation of transaminases are common side-effects to be looked after[16,27,105].

Sorafenib: This is a novel multikinase inhibitor against Raf kinase and vascular endothelial growth factor receptor, along with anti-proliferative and anti-angiogenic

Table 3 Changing outcome of children with hepatocellular carcinoma over last 4 decades

Study (yr) Ref.	Number of patients	Factors governing outcome	Intervention (s) done	Survival
Conservative treatment (Observation and resection)				
Lack *et al*[31] (1983)	32 (5FLHCC)	Higher resectability and overall survival with FLHCC	Observation Resection	5 yr 7% MST of HCC 4.2 mo and FLHCC 28.5 mo
Wu *et al*[96] (1987)	20	-	-	5 yr 0; MST 4.7 mo
Hsu *et al*[28] (1987)	51	Early HBeAg seroconversion with severe liver injury predispose to HCC	Observation Resection	1 yr 10.5%
Chen *et al*[29] (1988)	44	No difference in survival with chemotherapy	Observation Resection	5 yr 7%
Ni *et al*[97] (1991)	71	Favorable prognosis with resectability and absence of icterus	Observation Resection	1 yr 10%, 5 yr 4%
Lee *et al*[6] (1998)	28	-	-	5 yr 17%
Hsiao *et al*[98] (2009)	13	-	-	DFS 30%
Allan *et al*[8] (2014) SEER database	218	Reduced mortality associated with resectability (OR = 0.18), non-Hispanic (OR = 0.52), local disease (OR = 0.46)	-	5 yr 24%, 10 yr 23%, 20 yr 8%
Mixed treatments (chemotherapy/TACE/liver transplantation)				
Tagge *et al*[99] (1992)	21	Total hepatectomy and LT improved survival in those with unresectable disease	Surgery in 15 (6 PH, 7 LT, 2 Exenteration and MOT) Pre-operative CT in 2 Observation in 6	1 yr 29%
Chen *et al*[10] (1998)	55	Good outcome with resection, poor with unsatisfactory resection & metastases Distant metastases carries worst prognosis	Resection CT Observation	MST with resection 23 mo, CT 3 mo and no treatment 2 mo
Moore *et al*[7] (2004)	68	-	Resection ± CT TACE Observation	> 5y 11% MST 4 mo
Pham *et al*[17] (2007)	22	-	Surgery ± CT	OS 5 yr 30% MST 23 mo
Zhang *et al*[12] (2013)	45	Low overall survival with metastases & non-resectability, but unrelated to HBsAg positivity Large tumor size, early metastasis, bilateral involvement, and PV invasion precluded resection	Resection TACE Observation	1 yr 34%, 3 yr 4%, 5 yr 4% MST 6 mo (Resection 28.6 mo, TACE 4 mo, None 5 mo, presence of metastases 4 mo)
McAteer *et al*[15] (2013) SEER database	238	Lower hazard of death with surgery (HR = 0.23) and lymphadenectomy (HR = 0.26) More hazard of death with female gender (HR = 2.07), older age (> 5 yr, HR > 5) and distant metastases (HR = 3.4)	Surgery in 112 No surgery in 118 Unknown in 8	OS 5 yr for 0-4 yr age 53%, 5-19 yr age 32% OS 5 yr for males 40%, females 26% DFS 5 yr for localized 61%, regional 39% and metastatic 9% DFS 5 yr 70% with lymphadenectomy *vs* 57% without
McAteer *et al*[30] (2013) SEER database	80	Lower hazard of death with LT as compared to resection (HR = 0.05)	Surgery (LT 20, resection 60)	OS 5 y with LT 85%, Resection 53%
Wang *et al*[38] (2017)	65	Initial treatment allocation predicted OS (TACE HR = 0.298, Resection HR = 0.105 with No treatment as reference)	Resection TACE No treatment	For moderate stage disease: Median OS longer with resection (38 mo) *vs* TACE (13.6 mo) *vs* No treatment (1.8 mo). For advanced disease: Median OS longer with TACE (7.1 mo) *vs* no treatment (2.3 mo)
Chemotherapy				
Czauderna *et al*[27] (2002) SIOPEL 1	39	Poor outcome related to metastases and higher PRETEXT stage	CT in 37, followed by resection	OS 5 yr 28% EFS 5 yr 17% 93% deaths due to tumour progression
Katzenstein *et al*[16] (2002) CCG/POG	46	Poor outcome with recurrent disease Favourable prognosis with stage Ⅰ and normal AFP Comparable survival between 2 regimens	CT (CDDP + Vincristine + 5-FU *vs* CDDP + Doxo)	EFS 5 yr 19% (Stage Ⅰ 88%, Ⅲ 8%, Ⅳ 0) OS 5 yr 19% (Stage Ⅰ 88%, Ⅲ 23%, Ⅳ 10%)

Murawski *et al*[100] (2016) SIOPEL 2 and 3	85	Complete tumor resection and tumor free margins predict OS	Primary surgery (if feasible) à Super-PLADO (CDDP, Doxo and Carbo) à Assessment for LT	Response to CT in 40% OS at 5 yr 22% 5-yr OS with complete resection 63% *vs* 59% with LT 5-yr OS with macroscopically involved margins 14%
Liver transplantation				
Reyes *et al*[101] (2000)	19	Risk for recurrence with vascular and LN invasion, distant metastases, size of tumor and male gender	LT ± Systemic or intra-arterial neoadjuvant CT	1 yr 79% 3 yr 68% 5 yr 63%
Austin *et al*[24] (2006) UNOS database	41	Primary cause of death: Metastatic or recurrent disease Pretransplant medical disease and era of LT associated with graft and patient survival	All LT	1 yr 86% 3 yr 63% 5 yr 58%
Arikan *et al*[13] (2006)	13	-	LT in 7 Observation in 6	Overall 1 yr 53%, 4 yr 27% (With LT 1 yr 72%, 4 yr 72%) No recurrence at 36 mo with LT
Beaunoyer *et al*[23] (2007)	10	1 out of 7 outside MC had recurrence, died	LT in all Pre-LT CT in 5	OS 1 yR 100%, 5 yR 83% RFS 5 yr 89%
Sevmis *et al*[14] (2008)	9	1 out of 4 outside MC had recurrence, excised	LT in all Pre-LT CT in 3	100% survival at 19.8 ± 10.6 (7-32) mo Recurrence in 1 out of 4 outside MC, excised
Ismail *et al*[22] (2009)	21	Mortality related to recurrence and PRETEXT stage in the non-LT group, but not in the LT group	LT 11 Non-LT 10 (Resection in 8 - 4 after CT)	OS with LT 72% at median 43 mo and Non-LT 40% at median 66 mo Recurrence after LT in 1/11 and after resection in 6/8
Romano *et al*[18] (2011)	10	-	All primary LT No CT / resection	80% RFS at median FU of 4 y (1-11 y)
Palaniappan *et al*[26] (2016)	12	1 Multifocal + 2 with microvascular invasion 2 underwent TACE before LT	All primary LT (8 diagnosed incidentally in explant livers)	92% OS at a median of 5 (1-27) mo
Baumann *et al*[25] (2018) ELTR data	175	Survival better in children with inherited liver disease than without (HR = 0.29) and *vs* adults with HCC with inherited liver disease (HR = 0.27) Survival rate increased with increasing age in non-inherited group	All LT	OS at 5 yr: Patient 58% and Graft 56% Patient survival at 5 yr and 10 yr Inherited: 81% and 81% Non-inherited: 53% and 45%

AFP: Alpha-fetoprotein; Carbo: Carboplatin; CDDP: Cisplatin; CCG: Children's cancer group; CT: Chemotherapy; DFS: Disease free survival; EFS: Event-free survival; ELTR: European Liver Transplant Registry; FLHCC: Fibrolamellar variant of HCC; FU: Follow-up; 5-FU: 5-Fluorouracil; HR: Hazard ratio; LN: Lymph-node; LT: Liver transplantation; MC: Milan criteria; MOT: Multi-organ transplantation; MST: Median survival time; OR: Odd's ratio; OS: Overall survival; PH: Partial hepatectomy; POG: Pediatric Oncology group; PRETEXT: Pretreatment tumor extent evaluation; RFS: Recurrence free survival; SEER: Surveillance epidemiology and end-results; SIOPEL: Group for epithelial liver tumors of the international society of pediatric oncology; TACE: Trans-arterial chemoembolization.

properties. From the multi-center randomized placebo controlled phase Ⅲ study, usage of Sorafenib in adults with advanced HCC has been shown to improve time to tumor progression (median 5.5 mo *vs* 2.8 mo) and overall survival (10.7 mo *vs* 7.9 mo)[106]. Further a randomized controlled study found that the combination of Sorafenib and Doxorubicin in adults was superior to Doxorubicin alone in terms of better overall survival (6.4 mo *vs* 2.8 mo) and progression-free survival (6.0 mo *vs* 2.7 mo)[107]. From the German GPOH group, Sorafenib when used in combination with PLADO in children with HCC showed tumor regression in 4 out of 7 unresectable tumors. There was decrease in AFP levels in four children who had very high levels. Hand-foot-skin reaction was seen in 7 (58%) of 12 children[108].

Targeted therapies: Various targeted therapies based on the pathogenic mechanisms of oncogenesis and cell proliferation are under trials in adults with HCC. The common biological pathways for target are VEGF receptor (Sorafenib, Bevacizumab, Brivanib, Sunitinib), epidermal growth factor (Erlotinib), mammalian target of rapamycin (Everolimus), tyrosine kinase receptor for hepatocyte growth factor, cMET (Tivantinib), combined VEGF and cMET (Cabozantinib) and programmed cell death receptor (anti-PD-1, Nivolumab)[2].

Liver transplantation: Liver transplantation offers best cure for HCC in a cirrhotic liver in terms of oncological viewpoint by enabling the widest possible resection margins and simultaneously ensuring complete removal of the diseased liver at risk of developing HCC[90]. The commonest criterion used in adults to decide candidacy for LT is Milan criteria which includes single tumor with diameter less than 5 cm, or tumor foci up to 3 in number each one not exceeding 3 cm, without any vascular invasion or extrahepatic involvement. Application of Milan criteria has been shown to improve the survival after LT

in adults from 30% to 75%[91]. However in Asia, where living donors are the mainstay for LT and there are no restrictions imposed by the national organ allocation system, the criteria for LT are more liberal[90]. Most countries like Hong Kong and Taiwan are using extended criteria like University of California San Francisco (UCSF) criteria, *i.e.*, solitary tumor ⩽ 65 mm in diameter, or 2-3 tumors, each with diameter ⩽ 45 mm and total tumor diameter ⩽ 80 mm, and without radiological evidence of vascular invasion or distant metastasis, with acceptable long term survival rates[90,103]. Similarly in Japan, patients beyond Milan criteria or those with recurrent HCC with CTP A or B are sometimes considered for LT[102]. Usage of these criteria is limited to case series in children. Transplantation in children outside Milan criteria has a fairly good outcome of 72%-83% at 5 years[18,22,23]. Various series have shown that the outcome post LT in children with HCC is related to PRETEXT stage, recurrence of disease, vascular and lymph node invasion, size of tumor and distant metastases[10,12,15,16,22,24,27,30,38,99-101]. A recent analysis of ELTR data of 175 pediatric HCC showed that children with inherited liver disease (ILD) have better survival after LT in comparison to children without ILD and adults with ILD[25] (Table 3). There is no head to head prospective series comparing resection *vs* LT in children with HCC. However as per the SEER database, the 5-year survival rates are better with LT (85%) in contrast to resection (53%) (hazard ratio 0.05, 95%CI: 0.003-0.94)[30].

Radiological interventions: Radiological interventions like radiofrequency ablation (RFA), transarterial embolization (bland), transarterial chemo-embolization (TACE, with doxorubicin drug eluting beads), hepatic arterial infusion chemotherapy (HAIC, with low dose 5-fluorouracil and cisplatin with or without systemic interferon therapy) and transarterial radio-embolization (TARE, using yttrium-90 microspheres) are routinely used in adults with advanced HCC for downstaging and those listed for LT. AASLD suggests using one of the radiological techniques for adult patients (1) listed as per Milan criteria to decrease progression of disease and subsequent dropout from the waiting list, (2) outside Milan to bring them into the LT criteria, and (3) who are not candidates for resection or LT to improve their survival[91]. As HCC nodules receive their blood supply preferentially from the branches of hepatic artery, catheters placed into hepatic artery are used to obliterate tumor vascular supply and also to inject chemotherapeutic agents (TACE). Absolute contraindications for endovascular therapy include surgically resectable tumor, intractable systemic infection, advanced liver disease (CTP > 8 or BCLC stage C), hepatic encephalopathy, lung shunt fraction > 20% and hepatic encephalopathy. Relative contraindications are biliary obstruction, tumor > 10 cm or burden > 50% of liver, bilirubin > 3 mg/dL, albumin < 2 gm/dL, impaired renal function, AST > 5 times upper limit of normal and presence of extrahepatic metastases. Common side effects include elevation of bilirubin and transaminases, and post-embolization syndrome (pain, malaise, low grade fever). In Asian countries, TACE is frequently offered to adults with early HCC, or in combination with ablation when the nodules are more than 3 or exceed 3 cm in diameter[102,103]. There is limited data on radiological interventions in children with HCC[109]. Two series from China comprising 110 children with HCC have shown that overall survival with resection, TACE and no treatment is 29-38, 4-14 and 2-5 mo, respectively[12,38]. Moreover in children with advanced HCC, TACE offered 4.8 mo of survival benefit in comparison to standard treatment[38]. Special considerations related to sedation and procedure should be taken - like there is more risk of post-embolization syndrome and arterial spasm[109].

SURVEILLANCE

AASLD recommends surveillance of adults with cirrhosis using ultrasound with or without AFP every 6 mo in order to improve their survival[91]. Asia-Pacific consensus on HCC suggests an AFP cut-off of > 200 ng/mL for surveillance programs in combination with ultrasound[90]. Compliance to the surveillance guidelines has been shown to improve overall survival in adults (53 mo *vs* 25 mo) in comparison to non-compliance, and translates into low tumor burden and allocation of curative treatment[110]. For children, we suggest surveillance with ultrasound and AFP every 6 mo for all cirrhotic children and those with chronic HBV infection with elevated transaminases (HBeAg positive or negative), GSD types 1, 3 and 4, alpha-1 antitrypsin deficiency, Wilson disease, autoimmune hepatitis, congenital porto-systemic shunts and hepatic venous outflow tract obstruction. The duration should be reduced to 3 mo in children with BSEP deficiency and tyrosinemia, and increased to 1 year in inactive carriers of HBV[2].

FIBROLAMELLAR HCC

Fibrolamellar variant of HCC (FLHCC) is a distinct rare variant of HCC which is more commonly seen in the pediatric population[8,15,19]. Pathologically, it is characterized by large polygonal cells with abundant eosinophilic cytoplasm containing pale bodies and hyaline globules, and large nuclei surrounded by lamellar stroma and a central scar[19]. There is slight preponderance of females (52%-58%) with a median age ranging between 14 to 33 years. Elevation of AFP is seen in around 10%. A large proportion of these tumors are potentially removable (77%-88%) but recurrence rate as high as 77% has been reported. Up to 60% have extrahepatic metastases. 5-year survival rates after resection and LT range from 58% to 82% and 29% to 55%, respectively and is related to absence of metastases, resectability and old age[111,112]. FLHCC, in contrast to adult HCC, has been shown to have better survival when matched for age, gender, stage of disease and liver functions, but the data is controversial[112]. Pediatric FLHCC cases are older in age (age above 12 yr - 88% *vs* 29%), less multifocal but

more metastatic in contrast to pediatric HCC. Response to chemotherapy (super-PLADO) is partial in 31% and is comparable to Pediatric HCC. Complete resection is possible in 42%, however event free survival (22% *vs* 28%) and overall survival (42% *vs* 33%) at 3 years are comparable to pediatric HCC. In pediatric FLHCC younger age (< 12 years) and absence of multifocality are associated with a trend towards better outcome[112].

CONCLUSION

Pediatric HCC is a rare aggressive liver malignancy. Tyrosinemia, perinatal hepatitis-B, familial intrahepatic cholestasis, glycogen storage disorders and congenital portosystemic shunts are common predispositions. Management focuses primarily on early detection and surgery. Recurrence free survival after resection and liver transplantation has improved over last 3 decades. Prevention and surveillance strategies may help in improving the overall outcome. Fibrolamellar variant constitutes one-fourth of pediatric HCC with a variable prognosis.

REFERENCES

1 **Hadzic N**, Finegold MJ. Liver neoplasia in children. *Clin Liver Dis* 2011; **15**: 443-462, vii-vix [PMID: 21689623 DOI: 10.1016/j.cld.2011.03.011]
2 **Kelly D**, Sharif K, Brown RM, Morland B. Hepatocellular carcinoma in children. *Clin Liver Dis* 2015; **19**: 433-447 [PMID: 25921672 DOI: 10.1016/j.cld.2015.01.010]
3 **Otte JB**. Progress in the surgical treatment of malignant liver tumors in children. *Cancer Treat Rev* 2010; **36**: 360-371 [PMID: 20227190 DOI: 10.1016/j.ctrv.2010.02.013]
4 **Ng VL**, Fecteau A, Shepherd R, Magee J, Bucuvalas J, Alonso E, McDiarmid S, Cohen G, Anand R; Studies of Pediatric Liver Transplantation Research Group. Outcomes of 5-year survivors of pediatric liver transplantation: report on 461 children from a north american multicenter registry. *Pediatrics* 2008; **122**: e1128-e1135 [PMID: 19047213 DOI: 10.1542/peds.2008-1363]
5 **Darbari A**, Sabin KM, Shapiro CN, Schwarz KB. Epidemiology of primary hepatic malignancies in US children. *Hepatology* 2003; **38**: 560-566 [PMID: 12939582 DOI: 10.1053/jhep.2003.50375]
6 **Lee CL**, Ko YC. Survival and distribution pattern of childhood liver cancer in Taiwan. *Eur J Cancer* 1998; **34**: 2064-2067 [PMID: 10070312 DOI: 10.1016/S0959-8049(98)00281-0]
7 **Moore SW**, Millar AJ, Hadley GP, Ionescu G, Kruger M, Poole J, Stones D, Wainwright L, Chitnis M, Wessels G. Hepatocellular carcinoma and liver tumors in South African children: a case for increased prevalence. *Cancer* 2004; **101**: 642-649 [PMID: 15274079 DOI: 10.1002/cncr.20398]
8 **Allan BJ**, Wang B, Davis JS, Parikh PP, Perez EA, Neville HL, Sola JE. A review of 218 pediatric cases of hepatocellular carcinoma. *J Pediatr Surg* 2014; **49**: 166-171; discussion 171 [PMID: 24439603 DOI: 10.1016/j.jpedsurg.2013.09.050]
9 **Czauderna P**. Adult type vs. Childhood hepatocellular carcinoma--are they the same or different lesions? Biology, natural history, prognosis, and treatment. *Med Pediatr Oncol* 2002; **39**: 519-523 [PMID: 12228910 DOI: 10.1002/mpo.10178]
10 **Chen JC**, Chen CC, Chen WJ, Lai HS, Hung WT, Lee PH. Hepatocellular carcinoma in children: clinical review and comparison with adult cases. *J Pediatr Surg* 1998; **33**: 1350-1354 [PMID: 9766351 DOI: 10.1016/S0022-3468(98)90005-7]
11 **Choo SP**, Tan WL, Goh BK, Tai WM, Zhu AX. Comparison of hepatocellular carcinoma in Eastern versus Western populations. *Cancer* 2016 [PMID: 27622302 DOI: 10.1002/cncr.30237]
12 **Zhang XF**, Liu XM, Wei T, Liu C, Li MX, Long ZD, Lv Y. Clinical characteristics and outcome of hepatocellular carcinoma in children and adolescents. *Pediatr Surg Int* 2013; **29**: 763-770 [PMID: 23794023 DOI: 10.1007/s00383-013-3334-4]
13 **Arikan C**, Kilic M, Nart D, Ozgenc F, Ozkan T, Tokat Y, Yagci RV, Aydogdu S. Hepatocellular carcinoma in children and effect of living-donor liver transplantation on outcome. *Pediatr Transplant* 2006; **10**: 42-47 [PMID: 16499586 DOI: 10.1111/j.1399-3046.2005.00395.x]
14 **Sevmis S**, Karakayali H, Ozçay F, Canan O, Bilezikci B, Torgay A, Haberal M. Liver transplantation for hepatocellular carcinoma in children. *Pediatr Transplant* 2008; **12**: 52-56 [PMID: 18186889 DOI: 10.1111/j.1399-3046.2007.00777.x]
15 **McAteer JP**, Goldin AB, Healey PJ, Gow KW. Hepatocellular carcinoma in children: epidemiology and the impact of regional lymphadenectomy on surgical outcomes. *J Pediatr Surg* 2013; **48**: 2194-2201 [PMID: 24210185 DOI: 10.1016/j.jpedsurg.2013.05.007]
16 **Katzenstein HM**, Krailo MD, Malogolowkin MH, Ortega JA, Liu-Mares W, Douglass EC, Feusner JH, Reynolds M, Quinn JJ, Newman K, Finegold MJ, Haas JE, Sensel MG, Castleberry RP, Bowman LC. Hepatocellular carcinoma in children and adolescents: results from the Pediatric Oncology Group and the Children's Cancer Group intergroup study. *J Clin Oncol* 2002; **20**: 2789-2797 [PMID: 12065555 DOI: 10.1200/JCO.2002.06.155]
17 **Pham TH**, Iqbal CW, Grams JM, Zarroug AE, Wall JC, Ishitani MB, Nagorney DM, Moir C. Outcomes of primary liver cancer in children: an appraisal of experience. *J Pediatr Surg* 2007; **42**: 834-839 [PMID: 17502194 DOI: 10.1016/j.jpedsurg.2006.12.065]
18 **Romano F**, Stroppa P, Bravi M, Casotti V, Lucianetti A, Guizzetti M, Sonzogni A, Colledan M, D'Antiga L. Favorable outcome of primary liver transplantation in children with cirrhosis and hepatocellular carcinoma. *Pediatr Transplant* 2011; **15**: 573-579 [PMID: 21797955 DOI: 10.1111/j.1399-3046.2011.01528.x]
19 **Weeda VB**, Murawski M, McCabe AJ, Maibach R, Brugières L, Roebuck D, Fabre M, Zimmermann A, Otte JB, Sullivan M, Perilongo G, Childs M, Brock P, Zsíros J, Plaschkes J, Czauderna P, Aronson DC. Fibrolamellar variant of hepatocellular carcinoma does not have a better survival than conventional hepatocellular carcinoma--results and treatment recommendations from the Childhood Liver Tumour Strategy Group (SIOPEL) experience. *Eur J Cancer* 2013; **49**: 2698-2704 [PMID: 23683550 DOI: 10.1016/j.ejca.2013.04.012]
20 **Nault JC**, Zucman-Rossi J. Genetics of hepatocellular carcinoma: the next generation. *J Hepatol* 2014; **60**: 224-226 [PMID: 23994386 DOI: 10.1016/j.jhep.2013.08.019]
21 **Kim H**, Lee MJ, Kim MR, Chung IP, Kim YM, Lee JY, Jang JJ. Expression of cyclin D1, cyclin E, cdk4 and loss of heterozygosity of 8p, 13q, 17p in hepatocellular carcinoma: comparison study of childhood and adult hepatocellular carcinoma. *Liver* 2000; **20**: 173-178 [PMID: 10847487 DOI: 10.1034/j.1600-0676.2000.020002173.x]
22 **Ismail H**, Broniszczak D, Kaliciński P, Markiewicz-Kijewska M, Teisseyre J, Stefanowicz M, Szymczak M, Dembowska-Bagińska B, Kluge P, Perek D, Kościesza A, Dzik E, Lembas A, Teisserye M. Liver transplantation in children with hepatocellular carcinoma. Do Milan criteria apply to pediatric patients? *Pediatr Transplant* 2009; **13**: 682-692 [PMID: 19496985 DOI: 10.1111/j.1399-3046.2009.01062.x]
23 **Beaunoyer M**, Vanatta JM, Ogihara M, Strichartz D, Dahl G, Berquist WE, Castillo RO, Cox KL, Esquivel CO. Outcomes of transplantation in children with primary hepatic malignancy. *Pediatr Transplant* 2007; **11**: 655-660 [PMID: 17663690 DOI: 10.1111/j.1399-3046.2007.00751.x]
24 **Austin MT**, Leys CM, Feurer ID, Lovvorn HN 3rd, O'Neill JA Jr, Pinson CW, Pietsch JB. Liver transplantation for childhood hepatic malignancy: a review of the United Network for Organ Sharing (UNOS) database. *J Pediatr Surg* 2006; **41**: 182-186 [PMID: 16410130 DOI: 10.1016/j.jpedsurg.2005.10.091]
25 **Baumann U**, Adam R, Duvoux C, Mikolajczyk R, Karam V, D'Antiga L, Chardot C, Coker A, Colledan M, Ericzon BG, Line PD,

Hadzic N, Isoniemi H, Klempnauer JL, Reding R, McKiernan PJ, McLin V, Paul A, Salizzoni M, Furtado ESB, Schneeberger S, Karch A; European Liver and Intestine Transplant Association. Survival of children after liver transplantation for hepatocellular carcinoma. *Liver Transpl* 2018; **24**: 246-255 [PMID: 29222922 DOI: 10.1002/lt.24994]

26 **Palaniappan K**, Borkar VV, Safwan M, Vij M, Govil S, Shanmugam N, Rela M. Pediatric hepatocellular carcinoma in a developing country: Is the etiology changing? *Pediatr Transplant* 2016; **20**: 898-903 [PMID: 27392999 DOI: 10.1111/petr.12754]

27 **Czauderna P**, Mackinlay G, Perilongo G, Brown J, Shafford E, Aronson D, Pritchard J, Chapchap P, Keeling J, Plaschkes J, Otte JB; Liver Tumors Study Group of the International Society of Pediatric Oncology. Hepatocellular carcinoma in children: results of the first prospective study of the International Society of Pediatric Oncology group. *J Clin Oncol* 2002; **20**: 2798-2804 [PMID: 12065556 DOI: 10.1200/JCO.2002.06.102]

28 **Hsu HC**, Wu MZ, Chang MH, Su IJ, Chen DS. Childhood hepatocellular carcinoma develops exclusively in hepatitis B surface antigen carriers in three decades in Taiwan. Report of 51 cases strongly associated with rapid development of liver cirrhosis. *J Hepatol* 1987; **5**: 260-267 [PMID: 2828461 DOI: 10.1016/S0168-8278(87)80030-2]

29 **Chen WJ**, Lee JC, Hung WT. Primary malignant tumor of liver in infants and children in Taiwan. *J Pediatr Surg* 1988; **23**: 457-461 [PMID: 2837562 DOI: 10.1016/S0022-3468(88)80448-2]

30 **McAteer JP**, Goldin AB, Healey PJ, Gow KW. Surgical treatment of primary liver tumors in children: outcomes analysis of resection and transplantation in the SEER database. *Pediatr Transplant* 2013; **17**: 744-750 [PMID: 23992390 DOI: 10.1111/petr.12144]

31 **Lack EE**, Neave C, Vawter GF. Hepatocellular carcinoma. Review of 32 cases in childhood and adolescence. *Cancer* 1983; **52**: 1510-1515 [PMID: 6311396 DOI: 10.1002/1097-0142(19831015)52:8<1510::AID-CNCR2820520830>3.0.CO;2-7]

32 **Chang MH**, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM, Chu HC, Wu TC, Yang SS, Kuo HS, Chen DS; Taiwan Hepatoma Study Group. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. *J Natl Cancer Inst* 2009; **101**: 1348-1355 [PMID: 19759364 DOI: 10.1093/jnci/djp288]

33 **Chang MH**, Chen CJ, Lai MS, Hsu HM, Wu TC, Kong MS, Liang DC, Shau WY, Chen DS. Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group. *N Engl J Med* 1997; **336**: 1855-1859 [PMID: 9197213 DOI: 10.1056/NEJM199706263362602]

34 **Chang MH**, Shau WY, Chen CJ, Wu TC, Kong MS, Liang DC, Hsu HM, Chen HL, Hsu HY, Chen DS; Taiwan Childhood Hepatoma Study Group. Hepatitis B vaccination and hepatocellular carcinoma rates in boys and girls. *JAMA* 2000; **284**: 3040-3042 [PMID: 11122592 DOI: 10.1001/jama.284.23.3040]

35 **Ni YH**, Chang MH, Wang KJ, Hsu HY, Chen HL, Kao JH, Yeh SH, Jeng YM, Tsai KS, Chen DS. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 2004; **127**: 1733-1738 [PMID: 15578511 DOI: 10.1053/j.gastro.2004.09.048]

36 **Wu JF**, Chang MH. Natural history of chronic hepatitis B virus infection from infancy to adult life - the mechanism of inflammation triggering and long-term impacts. *J Biomed Sci* 2015; **22**: 92 [PMID: 26487087 DOI: 10.1186/s12929-015-0199-y]

37 **Takano T**, Tajiri H, Hosono S, Inui A, Murakami J, Ushijima K, Miyoshi Y, Etani Y, Abukawa D, Suzuki M, Brooks S. Natural history of chronic hepatitis B virus infection in children in Japan: a comparison of mother-to-child transmission with horizontal transmission. *J Gastroenterol* 2017; **52**: 1041-1050 [PMID: 28184998 DOI: 10.1007/s00535-017-1315-4]

38 **Wang J**, Mao Y, Liu Y, Chen Z, Chen M, Lao X, Li S. Hepatocellular Carcinoma in Children and Adolescents: Clinical Characteristics and Treatment. *J Gastrointest Surg* 2017; **21**: 1128-1135 [PMID: 28397025 DOI: 10.1007/s11605-017-3420-3]

39 **Satapathy SK**, Garg S, Chauhan R, Malhotra V, Sakhuja P, Sharma BC, Sarin SK. Profile of chronic hepatitis B virus in children in India: experience with 116 children. *J Gastroenterol Hepatol* 2006; **21**: 1170-1176 [PMID: 16824071 DOI: 10.1111/j.1440-1746.2006.04382.x]

40 **Iavarone M**, Colombo M. HBV infection and hepatocellular carcinoma. *Clin Liver Dis* 2013; **17**: 375-397 [PMID: 23905811 DOI: 10.1016/j.cld.2013.05.002]

41 **Liu S**, Zhang H, Gu C, Yin J, He Y, Xie J, Cao G. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J Natl Cancer Inst* 2009; **101**: 1066-1082 [PMID: 19574418 DOI: 10.1093/jnci/djp180]

42 **Li Z**, Xie Z, Ni H, Zhang Q, Lu W, Yin J, Liu W, Ding Y, Zhao Y, Zhu Y, Pu R, Zhang H, Dong H, Fu Y, Sun Q, Xu G, Cao G. Mother-to-child transmission of hepatitis B virus: evolution of hepatocellular carcinoma-related viral mutations in the post-immunization era. *J Clin Virol* 2014; **61**: 47-54 [PMID: 24973814 DOI: 10.1016/j.jcv.2014.06.010]

43 **Mayorandan S**, Meyer U, Gokcay G, Segarra NG, de Baulny HO, van Spronsen F, Zeman J, de Laet C, Spiekerkoetter U, Thimm E, Maiorana A, Dionisi-Vici C, Moeslinger D, Brunner-Krainz M, Lotz-Havla AS, Cocho de Juan JA, Couce Pico ML, Santer R, Scholl-Bürgi S, Mandel H, Bliksrud YT, Freisinger P, Aldamiz-Echevarria LJ, Hochuli M, Gautschi M, Endig J, Jordan J, McKiernan P, Ernst S, Morlot S, Vogel A, Sander J, Das AM. Cross-sectional study of 168 patients with hepatorenal tyrosinaemia and implications for clinical practice. *Orphanet J Rare Dis* 2014; **9**: 107 [PMID: 25081276 DOI: 10.1186/s13023-014-0107-7]

44 **Zeybek AC**, Kiykim E, Soyucen E, Cansever S, Altay S, Zubarioglu T, Erkan T, Aydin A. Hereditary tyrosinemia type 1 in Turkey: twenty year single-center experience. *Pediatr Int* 2015; **57**: 281-289 [PMID: 25223216 DOI: 10.1111/ped.12503]

45 **Larochelle J**, Alvarez F, Bussières JF, Chevalier I, Dallaire L, Dubois J, Faucher F, Fenyves D, Goodyer P, Grenier A, Holme E, Laframboise R, Lambert M, Lindstedt S, Maranda B, Melançon S, Merouani A, Mitchell J, Parizeault G, Pelletier L, Phan V, Rinaldo P, Scott CR, Scriver C, Mitchell GA. Effect of nitisinone (NTBC) treatment on the clinical course of hepatorenal tyrosinemia in Québec. *Mol Genet Metab* 2012; **107**: 49-54 [PMID: 22885033 DOI: 10.1016/j.ymgme.2012.05.022]

46 **Luijerink MC**, Jacobs SM, van Beurden EA, Koornneef LP, Klomp LW, Berger R, van den Berg IE. Extensive changes in liver gene expression induced by hereditary tyrosinemia type I are not normalized by treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). *J Hepatol* 2003; **39**: 901-909 [PMID: 14642604 DOI: 10.1016/S0168-8278(03)00433-1]

47 **Seda Neto J**, Leite KM, Porta A, Fonseca EA, Feier FH, Pugliese R, Miura IK, Chapchap P, Porta G. HCC prevalence and histopathological findings in liver explants of patients with hereditary tyrosinemia type 1. *Pediatr Blood Cancer* 2014; **61**: 1584-1589 [PMID: 24852359 DOI: 10.1002/pbc.25094]

48 **Bahador A**, Dehghani SM, Geramizadeh B, Nikeghbalian S, Bahador M, Malekhosseini SA, Kazemi K, Salahi H. Liver Transplant for Children With Hepatocellular Carcinoma and Hereditary Tyrosinemia Type 1. *Exp Clin Transplant* 2015; **13**: 329-332 [PMID: 24679101 DOI: 10.6002/ect.2013.0158]

49 **Knisely AS**, Strautnieks SS, Meier Y, Stieger B, Byrne JA, Portmann BC, Bull LN, Pawlikowska L, Bilezikçi B, Ozçay F, László A, Tiszlavicz L, Moore L, Raftos J, Arnell H, Fischler B, Németh A, Papadogiannakis N, Cielecka-Kuszyk J, Jankowska I, Pawłowska J, Melín-Aldana H, Emerick KM, Whitington PF, Mieli-Vergani G, Thompson RJ. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. *Hepatology* 2006; **44**: 478-486 [PMID: 16871584 DOI: 10.1002/hep.21287]

50 **Davit-Spraul A**, Fabre M, Branchereau S, Baussan C, Gonzales E, Stieger B, Bernard O, Jacquemin E. ATP8B1 and ABCB11 analysis in 62 children with normal gamma-glutamyl transferase progressive familial intrahepatic cholestasis (PFIC): phenotypic differences between PFIC1 and PFIC2 and natural history. *Hepatology* 2010; **51**: 1645-1655 [PMID: 20232290 DOI: 10.1002/hep.23539]

51 **Pawlikowska L**, Strautnieks S, Jankowska I, Czubkowski P,

Emerick K, Antoniou A, Wanty C, Fischler B, Jacquemin E, Wali S, Blanchard S, Nielsen IM, Bourke B, McQuaid S, Lacaille F, Byrne JA, van Eerde AM, Kolho KL, Klomp L, Houwen R, Bacchetti P, Lobritto S, Hupertz V, McClean P, Mieli-Vergani G, Shneider B, Nemeth A, Sokal E, Freimer NB, Knisely AS, Rosenthal P, Whitington PF, Pawlowska J, Thompson RJ, Bull LN. Differences in presentation and progression between severe FIC1 and BSEP deficiencies. *J Hepatol* 2010; **53**: 170-178 [PMID: 20447715 DOI: 10.1016/j.jhep.2010.01.034]

52 **Strautnieks SS**, Byrne JA, Pawlikowska L, Cebecauerová D, Rayner A, Dutton L, Meier Y, Antoniou A, Stieger B, Arnell H, Ozçay F, Al-Hussaini HF, Bassas AF, Verkade HJ, Fischler B, Németh A, Kotalová R, Shneider BL, Cielecka-Kuszyk J, McClean P, Whitington PF, Sokal E, Jirsa M, Wali SH, Jankowska I, Pawłowska J, Mieli-Vergani G, Knisely AS, Bull LN, Thompson RJ. Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families. *Gastroenterology* 2008; **134**: 1203-1214 [PMID: 18395098 DOI: 10.1053/j.gastro.2008.01.038]

53 **Iannelli F**, Collino A, Sinha S, Radaelli E, Nicoli P, D'Antiga L, Sonzogni A, Faivre J, Buendia MA, Sturm E, Thompson RJ, Knisely AS, Natoli G, Ghisletti S, Ciccarelli FD. Massive gene amplification drives paediatric hepatocellular carcinoma caused by bile salt export pump deficiency. *Nat Commun* 2014; **5**: 3850 [PMID: 24819516 DOI: 10.1038/ncomms4850]

54 **Chen Y**, Song X, Valanejad L, Vasilenko A, More V, Qiu X, Chen W, Lai Y, Slitt A, Stoner M, Yan B, Deng R. Bile salt export pump is dysregulated with altered farnesoid X receptor isoform expression in patients with hepatocellular carcinoma. *Hepatology* 2013; **57**: 1530-1541 [PMID: 23213087 DOI: 10.1002/hep.26187]

55 **Zhou S**, Hertel PM, Finegold MJ, Wang L, Kerkar N, Wang J, Wong LJ, Plon SE, Sambrotta M, Foskett P, Niu Z, Thompson RJ, Knisely AS. Hepatocellular carcinoma associated with tight-junction protein 2 deficiency. *Hepatology* 2015; **62**: 1914-1916 [PMID: 25921221 DOI: 10.1002/hep.27872]

56 **Sambrotta M**, Strautnieks S, Papouli E, Rushton P, Clark BE, Parry DA, Logan CV, Newbury LJ, Kamath BM, Ling S, Grammatikopoulos T, Wagner BE, Magee JC, Sokol RJ, Mieli-Vergani G; University of Washington Center for Mendelian Genomics, Smith JD, Johnson CA, McClean P, Simpson MA, Knisely AS, Bull LN, Thompson RJ. Mutations in TJP2 cause progressive cholestatic liver disease. *Nat Genet* 2014; **46**: 326-328 [PMID: 24614073 DOI: 10.1038/ng.2918]

57 **Vij M**, Shanmugam NP, Reddy MS, Govil S, Rela M. Hepatocarcinogenesis in multidrug-resistant P-glycoprotein 3 deficiency. *Pediatr Transplant* 2017; **21** [PMID: 28127842 DOI: 10.1111/petr.12889]

58 **Reddy SK**, Austin SL, Spencer-Manzon M, Koeberl DD, Clary BM, Desai DM, Smith AD, Kishnani PS. Liver transplantation for glycogen storage disease type Ia. *J Hepatol* 2009; **51**: 483-490 [PMID: 19596478 DOI: 10.1016/j.jhep.2009.05.026]

59 **Kishnani PS**, Austin SL, Abdenur JE, Arn P, Bali DS, Boney A, Chung WK, Dagli AI, Dale D, Koeberl D, Somers MJ, Wechsler SB, Weinstein DA, Wolfsdorf JI, Watson MS; American College of Medical Genetics and Genomics. Diagnosis and management of glycogen storage disease type I: a practice guideline of the American College of Medical Genetics and Genomics. *Genet Med* 2014; **16**: e1 [PMID: 25356975 DOI: 10.1038/gim.2014.128]

60 **Bianchi L**. Glycogen storage disease Ⅰ and hepatocellular tumours. *Eur J Pediatr* 1993; **152** Suppl 1: S63-S70 [PMID: 8391447 DOI: 10.1007/BF02072092]

61 **Labrune P**, Trioche P, Duvaltier I, Chevalier P, Odièvre M. Hepatocellular adenomas in glycogen storage disease type Ⅰ and Ⅲ: a series of 43 patients and review of the literature. *J Pediatr Gastroenterol Nutr* 1997; **24**: 276-279 [PMID: 9138172 DOI: 10.1097/00005176-199703000-00008]

62 **Kishnani PS**, Chuang TP, Bali D, Koeberl D, Austin S, Weinstein DA, Murphy E, Chen YT, Boyette K, Liu CH, Chen YT, Li LH. Chromosomal and genetic alterations in human hepatocellular adenomas associated with type Ia glycogen storage disease. *Hum Mol Genet* 2009; **18**: 4781-4790 [PMID: 19762333 DOI: 10.1093/hmg/ddp441]

63 **Kishnani PS**, Austin SL, Arn P, Bali DS, Boney A, Case LE, Chung WK, Desai DM, El-Gharbawy A, Haller R, Smit GP, Smith AD, Hobson-Webb LD, Wechsler SB, Weinstein DA, Watson MS; ACMG. Glycogen storage disease type Ⅲ diagnosis and management guidelines. *Genet Med* 2010; **12**: 446-463 [PMID: 20631546 DOI: 10.1097/GIM.0b013e3181e655b6]

64 **Demo E**, Frush D, Gottfried M, Koepke J, Boney A, Bali D, Chen YT, Kishnani PS. Glycogen storage disease type Ⅲ-hepatocellular carcinoma a long-term complication? *J Hepatol* 2007; **46**: 492-498 [PMID: 17196294 DOI: 10.1016/j.jhep.2006.09.022]

65 **Townsend SA**, Edgar RG, Ellis PR, Kantas D, Newsome PN, Turner AM. Systematic review: the natural history of alpha-1 antitrypsin deficiency, and associated liver disease. *Aliment Pharmacol Ther* 2018; **47**: 877-885 [PMID: 29446109 DOI: 10.1111/apt.14537]

66 **Antoury C**, Lopez R, Zein N, Stoller JK, Alkhouri N. Alpha-1 antitrypsin deficiency and the risk of hepatocellular carcinoma in end-stage liver disease. *World J Hepatol* 2015; **7**: 1427-1432 [PMID: 26052388 DOI: 10.4254/wjh.v7.i10.1427]

67 **Elzouki AN**, Eriksson S. Risk of hepatobiliary disease in adults with severe alpha 1-antitrypsin deficiency (PiZZ): is chronic viral hepatitis B or C an additional risk factor for cirrhosis and hepatocellular carcinoma? *Eur J Gastroenterol Hepatol* 1996; **8**: 989-994 [PMID: 8930564 DOI: 10.1097/00042737-199610000-00010]

68 **Marcus NY**, Brunt EM, Blomenkamp K, Ali F, Rudnick DA, Ahmad M, Teckman JH. Characteristics of hepatocellular carcinoma in a murine model of alpha-1-antitrypsin deficiency. *Hepatol Res* 2010; **40**: 641-653 [PMID: 20618460 DOI: 10.1111/j.1872-034X.2010.00663.x]

69 **Marcus NY**, Blomenkamp K, Ahmad M, Teckman JH. Oxidative stress contributes to liver damage in a murine model of alpha-1-antitrypsin deficiency. *Exp Biol Med* (Maywood) 2012; **237**: 1163-1172 [PMID: 23104507 DOI: 10.1258/ebm.2012.012106]

70 **Kamath BM**, Baker A, Houwen R, Todorova L, Kerkar N. Systematic Review: the Epidemiology, Natural History and Burden of Alagille Syndrome. *J Pediatr Gastroenterol Nutr* 2018; 67: 148-156 [PMID: 29543694 DOI: 10.1097/MPG.0000000000001958]

71 **Bhadri VA**, Stormon MO, Arbuckle S, Lam AH, Gaskin KJ, Shun A. Hepatocellular carcinoma in children with Alagille syndrome. *J Pediatr Gastroenterol Nutr* 2005; **41**: 676-678 [PMID: 16254531 DOI: 10.1097/01.mpg.0000179759.60048.c4]

72 **Dill MT**, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, Heim MH, Tchorz JS. Constitutive Notch2 signaling induces hepatic tumors in mice. *Hepatology* 2013; **57**: 1607-1619 [PMID: 23175466 DOI: 10.1002/hep.26165]

73 **Tschaharganeh DF**, Chen X, Latzko P, Malz M, Gaida MM, Felix K, Ladu S, Singer S, Pinna F, Gretz N, Sticht C, Tomasi ML, Delogu S, Evert M, Fan B, Ribback S, Jiang L, Brozzetti S, Bergmann F, Dombrowski F, Schirmacher P, Calvisi DF, Breuhahn K. Yes-associated protein up-regulates Jagged-1 and activates the Notch pathway in human hepatocellular carcinoma. *Gastroenterology* 2013; **144**: 1530-1542.e12 [PMID: 23419361 DOI: 10.1053/j.gastro.2013.02.009]

74 **Bernard O**, Franchi-Abella S, Branchereau S, Pariente D, Gauthier F, Jacquemin E. Congenital portosystemic shunts in children: recognition, evaluation, and management. *Semin Liver Dis* 2012; **32**: 273-287 [PMID: 23397528 DOI: 10.1055/s-0032-1329896]

75 **Franchi-Abella S**, Branchereau S, Lambert V, Fabre M, Steimberg C, Losay J, Riou JY, Pariente D, Gauthier F, Jacquemin E, Bernard O. Complications of congenital portosystemic shunts in children: therapeutic options and outcomes. *J Pediatr Gastroenterol Nutr* 2010; **51**: 322-330 [PMID: 20601902 DOI: 10.1097/MPG.0b013e3181d9cb92]

76 **Sokollik C**, Bandsma RH, Gana JC, van den Heuvel M, Ling SC. Congenital portosystemic shunt: characterization of a multisystem disease. *J Pediatr Gastroenterol Nutr* 2013; **56**: 675-681 [PMID: 23412540 DOI: 10.1097/MPG.0b013e31828b3750]

77 **Hadžić N**, Quaglia A, Portmann B, Paramalingam S, Heaton ND, Rela M, Mieli-Vergani G, Davenport M. Hepatocellular carcinoma in biliary atresia: King's College Hospital experience. *J*

Pediatr 2011; **159**: 617-622.e1 [PMID: 21489554 DOI: 10.1016/j.jpeds.2011.03.004]
78 **Tatekawa Y**, Asonuma K, Uemoto S, Inomata Y, Tanaka K. Liver transplantation for biliary atresia associated with malignant hepatic tumors. *J Pediatr Surg* 2001; **36**: 436-439 [PMID: 11226990 DOI: 10.1053/jpsu.2001.21600]
79 **Moucari R**, Rautou PE, Cazals-Hatem D, Geara A, Bureau C, Consigny Y, Francoz C, Denninger MH, Vilgrain V, Belghiti J, Durand F, Valla D, Plessier A. Hepatocellular carcinoma in Budd-Chiari syndrome: characteristics and risk factors. *Gut* 2008; **57**: 828-835 [PMID: 18218675 DOI: 10.1136/gut.2007.139477]
80 **Khanna R**, Alam S, Mukund A, Ahuja A, Rastogi A. Hepatocellular carcinoma in an adolescent with celiac disease. *J Pediatr Gastroenterol Nutr* 2013; **57**: e16-e18 [PMID: 22785418 DOI: 10.1097/MPG.0b013e3182680d55]
81 **Alam S**, Lal BB, Sood V, Rawat D. Pediatric Acute-on-Chronic Liver Failure in a Specialized Liver Unit: Prevalence, Profile, Outcome, and Predictive Factors. *J Pediatr Gastroenterol Nutr* 2016; **63**: 400-405 [PMID: 26967824 DOI: 10.1097/MPG.0000000000001179]
82 **Tansel A**, Katz LH, El-Serag HB, Thrift AP, Parepally M, Shakhatreh MH, Kanwal F. Incidence and Determinants of Hepatocellular Carcinoma in Autoimmune Hepatitis: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2017; **15**: 1207-1217.e4 [PMID: 28215616 DOI: 10.1016/j.cgh.2017.02.006]
83 **Hino-Arinaga T**, Ide T, Kuromatsu R, Miyajima I, Ogata K, Kuwahara R, Hisamochi A, Torimura T, Sata M; Autoimmune Hepatitis Study Group. Risk factors for hepatocellular carcinoma in Japanese patients with autoimmune hepatitis type 1. *J Gastroenterol* 2012; **47**: 569-576 [PMID: 22187167 DOI: 10.1007/s00535-011-0519-2]
84 **van Meer S**, de Man RA, van den Berg AP, Houwen RH, Linn FH, van Oijen MG, Siersema PD, van Erpecum KJ. No increased risk of hepatocellular carcinoma in cirrhosis due to Wilson disease during long-term follow-up. *J Gastroenterol Hepatol* 2015; **30**: 535-539 [PMID: 25160780 DOI: 10.1111/jgh.12716]
85 **Pfeiffenberger J**, Mogler C, Gotthardt DN, Schulze-Bergkamen H, Litwin T, Reuner U, Hefter H, Huster D, Schemmer P, Członkowska A, Schirmacher P, Stremmel W, Cassiman D, Weiss KH. Hepatobiliary malignancies in Wilson disease. *Liver Int* 2015; **35**: 1615-1622 [PMID: 25369181 DOI: 10.1111/liv.12727]
86 **Dragani TA**. Risk of HCC: genetic heterogeneity and complex genetics. *J Hepatol* 2010; **52**: 252-257 [PMID: 20022654 DOI: 10.1016/j.jhep.2009.11.015]
87 **Zen Y**, Vara R, Portmann B, Hadzic N. Childhood hepatocellular carcinoma: a clinicopathological study of 12 cases with special reference to EpCAM. *Histopathology* 2014; **64**: 671-682 [PMID: 24138022 DOI: 10.1111/his.12312]
88 **Choi JY**, Lee JM, Sirlin CB. CT and MR imaging diagnosis and staging of hepatocellular carcinoma: part Ⅱ. Extracellular agents, hepatobiliary agents, and ancillary imaging features. *Radiology* 2014; **273**: 30-50 [PMID: 25247563 DOI: 10.1148/radiol.14132362]
89 **Roberts LR**, Sirlin CB, Zaiem F, Almasri J, Prokop LJ, Heimbach JK, Murad MH, Mohammed K. Imaging for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. *Hepatology* 2018; **67**: 401-421 [PMID: 28859233 DOI: 10.1002/hep.29487]
90 **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]
91 **Heimbach JK**, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, Zhu AX, Murad MH, Marrero JA. AASLD guidelines for the treatment of hepatocellular carcinoma. *Hepatology* 2018; **67**: 358-380 [PMID: 28130846 DOI: 10.1002/hep.29086]
92 **Jacob J**, Deganello A, Sellars ME, Hadzic N, Sidhu PS. Contrast enhanced ultrasound (CEUS) characterization of grey-scale sonographic indeterminate focal liver lesions in pediatric practice. *Ultraschall Med* 2013; **34**: 529-540 [PMID: 24132647 DOI: 10.1055/s-0033-1355785]
93 **Darge K**, Papadopoulou F, Ntoulia A, Bulas DI, Coley BD, Fordham LA, Paltiel HJ, McCarville B, Volberg FM, Cosgrove DO, Goldberg BB, Wilson SR, Feinstein SB. Safety of contrast-enhanced ultrasound in children for non-cardiac applications: a review by the Society for Pediatric Radiology (SPR) and the International Contrast Ultrasound Society (ICUS). *Pediatr Radiol* 2013; **43**: 1063-1073 [PMID: 23843130 DOI: 10.1007/s00247-013-2746-6]
94 **López-Terrada D**, Alaggio R, de Dávila MT, Czauderna P, Hiyama E, Katzenstein H, Leuschner I, Malogolowkin M, Meyers R, Ranganathan S, Tanaka Y, Tomlinson G, Fabrè M, Zimmermann A, Finegold MJ; Children's Oncology Group Liver Tumor Committee. Towards an international pediatric liver tumor consensus classification: proceedings of the Los Angeles COG liver tumors symposium. *Mod Pathol* 2014; **27**: 472-491 [PMID: 24008558 DOI: 10.1038/modpathol.2013.80]
95 **Towbin AJ**, Meyers RL, Woodley H, Miyazaki O, Weldon CB, Morland B, Hiyama E, Czauderna P, Roebuck DJ, Tiao GM. 2017 PRETEXT: radiologic staging system for primary hepatic malignancies of childhood revised for the Paediatric Hepatic International Tumour Trial (PHITT). *Pediatr Radiol* 2018; **48**: 536-554 [PMID: 29427028 DOI: 10.1007/s00247-018-4078-z]
96 **Wu TC**, Tong MJ, Hwang B, Lee SD, Hu MM. Primary hepatocellular carcinoma and hepatitis B infection during childhood. *Hepatology* 1987; **7**: 46-48 [PMID: 2433204 DOI: 10.1002/hep.1840070111]
97 **Ni YH**, Chang MH, Hsu HY, Hsu HC, Chen CC, Chen WJ, Lee CY. Hepatocellular carcinoma in childhood. Clinical manifestations and prognosis. *Cancer* 1991; **68**: 1737-1741 [PMID: 1655224 DOI: 10.1002/1097-0142(19911015)68:8<1737::AID-CNCR2820680815>3.0.CO;2-G]
98 **Hsiao CC**, Chuang JH, Tiao MM, Sheen JM, Shieh CS. Patterns of hepatoblastoma and hepatocellular carcinoma in children after universal hepatitis B vaccination in taiwan: a report from a single institution in southern Taiwan. *J Pediatr Hematol Oncol* 2009; **31**: 91-96 [PMID: 19194190 DOI: 10.1097/MPH.0b013e31818b3784]
99 **Tagge EP**, Tagge DU, Reyes J, Tzakis A, Iwatsuki S, Starzl TE, Wiener ES. Resection, including transplantation, for hepatoblastoma and hepatocellular carcinoma: impact on survival. *J Pediatr Surg* 1992; **27**: 292-296; discussion 297 [PMID: 1323649 DOI: 10.1016/0022-3468(92)90849-3]
100 **Murawski M**, Weeda VB, Maibach R, Morland B, Roebuck DJ, Zimmerman A, Casanova M, Perilongo G, Laithier V, Kebudi R, Scopinaro MJ, Shun A, Brichard B, de Camargo B, Childs M, Aronson DC, Czauderna P. Hepatocellular Carcinoma in Children: Does Modified Platinum- and Doxorubicin-Based Chemotherapy Increase Tumor Resectability and Change Outcome? Lessons Learned From the SIOPEL 2 and 3 Studies. *J Clin Oncol* 2016; **34**: 1050-1056 [PMID: 26811523 DOI: 10.1200/JCO.2014.60.2250]
101 **Reyes JD**, Carr B, Dvorchik I, Kocoshis S, Jaffe R, Gerber D, Mazariegos GV, Bueno J, Selby R. Liver transplantation and chemotherapy for hepatoblastoma and hepatocellular cancer in childhood and adolescence. *J Pediatr* 2000; **136**: 795-804 [PMID: 10839879 DOI: 10.1016/S0022-3476(00)44469-0]
102 **Kudo M**, Matsui O, Izumi N, Iijima H, Kadoya M, Imai Y, Okusaka T, Miyayama S, Tsuchiya K, Ueshima K, Hiraoka A, Ikeda M, Ogasawara S, Yamashita T, Minami T, Yamakado K; Liver Cancer Study Group of Japan. JSH Consensus-Based Clinical Practice Guidelines for the Management of Hepatocellular Carcinoma: 2014 Update by the Liver Cancer Study Group of Japan. *Liver Cancer* 2014; **3**: 458-468 [PMID: 26280007 DOI: 10.1159/000343875]
103 **Poon RT**, Cheung TT, Kwok PC, Lee AS, Li TW, Loke KL, Chan SL, Cheung MT, Lai TW, Cheung CC, Cheung FY, Loo CK, But YK, Hsu SJ, Yu SC, Yau T. Hong Kong consensus recommendations on the management of hepatocellular carcinoma. *Liver Cancer* 2015; **4**: 51-69 [PMID: 26020029 DOI: 10.1159/000367728]
104 **von Schweinitz D**, Bürger D, Bode U, Weinel P, Erttmann R, Hecker H, Mildenberger H. [Results of the HB-89 Study in treatment of malignant epithelial liver tumors in childhood and concept of a new

HB-94 protocol]. *Klin Padiatr* 1994; **206**: 282-288 [PMID: 7526028 DOI: 10.1055/s-2008-1046615]
105 **Schmid I**, Albert MH, Ha¨berle B. HB99-Hepatozellula¨re Karzinome: Behandlungsergebnisse und neue Konzepte. *Monatsschr Kinderheilkd* 2008; **156**: 412
106 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]
107 **Abou-Alfa GK**, Johnson P, Knox JJ, Capanu M, Davidenko I, Lacava J, Leung T, Gansukh B, Saltz LB. Doxorubicin plus sorafenib vs doxorubicin alone in patients with advanced hepatocellular carcinoma: a randomized trial. *JAMA* 2010; **304**: 2154-2160 [PMID: 21081728 DOI: 10.1001/jama.2010.1672]
108 **Schmid I**, Häberle B, Albert MH, Corbacioglu S, Fröhlich B, Graf N, Kammer B, Kontny U, Leuschner I, Scheel-Walter HG, Scheurlen W, Werner S, Wiesel T, von Schweinitz D. Sorafenib and cisplatin/doxorubicin (PLADO) in pediatric hepatocellular carcinoma. *Pediatr Blood Cancer* 2012; **58**: 539-544 [PMID: 21922643 DOI: 10.1002/pbc.23295]
109 **Lungren MP**, Towbin AJ, Roebuck DJ, Monroe EJ, Gill AE, Thakor A, Towbin RB, Cahill AM, Matthew Hawkins C. Role of interventional radiology in managing pediatric liver tumors : Part 1: Endovascular interventions. *Pediatr Radiol* 2018; **48**: 555-564 [PMID: 29362840 DOI: 10.1007/s00247-018-4068-1]
110 **Costentin C**, Layese R, Bourcier V, Cagnot C, Marcellin P, Guyader D, Pol S, Larrey D, De Lédinghen V, Ouzan D, Zoulim F, Roulot D, Tran A, Bronowicki JP, Zarski JP, Riachi G, Calès P, Péron JM, Alric L, Bourlière M, Mathurin P, Blanc JF, Abergel A, Serfaty L, Mallat A, Grangé JD, Attali P, Bacq Y, Wartelle C, Dao T, Thabut D, Pilette C, Silvain C, Christidis C, Nguyen-Khac E, Bernard-Chabert B, Zucman D, Di Martino V, Sutton A, Letouzé E, Imbeaud S, Zucman-Rossi J, Audureau E, Roudot-Thoraval F, Nahon P; ANRS CO12 CirVir Group. Compliance With Hepatocellular Carcinoma Surveillance Guidelines Associated With Increased Lead-Time Adjusted Survival of Patients With Compensated Viral Cirrhosis: A Multi-Center Cohort Study. *Gastroenterology* 2018; **155**: 431-442.e10 [PMID: 29729258 DOI: 10.1053/j.gastro.2018.04.027]
111 **Ang CS**, Kelley RK, Choti MA, Cosgrove DP, Chou JF, Klimstra D, Torbenson MS, Ferrell L, Pawlik TM, Fong Y, O'Reilly EM, Ma J, McGuire J, Vallarapu GP, Griffin A, Stipa F, Capanu M, Dematteo RP, Venook AP, Abou-Alfa GK. Clinicopathologic characteristics and survival outcomes of patients with fibrolamellar carcinoma: data from the fibrolamellar carcinoma consortium. *Gastrointest Cancer Res* 2013; **6**: 3-9 [PMID: 23505572]
112 **Mavros MN**, Mayo SC, Hyder O, Pawlik TM. A systematic review: treatment and prognosis of patients with fibrolamellar hepatocellular carcinoma. *J Am Coll Surg* 2012; **215**: 820-830 [PMID: 22981432 DOI: 10.1016/j.jamcollsurg.2012.08.001]

P- Reviewer: Giorgio A, Streba LL, Zhao HT **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4000-4013

DOI: 10.3748/wjg.v24.i35.4000

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

REVIEW

Changing role of histopathology in the diagnosis and management of hepatocellular carcinoma

Archana Rastogi

Archana Rastogi, Department of Pathology, Institute of Liver & Biliary Sciences, New Delhi 110070, India

ORCID number: Archana Rastogi (0000-0001-9669-3961).

Author contributions: Rastogi A conceptualized and designed the study, performed literature review and drafting, editing, of the manuscript.

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Archana Rastogi DNB, MBBS, MD, Professor, Department of Pathology, Institute of Liver & Biliary Sciences (ILBS), D-1, Vasant Kunj, New Delhi 110070, India. drarchanarastogi@gmail.com
Telephone: +91-11-46300000

Received: June 23, 2018
Peer-review started: June 24, 2018
First decision: July 6, 2018
Revised: July 23, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 21, 2018

Abstract

Hepatocellular carcinoma (HCC) is one of the most common and fatal cancer in the world. HCC frequently presents with advanced disease, has a high recurrence rate and limited treatment options, which leads to very poor prognosis. This warrants urgent improvement in the diagnosis and treatment. Liver biopsy plays very important role in the diagnosis and prognosis of HCC, but with technical advancements and progression in the field of imaging, clinical guidelines have restricted the role of biopsy to very limited situations. Biopsy also has its own problems of needle tract seeding of tumor, small risk of complications, technical and sampling errors along with interpretative errors. Despite this, tissue analysis is often required because imaging is not always specific, limited expertise and lack of advanced imaging in many centers and limitations of imaging in the diagnosis of small, mixed and other variant forms of HCC. In addition, biopsy confirmation is often required for clinical trials of new drugs and targeted therapies. Tissue biomarkers along with certain morphological features, phenotypes and immune-phenotypes that serve as important prognostic and outcome predictors and as decisive factors for therapy decisions, add to the continuing role of histopathology. Advancements in cancer biology and development of molecular classification of HCC with clinic pathological correlation, lead to discovery of HCC phenotypic surrogates of prognostic and therapeutically significant molecular signatures. Thus tissue characteristics and morphology based correlates of molecular subtypes provide invaluable information for management and prognosis. This review thus focuses on the importance of histopathology and resurgence of role of biopsy in the diagnosis, management and prognostication of HCC.

Key words: Hepatocellular carcinoma; Biomarker; Biopsy; Histopathology; Immunohistochemistry; Targeted therapy; Molecular; Diagnosis; Prognosis

Core tip: Liver biopsy plays important roles in the diagnosis and prognosis of hepatocellular carcinoma. However biopsy related complications and limitations

along with advancements in imaging have restricted its role to very limited situations. In recent time, studies on tissue biomarkers, molecular classifications and targeted therapies for hepatocellular carcinoma (HCC) with their clinic-pathologic correlations have highlighted that morphologic variants and subtypes can serve as importance surrogates of molecular signatures, thus renewing the interest in tissue analysis. Tumor biopsy thus is increasingly being recognized as an invaluable tool for the diagnosis, management and prognostication of HCC.

Rastogi A. Changing role of histopathology in the diagnosis and management of hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(35): 4000-4013 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4000.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4000

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world[1-4], with an increasing incidence each year. It is also one of the most lethal human malignant tumor with > 600000 deaths per year worldwide[1,4] making it the third leading cause of cancer related death[5,6]. Dismal prognosis of HCC is attributable to advanced disease at presentation, high rates of metastases and recurrence along with the limited and unsuccessful treatment options available[7,8]. Also, Amongst the primary liver cancers, HCC is the most common, accounting for 70%-85% of all the histological types[9,10]. Major risk factors of HCC are infection with hepatitis B and hepatitis C, excess alcohol intake, obesity, diabetes and metabolic diseases[7,11]. These risk factors cause repeated episodes or sustained state of inflammation, resulting in progressive fibrosis and cirrhosis, along with development of preneoplastic lesions with stem cells acting as a nidus for HCC[12,13]. Literature indicates that 70%-97% of patients with HCC have underlying cirrhosis of the liver at the time of diagnosis[14]. Poor clinical outcome makes it imperative to advance our understanding of HCC at the cellular level and improve methods for the early diagnosis and treatment particularly targeted therapies. HCC is diagnosed by the non-invasive methods of imaging and tumor markers and by the invasive techniques of biopsy and aspiration. Lesion biopsy in HCC, like other solid organs provide valuable information about the diagnosis, prognosis and in certain circumstances, guide about treatment decisions, however biopsy in the HCC and cirrhotic milieu is controversial and is superseded by imaging[6,15]. Certain biopsy limitations especially needle tract seeding, sampling errors and small risk of morbidity along with the technical advancements in imaging, undermined the importance of tissue analysis. This led most of the international guidelines on HCC to restrict the role of liver biopsy to characterize the lesions in non-cirrhotic liver or those with equivocal imaging. However, imaging technologies also have certain caveats, cautioning against abandon of histopathology assessment for HCC. Tumor histopathology, besides being an important diagnostic tool, plays numerous other important roles such as distinguishing from metastasis and other primary benign or preneoplastic lesions, in prognostication and influencing treatment decisions, which cannot be substituted by imaging techniques or tumor markers. With increasingly accumulating data on prognostic and therapeutic importance of specific phenotypes and distinct molecular-morphologic correlates, there is resurgence of interest in the role of tissue evaluation.

ROLE OF IMAGING IN THE DIAGNOSIS OF HCC

The recent diagnostic approach for HCC is based on imaging studies, restricting the role of histopathology to only certain situations. Clinical guidelines of American Association for the Study of Liver disease, European Association for the Study of the Liver (EASL) and Asian-Pacific Association for the Study of Liver have changed the diagnostic criteria for HCC[2,16-18], recommending radiology in view of the remarkable advances in techniques that have led to very high sensitivity and specificity for the diagnosis of HCC[19]. A recent systematic review and meta-analysis for studies comparing CT with extracellular contrast-enhanced MRI or gadoxetate-enhanced MRI in adults with cirrhosis and suspected HCC, found that all them performed better for HCC $\geqslant$ 2 cm in comparison to lesions < 1 cm. For all tumor sizes, studies showed significantly higher sensitivity for MRI over CT, with no difference in the specificity between techniques[20]. Typical imaging findings of intense uptake of contrast during the arterial phase followed by decreased enhancement and washout during the portal phases, based on the neo-arterial supply feeding the HCC, by even a single contrast enhanced imaging study is considered sufficiently specific for the diagnosis of HCC[2,16-18,21]. Thus, biopsy is not advocated for the diagnosis of HCC, if typical features are present on dynamic imaging technique[16-18,22].

IMAGING LIMITATIONS

Imaging techniques falter in certain situations and histopathology assessment becomes mandatory for the diagnosis of all equivocal lesions, irrespective of the size[6], reported in 10%-15% of patients[23]. Lesion in a cirrhotic patient that lacks typical imaging characteristics, histopathological evaluation is the recommended diagnostic tool. Imaging alone has been found to be insufficient to diagnose well-differentiated HCC.

Biopsy has advantage over imaging as comparison with non-lesional liver tissue provides vital information, particularly for the diagnosis of well-differentiated HCC. Horigome *et al*[24] reported that digital subtraction

angiography and magnetic resonance imaging in the absence of biopsy, could diagnose HCC in only 58% of well-differentiated HCC. Dynamic imaging has been shown to have a sensitivity of 35%-71% in different series for tumors of less than 2 cm size[25-29], attributable to hypovascularity of small HCC. Another limitation of imaging is the requirement of pathologic diagnosis for all nodules developing in the non-cirrhotic background[6]. Also, false positives, as high as 33%[30,31], have been reported with the radiology techniques for diagnosing HCC. A study reported that many nodules detected by ultrasound were not found on computed tomography[32,33]. Similarly, 41% and 30.8% of the imaging based cases turned out to be non-HCC on biopsy or follow-up[30]. Without biopsy confirmation, these cases might get subjected to unnecessary transplantation, resection and other therapies. Strength of tissue analysis to determine the malignant potential and histogenesis of liver lesions is another major constraint with imaging. Combined hepato-cholangiocarcinoma (cHCC-CC) is difficult to diagnose and characterize by the imaging alone[34]. Lack of skill and absence of advance imaging technologies in many centers, are other constraints, requiring histopathology assessment for confirmation. Such limitations of imaging compel tissue confirmation in a significant number of cases.

Role of serum α-feto protein (AFP) in the diagnosis of HCC although controversial, is still recommended by certain guidelines[35]. In situations, where imaging is atypical, AFP levels are useful for diagnosing HCC when biopsy needs to be avoided in view of the risk of tumour seeding[36-38]. Studies have shown that increasing AFP levels before liver transplantation are associated with an increased risk of tumor recurrence and decreased survival following transplantation[39,40]. Serum AFP levels are not influenced by technical factors, skill, observer variability thus still has an important role in surveillance, diagnosis, prediction of outcome and monitoring treatment response[41].

BIOPSY SHORTCOMINGS

The declining interest for biopsy is due to several issues. There are risks associated with the procedure, that include morbidity due to the most frequent complication, *i.e.*, pain[42,43] and bleeding especially in patients with cirrhosis who are at a higher risk of this complication. Incidence of such minor complications is 5.9%[44]. Significant hemorrhage occurs in 0.5%[45]. There is slight risk of mortality[46,47], with incidence of 0.11% reported in experienced centers[48]. Evaluation of sixty-four series reporting 7649 TJLBs revealed minor and major complication rates to be 6.5% and 0.56%, respectively with mortality in adults to be 0.09%[49]. However, a large series of 16648 guided biopsies and 3035 therapeutic procedures performed in 13222 patients, overall mortality was reported in 0.06%[50].

Needle track seeding of malignant cells is another important concern, especially in patients who might otherwise benefit from liver transplantation. The reported incidence of tumor seeding following a liver biopsy ranges between 1.6%-5.1%[51-55], however one of the largest series has reported this complication in only 0.76% in their experience[19,56] In a systematic review and meta-analysis of eight observational studies, it was shown that the incidence of needle tract tumour seeding following biopsy of a HCC is 2.7% overall, or 0.9% per year[57]. Other studies have emphasized not to preclude the biopsy if management can be altered based on the biopsy interpretation, supporting this is the fact that 2.5% unnecessary surgery are conducted if the patient is not biopsied[58].

Technical challenges are another section which limits the utility of biopsy. Small lesions which are < 2 cm are often difficult to target, leading to high false-negatives[19]. Distinction of well-differentiated HCC from preneoplastic and regenerative focus is also a problem area. Characteristic histomorphologic profile in combination with reticulin stain and immunohistochemistry for HSP70 and Glutamine synthetase, can differentiate hepatocellular adenoma from HCC, however well-differentiated HCC is often difficult to distinguish[59-61]. Tumor heterogeneity, necrosis and inadequacy or failed sampling of the suspected lesion, all add to the inferior results of biopsy with the risk of mismanagement after diagnostic errors. Negative predictive value is very low in such setting (14%)[51,62,63].

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY FOR THE DIAGNOSIS AND PROGNOSTICATION OF HCC

Tumor histopathology, besides being an important diagnostic tool, plays numerous other important roles such as distinguishing from other lesions, prognostication and influencing treatment decisions, which cannot be substituted by imaging techniques or tumour markers. Assessment of histological parameters in tumour resection specimens has been shown to predict recurrence and metastatic potential and thus indicate the need for salvage transplantation[64-67]. Biopsy tissue and archived blocks are important source for teaching, knowledge sharing, correlation with translational research and biomarker development. Several histopathology parameters had been extensively studied and shown to be significant predictors of prognosis, highlighting the role of tissue analysis in HCC. The most studied parameters which are also linked to prognosis are tumor number, size, cell differentiation and grade, presence of satellite nodules, pTNM stage[68]. (Figure 1)

IMPORTANCE OF GROSS PATHOLOGY DESCRIPTION

HCC is a heterogeneous tumor with varied gross and

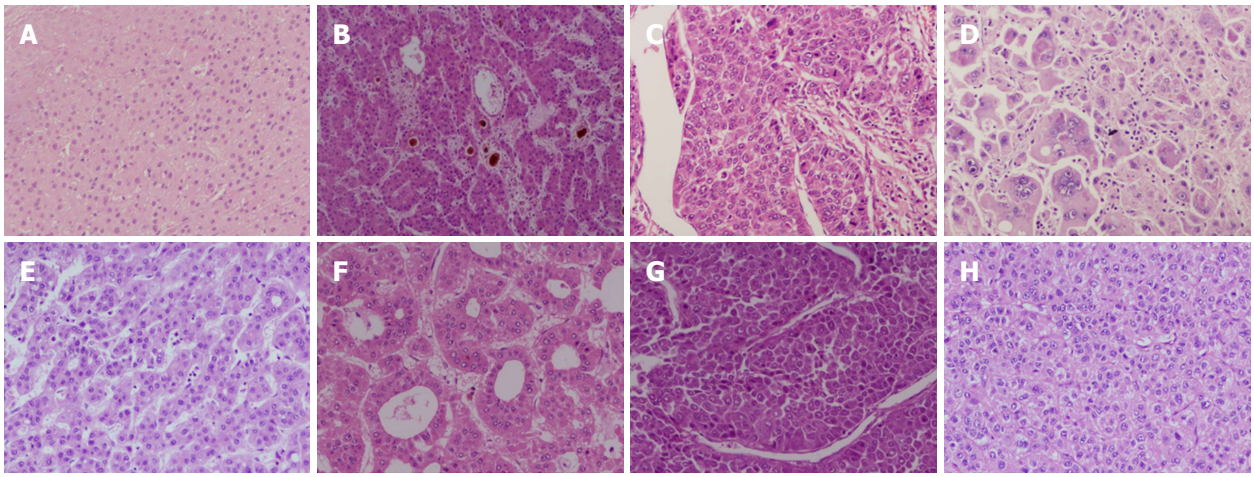


Figure 1 Hepatocellular carcinoma Edmondson and Steiner grading. Grade 1 (A); grade 2 (B); grade 3 (C); and grade 4 (D). Most common patterns in histopathology of hepatocellular carcinoma: Microtrabecular (E); pseudoglandular (F); macrotrabecular (G); and compact (H). (HE stain).

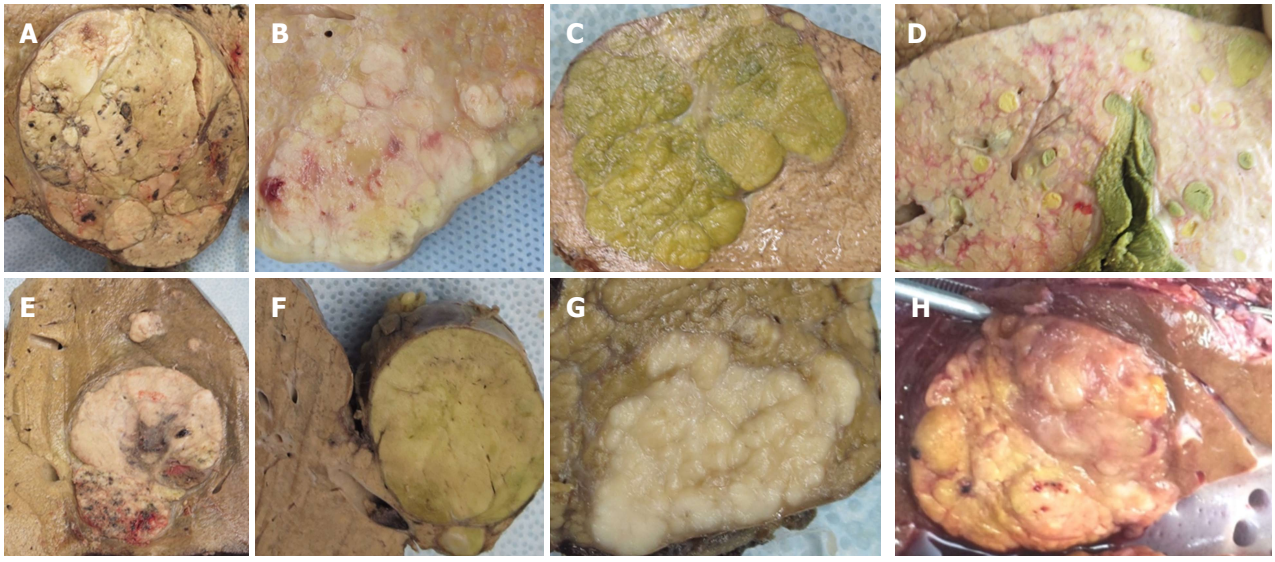


Figure 2 Gross morphology of hepatocellular carcinoma. Single expanding nodular hepatocellular carcinoma (A); vaguely nodular with perinodular extension (B); Multinodular (C); multicentric with cirrhotomimetic appearance (D); nodular with satellite nodules (E); pedunculated (F); infiltrative (G); and hepatocellular carcinoma in non-cirrhotic background (H).

microscopic appearances. A study from Seoul had shown that gross features are also independent predictor of overall and disease-free survival regardless of tumor size. They classified 242 HCC resection specimens based on the gross appearance into vaguely nodular, expanding nodular, multinodular confluent, nodular with perinodular extension and infiltrative types[5]. Infiltrative type had the worse prognosis whereas vaguely nodular and expanding nodular had more favorable prognosis. Similar results in small HCC and in HCC > 10 cm[69,70] and in patients treated with RFA[5,71], verify that gross morphology is an important predictor of prognosis. (Figure 2)

ROLE OF MORPHOLOGICAL PARAMETERS AND HISTOLOGICAL SUBTYPES

Four major growth patterns in HCC are trabecular (70%), solid (20%), pseudo glandular (10%) and macrotrabecular (1%)[72]. Of these, macrotrabecular pattern has recently been shown to have significant clinical relevance[73,74]. Morphology based segregation into histological subtypes[72,75] such as fibro lamellar carcinoma[76,77], lymphoepithelioma like carcinoma[78], steatohepatitis HCC[79], combined hepatocholangio-carcinoma[75], and histological subtype with stem cell markers[80,81] have independent prognostic importance. Others such as Clear cell HCC has been demonstrated to be smaller, better differentiated with lower rates of vascular invasion[82,83], whereas the sarcomatoid HCC is a poorly differentiated subtype[72]. Several recent studies have also highlighted distinctive clinical, biological and molecular characteristics associated with these phenotypes and subtypes, with creditable prognostic implications[84,85] (Figure 3).

In a recent study by Ziol *et al*[74] Macrotrabecular-massive is a newly described subtype of HCC, found in

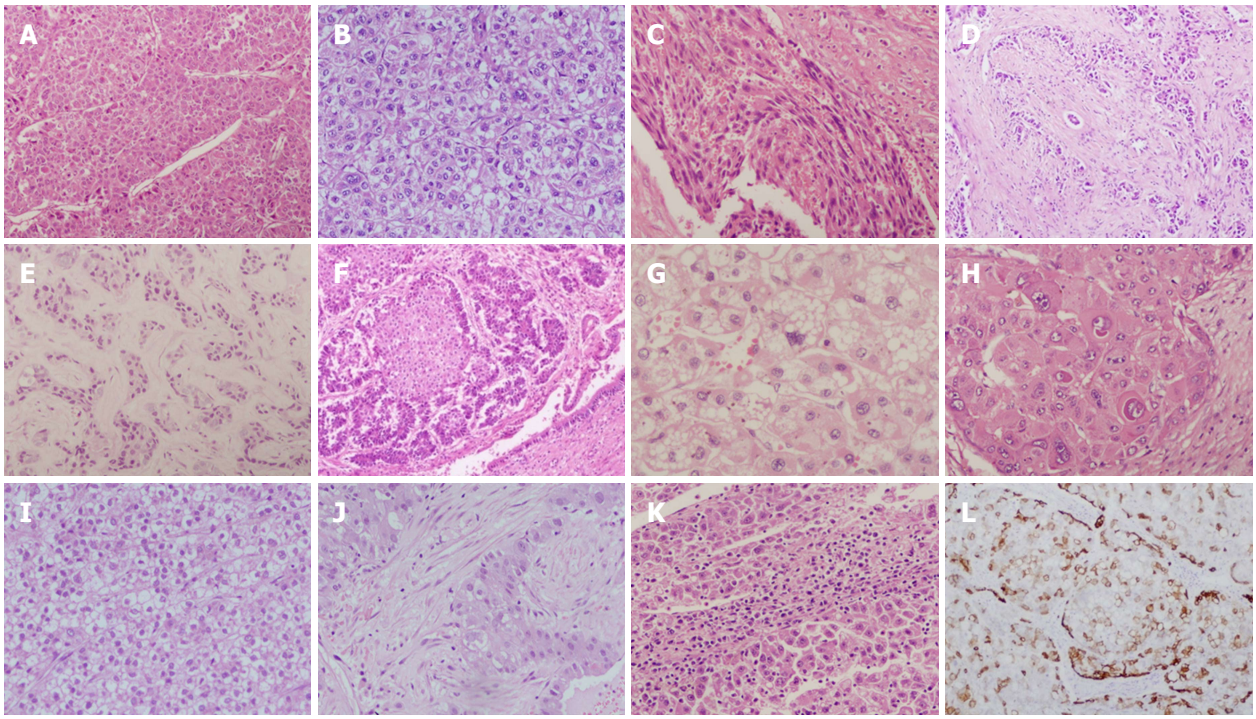


Figure 3 Hepatocellular carcinoma variants, subtypes and histological features. Macrotrabecular (A); steatohepatitic (B); sarcomatoid (C); cholangiocellular (D); sclerosing (E); combined HCC-CC (F); HCC with foam cells (G); HCC with giant cells and hyaline bodies (H); clear cell (I); fibrolamellar (J); HCC with immune cells (K); CK19 positive stem cells (L). HCC: Hepatocellular carcinoma.

12% of the cohort of 237 HCC surgical samples and 284 HCC liver biopsies. Authors have defined this entity by the presence of a predominant (> 50%) macrotrabecular architecture (more than 6 cells thick). This phenotype was associated with poor prognostic factors like tumor size, α-Feto protein (AFP) level, satellite nodules, and vascular invasion and was found to be an independent predictor of early and overall recurrence[74]. Lauwers *et al*[86] noted macrotrabecular -predominant architecture in 26.6% of their 425 cases of resected HCC. In comparison to the compact architecture they had worse overall survival. Prognostic information derived from phenotypes reemphasizes the potential benefits of biopsy add to reviving its importance[74].

Combined hepatocellular-cholangiocarcinoma (cHCC-CC) representing 0.4%-14.2% of primary liver cancers is an aggressive tumor associated with poor outcome[34,87-90]. Histopathological evaluation is crucial for diagnosis, as this entity lacks the typical imaging characteristics thus often misdiagnosed by radiology. cHCC-CC was first described by Allen and Lisa in 1949[91] was updated by Goodman in 1985[92]. In 2010, WHO classified it into classical subtype and subtypes with stem cell features[92]. In a study of sixty-two patients of cHCC-CC, stem cell subtypes (WHO criteria), were found in various amount and combinations in all of their patients with typical subtype in 16%, intermediate cell type in 83.9% and cholangiolocellular type (CLC) in 71%[93]. cHCC-CC has been shown in a study, to have 1-year and 3-year survival rates of 81.9% and 47% respectively, which suggest a better prognosis than CC but worse compared with HCC[88]. Similarly, another study has found 1, 3 and 5 year overall survival rates of 53%, 26% and 12% respectively, supporting their biological behavior intermediate between HCC and CC[90,94]. Knowledge of mixed tumor by biopsy evaluation prior to surgery, can guide the type of resection including the lymph node dissection. Recently, consensus terminology for primary liver carcinomas with both hepatocytic and cholangiocytic differentiation has been published[8] which emphasizes that stem cell phenotypes and features can coexist within combined HCC-CC and should be reported in a descriptive report. Sub classifying stem cells is not necessary. Presence of two other types of primary liver cancers - CLC and intermediate cell carcinoma were described, which may coexist with HCC, intrahepatic cholangiocarcinoma or cHCC-CC. Although the minimum cut-off of HCC and CC to qualify for the diagnosis of cHCC-CC is uncertain, the accepted criteria to qualify as CLC is > 80% of the tumor comprising CLC[8]. This phenotype is very important, with prognostic implications[93]. CLC exhibits increased expression of ABC transporters and is linked with a worse prognosis, chemo resistance, and an aggressive behavior[95]. Meta-analyses of twelve articles involving 1344 patients showed that the presence of cancer stem cells (CSCs) was significantly associated with a poor histological grade[96]. HCC expressing stem cells marker keratin 19 (K19), also known as HCC with "stem cell features" or "progenitor features" display immunohistochemical expression of K19 in > 5% of tumor cells[97,98]. This particular tumor subtype gene expression profile with oval cells and fetal

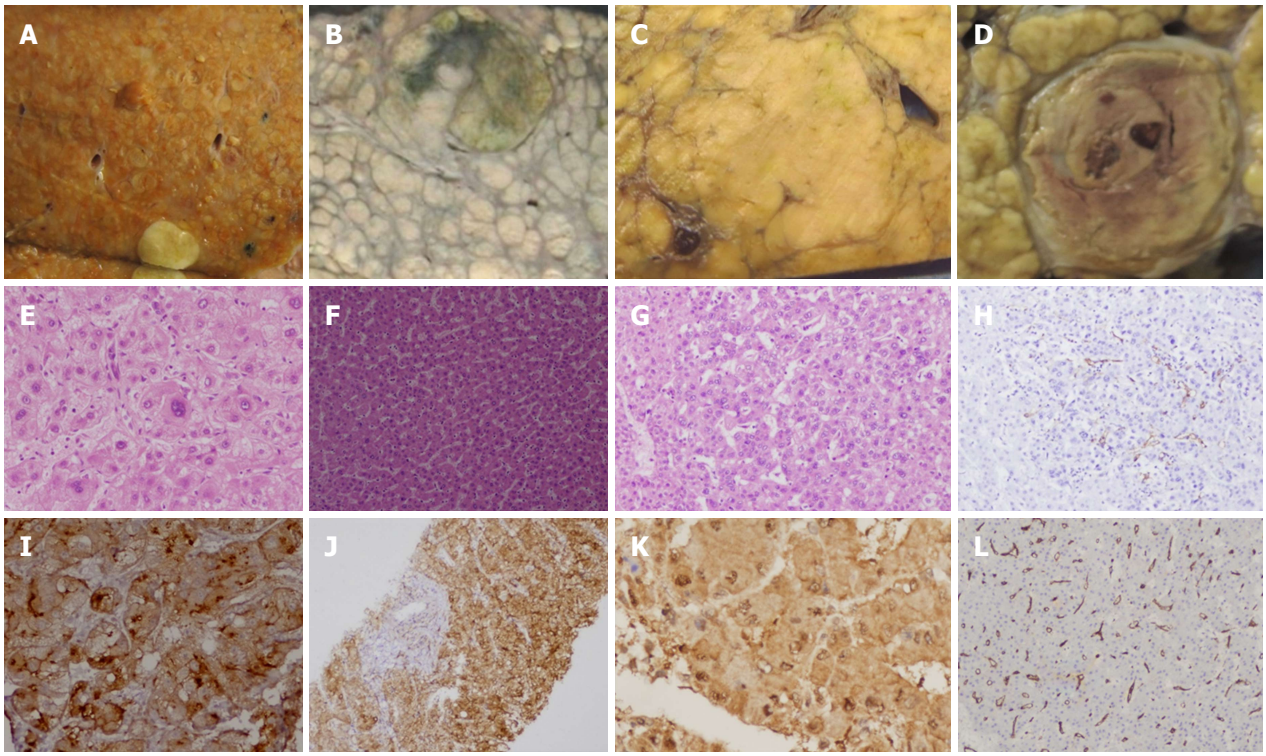


Figure 4 Dysplastic lesions and early hepatocellular carcinoma. Gross morphology of small distinctly nodular HCC (A); small vaguely nodular HCC (B, C); HCC with nodule in nodule appearance (D). Microphotographs of large cell change (E) and small cell change (F) in dysplastic nodules. Nodule in nodule with low grade dysplasia surrounding central high grade dysplastic nodule (G) on HE stain and focal CD34 positive (H) on immunohistochemistry. Glypican-3 (I), glutamine synthetase (J), HSP-70 (K) and diffuse CD34 (L) immunostaining in well-differentiated HCC. HCC: Hepatocellular carcinoma.

hepatoblasts[97-99]. It has adverse clinical outcome with worse postoperative survival rate.

ROLE OF TISSUE ANALYSIS FOR SMALL HCC

Small suspicious nodules are difficult to detect and characterize by the imaging modalities. Vascular characteristics and capsule formation are not completely developed hence such lesions lack the typical imaging characteristics. Histopathology evaluation is required in around 60% of such cases, for early and correct diagnosis[24]. Hepatocellular nodules were classified by the International working party in 1995[100] and further characterized by the International consensus group for hepatocellular neoplasia in 2009[101], which classified small HCC (< 2 cm in size) as early HCC and progressed HCC. (Figure 4)

Pathology based differentiation of HCC from other nodular lesion found in chronic liver disease such as large regenerative nodule, low grade dysplastic nodule (LGDN) and high grade dysplastic nodule (HGDN) has become one of the most important accomplishments and indications of biopsy, gaining importance in view of surveillance programs and early detection of HCC. Biopsy is often recommended for nodules 1.0 cm or larger to make a differential diagnosis between early HCC and a DN[102]. Immunohistochemsitry provides crucial support in such diagnostic dilemmas. Glypican-3, heat shock protein 70 and glutamine synthetase are used as single panel for delineation of HCC from other suspicious nodules[72]. LGDNs and large regenerative nodules (LRNs) are negative for the panel. In a study, all negative phenotype was noted in 100% of LRNs, 100% of LGDNs, 72.7% of HGDNs and 3.1% of early HCC[103-105]. Studies have shown that if two of these three stains are positive, the sensitivity and specificity for the detection of HCC is 60%-70% and 100% respectively[10]. Tommaso *et al*[104], has reported that addition of clathrin heavy chain to this panel, improved diagnostic accuracy to 84.3% for small HCCs. Hepatocyte paraffin 1, arginase 1, polyclonal CEA, CD10 and several other tissue markers that are frequently performed for confirmation of diagnosis, differentiation of HCC from metastatic carcinoma, identifying HCC in poorly differentiated and necrotic tumors as well as a protocol tissue based diagnosis confirmation before enrolment in phase Ⅲ clinical trials of novel drugs[68,106,107].

BIOPSY FOR PROGNOSTICATION

Grading of HCC cellular differentiation, pathological tumor node metastasis stage and vessel invasion are reported as the most important histoprognostic features[22,72,108].

Edmondson and Steiner system of HCC (ES) grading, published in Cancer in 1954[109], is the most widely adopted classification[86]. It divides HCC into four grades based on histological differentiation with grade 1 being

the best differentiated. Current three-tier classification of HCC into well-, moderately- and poorly differentiated HCC is also based on this system, combining architectural and nuclear features[110,111], pTNM staging based on 8th edition of AJCC staging, which is based on number, size and vessel invasion, provides crucial prognostic information, guides about therapeutic decisions and is an essential component of research and clinical studies. 8th ed. of pTNM lays emphasizes on separation of small HCC and on vessel invasion by modifying pT1 and pT4 respectively[112].

Macro vascular and Micro vascular (MVI) invasion is the major predictor of prognosis of HCC and are associated with more advanced tumor stage, disease progression, local invasion and distant metastasis[86,113]. Identification of MVI is feasible only on histopathological examination of resected surgical specimens[114]. Incidence of MVI after surgical resections and liver transplantation has been shown to be between 15% and 57%[114-116]. Pawlik *et al*[115], reported MVI to occur at the rates of 25%, 40%, 55% and 63% in HCC < 3, 3-5, 5-6.5, and > 6.5 cm. Similarly, Yamashita *et al*[117] has also shown 28.9% of the small HCC has MVI, with 1-year recurrence rates of 7.5% and 23.3% for patients without and with MVI. A systematic review of 20 observation studies investigating prognostic role of MVI in patients who had undergone liver transplantation or resection, highlight the adverse impact of MVI on disease free and overall survival[118]. Different studies have graded MVI based on the number of vessels invaded[119] as well as have sub typed them into adhesion, invasion and breakthrough types and found association with long-term survival[120]. Distance of embolized vessel from the main tumor has prognostic significance with 1 cm cut-off shown in studies to predict very poor outcome[121]. MVI detection helps to identify patients at risk of development of distant metastasis post-resection and guides for the need of adjuvant therapy[114]. There is an urgent need for prediction of MVI in biopsies to guide therapeutic strategy. However, currently neither the detection of MVI in biopsy is possible nor any validated surrogate markers of MVI are available[122]. A recent pilot study assessed the performance of IHC panel of three biomarkers of MVI (H4K16ac, H4K20me2, PIVKA-Ⅱ) in a test set of 64 HCC surgical specimens and 42 core needle biopsies of HCC. In this study combination of PIVKA-Ⅱ with H4K20me2 showed the best accuracy for prediction of mVI, with very high specificity and PPV in HCC core needle biopsies[123].

Several studies have highlighted the prognostic relevance of histological grade, stage and MVI[124-126]. Analysis of eighteen registries comprising 570 transplanted patients revealed MVI in 16% and poor differentiation of tumor in 12%. These features were significant risk factors for dismal cancer specific survival[127]. Similar, prognostic significance of MVI and grade 3, in predicting overall survival, disease free survival and recurrence, had been found in 151 patients transplanted for HCC, by Donat *et al*[124] and by Lauwers *et al*[86] in their 425 HCC resections. In a study of 116 patients of large HCC resection, MVI and ES grade were reported as predictors of early recurrence[128]. Poor differentiation in a single HCC or in the largest HCC in a pre-transplant biopsy indicates aggressive tumour biology associated with poor prognosis. Certain centers exclude transplantation and assign patients to other treatment modalities, based on such prognosis and management related effects of biopsy[129,130].

ROLE OF IMMUNOHISTOCHEMISTRY IN PREDICTING PROGNOSIS

Several biomarkers have been investigated in HCC for prognostication and treatment decisions. K19 expressing HCCs show aggressive behavior, poor differentiation, high proliferative index and high recurrence rate[131-133]. Similarly, expression of stem cell marker CD133 is associated with higher tumor grade, advanced disease stage, higher recurrence rates and shorter overall survival[134]. EpCAM expression also demonstrates poor prognosis[5,134]. In a recent study, progressed HCC with > 5% tumor fraction expressing stem/progenitor cell markers CK19, EpCAM and CD133, displayed more aggressive behavior with increased likelihood of recurrence, chemo-resistance and metastasis[135].

Tumor tissue expression of high Wnt-1 shows correlation with nuclear B-catenin accumulation and increased rate of tumor recurrence. In a study of 142 patients of HCC, correlation of 3 groups: Biliary/stem cell marker positive group, Wnt/B-catenin signaling related marker positive, and both negative group was done with other prognostic features. Biliary/stem cell marker positive group demonstrated poor tumor differentiation, high frequency of portal vein invasion, intrahepatic metastasis and high proliferative activity[132].

HCC are hyper vascular and tumor angiogenesis is a known important prognostic factor. Immunohistochemical staining for endothelium specific markers CD31, CD34 or vWF allow semi quantitative assessment of micro vessel density, which is a significant prognostic indicator[136,68]. Li *et al*[137] performed meta-analysis of 12 articles comprising a total 1138 HCC patients. Survival outcomes showed positive correlation between poor prognosis and high micro vessel density levels.

Molecular markers with prognostic significance are analyzed by tissue markers and abundant literature is available on studies of phenotypic correlates. DNA ploidy, cell proliferation markers, Cell surface proteins Glypican-3, cytoskeleton proteins Fascin, enzymes Histone deacetylase, transcription factors BATF2, tumor suppressor genes TP53, adhesion molecule E-cadherin, cell cycle regulators, oncogenes, tumor angiogenesis related markers and several others related to Notch, Hippo, Hedgehog and other signaling pathways, have been analyzed in tissues and shown to be of prognostic significance[103,138].

IHC markers are also increasingly being used for the decisions of molecular targeted therapy and as a

predictor of therapeutic response[22,139]. Six areas of genomic alterations which have targetable potential for HCC are TERT promoter mutation, p53 pathway, oxidative stress pathway, Wnt-B catenin pathway, epigenetic, AKT/mTOR and MAP kinase pathway[22]. Evaluation of c-MET at the tissue level had been related to response to Tivantinib[106,140,141] with several such phase Ⅱ and phase Ⅲ ongoing trials are linked to molecular analysis of tissue samples.

Immune biomarkers such as immune checkpoint-inhibiting antibodies anti-PD-1, anti-PD-L1 and anti-CTLA-4 are useful for decisions regarding adjuvant therapy[142-144]. PD-L1 can predict response to anti-PD-1 antibody. A recent study assessed 294 HCC samples for the expression of PD-1, PD-L1 and CTLA-4. High PD-L1 staining was associated with poor disease-free survival and simultaneous increased expression of PD-L1 and CD68+ TIL was reported to be an important prognostic factor related to immune checkpoint pathway in HCC[145,146]. Advances related to determination and response assessment to the targeted therapies are dependent on archived tissues in institutions and biobanks. This further highlights the revival of importance of tissue procurement and analysis. Phase Ⅲ studies of novel targeted therapies require biopsy confirmation of diagnosis, and assessment of treatment response as in a study of 30 patients of HCC treated by Radiofrequency ablation (RFA) and check point inhibitor[147].

MORPHOMOLECULAR CLASSIFICATIONS: REINCARNATION OF TISSUE ANALYSIS IN HCC

Histopathological features can predict prognosis and lately, linkage of prognosis to distinct biological phenotypes has been demonstrated[84,85]. HCC phenotype may be associated with activation of specific oncogenic pathways thus histopathological parameters and molecular markers acting in concert can be very useful prognostic predictors[85]. HCC phenotypes are associated with distinct molecular pathways and such association is recently being the focus point due to the tremendous scope for advancement in HCC therapy. Transcriptomic classifications by Tan *et al*[84] and Calderaro *et al*[85] deserve special mention in this context.

In the study by Calderaro *et al*[85], CTNNB1 and TP53 defined two distinct tumor phenotypes in a large series of 343 cases of surgically resected HCC. Using pathology, immunohistochemistry, gene expression profiling and sequencing; patho-molecular correlates were classified into G1-G6 based on transcriptomics. Tumors associated with CTNNB1 mutations were large, well-differentiated, cholestatic, with microtrabecular/pseudo glandular pattern whereas TP53 mutated tumors were poorly differentiated, with multinucleated, pleomorphic cells arranged in compact pattern and displayed frequent vascular invasion. G1-G3 subclasses demonstrated correlation with histological features of poor differentiation, frequent macro vascular invasion, foci of clear cells, sarcomatous change, compact and macrotrabecular pattern, and foci of pleomorphic and multinucleated cells. Whereas G4-G6 subclasses, revealed low cell proliferation, association with small tumor size, lack of satellite nodules or micro vascular invasion, and tumors were well-differentiated.

In the study by Tan *et al*[84], 96 tumor tissues were used for the development of clinic pathological indices predictive of HCC molecular subclass. HCC transcriptome had been characterized into 3 subtypes S1-S3[148], determined by genome-wide transcriptome profiling, with potential therapeutic targets. S1 reflected aberrant activation of the WNT signaling pathway, S2 was characterized by proliferation as well as MYC and AKT activation, and S3 was associated with hepatocyte differentiation[46,148]. Predictive indices in the study by Tan *et al*[84] were validated in 99 HCC tumors. Phenotypic molecular correlation with S1-S3 based on transcriptomic analysis, revealed steatohepatitic HCC and immune cell infiltrates represented S1, macrotrabecular or compact pattern, lack of pseudo glands belonged to S2 and microtrabecular low histological grade and lack of steatohepatitis and clear cell patterns constituted S3 subclass. Macrotrabecular pattern/S2 showed activation of therapeutically targetable oncogene YAP and stemness markers EPCAM, keratin 19[84].

Similar histomorphology correlates of molecular characteristics were reported in scirrhous subtype and TSC1/TSC2 mutations and steatohepatitis subtype with IL-6/JAK/ STAT activation[85].

SUMMARY: RENEWED INTEREST IN BIOPSY WITH ROLE REDEFINED

Histopathological evaluation of HCC tissue has time tested applications in the diagnosis and prognosis (Table 1). Pathology combined with immunohistochemistry is essential for the differentiation of HCC from preneoplastic lesions, from metastatic diseases and other primary liver tumors. Certain biopsy limitations especially needle tract seeding, sampling errors and small risk of morbidity along with the technical advancements in imaging, undermined the importance of tissue analysis. This led most of the international guidelines on HCC to restrict the role of liver biopsy to characterize the lesions in non-cirrhotic liver or those with equivocal imaging. Other advantages of biopsy including the role in prognostication, therapy decision, research and clinical trials along with teaching and archiving in biobank, all suffered indirectly due to lesser availability of tissue specimens. With increasingly accumulating data on prognostic and therapeutic importance of specific phenotypes and distinct molecular-morphologic correlates, there is resurgence of interest in the role of tissue evaluation. Analysis of tumor biopsies allows clinicians to fine tune therapies. Molecular subclasses with phenotypic surrogates will be valuable for predicting response to specific targeted therapies

Table 1 Role of tumour tissue analysis in hepatocellular carcinoma

Diagnostic	Prognostic/theragnostic
Distinguishing HCC from metastasis	Prognostic histomorpholic parameters-tumour grade, vessel invasion, pTNM stage
Distinguishing benign/preneoplastic lesions from HCC	Identification of Histological variants of prognostic importance
Diagnosis confirmation of small HCC	Tissue biomarkers-for prognostication
Diagnosis of liver nodules in non-cirrhotic background	Tissue biomarkers-for assessing presence of therapeutic targets and drug development
Diagnosis of atypical variants of HCC, which have atypical Imaging findings	Histologic surrogates of clinically relevant molecular signatures-for predicting prognosis
Combined HCC-CC	Histologic surrogates of clinically relevant molecular signatures-as predictors of potential responders for targeted therapies
Diagnosis confirmation of HCC in phase Ⅲ trials of new drugs	

HCC: Hepatocellular carcinoma.

Table 2 Based on the evidence from published literature, algorithm based on the role of tissue diagnosis is inserted

Mandatory	Potentially necessary/helpful	Unwarranted
Lesion in a cirrhotic patient that lacks typical imaging characteristics	Well-differentiated HCC	HCC, if typical features are present on dynamic imaging technique
All nodules developing in the non-cirrhotic background	< 2 cm sized HCC, due to hypovascularity in small HCC and lack of typical imaging findings	AFP levels very high in the absence of other known causative tumours
Combined hepato-cholangiocarcinoma	Predictors of prognosis	Curative resection possible due to risk of needle tract seeding, if biopsied
Atypical variants of HCC	Teaching and biobanking	
Identifying stem cell phenotypes	Distinguishing HCC from regenerative nodule, dysplastic nodule, hepatic adenoma, FNH	
Phase Ⅲ trials of novel drugs	Morphologic surrogates of molecular signatures	
Tissue biomarker development and studies	Surrogate biomarkers of MVI in liver biopsy	

HCC: Hepatocellular carcinoma; MVI: Micro vascular; AFP: α-fetoprotein.

for HCC. HCC morphologic correlates of prognostically important molecular signatures need to be explored further to alleviate the HCC complications of recurrence, intra-hepatic and distant metastasis, responsible for dismal prognosis of HCC and to discover useful biomarkers (Table 2).

CONCLUSION

Histopathological analysis of HCC plays very important role in the diagnosis, prognosis and management decisions. Despite the shortcomings of biopsy and advancements in imaging and molecular characterization of HCC, value of biopsy is unshaken, with several recent facets further empowering tissue analysis.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *Cancer J Clin* 2005; **55**: 74-108 [DOI: 10.3322/canjclin.55.2.74]

2 **Song DS**, Bae SH. Changes of guidelines diagnosing hepatocellular carcinoma during the last ten-year period. *Clin Mol Hepatol* 2012; **18**: 258-267 [PMID: 23091805 DOI: 10.3350/cmh.2012.18.3.258]

3 **Berretta M**, Cavaliere C, Alessandrini L, Stanzione B, Facchini G, Balestreri L, Perin T, Canzonieri V. Serum and tissue markers in hepatocellular carcinoma and cholangiocarcinoma: clinical and prognostic implications. *Oncotarget* 2017; **8**: 14192-14220 [PMID: 28077782 DOI: 10.18632/oncotarget.13929]

4 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

5 **Lee Y**, Park H, Lee H, Cho JY, Yoon YS, Choi YR, Han HS, Jang ES, Kim JW, Jeong SH, Ahn S, Kim H. The Clinicopathological and Prognostic Significance of the Gross Classification of Hepatocellular Carcinoma. *J Pathol Transl Med* 2018; **52**: 85-92 [PMID: 29172395 DOI: 10.4132/jptm.2017.11.13]

6 **Agni RM**. Diagnostic histopathology of hepatocellular carcinoma: A case-based review. *Semin Diagn Pathol* 2017; **34**: 126-137 [PMID: 28143798 DOI: 10.1053/j.semdp.2016.12.008]

7 **Llovet JM**, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016; **2**: 16018 [PMID: 27158749 DOI: 10.1038/nrdp.2016.18]

8 **Brunt E**, Aishima S, Clavien PA, Fowler K, Goodman Z, Gores G, Gouw A, Kagen A, Klimstra D, Komuta M, Kondo F, Miksad R, Nakano M, Nakanuma Y, Ng I, Paradis V, Nyun Park Y, Quaglia A, Roncalli M, Roskams T, Sakamoto M, Saxena R, Sempoux C, Sirlin C, Stueck A, Thung S, Tsui WMS, Wang XW, Wee A, Yano H, Yeh M, Zen Y, Zucman-Rossi J, Theise N. cHCC-CCA: Consensus terminology for primary liver carcinomas with both hepatocytic and cholangiocytic differentation. *Hepatology* 2018; **68**: 113-126 [PMID: 29360137 DOI: 10.1002/hep.29789]

9 **McGlynn KA**, London WT. The global epidemiology of hepatocellular carcinoma: present and future. *Clin Liver Dis* 2011; **15**: 223-243 [PMID: 21689610 DOI: 10.1016/j.cld.2011.03.006]

10 **Pittman ME**, Brunt EM. Anatomic pathology of hepatocellular carcinoma: histopathology using classic and new diagnostic tools. *Clin Liver Dis* 2015; **19**: 239-259 [PMID: 25921661 DOI: 10.1016/j.cld.2015.01.003]

11 **El-Serag HB**. Hepatocellular carcinoma. *N Engl J Med* 2011; **365**: 1118-1127 [PMID: 21992124 DOI: 10.1056/NEJMra1001683]

12 **Ramakrishna G**, Rastogi A, Trehanpati N, Sen B, Khosla R, Sarin SK. From cirrhosis to hepatocellular carcinoma: new molecular insights on inflammation and cellular senescence. *Liver Cancer* 2013; **2**: 367-383 [PMID: 24400224 DOI: 10.1159/000343852]

13 **Khosla R**, Rastogi A, Ramakrishna G, Pamecha V, Mukhopadhyay A, Vasudevan M, Sarin SK, Trehanpati N. EpCAM+ Liver Cancer Stem-Like Cells Exhibiting Autocrine Wnt Signaling Potentially Originate in Cirrhotic Patients. *Stem Cells Transl Med* 2017; **6**: 807-818 [PMID: 28176469 DOI: 10.1002/sctm.16-0248]

14 **Sarin SK**, Thakur V, Guptan RC, Saigal S, Malhotra V, Thyagarajan SP, Das BC. Profile of hepatocellular carcinoma in India: an insight into the possible etiologic associations. *J Gastroenterol Hepatol* 2001; **16**: 666-673 [PMID: 11422620 DOI: 10.1046/j.1440-1746.2001.02476.x]

15 **Blechacz B**, Mishra L. Biopsy for liver cancer: How to balance research needs with evidence-based clinical practice. *Hepatology* 2015; **62**: 1645 [PMID: 25678381 DOI: 10.1002/hep.27746]

16 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

17 **Omata M**, Lesmana LA, Tateishi R, Chen PJ, Lin SM, Yoshida H, Kudo M, Lee JM, Choi BI, Poon RT, Shiina S, Cheng AL, Jia JD, Obi S, Han KH, Jafri W, Chow P, Lim SG, Chawla YK, Budihusodo U, Gani RA, Lesmana CR, Putranto TA, Liaw YF, Sarin SK. Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. *Hepatol Int* 2010; **4**: 439-474 [PMID: 20827404 DOI: 10.1007/s12072-010-9165-7]

18 **European Association for Study of Liver.** European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *Eur J Cancer* 2012; **48**: 599-641 [PMID: 22424278 DOI: 10.1016/j.ejca.2011.12.021]

19 **Gonzalez SA**, Keeffe EB. Diagnosis of hepatocellular carcinoma: role of tumor markers and liver biopsy. *Clin Liver Dis* 2011; **15**: 297-306, vii-vix [PMID: 21689614 DOI: 10.1016/j.cld.2011.03.012]

20 **Canzonieri V**, Alessandrini L, Caggiari L, Perini T, Berretta M, Cannizzaro R, De Re V. Hepatocellular carcinoma: an overview of clinic-pathological and molecular perspectives. *WCRJ* 2015; **2**: e485

21 **Roberts LR**, Sirlin CB, Zaiem F, Almasri J, Prokop LJ, Heimbach JK, Murad MH, Mohammed K. Imaging for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. *Hepatology* 2018; **67**: 401-421 [PMID: 28859233 DOI: 10.1002/hep.29487]

22 **Gerbes A**, Zoulim F, Tilg H, Dufour JF, Bruix J, Paradis V, Salem R, Peck-Radosavljevic M, Galle PR, Greten TF, Nault JC, Avila MA. Gut roundtable meeting paper: selected recent advances in hepatocellular carcinoma. *Gut* 2018; **67**: 380-388 [PMID: 29150490 DOI: 10.1136/gutjnl-2017-315068]

23 **Forner A**, Vilana R, Ayuso C, Bianchi L, Solé M, Ayuso JR, Boix L, Sala M, Varela M, Llovet JM, Brú C, Bruix J. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: Prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. *Hepatology* 2008; **47**: 97-104 [PMID: 18069697 DOI: 10.1002/hep.21966]

24 **Horigome H**, Nomura T, Saso K, Itoh M, Joh T, Ohara H. Limitations of imaging diagnosis for small hepatocellular carcinoma: comparison with histological findings. *J Gastroenterol Hepatol* 1999; **14**: 559-565 [PMID: 10385065 DOI: 10.1046/j.1440-1746.1999.01915.x]

25 **Peterson MS**, Baron RL, Marsh JW Jr, Oliver JH 3rd, Confer SR, Hunt LE. Pretransplantation surveillance for possible hepatocellular carcinoma in patients with cirrhosis: epidemiology and CT-based tumor detection rate in 430 cases with surgical pathologic correlation. *Radiology* 2000; **217**: 743-749 [PMID: 11110938 DOI: 10.1148/radiology.217.3.r00dc28743]

26 **Krinsky GA**, Lee VS, Theise ND, Weinreb JC, Morgan GR, Diflo T, John D, Teperman LW, Goldenberg AS. Transplantation for hepatocellular carcinoma and cirrhosis: sensitivity of magnetic resonance imaging. *Liver Transpl* 2002; **8**: 1156-1164 [PMID: 12474156 DOI: 10.1053/jlts.2002.35670]

27 **Zacherl J**, Pokieser P, Wrba F, Scheuba C, Prokesch R, Zacherl M, Längle F, Berlakovich GA, Mühlbacher F, Steininger R. Accuracy of multiphasic helical computed tomography and intraoperative sonography in patients undergoing orthotopic liver transplantation for hepatoma: what is the truth? *Ann Surg* 2002; **235**: 528-532 [PMID: 11923609 DOI: 10.1097/00000658-200204000-00011]

28 **Bhartia B**, Ward J, Guthrie JA, Robinson PJ. Hepatocellular carcinoma in cirrhotic livers: double-contrast thin-section MR imaging with pathologic correlation of explanted tissue. *AJR Am J Roentgenol* 2003; **180**: 577-584 [PMID: 12591657 DOI: 10.2214/ajr.180.3.1800577]

29 **Noguchi Y**, Murakami T, Kim T, Hori M, Osuga K, Kawata S, Kumano S, Okada A, Sugiura T, Nakamura H. Detection of hepatocellular carcinoma: comparison of dynamic MR imaging with dynamic double arterial phase helical CT. *AJR Am J Roentgenol* 2003; **180**: 455-460 [PMID: 12540451 DOI: 10.2214/ajr.180.2.1800455]

30 **Bialecki ES**, Ezenekwe AM, Brunt EM, Collins BT, Ponder TB, Bieneman BK, Di Bisceglie AM. Comparison of liver biopsy and noninvasive methods for diagnosis of hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4**: 361-368 [PMID: 16527701 DOI: 10.1016/S1542-3565(05)00977-8]

31 **Hayashi PH**, Trotter JF, Forman L, Kugelmas M, Steinberg T, Russ P, Wachs M, Bak T, Kam I, Everson GT. Impact of pretransplant diagnosis of hepatocellular carcinoma on cadveric liver allocation in the era of MELD. *Liver Transpl* 2004; **10**: 42-48 [PMID: 14755776 DOI: 10.1002/lt.20020]

32 **Schölmerich J**, Schacherer D. Diagnostic biopsy for hepatocellular carcinoma in cirrhosis: useful, necessary, dangerous, or academic sport? *Gut* 2004; **53**: 1224-1226 [PMID: 15306574 DOI: 10.1136/gut.2004.040816]

33 **Tanaka Y**, Sasaki Y, Katayama K, Hiramatsu N, Ito A, Murata H, Enomoto N, Oshita M, Mochizuki K, Tsujii M, Tsuji S, Kasahara A, Tomoda K, Nakamura H, Hayashi N, Hori M. Probability of hepatocellular carcinoma of small hepatocellular nodules undetectable by computed tomography during arterial portography. *Hepatology* 2000; **31**: 890-898 [PMID: 10733545 DOI: 10.1053/he.2000.5979]

34 **Wang AQ**, Zheng YC, Du J, Zhu CP, Huang HC, Wang SS, Wu LC, Wan XS, Zhang HH, Miao RY, Sang XT, Zhao HT. Combined hepatocellular cholangiocarcinoma: Controversies to be addressed. *World J Gastroenterol* 2016; **22**: 4459-4465 [PMID: 27182157 DOI: 10.3748/wjg.v22.i18.4459]

35 **Korean Liver Cancer Study Group and National Cancer Center, Korea**. Practice guidelines for management of hepatocellular carcinoma 2009. *Korean J Hepatol* 2009; **15**: 391-423 [PMID: 19783891 DOI: 10.3350/kjhep.2009.15.3.391]

36 **Jang HJ**, Kim TK, Burns PN, Wilson SR. Enhancement patterns of hepatocellular carcinoma at contrast-enhanced US: comparison with histologic differentiation. *Radiology* 2007; **244**: 898-906 [PMID: 17709836 DOI: 10.1148/radiol.2443061520]

37 **Demirjian A**, Peng P, Geschwind JF, Cosgrove D, Schutz J, Kamel IR, Pawlik TM. Infiltrating hepatocellular carcinoma: seeing the tree through the forest. *J Gastrointest Surg* 2011; **15**: 2089-2097 [PMID: 21725699 DOI: 10.1007/s11605-011-1614-7]

38 **Myung SJ**, Yoon JH, Kim KM, Gwak GY, Kim YJ, Yu JW, Chung JW, Lee HS. Diffuse infiltrative hepatocellular carcinomas in a hepatitis B-endemic area: diagnostic and therapeutic impediments. *Hepatogastroenterology* 2006; **53**: 266-270 [PMID: 16608037]

39 **Farinati F**, Marino D, De Giorgio M, Baldan A, Cantarini M, Cursaro C, Rapaccini G, Del Poggio P, Di Nolfo MA, Benvegnù L, Zoli M, Borzio F, Bernardi M, Trevisani F. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? *Am J Gastroenterol* 2006; **101**: 524-532 [PMID: 16542289 DOI: 10.1111/j.1572-0241.2006.00443.x]

40 **Vibert E**, Azoulay D, Hoti E, Iacopinelli S, Samuel D, Salloum C, Lemoine A, Bismuth H, Castaing D, Adam R. Progression of alphafetoprotein before liver transplantation for hepatocellular carcinoma in cirrhotic patients: a critical factor. *Am J Transplant* 2010; **10**: 129-137 [PMID: 20070666 DOI: 10.1111/

j.1600-6143.2009.02750.x]
41 **Toyoda H**, Kumada T, Tada T, Sone Y, Kaneoka Y, Maeda A. Tumor Markers for Hepatocellular Carcinoma: Simple and Significant Predictors of Outcome in Patients with HCC. *Liver Cancer* 2015; **4**: 126-136 [PMID: 26020034 DOI: 10.1159/000367735]
42 **Cresswell AB**, Welsh FK, Rees M. A diagnostic paradigm for resectable liver lesions: to biopsy or not to biopsy? *HPB* (Oxford) 2009; **11**: 533-540 [PMID: 20495704 DOI: 10.1111/j.1477-2574.2009.00081.x]
43 **Gilmore IT**, Burroughs A, Murray-Lyon IM, Williams R, Jenkins D, Hopkins A. Indications, methods, and outcomes of percutaneous liver biopsy in England and Wales: an audit by the British Society of Gastroenterology and the Royal College of Physicians of London. *Gut* 1995; **36**: 437-441 [PMID: 7698705 DOI: 10.1136/gut.36.3.437]
44 **Perrault J**, McGill DB, Ott BJ, Taylor WF. Liver biopsy: complications in 1000 inpatients and outpatients. *Gastroenterology* 1978; **74**: 103-106 [PMID: 618417]
45 **Knauer CM**. Percutaneous biopsy of the liver as a procedure for outpatients. *Gastroenterology* 1978; **74**: 101-102 [PMID: 618416]
46 **Kleiner DE**. Hepatocellular carcinoma: Liver biopsy in the balance. *Hepatology* 2018; **68**: 13-15 [PMID: 29405373 DOI: 10.1002/hep.29831]
47 **Rockey DC**, Caldwell SH, Goodman ZD, Nelson RC, Smith AD; American Association for the Study of Liver Diseases. Liver biopsy. *Hepatology* 2009; **49**: 1017-1044 [PMID: 19243014 DOI: 10.1002/hep.22742]
48 **McGill DB**, Rakela J, Zinsmeister AR, Ott BJ. A 21-year experience with major hemorrhage after percutaneous liver biopsy. *Gastroenterology* 1990; **99**: 1396-1400 [PMID: 2101588 DOI: 10.1016/0016-5085(90)91167-5]
49 **Kalambokis G**, Manousou P, Vibhakorn S, Marelli L, Cholongitas E, Senzolo M, Patch D, Burroughs AK. Transjugular liver biopsy--indications, adequacy, quality of specimens, and complications--a systematic review. *J Hepatol* 2007; **47**: 284-294 [PMID: 17561303 DOI: 10.1016/j.jhep.2007.05.001]
50 **Giorgio A**, Tarantino L, de Stefano G, Francica G, Esposito F, Perrotta A, Aloisio V, Farella N, Mariniello N, Coppola C, Caturelli E. Complications after interventional sonography of focal liver lesions: a 22-year single-center experience. *J Ultrasound Med* 2003; **22**: 193-205 [PMID: 12562124 DOI: 10.7863/jum.2003.22.2.193]
51 **Durand F**, Regimbeau JM, Belghiti J, Sauvanet A, Vilgrain V, Terris B, Moutardier V, Farges O, Valla D. Assessment of the benefits and risks of percutaneous biopsy before surgical resection of hepatocellular carcinoma. *J Hepatol* 2001; **35**: 254-258 [PMID: 11580148 DOI: 10.1016/S0168-8278(01)00108-8]
52 **Kim SH**, Lim HK, Lee WJ, Cho JM, Jang HJ. Needle-tract implantation in hepatocellular carcinoma: frequency and CT findings after biopsy with a 19.5-gauge automated biopsy gun. *Abdom Imaging* 2000; **25**: 246-250 [PMID: 10823443 DOI: 10.1007/s002610000025]
53 **Kosugi C**, Furuse J, Ishii H, Maru Y, Yoshino M, Kinoshita T, Konishi M, Nakagohri T, Inoue K, Oda T. Needle tract implantation of hepatocellular carcinoma and pancreatic carcinoma after ultrasound-guided percutaneous puncture: clinical and pathologic characteristics and the treatment of needle tract implantation. *World J Surg* 2004; **28**: 29-32 [PMID: 14648043 DOI: 10.1007/s00268-003-7003-y]
54 **Liu YW**, Chen CL, Chen YS, Wang CC, Wang SH, Lin CC. Needle tract implantation of hepatocellular carcinoma after fine needle biopsy. *Dig Dis Sci* 2007; **52**: 228-231 [PMID: 17151805 DOI: 10.1007/s10620-006-9354-3]
55 **Takamori R**, Wong LL, Dang C, Wong L. Needle-tract implantation from hepatocellular cancer: is needle biopsy of the liver always necessary? *Liver Transpl* 2000; **6**: 67-72 [PMID: 10648580 DOI: 10.1002/lt.500060103]
56 **Chang S**, Kim SH, Lim HK, Lee WJ, Choi D, Lim JH. Needle tract implantation after sonographically guided percutaneous biopsy of hepatocellular carcinoma: evaluation of doubling time, frequency, and features on CT. *AJR Am J Roentgenol* 2005; **185**: 400-405 [PMID: 16037512 DOI: 10.2214/ajr.185.2.01850400]
57 **Silva MA**, Hegab B, Hyde C, Guo B, Buckels JA, Mirza DF. Needle track seeding following biopsy of liver lesions in the diagnosis of hepatocellular cancer: a systematic review and meta-analysis. *Gut* 2008; **57**: 1592-1596 [PMID: 18669577 DOI: 10.1136/gut.2008.149062]
58 **Torzilli G**, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, Ohtomo K, Makuuchi M. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. *Hepatology* 1999; **30**: 889-893 [PMID: 10498639 DOI: 10.1002/hep.510300411]
59 **Roncalli M**, Sciarra A, Tommaso LD. Benign hepatocellular nodules of healthy liver: focal nodular hyperplasia and hepatocellular adenoma. *Clin Mol Hepatol* 2016; **22**: 199-211 [PMID: 27189732 DOI: 10.3350/cmh.2016.0101]
60 **Nguyen TB**, Roncalli M, Di Tommaso L, Kakar S. Combined use of heat-shock protein 70 and glutamine synthetase is useful in the distinction of typical hepatocellular adenoma from atypical hepatocellular neoplasms and well-differentiated hepatocellular carcinoma. *Mod Pathol* 2016; **29**: 283-292 [PMID: 26769138 DOI: 10.1038/modpathol.2015.162]
61 **Lagana SM**, Salomao M, Bao F, Moreira RK, Lefkowitch JH, Remotti HE. Utility of an immunohistochemical panel consisting of glypican-3, heat-shock protein-70, and glutamine synthetase in the distinction of low-grade hepatocellular carcinoma from hepatocellular adenoma. *Appl Immunohistochem Mol Morphol* 2013; **21**: 170-176 [PMID: 22914605 DOI: 10.1097/PAI.0b013e31825d527f]
62 **Daneo V**, Robecchi A Jr, Secondo G. [Monoarthritis of the knee, sacroileitis and ulcero-hemorrhagic colitis. Chronicity of the arthropathy and appearance of the rheumatoid factor in serum]. *Reumatismo* 1967; **19**: 340-345 [PMID: 5306600]
63 **Durand F**, Belghiti J, Paradis V. Liver transplantation for hepatocellular carcinoma: role of biopsy. *Liver Transpl* 2007; **13**: S17-S23 [PMID: 17969095 DOI: 10.1002/lt.21326]
64 **Kelley RK**, Yao F. Salvage liver transplantation for recurrent hepatocellular carcinoma after radiofrequency ablation: a new strategy? *J Hepatol* 2012; **56**: 14-16 [PMID: 21872558 DOI: 10.1016/j.jhep.2011.06.029]
65 **N'Kontchou G**, Aout M, Laurent A, Nahon P, Ganne-Carrié N, Grando V, Baghad I, Roulot D, Trinchet JC, Sellier N, Cherqui D, Vicaut E, Beaugrand M, Seror O. Survival after radiofrequency ablation and salvage transplantation in patients with hepatocellular carcinoma and Child-Pugh A cirrhosis. *J Hepatol* 2012; **56**: 160-166 [PMID: 21703186 DOI: 10.1016/j.jhep.2011.03.026]
66 **Sala M**, Fuster J, Llovet JM, Navasa M, Solé M, Varela M, Pons F, Rimola A, García-Valdecasas JC, Brú C, Bruix J; Barcelona Clinic Liver Cancer (BCLC) Group. 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]
67 **Scatton O**, Zalinski S, Terris B, Lefevre JH, Casali A, Massault PP, Conti F, Calmus Y, Soubrane O. Hepatocellular carcinoma developed on compensated cirrhosis: resection as a selection tool for liver transplantation. *Liver Transpl* 2008; **14**: 779-788 [PMID: 18508370 DOI: 10.1002/lt.21431]
68 **Qin LX**, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 385-392 [PMID: 12046056 DOI: 10.3748/wjg.v8.i3.385]
69 **Choi GH**, Han DH, Kim DH, Choi SB, Kang CM, Kim KS, Choi JS, Park YN, Park JY, Kim DY, Han KH, Chon CY, Lee WJ. Outcome after curative resection for a huge hepatocellular carcinoma and prognostic significance of gross tumor classification. *Am J Surg* 2009; **198**: 693-701 [PMID: 19268907 DOI: 10.1016/j.amjsurg.2008.09.019]
70 **Shimada M**, Rikimaru T, Hamatsu T, Yamashita Y, Terashi T, Taguchi K, Tanaka S, Shirabe K, Sugimachi K. The role of macroscopic classification in nodular-type hepatocellular carcinoma. *Am J Surg* 2001; **182**: 177-182 [PMID: 11574092 DOI: 10.1016/S0002-9610(01)00682-1]
71 **Fu X**, Mao L, Tang M, Yan X, Qiu Y, He J, Zhou T. Gross classification of solitary small hepatocellular carcinoma on preoperative computed tomography: Prognostic significance after radiofrequency ablation. *Hepatol Res* 2016; **46**: 298-305 [PMID: 26041379 DOI: 10.1111/hepr.12540]

72 **Martins-Filho SN**, Paiva C, Azevedo RS, Alves VAF. Histological Grading of Hepatocellular Carcinoma-A Systematic Review of Literature. *Front Med* (Lausanne) 2017; **4**: 193 [PMID: 29209611 DOI: 10.3389/fmed.2017.00193]

73 **Mounajjed T**, Chandan VS, Torbenson MS, SpringerLink (Online service). Surgical Pathology of Liver Tumors

74 **Ziol M**, Poté N, Amaddeo G, Laurent A, Nault JC, Oberti F, Costentin C, Michalak S, Bouattour M, Francoz C, Pageaux GP, Ramos J, Decaens T, Luciani A, Guiu B, Vilgrain V, Aubé C, Derman J, Charpy C, Zucman-Rossi J, Barget N, Seror O, Ganne-Carrié N, Paradis V, Calderaro J. Macrotrabecular-massive hepatocellular carcinoma: A distinctive histological subtype with clinical relevance. *Hepatology* 2018; **68**: 103-112 [PMID: 29281854 DOI: 10.1002/hep.29762]

75 **Torbenson MS**. Morphologic Subtypes of Hepatocellular Carcinoma. *Gastroenterol Clin North Am* 2017; **46**: 365-391 [PMID: 28506370 DOI: 10.1016/j.gtc.2017.01.009]

76 **Craig JR**, Peters RL, Edmondson HA, Omata M. Fibrolamellar carcinoma of the liver: a tumor of adolescents and young adults with distinctive clinico-pathologic features. *Cancer* 1980; **46**: 372-379 [PMID: 6248194 DOI: 10.1002/1097-0142(19800715)46:2<372::AID-CNCR2820460227>3.0.CO;2-S]

77 **Klein WM**, Molmenti EP, Colombani PM, Grover DS, Schwarz KB, Boitnott J, Torbenson MS. Primary liver carcinoma arising in people younger than 30 years. *Am J Clin Pathol* 2005; **124**: 512-518 [PMID: 16146811 DOI: 10.1309/TT0R7KAL32228E99]

78 **Goldie AS**, Fearon KC, Ross JA, Barclay GR, Jackson RE, Grant IS, Ramsay G, Blyth AS, Howie JC. Natural cytokine antagonists and endogenous antiendotoxin core antibodies in sepsis syndrome. The Sepsis Intervention Group. *JAMA* 1995; **274**: 172-177 [PMID: 7596007 DOI: 10.1001/jama.1995.03530020090038]

79 **Salomao M**, Yu WM, Brown RS Jr, Emond JC, Lefkowitch JH. Steatohepatitic hepatocellular carcinoma (SH-HCC): a distinctive histological variant of HCC in hepatitis C virus-related cirrhosis with associated NAFLD/NASH. *Am J Surg Pathol* 2010; **34**: 1630-1636 [PMID: 20975341 DOI: 10.1097/PAS.0b013e3181f31caa]

80 **Kim H**, Choi GH, Na DC, Ahn EY, Kim GI, Lee JE, Cho JY, Yoo JE, Choi JS, Park YN. Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis. *Hepatology* 2011; **54**: 1707-1717 [PMID: 22045674 DOI: 10.1002/hep.24559]

81 **Fatourou E**, Koskinas J, Karandrea D, Palaiologou M, Syminelaki T, Karanikolas M, Felekouras E, Antoniou E, Manesis EK, Delladetsima J, Tiniakos D. Keratin 19 protein expression is an independent predictor of survival in human hepatocellular carcinoma. *Eur J Gastroenterol Hepatol* 2015; **27**: 1094-1102 [PMID: 26011233 DOI: 10.1097/MEG.0000000000000398]

82 **Li T**, Fan J, Qin LX, Zhou J, Sun HC, Qiu SJ, Ye QH, Wang L, Tang ZY. Risk factors, prognosis, and management of early and late intrahepatic recurrence after resection of primary clear cell carcinoma of the liver. *Ann Surg Oncol* 2011; **18**: 1955-1963 [PMID: 21240562 DOI: 10.1245/s10434-010-1540-z]

83 **Xu W**, Ge P, Liao W, Ren J, Yang H, Xu H, Sang X, Lu X, Zhong S, Mao Y. Edmondson grade predicts survival of patients with primary clear cell carcinoma of liver after curative resection: A retrospective study with long-term follow-up. *Asia Pac J Clin Oncol* 2017; **13**: e312-e320 [PMID: 27098441 DOI: 10.1111/ajco.12494]

84 **Tan PS**, Nakagawa S, Goossens N, Venkatesh A, Huang T, Ward SC, Sun X, Song WM, Koh A, Canasto-Chibuque C, Deshmukh M, Nair V, Mahajan M, Zhang B, Fiel MI, Kobayashi M, Kumada H, Hoshida Y. Clinicopathological indices to predict hepatocellular carcinoma molecular classification. *Liver Int* 2016; **36**: 108-118 [PMID: 26058462 DOI: 10.1111/liv.12889]

85 **Calderaro J**, Couchy G, Imbeaud S, Amaddeo G, Letouzé E, Blanc JF, Laurent C, Hajji Y, Azoulay D, Bioulac-Sage P, Nault JC, Zucman-Rossi J. Histological subtypes of hepatocellular carcinoma are related to gene mutations and molecular tumour classification. *J Hepatol* 2017; **67**: 727-738 [PMID: 28532995 DOI: 10.1016/j.jhep.2017.05.014]

86 **Lauwers GY**, Terris B, Balis UJ, Batts KP, Regimbeau JM, Chang Y, Graeme-Cook F, Yamabe H, Ikai I, Cleary KR, Fujita S, Flejou JF, Zukerberg LR, Nagorney DM, Belghiti J, Yamaoka Y, Vauthey JN; International Cooperative Study Group on Hepatocellular Carcinoma. Prognostic histologic indicators of curatively resected hepatocellular carcinomas: a multi-institutional analysis of 425 patients with definition of a histologic prognostic index. *Am J Surg Pathol* 2002; **26**: 25-34 [PMID: 11756766 DOI: 10.1097/00000478-200201000-00003]

87 **Kassahun WT**, Hauss J. Management of combined hepatocellular and cholangiocarcinoma. *Int J Clin Pract* 2008; **62**: 1271-1278 [PMID: 18284443 DOI: 10.1111/j.1742-1241.2007.01694.x]

88 **Koh KC**, Lee H, Choi MS, Lee JH, Paik SW, Yoo BC, Rhee JC, Cho JW, Park CK, Kim HJ. Clinicopathologic features and prognosis of combined hepatocellular cholangiocarcinoma. *Am J Surg* 2005; **189**: 120-125 [PMID: 15701504 DOI: 10.1016/j.amjsurg.2004.03.018]

89 **Goodman ZD**, Ishak KG, Langloss JM, Sesterhenn IA, Rabin L. Combined hepatocellular-cholangiocarcinoma. A histologic and immunohistochemical study. *Cancer* 1985; **55**: 124-135 [PMID: 2578078 DOI: 10.1002/1097-0142(19850101)55:1<124::AID-CNCR2820550120>3.0.CO;2-Z]

90 **Lin G**, Toh CH, Wu RC, Ko SF, Ng SH, Chou WC, Tseng JH. Combined hepatocellular cholangiocarcinoma: prognostic factors investigated by computed tomography/magnetic resonance imaging. *Int J Clin Pract* 2008; **62**: 1199-1205 [PMID: 17537192 DOI: 10.1111/j.1742-1241.2007.01291.x]

91 **ALLEN RA**, LISA JR. Combined liver cell and bile duct carcinoma. *Am J Pathol* 1949; **25**: 647-655 [PMID: 18152860]

92 **Theise ND**, Park YN, Nakanuma Y. Combined hepatocellular cholangiocarcinoma. WHO Classification of Tumours of the Digestive System, 4th ed. IARC Press, **2010**: 225-227

93 **Sasaki M**, Sato H, Kakuda Y, Sato Y, Choi JH, Nakanuma Y. Clinicopathological significance of 'subtypes with stem-cell feature' in combined hepatocellular-cholangiocarcinoma. *Liver Int* 2015; **35**: 1024-1035 [PMID: 24712771 DOI: 10.1111/liv.12563]

94 **Zhou L**, Rui JA, Wang SB, Chen SG, Qu Q, Chi TY, Wei X, Han K, Zhang N, Zhao HT. Outcomes and prognostic factors of cirrhotic patients with hepatocellular carcinoma after radical major hepatectomy. *World J Surg* 2007; **31**: 1782-1787 [PMID: 17610113 DOI: 10.1007/s00268-007-9029-z]

95 **Moeini A**, Sia D, Zhang Z, Camprecios G, Stueck A, Dong H, Montal R, Torrens L, Martinez-Quetglas I, Fiel MI, Hao K, Villanueva A, Thung SN, Schwartz ME, Llovet JM. Mixed hepatocellular cholangiocarcinoma tumors: Cholangiolocellular carcinoma is a distinct molecular entity. *J Hepatol* 2017; **66**: 952-961 [PMID: 28126467 DOI: 10.1016/j.jhep.2017.01.010]

96 **Ma YC**, Yang JY, Yan LN. Relevant markers of cancer stem cells indicate a poor prognosis in hepatocellular carcinoma patients: a meta-analysis. *Eur J Gastroenterol Hepatol* 2013; **25**: 1007-1016 [PMID: 23478672 DOI: 10.1097/MEG.0b013e32836019d8]

97 **Govaere O**, Roskams T. Pathogenesis and prognosis of hepatocellular carcinoma at the cellular and molecular levels. *Clin Liver Dis* 2015; **19**: 261-276 [PMID: 25921662 DOI: 10.1016/j.cld.2015.01.002]

98 **Bruix J**, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut* 2014; **63**: 844-855 [PMID: 24531850 DOI: 10.1136/gutjnl-2013-306627]

99 **Sherman M**. Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis. *Semin Liver Dis* 2010; **30**: 3-16 [PMID: 20175029 DOI: 10.1002/hep.1840220341]

100 **International Working Party**. Terminology of nodular hepatocellular lesions. *Hepatology* 1995; **22**: 983-993 [PMID: 7657307 DOI: 10.1055/s-0030-1247128]

101 **International Consensus Group for Hepatocellular Neoplasia**. Pathologic diagnosis of early hepatocellular carcinoma: a report of the international consensus group for hepatocellular neoplasia. *Hepatology* 2009; **49**: 658-64 [PMID: 19177576 DOI: 10.1002/hep.22709]

102 **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]

103 **Lo RC**, Ng IO. Hepatocellular tumors: immunohistochemical analyses for classification and prognostication. *Chin J Cancer Res* 2011; **23**: 245-253 [PMID: 23359751 DOI: 10.1007/s11670-011-0245-6]

104 **Di Tommaso L**, Destro A, Seok JY, Balladore E, Terracciano L, Sangiovanni A, Iavarone M, Colombo M, Jang JJ, Yu E, Jin SY, Morenghi E, Park YN, Roncalli M. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. *J Hepatol* 2009; **50**: 746-754 [PMID: 19231003 DOI: 10.1016/j.jhep.2008.11.014]

105 **Yamauchi N**, Watanabe A, Hishinuma M, Ohashi K, Midorikawa Y, Morishita Y, Niki T, Shibahara J, Mori M, Makuuchi M, Hippo Y, Kodama T, Iwanari H, Aburatani H, Fukayama M. The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. *Mod Pathol* 2005; **18**: 1591-1598 [PMID: 15920546 DOI: 10.1038/modpathol.3800436]

106 **Di Tommaso L**, Roncalli M. Tissue Biomarkers in Hepatocellular Tumors: Which, When, and How. *Front Med* (Lausanne) 2017; **4**: 10 [PMID: 28280721 DOI: 10.3389/fmed.2017.00010]

107 **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 DOI: 10.1016/j.jhep.2011.12.001]

108 **Tamura S**, Kato T, Berho M, Misiakos EP, O'Brien C, Reddy KR, Nery JR, Burke GW, Schiff ER, Miller J, Tzakis AG. Impact of histological grade of hepatocellular carcinoma on the outcome of liver transplantation. *Arch Surg* 2001; **136**: 25-30; discussion 31 [PMID: 11146770 DOI: 10.1001/archsurg.136.1.25]

109 **EDMONDSON HA**, STEINER PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954; **7**: 462-503 [PMID: 13160935 DOI: 10.1002/1097-0142(195405)7:3<462::AID-CNCR2820070308>3.0.CO;2-E]

110 **Bosman FT**, World Health Organization. International Agency for Research on Cancer. WHO classification of tumours of the digestive system. 4th ed. International Agency for Research on Cancer, **2010**: 417

111 **Alastair Burt**, Linda Ferrell, Stefan Hubscher. MacSween's pathology of the liver. In 7th ed. Churchill Livingstone 2017

112 **Amin MB**, Edge SB, Greene FL. AJCC Cancer Staging Manual. 8th ed. New York, Springer 2017 [DOI: 10.1007/978-3-319-40618-3]

113 **Qin LX**, Tang ZY. The prognostic significance of clinical and pathological features in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 193-199 [PMID: 11925590 DOI: 10.3748/wjg.v8.i2.193]

114 **Zhang X**, Li J, Shen F, Lau WY. Significance of presence of microvascular invasion in specimens obtained after surgical treatment of hepatocellular carcinoma. *J Gastroenterol Hepatol* 2018; **33**: 347-354 [PMID: 28589639 DOI: 10.1111/jgh.13843]

115 **Pawlik TM**, Delman KA, Vauthey JN, Nagorney DM, Ng IO, Ikai I, Yamaoka Y, Belghiti J, Lauwers GY, Poon RT, Abdalla EK. Tumor size predicts vascular invasion and histologic grade: Implications for selection of surgical treatment for hepatocellular carcinoma. *Liver Transpl* 2005; **11**: 1086-1092 [PMID: 16123959 DOI: 10.1002/lt.20472]

116 **Du M**, Chen L, Zhao J, Tian F, Zeng H, Tan Y, Sun H, Zhou J, Ji Y. Microvascular invasion (MVI) is a poorer prognostic predictor for small hepatocellular carcinoma. *BMC Cancer* 2014; **14**: 38 [PMID: 24460749 DOI: 10.1186/1471-2407-14-38]

117 **Yamashita Y**, Tsuijita E, Takeishi K, Fujiwara M, Kira S, Mori M, Aishima S, Taketomi A, Shirabe K, Ishida T, Maehara Y. Predictors for microinvasion of small hepatocellular carcinoma ⩽ 2 cm. *Ann Surg Oncol* 2012; **19**: 2027-2034 [PMID: 22203184 DOI: 10.1245/s10434-011-2195-0]

118 **Rodríguez-Perálvarez M**, Luong TV, Andreana L, Meyer T, Dhillon AP, Burroughs AK. A systematic review of microvascular invasion in hepatocellular carcinoma: diagnostic and prognostic variability. *Ann Surg Oncol* 2013; **20**: 325-339 [PMID: 23149850 DOI: 10.1245/s10434-012-2513-1]

119 **Shindoh J**, Andreou A, Aloia TA, Zimmitti G, Lauwers GY, Laurent A, Nagorney DM, Belghiti J, Cherqui D, Poon RT, Kokudo N, Vauthey JN. Microvascular invasion does not predict long-term survival in hepatocellular carcinoma up to 2 cm: reappraisal of the staging system for solitary tumors. *Ann Surg Oncol* 2013; **20**: 1223-1229 [PMID: 23179993 DOI: 10.1245/s10434-012-2739-y]

120 **Feng LH**, Dong H, Lau WY, Yu H, Zhu YY, Zhao Y, Lin YX, Chen J, Wu MC, Cong WM. Novel microvascular invasion-based prognostic nomograms to predict survival outcomes in patients after R0 resection for hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2017; **143**: 293-303 [PMID: 27743138 DOI: 10.1007/s00432-016-2286-1]

121 **Cong WM**, Bu H, Chen J, Dong H, Zhu YY, Feng LH, Chen J; Guideline Committee. Practice guidelines for the pathological diagnosis of primary liver cancer: 2015 update. *World J Gastroenterol* 2016; **22**: 9279-9287 [PMID: 27895416 DOI: 10.3748/wjg.v22.i42.9279]

122 **Gouw AS**, Balabaud C, Kusano H, Todo S, Ichida T, Kojiro M. Markers for microvascular invasion in hepatocellular carcinoma: where do we stand? *Liver Transpl* 2011; **17** Suppl 2: S72-S80 [PMID: 21714066 DOI: 10.1002/lt.22368]

123 **Poté N**, Cauchy F, Albuquerque M, Cros J, Soubrane O, Bedossa P, Paradis V. Contribution of virtual biopsy to the screening of microvascular invasion in hepatocellular carcinoma: A pilot study. *Liver Int* 2018; **38**: 687-694 [PMID: 28872754 DOI: 10.1111/liv.13585]

124 **Donat M**, Alonso S, Pereira F, Ferrero E, Carrión L, Acin-Gándara D, Moreno E. Impact of Histological Factors of Hepatocellular Carcinoma on the Outcome of Liver Transplantation. *Transplant Proc* 2016; **48**: 1968-1977 [PMID: 27569930 DOI: 10.1016/j.transproceed.2016.04.002]

125 **Li WX**, Li Z, Gao PJ, Gao J, Zhu JY. Histological differentiation predicts post-liver transplantation survival time. *Clin Res Hepatol Gastroenterol* 2014; **38**: 201-208 [PMID: 24388339 DOI: 10.1016/j.clinre.2013.11.002]

126 **Yip VS**, Gomez D, Tan CY, Staettner S, Terlizzo M, Fenwick S, Malik HZ, Ghaneh P, Poston G. Tumour size and differentiation predict survival after liver resection for hepatocellular carcinoma arising from non-cirrhotic and non-fibrotic liver: a case-controlled study. *Int J Surg* 2013; **11**: 1078-1082 [PMID: 24129124 DOI: 10.1016/j.ijsu.2013.10.001]

127 **El-Fattah MA**. Hepatocellular carcinoma biology predicts survival outcome after liver transplantation in the USA. *Indian J Gastroenterol* 2017; **36**: 117-125 [PMID: 28194604 DOI: 10.1007/s12664-017-0732-x]

128 **Zhou L**, Rui JA, Wang SB, Chen SG, Qu Q. Early recurrence in large hepatocellular carcinoma after curative hepatic resection: prognostic significance and risk factors. *Hepatogastroenterology* 2014; **61**: 2035-2041 [PMID: 25713907]

129 **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]

130 **Cillo U**, Vitale A, Bassanello M, Boccagni P, Brolese A, Zanus G, Burra P, Fagiuoli S, Farinati F, Rugge M, D'Amico DF. Liver transplantation for the treatment of moderately or well-differentiated hepatocellular carcinoma. *Ann Surg* 2004; **239**: 150-159 [PMID: 14745321 DOI: 10.1097/01.sla.0000109146.72827.76]

131 **Lee JS**, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006; **12**: 410-416 [PMID: 16532004 DOI: 10.1038/nm1377]

132 **Tsujikawa H**, Masugi Y, Yamazaki K, Itano O, Kitagawa Y, Sakamoto M. Immunohistochemical molecular analysis indicates hepatocellular carcinoma subgroups that reflect tumor aggressiveness. *Hum Pathol* 2016; **50**: 24-33 [PMID: 26997435 DOI: 10.1016/j.humpath.2015.10.014]

133 **Kim H**, Park YN. Hepatocellular carcinomas expressing 'stemness'-related markers: clinicopathological characteristics. *Dig Dis* 2014; **32**: 778-785 [PMID: 25376296 DOI: 10.1159/000368021]

134 **Chan AW**, Tong JH, Chan SL, Lai PB, To KF. Expression of stemness markers (CD133 and EpCAM) in prognostication of hepatocellular carcinoma. *Histopathology* 2014; **64**: 935-950 [PMID: 24506513 DOI: 10.1111/his.12342]

135 **Guo Z**, Li LQ, Jiang JH, Ou C, Zeng LX, Xiang BD. Cancer stem cell markers correlate with early recurrence and survival in hepatocellular carcinoma. *World J Gastroenterol* 2014; **20**: 2098-2106 [PMID: 24616575 DOI: 10.3748/wjg.v20.i8.2098]

136 **Srivastava S**, Wong KF, Ong CW, Huak CY, Yeoh KG, Teh M, Luk JM, Salto-Tellez M. A morpho-molecular prognostic model for hepatocellular carcinoma. *Br J Cancer* 2012; **107**: 334-339 [PMID: 22713659 DOI: 10.1038/bjc.2012.230]

137 **Li Y**, Ma X, Zhang J, Liu X, Liu L. Prognostic value of microvessel density in hepatocellular carcinoma patients: a meta-analysis. *Int J Biol Markers* 2014; **29**: e279-e287 [PMID: 24803279 DOI: 10.5301/jbm.5000087]

138 **Zucman-Rossi J**, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015; **149**: 1226-1239.e4 [PMID: 26099527 DOI: 10.1053/j.gastro.2015.05.061]

139 **Mekuria AN**, Abdi AD. Potential Molecular Targets and Drugs for Treatment of Hepatocellular Carcinoma. *J Cancer Sci Ther* 2017, **9**:12 [DOI: 10.4172/1948-5956.1000501]

140 **Rimassa L**, Abbadessa G, Personeni N, Porta C, Borbath I, Daniele B, Salvagni S, Van Laethem JL, Van Vlierberghe H, Trojan J, De Toni EN, Weiss A, Miles S, Gasbarrini A, Lencioni M, Lamar ME, Wang Y, Shuster D, Schwartz BE, Santoro A. Tumor and circulating biomarkers in patients with second-line hepatocellular carcinoma from the randomized phase Ⅱ study with tivantinib. *Oncotarget* 2016; **7**: 72622-72633 [PMID: 27579536 DOI: 10.18632/oncotarget.11621]

141 **Rimassa L**, Assenat E, Peck-Radosavljevic M, Pracht M, Zagonel V, Mathurin P, Rota Caremoli E, Porta C, Daniele B, Bolondi L, Mazzaferro V, Harris W, Damjanov N, Pastorelli D, Reig M, Knox J, Negri F, Trojan J, López López C, Personeni N, Decaens T, Dupuy M, Sieghart W, Abbadessa G, Schwartz B, Lamar M, Goldberg T, Shuster D, Santoro A, Bruix J. Tivantinib for second-line treatment of MET-high, advanced hepatocellular carcinoma (METIV-HCC): a final analysis of a phase 3, randomised, placebo-controlled study. *Lancet Oncol* 2018; **19**: 682-693 [PMID: 29625879 DOI: 10.1016/S1470-2045(18)30146-3]

142 **Sia D**, Jiao Y, Martinez-Quetglas I, Kuchuk O, Villacorta-Martin C, Castro de Moura M, Putra J, Camprecios G, Bassaganyas L, Akers N, Losic B, Waxman S, Thung SN, Mazzaferro V, Esteller M, Friedman SL, Schwartz M, Villanueva A, Llovet JM. Identification of an Immune-specific Class of Hepatocellular Carcinoma, Based on Molecular Features. *Gastroenterology* 2017; **153**: 812-826 [PMID: 28624577 DOI: 10.1053/j.gastro.2017.06.007]

143 **Sideras K**, Biermann K, Verheij J, Takkenberg BR, Mancham S, Hansen BE, Schutz HM, de Man RA, Sprengers D, Buschow SI, Verseput MC, Boor PP, Pan Q, van Gulik TM, Terkivatan T, Ijzermans JN, Beuers UH, Sleijfer S, Bruno MJ, Kwekkeboom J. PD-L1, Galectin-9 and CD8+ tumor-infiltrating lymphocytes are associated with survival in hepatocellular carcinoma. *Oncoimmunology* 2017; **6**: e1273309 [PMID: 28344887 DOI: 10.1080/2162402X.2016.1273309]

144 **Kudo M**. Immune Checkpoint Blockade in Hepatocellular Carcinoma: 2017 Update. *Liver Cancer* 2016; **6**: 1-12 [PMID: 27995082 DOI: 10.1159/000449342]

145 **Chang H**, Jung W, Kim A, Kim HK, Kim WB, Kim JH, Kim BH. Expression and prognostic significance of programmed death protein 1 and programmed death ligand-1, and cytotoxic T lymphocyte-associated molecule-4 in hepatocellular carcinoma. *APMIS* 2017; **125**: 690-698 [PMID: 28493410 DOI: 10.1111/apm.12703]

146 **Calderaro J**, Rousseau B, Amaddeo G, Mercey M, Charpy C, Costentin C, Luciani A, Zafrani ES, Laurent A, Azoulay D, Lafdil F, Pawlotsky JM. Programmed death ligand 1 expression in hepatocellular carcinoma: Relationship With clinical and pathological features. *Hepatology* 2016; **64**: 2038-2046 [PMID: 27359084 DOI: 10.1002/hep.28710]

147 **Duffy AG**, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, Davis JL, Hughes MS, Heller T, ElGindi M, Uppala A, Korangy F, Kleiner DE, Figg WD, Venzon D, Steinberg SM, Venkatesan AM, Krishnasamy V, Abi-Jaoudeh N, Levy E, Wood BJ, Greten TF. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J Hepatol* 2017; **66**: 545-551 [PMID: 27816492 DOI: 10.1016/j.jhep.2016.10.029]

148 **Hoshida Y**, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, Watanabe G, Gabriel S, Friedman SL, Kumada H, Llovet JM, Golub TR. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009; **69**: 7385-7392 [PMID: 19723656 DOI: 10.1158/0008-5472.CAN-09-1089]

P- Reviewer: Kanda T, Rodríguez-Perálvarez M, Tsoulfas G
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4014-4020

DOI: 10.3748/wjg.v24.i35.4014

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

MINIREVIEWS

Endoscopy in inflammatory bowel disease: Role in diagnosis, management, and treatment

Clayton M Spiceland, Nilesh Lodhia

Clayton M Spiceland, Division of Gastroenterology and Hepatology, Medical University of South Carolina, Charleston, SC 29425, United States

Nilesh Lodhia, Division of Gastroenterology and Hepatology, University of North Carolina, Charlotte, NC 28204, United States

ORCID number: Clayton M Spiceland (0000-0002-7633-2568); Nilesh Lodhia (0000-0003-4177-7548).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and approval of the final version.

Conflict-of-interest statement: Lodhia N has served as a consultant for Medtronic. No potential conflicts of interest for Spiceland CM. No financial support.

Manuscript source: Invited manuscript

Correspondence to: Nilesh Lodhia, MD, Associate Professor, Division of Gastroenterology and Hepatology, University of North Carolina, Charlotte, CHS Digestive Health 1025 Morehead Medical Drive, Suite 300 Charlotte, NC 28204, United States. Nilesh.lodhia@atriumhealth.org
Telephone: +1-843-7043554593
Fax: +1-843-8760372

Received: March 29, 2018
Peer-review started: March 30, 2018
First decision: May 17, 2018
Revised: July 29, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018

Published online: September 21, 2018

Abstract

Endoscopy plays a fundamental role in the diagnosis, management, and treatment of inflammatory bowel disease (IBD). Colonoscopy, flexible sigmoidoscopy, and esophagogastroduodenoscopy have long been used in the care of patients with IBD. As endoscopic technologies have progressed, tools such as endoscopic ultrasound, capsule endoscopy, and balloon-assisted enteroscopy have expanded the role of endoscopy in IBD. Furthermore, chromoendoscopy has enhanced our ability to detect dysplasia in IBD. In this review article, we will focus on the roles, indications, and limitations of these tools in IBD. We will also discuss the most commonly used endoscopic scoring systems, as well as special considerations in post-surgical patients. Lastly, we will discuss the role of endoscopy in the diagnosis and management of fistulae and strictures.

Key words: Endoscopy; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Colonoscopy; Capsule endoscopy; Dysplasia detection; Stricture dilation; Fistula management

Core tip: Although endoscopy has long been used in the diagnosis and management of inflammatory bowel disease (IBD), technologic advances have allowed for additional tools to assist in the management and treatment of IBD patients. This review article discusses the roles, indications, and limitations of endoscopy in IBD.

Spiceland CM, Lodhia N. Endoscopy in inflammatory bowel disease: Role in diagnosis, management, and treatment. *World J Gastroenterol* 2018; 24(35): 4014-4020 Available from: URL:

http://www.wjgnet.com/1007-9327/full/v24/i35/4014.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4014

INTRODUCTION

Endoscopy plays a fundamental role in the diagnosis, management, and treatment of inflammatory bowel disease (IBD). Endoscopy is essential in excluding other etiologies, establishing diagnoses, differentiating between Crohn's disease (CD) and ulcerative colitis (UC), monitoring disease activity and response to treatment, and assessing for and treating complications. Colonoscopy, flexible sigmoidoscopy, and esophagogastroduodenoscopy (EGD) have long been used in the care of patients with IBD. As endoscopic technologies have progressed, tools such as endoscopic ultrasound, capsule endoscopy, and balloon-assisted enteroscopy have expanded the role of endoscopy in IBD. This review will focus on the role, indication, and contraindication of these tools in the management of IBD.

ENDOSCOPY IN DISEASE DIAGNOSIS

Diagnostic lower endoscopy (colonoscopy, flexible sigmoidoscopy)

In patients with clinical presentations suggestive of IBD, the initial evaluation should include a colonoscopy with intubation and examination of the terminal ileum[1]. Colonoscopy with ileoscopy not only allows for direct visualization of the colon and terminal ileum, but also allows for necessary biopsies to be performed. When IBD is suspected, two biopsy specimens from five sites, including the ileum and rectum, are recommended[2]. Biopsy specimens should be obtained from both affected and normal-appearing mucosa; specimens from different locations should be labeled and submitted separately[1]. The combination of endoscopic and histologic features assists in IBD diagnosis, the differentiation of CD *vs* UC, as well as in the exclusion of other disease entities with similar presentations (*e.g.*, drug-induced colitis, infectious colitis, ischemic colitis, and segmental colitis associated with diverticulosis). Although flexible sigmoidoscopy is inadequate for a complete initial evaluation of suspected IBD, it can be useful in a few specific circumstances. Overall, colonoscopy is a safe procedure with a low rate of adverse events in patients with IBD. However, it is relatively contraindicated in patients with severe colitis and toxic megacolon. Therefore, in cases where a full colonoscopy is contraindicated, flexible sigmoidoscopy can provide a safer alternative that allows for distal bowel examination and biopsy acquisition. Additionally, flexible sigmoidoscopy can be used in patients with established IBD to assess disease activity and/or to rule out concomitant infection.

While patient presentation, history, laboratory information, and radiologic data can assist, colonoscopy is essential in differentiating UC from CD. Classically described endoscopic findings in UC include edema, loss of vascularity, erythema, mucosal granularity and friability, erosions and ulcers, and pseudopolyps. In treatment-naïve patients, these findings typically begin at the rectum and extend proximally in a continuous manner with a gradual transition to normal-appearing mucosa[3]. It is important to note that UC patients on treatment can have patchy inflammation and rectal sparing[4]. Additionally, approximately 5% of patients may also have an area of isolated peri-appendiceal inflammation, commonly known as a cecal patch, which does not have any correlation with disease activity or clinical course[5,6]. While many of the classic findings of UC can also been seen in CD, three major endoscopic findings that can aid in distinguishing CD from UC are the presence of apthous ulcers, cobblestoning, and discontinuous or "skip" lesions[7]. Although isolated involvement of the terminal ileum is highly suggestive of CD, "backwash ileitis" can occur in UC in the setting of pancolitis[8]. Mucosal biopsies with histologic examination, upper gastrointestinal (GI) and small bowel endoscopy, small bowel imaging, and serologic markers can further assist when diagnostic uncertainty remains.

Diagnostic upper endoscopy

Although upper GI tract involvement can occur in up to 16% of patients with CD[9], routine EGD is currently not recommended in adult patients suspected of having CD. However, EGD is often included in the diagnostic evaluation of suspected IBD secondary to the overlap of IBD symptoms and indications for upper endoscopy such as abdominal pain, weight loss, nausea, and vomiting. Additionally, in adults with unclassified IBD, upper endoscopy can aid in the diagnosis of CD if upper GI involvement is found. At least two biopsies should be taken from the esophagus, stomach, and duodenum during EGD for suspected upper tract IBD[10]. Endoscopic findings of upper GI CD include aphthous ulcers, strictures, fistulas, and erythema. Upper GI tract disease can present simultaneously with distal disease or later in the disease course[11]. In pediatric populations, isolated upper GI CD occurs more commonly than in adults. Thus, an EGD is recommended as part of the initial evaluation of children with suspected IBD, regardless of upper GI symptoms[12]. Upper endoscopy is also useful in evaluation for celiac disease which can have a similar presentation to IBD in both the adult and pediatric populations.

Wireless video capsule endoscopy

Wireless video capsule endoscopy (VCE), first approved in 2001, has developed into a safe and effective technology to image the small intestine. The noninvasive nature of the VCE is a significant advantage over enteroscopy, and the ability to detect early mucosal lesions allows for a greater sensitivity than radiologic studies. The diagnostic yield of VCE can be as high as 71%, depending on the clinical setting[13]. Typical findings of CD on VCE include

Table 1 Mayo endoscopic subscore

Normal (0): No inflammatory signs
Mild (1): Erythema
Moderate (2): Friability, erosions
Severe (3): Spontaneous bleeding, ulcerations

Four-grade scale (0-3)[22].

Table 2 Simple endoscopic score in Crohn's disease

Ulcer: None (0), 0.1-0.5 cm (1), 0.5-2 cm (2), > 2 cm (3)
Ulcerated surface: None (0), < 10% (1), 10%-30% (2), > 30% (3)
Affected surface: None (0), < 50% (1), 50%-75% (2), > 75% (3)
Narrowing: None (0), single passable (1), multiple passable (2), impassable (3)

Sum of five segments scores for a total score (0-56)[23].

erythema, erosions, ulcerations, and strictures[14]. VCE should not be used in patients with known or suspected strictures, as capsule retention has been described in up to 13% of patients who underwent a capsule study for CD[15]. Therefore, it is recommended that patients with known small bowel CD have small bowel imaging or a patency study prior to VCE. The aforementioned mucosal findings are not specific to CD and can be found in patients with other etiologies, including NSAID use. Therefore, an important limitation to VCE is the inability to obtain tissue for histologic diagnosis. The PillCam® SB3 is a new capsule designed to provide improved diagnostic accuracy due to better image quality and creating more images with an adaptive frame rate of 2-6 frames per second. Additionally, using the automatic mode of Rapid Reader® software version 8.0 is expected to reduce the reading time and minimize the possibility of missing lesions[16]. Given these improvements, this is being marketed for use in the evaluation of CD.

Balloon assisted enteroscopy

Given the high diagnostic yields of less-invasive modalities such as radiologic small bowel imaging and VCE, enteroscopy has a limited role in the initial evaluation of patients with suspected IBD. However, when small bowel abnormalities are identified by less invasive studies, endoscopic and histologic evaluation is often a necessary next step. When the location lies outside the reach of standard endoscopy, balloon-assisted anterograde or retrograde enteroscopy can be used to access the area of interest. Additionally, enteroscopy allows for therapeutic interventions such as hemostasis, stricture dilation, or foreign body retrieval. However, deep enteroscopy can be time consuming and technically challenging which often limits use to specialized providers and centers.

ENDOSCOPY IN DISEASE MANAGEMENT

Assessment of disease activity and response to treatment

Endoscopy allows for the visual assessment and histologic confirmation of treatment response, thus playing a critical role in the management of IBD. Clinical trials have shown that mucosal healing has been associated with improved outcomes in IBD, including sustained remission, fewer hospitalizations, and a decreased need for surgery[17,18]. Therefore, endoscopic outcomes, such as mucosal healing, are now recommended treatment goals in clinical practice[19]. In 2015, the Selecting Therapeutic Targets in Inflammatory Bowel Disease initiative set forth consensus recommendations for treat-to-target goals[19]. The international consensus group recommended that disease activity be reassessed at 6 to 9 mo by endoscopy in CD and at 3 to 6 mo after the start of therapy in UC[19]. Although no consensus exists for endoscopic disease severity scores, numerous endoscopic classification and scoring systems have been developed to standardize endoscopic assessment[20,21]. The Mayo Endoscopic Subscore for UC[22] and the Simple Endoscopic Score for CD (SES-CD)[23] are often used both in clinic trials and clinical practice and can be seen in Tables 1 and 2. Familiarity with and regular use of a disease classification and severity scoring system allows for a more standardized monitoring protocol among providers.

CRC screening and surveillance

Patients with IBD have an increased risk of developing colorectal cancer (CRC) compared to the general population[24]. Therefore, surveillance endoscopy to detect dysplasia has been recommended in patients with UC and CD with colon involvement beginning eight years after diagnosis. Additionally, surveillance colonoscopy is recommended annually, beginning at the time of diagnosis, in patients with concomitant primary sclerosing cholangitis given the high risk of CRC in this population[1,25,26].

Prior to the use of high imaging quality endoscopes, IBD-related dysplasia was thought to be an endoscopically invisible entity. Therefore, a random biopsy technique was recommended by societies and became common practice. In the era of high definition endoscopes and chromoendoscopy, most IBD-related dysplasia is now believed to be visible and endoscopically identifiable. Rather than random biopsies, a targeted biopsy sampling method is now recommended[27]. In 2015, the Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients: International Consensus (SCENIC)[28] published a consensus statement on surveillance and management of dysplasia in IBD; key recommendations can be seen in Table 3. Highlights of the SCENIC recommendations include the use of chromoendoscopy with targeted biopsy sampling rather than random biopsy sampling and the use of high-definition instead of standard-definition colonoscopes.

Along with the advances in dysplasia detection, the management of IBD-related dysplasia has also progressed. Historically, patients with dysplasia were primarily managed with colectomy. However, it is now

Table 3 Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients: International Consensus recommendations for optimizing detection and management of dysplasia in inflammatory bowel disease[28]

Detection of dysplasia on surveillance colonoscopy	When performing surveillance with white-light colonoscopy, high definition is recommended rather than standard definition
	When performing surveillance with standard-definition colonoscopy, chromoendoscopy is recommended rather than white-light colonoscopy
	When performing surveillance with high-definition colonoscopy, chromoendoscopy is suggested rather than white-light colonoscopy
	When performing surveillance with standard-definition colonoscopy, narrow-band imaging is not suggested in place of white-light colonoscopy
	When performing surveillance with high-definition colonoscopy, narrow-band imaging is not suggested in place of white-light colonoscopy
	When performing surveillance with image-enhanced high-definition colonoscopy, narrow-band imaging is not suggested in place of chromoendoscopy
Management of dysplasia discovered on surveillance colonoscopy	After complete removal of endoscopically resectable polypoid dysplastic lesions, surveillance colonoscopy is recommended rather than colectomy
	After complete removal of endoscopically resectable nonpolypoid dysplastic lesions, surveillance colonoscopy is suggested rather than colectomy
	For patients with endoscopically invisible dysplasia (confirmed by a GI pathologist) referral is suggested to an endoscopist with expertise in IBD surveillance using chromoendoscopy with high-definition colonoscopy

IBD: Inflammatory bowel disease.

Table 4 Rutgeerts score for Crohn's disease recurrence at ileocolonic anastomoses[35]

i0 no lesions in neoterminal ileum
i1 < 5 aphthous lesions in neoterminal ileum
i2 > 5 aphthous lesions with normal mucosa, skip areas with larger lesions, anastomotic lesions
i3 diffuse aphthous ileitis
i4 diffuse inflammation with ulcer, nodules, and/or stenosis

recommended that dysplasia determined to be "endoscopically resectable" be managed with polypectomy, endoscopic mucosal resection, or submucosal dissection[28]. Colectomy is now reserved for patients with "endoscopically unresectable" lesions, endoscopically invisible high-grade dysplasia despite chromoendoscopy, or multifocal dysplasia[29].

Post-surgical management

Special considerations are necessary in the care of patients who undergo an IBD-related surgery. Familiarity with post-surgical anatomy and the diagnosis and treatment of common postoperative complications are required for adequate treatment of the post-surgical patient population.

For patients with UC, ileal pouch anal anastomosis (IPAA) has become the surgical treatment of choice in patients who require colectomy. During ileal pouch endoscopy, or pouchoscopy, a gastroscope is most commonly used to examine the pouch, anastomosis, and afferent small bowel. The gastroscope allows for greater maneuverability than a colonoscope and its smaller caliber allows it to pass through a stricture or tight anastomosis more easily than the larger adult or pediatric colonoscopes. Pouchitis is the most common complication of IPAA and occurs in up to 50% of patients over 10 years of follow-up[30]. Endoscopic findings of pouchitis include mucosal erythema, edema, granularity, erosions, and ulcerations. Other possible complications after IPAA include cuffitis, pouch leakage, CD of the pouch, and pouch failure. Pouch strictures are relatively common and endoscopic therapy with balloon dilation has been found to be both safe and effective[31].

Patients with CD who undergo partial colectomy or partial ileocolectomy should have an endoscopic evaluation of the neoterminal ileum six months to one year after surgery[32,33] to evaluate for and stratify risk of recurrence. CD can recur after surgery, most commonly at the surgical anastomosis and neoterminal ileum[34]. The Rutgeerts Score[35] may be used to classify the risk of CD recurrence after ileocolonic anastomosis (Table 4). In patients who have undergone total colectomy and end-ileostomy (patients with CD, or older UC patients with poor functional outcomes after IPAA), ileoscopy can be performed to visualize the ileum and assess for disease response/recurrence.

ENDOSCOPIC THERAPY IN IBD

Stricture evaluation and management

Modern IBD medical therapies, including anti-tumor necrosis factor agents (ant-TNFs), have improved the natural history of CD. This is especially true when used before the development of irreversible fibrotic intestinal damage. Despite this, stricturing complications remain a significant cause of surgery, disability, and reduced quality of life in IBD patients[36]. Endoscopy is essential in the diagnosis and assessment of IBD-related strictures and is an evolving option for treatment.

Radiologic investigation is often an initial step when a patient with CD presents with obstructive symptoms suspicious for stricturing disease. Whether a CT scan is obtained in an emergent setting due to concern for pending small bowel obstruction, or a planned entero-

graphy is obtained prior to endoscopy, radiologic studies can provide vital information about IBD-related strictures. However, endoscopy is indispensable to allow for visual assessment of the stricture, biopsy to exclude malignancy, and to provide therapy in select cases.

In patients with CD, strictures are most often located in the terminal ileum and colon. In post-surgical patients, the site of ileo-colonic surgical anastomosis is a common location for stricturing[37]. Surgery has been a long-standing treatment option for CD-related strictures with stricturoplasty or small bowel resection. However, endoscopic balloon dilation (EBD) is increasingly used in patients with CD strictures to avoid surgery. The symptomatic benefits of endoscopic treatment can be sustained, with the ability to avoid surgery at 1, 3, and 5 years in 80%, 57%, and 52% of patients, respectively, based on a retrospective series[38]. In general, EBD is most successful in patients with a short stricture (4 cm or less) with minimal inflammation, straight angle of stricture (in line with the bowel lumen), and when there is a narrowing attributable to a single surgical anastomosis without a fistula orifice nearby[39,40]. Additionally, EBD has been found to be safe, with only a 3% rate of perforation[41]. Typically, the maximum balloon diameter used for stricture dilatation is 20 mm and stricture with fistula is classically a contraindication for endoscopic dilatation. Endoscopic balloon dilatation of ileoanal pouch strictures is safe and effective and is often recommended as the first line strategy to treat ileoanal pouch strictures[42].

Although endoscopic injection of steroids and anti-TNFs have been attempted, there is no clear evidence to support this as an adjunct to EBD. Needle-knife stricturotomy[43] and self-expanding metal stent placement[44] are additional described techniques of endoscopic stricture management. However, the indication, efficacy, and safety are still being determined and future studies will be necessary before mainstream use can be recommended. Lastly, it should be noted that a colonic stricture in UC should be considered malignant until proven otherwise. If adequate biopsies are not possible, surgery should be considered[45].

Fistula evaluation and management

Endoscopic ultrasound (EUS) has developed a role in the management of IBD, most commonly in the evaluation of perianal CD. Traditionally, surgical exam under anesthesia (EUA) or magnetic resonance imaging (MRI) were used in evaluation for suspected fistulae. However, EUS has been found to be equivalent to MRI or EUA[46] for the evaluation of perianal fistulae. EUS provides the additional benefit of being able to be performed in real-time in combination with traditional endoscopy. Endoscopic fistula treatment, including endoscopic suturing, over-the-scope clipping, endoscopic fistulotomy, and endoscopic drainage and seton placement have been described[47]. However, until recently, the treatment of fistulae refractory to medical therapy has been predominantly surgical. A recent study which included 29 consecutive patients with fistulas and IBD showed that endoscopic fistulotomy with a needle-knife appears to be safe and effective in treating IBD-related fistulas. Twenty-six patients (89.6%) achieved complete resolution of the fistula, while three patients (10.3%) had a persistent fistula and required surgical intervention[48].

CONCLUSION

Endoscopy plays an essential role in the diagnosis of IBD, differentiating between CD and UC, monitoring disease activity, assessing for and treating complications, and for colorectal cancer surveillance. New technologies and endoscopic techniques allow for an evolving role of endoscopic management of complications, such as strictures, that traditionally required surgery.

REFERENCES

1 **American Society for Gastrointestinal Endoscopy Standards of Practice Committee**, Shergill AK, Lightdale JR, Bruining DH, Acosta RD, Chandrasekhara V, Chathadi KV, Decker GA, Early DS, Evans JA, Fanelli RD, Fisher DA, Fonkalsrud L, Foley K, Hwang JH, Jue TL, Khashab MA, Muthusamy VR, Pasha SF, Saltzman JR, Sharaf R, Cash BD, DeWitt JM. The role of endoscopy in inflammatory bowel disease. *Gastrointest Endosc* 2015; **81**: 1101-1121.e1-e13 [PMID: 25800660 DOI: 10.1016/j.gie.2014.10.030]

2 **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; IBD Section of the British Society of Gastroenterology. Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2011; **60**: 571-607 [PMID: 21464096 DOI: 10.1136/gut.2010.224154]

3 **Waye JD**. The role of colonoscopy in the differential diagnosis of inflammatory bowel disease. *Gastrointest Endosc* 1977; **23**: 150-154 [PMID: 838244 DOI: 10.1016/S0016-5107(77)73622-3]

4 **Bernstein CN**, Shanahan F, Anton PA, Weinstein WM. Patchiness of mucosal inflammation in treated ulcerative colitis: a prospective study. *Gastrointest Endosc* 1995; **42**: 232-237 [PMID: 7498688 DOI: 10.1016/S0016-5107(95)70097-8]

5 5 **Bakman Y**, Katz J, Shepela C. Clinical Significance of Isolated Peri-Appendiceal Lesions in Patients With Left Sided Ulcerative Colitis. *Gastroenterology Res* 2011; **4**: 58-63 [PMID: 27942315 DOI: 10.4021/gr302w]

6 **D'Haens G**, Geboes K, Peeters M, Baert F, Ectors N, Rutgeerts P. Patchy cecal inflammation associated with distal ulcerative colitis: a prospective endoscopic study. *Am J Gastroenterol* 1997; **92**: 1275-1279 [PMID: 9260788]

7 **Pera A**, Bellando P, Caldera D, Ponti V, Astegiano M, Barletti C, David E, Arrigoni A, Rocca G, Verme G. Colonoscopy in inflammatory bowel disease. Diagnostic accuracy and proposal of an endoscopic score. *Gastroenterology* 1987; **92**: 181-185 [PMID: 3781186 DOI: 10.1016/0016-5085(87)90856-0]

8 **Haskell H**, Andrews CW Jr, 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 DOI: 10.1097/01.pas.0000176435.19197.88]

9 **Annunziata ML**, Caviglia R, Papparella LG, Cicala M. Upper gastrointestinal involvement of Crohn's disease: a prospective study on the role of upper endoscopy in the diagnostic work-up. *Dig Dis Sci* 2012; **57**: 1618-1623 [PMID: 22350786 DOI: 10.1007/s10620-012-2072-0]

10 **Paerregaard A**. What does the IBD patient hide in the upper

gastrointestinal tract? *Inflamm Bowel Dis* 2009; **15**: 1101-1104 [PMID: 19202573 DOI: 10.1002/ibd.20862]
11 **Nugent FW**, Roy MA. Duodenal Crohn's disease: an analysis of 89 cases. *Am J Gastroenterol* 1989; **84**: 249-254 [PMID: 2919581]
12 **Levine A**, Koletzko S, Turner D, Escher JC, Cucchiara S, de Ridder L, Kolho KL, Veres G, Russell RK, Paerregaard A, Buderus S, Greer ML, Dias JA, Veereman-Wauters G, Lionetti P, Sladek M, Martin de Carpi J, Staiano A, Ruemmele FM, Wilson DC; European Society of Pediatric Gastroenterology, Hepatology, and Nutrition. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014; **58**: 795-806 [PMID: 24231644 DOI: 10.1097/MPG.0000000000000239]
13 **Doherty GA**, Moss AC, Cheifetz AS. Capsule endoscopy for small-bowel evaluation in Crohn's disease. *Gastrointest Endosc* 2011; **74**: 167-175 [PMID: 21497806 DOI: 10.1016/j.gie.2011.01.067]
14 **Bourreille A**, Ignjatovic A, Aabakken L, Loftus EV Jr, Eliakim R, Pennazio M, Bouhnik Y, Seidman E, Keuchel M, Albert JG, Ardizzone S, Bar-Meir S, Bisschops R, Despott EJ, Fortun PF, Heuschkel R, Kammermeier J, Leighton JA, Mantzaris GJ, Moussata D, Lo S, Paulsen V, Panés J, Radford-Smith G, Reinisch W, Rondonotti E, Sanders DS, Swoger JM, Yamamoto H, Travis S, Colombel JF, Van Gossum A; World Organisation of Digestive Endoscopy (OMED) and the European Crohn's and Colitis Organisation (ECCO). Role of small-bowel endoscopy in the management of patients with inflammatory bowel disease: an international OMED-ECCO consensus. *Endoscopy* 2009; **41**: 618-637 [PMID: 19588292 DOI: 10.1055/s-0029-1214790]
15 **Kornbluth A**, Colombel JF, Leighton JA, Loftus E; ICCE. ICCE consensus for inflammatory bowel disease. *Endoscopy* 2005; **37**: 1051-1054 [PMID: 16189789 DOI: 10.1055/s-2005-870315]
16 **Monteiro S**, de Castro FD, Carvalho PB, Moreira MJ, Rosa B, Cotter J. PillCam® SB3 capsule: Does the increased frame rate eliminate the risk of missing lesions? *World J Gastroenterol* 2016; **22**: 3066-3068 [PMID: 26973404 DOI: 10.3748/wjg.v22.i10.3066]
17 **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 DOI: 10.1053/j.gastro.2011.06.054]
18 **Peyrin-Biroulet L**, Ferrante M, Magro F, Campbell S, Franchimont D, Fidder H, Strid H, Ardizzone S, Veereman-Wauters G, Chevaux JB, Allez M, Danese S, Sturm A; Scientific Committee of the European Crohn's and Colitis Organization. Results from the 2nd Scientific Workshop of the ECCO. I: Impact of mucosal healing on the course of inflammatory bowel disease. *J Crohns Colitis* 2011; **5**: 477-483 [PMID: 21939925 DOI: 10.1016/j.crohns.2011.06.009]
19 **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV Jr, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]
20 **Mohammed Vashist N**, Samaan M, Mosli MH, Parker CE, MacDonald JK, Nelson SA, Zou GY, Feagan BG, Khanna R, Jairath V. Endoscopic scoring indices for evaluation of disease activity in ulcerative colitis. *Cochrane Database Syst Rev* 2018; **1**: CD011450 [PMID: 29338066 DOI: 10.1002/14651858.CD011450.pub2]
21 **Khanna R**, Nelson SA, Feagan BG, D'Haens G, Sandborn WJ, Zou GY, MacDonald JK, Parker CE, Jairath V, Levesque BG. Endoscopic scoring indices for evaluation of disease activity in Crohn's disease. *Cochrane Database Syst Rev* 2016; CD010642 [PMID: 27501379 DOI: 10.1002/14651858.CD010642.pub2]
22 **Travis SP**, Schnell D, Krzeski P, Abreu MT, Altman DG, Colombel JF, Feagan BG, Hanauer SB, Lichtenstein GR, Marteau PR, Reinisch W, Sands BE, Yacyshyn BR, Schnell P, Bernhardt CA, Mary JY, Sandborn WJ. Reliability and initial validation of the ulcerative colitis endoscopic index of severity. *Gastroenterology* 2013; **145**: 987-995 [PMID: 23891974 DOI: 10.1053/j.gastro.2013.07.024]
23 **Daperno M**, D'Haens G, Van Assche G, Baert F, Bulois P, Maunoury V, Sostegni R, Rocca R, Pera A, Gevers A, Mary JY, Colombel JF, Rutgeerts P. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc* 2004; **60**: 505-512 [PMID: 15472670 DOI: 10.1016/S0016-5107(04)01878-4]
24 **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.2012.01.010]
25 **Annese V**, Daperno M, Rutter MD, Amiot A, Bossuyt P, East J, Ferrante M, Götz M, Katsanos KH, Kießlich R, Ordás I, Repici A, Rosa B, Sebastian S, Kucharzik T, Eliakim R; European Crohn's and Colitis Organisation. European evidence based consensus for endoscopy in inflammatory bowel disease. *J Crohns Colitis* 2013; **7**: 982-1018 [PMID: 24184171 DOI: 10.1016/j.crohns.2013.09.016]
26 **Farraye FA**, Odze RD, Eaden J, Itzkowitz SH. AGA technical review on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease. *Gastroenterology* 2010; **138**: 746-774, 774.e1-e4; quiz e12-e13 [PMID: 20141809 DOI: 10.1053/j.gastro.2009.12.035]
27 **Soetikno R**, Kaltenbach T, McQuaid KR, Subramanian V, Kumar R, Barkun AN, Laine L. Paradigm Shift in the Surveillance and Management of Dysplasia in Inflammatory Bowel Disease (West). *Dig Endosc* 2016; **28**: 266-273 [PMID: 26866420 DOI: 10.1111/den.12634]
28 **Laine L**, Kaltenbach T, Barkun A, McQuaid KR, Subramanian V, Soetikno R; SCENIC Guideline Development Panel. SCENIC international consensus statement on surveillance and management of dysplasia in inflammatory bowel disease. *Gastroenterology* 2015; **148**: 639-651.e28 [PMID: 25702852 DOI: 10.1053/j.gastro.2015.01.031]
29 **Kaltenbach T**, Sandborn WJ. Endoscopy in inflammatory bowel disease: advances in dysplasia detection and management. *Gastrointest Endosc* 2017; **86**: 962-971 [PMID: 28987547 DOI: 10.1016/j.gie.2017.09.030]
30 **Shen B**, Achkar JP, Lashner BA, Ormsby AH, Remzi FH, Bevins CL, Brzezinski A, Petras RE, Fazio VW. Endoscopic and histologic evaluation together with symptom assessment are required to diagnose pouchitis. *Gastroenterology* 2001; **121**: 261-267 [PMID: 11487535 DOI: 10.1053/gast.2001.26290]
31 **Shen B**, Lian L, Kiran RP, Queener E, Lavery IC, Fazio VW, Remzi FH. Efficacy and safety of endoscopic treatment of ileal pouch strictures. *Inflamm Bowel Dis* 2011; **17**: 2527-2535 [PMID: 21351202 DOI: 10.1002/ibd.21644]
32 **Van Assche G**, Dignass A, Reinisch W, van der Woude CJ, Sturm A, De Vos M, Guslandi M, Oldenburg B, Dotan I, Marteau P, Ardizzone A, Baumgart DC, D'Haens G, Gionchetti P, Portela F, Vucelic B, Söderholm J, Escher J, Koletzko S, Kolho KL, Lukas M, Mottet C, Tilg H, Vermeire S, Carbonnel F, Cole A, Novacek G, Reinshagen M, Tsianos E, Herrlinger K, Oldenburg B, Bouhnik Y, Kiesslich R, Stange E, Travis S, Lindsay J; European Crohn's and Colitis Organisation (ECCO). The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Special situations. *J Crohns Colitis* 2010; **4**: 63-101 [PMID: 21122490 DOI: 10.1016/j.crohns.2009.09.009]
33 **Nguyen GC**, Loftus EV Jr, Hirano I, Falck-Ytter Y, Singh S, Sultan S; AGA Institute Clinical Guidelines Committee. American Gastroenterological Association Institute Guideline on the Management of Crohn's Disease After Surgical Resection. *Gastroenterology* 2017; **152**: 271-275 [PMID: 27840074 DOI: 10.1053/j.gastro.2016.10.038]
34 **Rutgeerts P**, Geboes K, Vantrappen G, Kerremans R, Coenegrachts JL, Coremans G. Natural history of recurrent Crohn's disease at the ileocolonic anastomosis after curative surgery. *Gut* 1984; **25**:

665-672 [PMID: 6735250 DOI: 10.1136/gut.25.6.665]
35 **Rutgeerts P**, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963 [PMID: 2394349 DOI: 10.1016/0016-5085(90)90613-6]
36 **Colombel JF**, Narula N, Peyrin-Biroulet L. Management Strategies to Improve Outcomes of Patients With Inflammatory Bowel Diseases. *Gastroenterology* 2017; **152**: 351-361.e5 [PMID: 27720840 DOI: 10.1053/j.gastro.2016.09.046]
37 **Cosnes J**, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, Gendre JP. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis* 2002; **8**: 244-250 [PMID: 12131607 DOI: 10.1097/00054725-200207000-00002]
38 **Gustavsson A**, Magnuson A, Blomberg B, Andersson M, Halfvarson J, Tysk C. Endoscopic dilation is an efficacious and safe treatment of intestinal strictures in Crohn's disease. *Aliment Pharmacol Ther* 2012; **36**: 151-158 [PMID: 22612326 DOI: 10.1111/j.1365-2036.2012.05146.x]
39 **Chen M**, Shen B. Endoscopic Therapy in Crohn's Disease: Principle, Preparation, and Technique. *Inflamm Bowel Dis* 2015; **21**: 2222-2240 [PMID: 26284298 DOI: 10.1097/MIB.0000000000000433]
40 **Rieder F**, Latella G, Magro F, Yuksel ES, Higgins PD, Di Sabatino A, de Bruyn JR, Rimola J, Brito J, Bettenworth D, van Assche G, Bemelman W, d'Hoore A, Pellino G, Dignass AU. European Crohn's and Colitis Organisation Topical Review on Prediction, Diagnosis and Management of Fibrostenosing Crohn's Disease. *J Crohns Colitis* 2016; **10**: 873-885 [PMID: 26928961 DOI: 10.1093/ecco-jcc/jjw055]
41 **Navaneethan U**, Lourdusamy V, Njei B, Shen B. Endoscopic balloon dilation in the management of strictures in Crohn's disease: a systematic review and meta-analysis of non-randomized trials. *Surg Endosc* 2016; **30**: 5434-5443 [PMID: 27126619 DOI: 10.1007/s00464-016-4902-1]
42 **Fumery M**, Patel NS, Boland BS, Dulai PS, Singh S, Sandborn WJ. Efficacy and Safety of Endoscopic Balloon Dilatation of Ileoanal Pouch Strictures. *Inflamm Bowel Dis* 2018; **24**: 1316-1320 [PMID: 29697797 DOI: 10.1093/ibd/izy006]
43 **Lan N**, Shen B. Endoscopic Stricturotomy with Needle Knife in the Treatment of Strictures from Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2017; **23**: 502-513 [PMID: 28296818 DOI: 10.1097/MIB.0000000000001044]
44 **Loras C**, Pérez-Roldan F, Gornals JB, Barrio J, Igea F, González-Huix F, González-Carro P, Pérez-Miranda M, Espinós JC, Fernández-Bañares F, Esteve M. Endoscopic treatment with self-expanding metal stents for Crohn's disease strictures. *Aliment Pharmacol Ther* 2012; **36**: 833-839 [PMID: 22966851 DOI: 10.1111/apt.12039]
45 **Rutter MD**, Saunders BP, Wilkinson KH, Rumbles S, Schofield G, Kamm MA, Williams CB, Price AB, Talbot IC, Forbes A. Cancer surveillance in longstanding ulcerative colitis: endoscopic appearances help predict cancer risk. *Gut* 2004; **53**: 1813-1816 [PMID: 15542520 DOI: 10.1136/gut.2003.038505]
46 **Schwartz DA**, Wiersema MJ, Dudiak KM, Fletcher JG, Clain JE, Tremaine WJ, Zinsmeister AR, Norton ID, Boardman LA, Devine RM, Wolff BG, Young-Fadok TM, Diehl NN, Pemberton JH, Sandborn WJ. A comparison of endoscopic ultrasound, magnetic resonance imaging, and exam under anesthesia for evaluation of Crohn's perianal fistulas. *Gastroenterology* 2001; **121**: 1064-1072 [PMID: 11677197 DOI: 10.1053/gast.2001.28676]
47 **Shen B**. Exploring endoscopic therapy for the treatment of Crohn's disease-related fistula and abscess. *Gastrointest Endosc* 2017; **85**: 1133-1143 [PMID: 28153572 DOI: 10.1016/j.gie.2017.01.025]
48 **Kochhar G**, Shen B. Endoscopic fistulotomy in inflammatory bowel disease (with video). *Gastrointest Endosc* 2018; **88**: 87-94 [PMID: 29501418 DOI: 10.1016/j.gie.2018.02.034]

P- Reviewer: Dhaliwal HS, Ogata H, Sergi CM
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4021-4027

DOI: 10.3748/wjg.v24.i35.4021

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

MINIREVIEWS

Biosimilars in paediatric inflammatory bowel disease

Joanna Sieczkowska-Golub, Dorota Jarzebicka, Grzegorz Oracz, Jaroslaw Kierkus

Joanna Sieczkowska-Golub, Dorota Jarzebicka, Grzegorz Oracz, Jaroslaw Kierkus, The Department of Gastroenterology, Hepatology, Feeding Disorders and Paediatrics, The Children's Memorial Health Institute, Warsaw 04-730, Poland

ORCID number: Joanna Sieczkowska-Golub (0000-0003-1381-0742); Dorota Jarzebicka (0000-0002-3977-7541); Grzegorz Oracz (0000-0003-1938-7519); Jaroslaw Kierkus (0000-0003-2272-1581).

Author contributions: All authors contributed equally to this paper including concept and design, literature review and analysis, preparation of the draft manuscript, critical revision and editing, and approval of the final version.

Conflict-of-interest statement: Kierkus J has received speaker fees from Egis and AbbVie.

Manuscript source: Invited manuscript

Correspondence to: Jaroslaw Kierkus, MD, PhD, Full Professor, The Department of Gastroenterology, Hepatology, Feeding Disorders and Paediatrics, The Children's Memorial Health Institute, Aleja Dzieci Polskich 20, Warsaw 04-730, Poland. j.kierkus@med-net.pl
Telephone: +48-22-8157392
Fax: +48-22-8157382

Received: June 7, 2018
Peer-review started: June 7, 2018
First decision: July 12, 2018
Revised: August 14, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 21, 2018

Abstract

The introduction of biological treatments has changed disease outcomes for patients with inflammatory bowel disease. Biologicals have high efficacy, and can induce and maintain remission after failed responses to conventional immunosuppressive and/or steroid therapy. The increasing occurrence of severe disease at diagnosis has resulted in infliximab being more often introduced as the first-line treatment in a "top-down" approach. Besides their favourable efficacy and safety profile, biologicals have one significant disadvantage, which is their high cost. This results in many patients stopping therapy prematurely, with the maintenance phase being too short. This often leads to disease exacerbation shortly after treatment cessation. Every newly started course of biological therapy can induce production of anti-drug antibodies, which can result in treatment failure and possible allergic/anaphylactic reactions. The introduction of biological biosimilars was intended to greatly reduce therapy costs thus increasing the availability of these agents to more patients. It was also anticipated that biosimilars would prevent premature termination of therapy. Analyses of paediatric data suggest that biosimilar infliximabs are equally effective as the reference infliximab. Safety patterns also seem to be similar. Paediatric experience places cost-therapy reductions at around 10%-30%.

Key words: Biosimilars; Paediatric inflammatory bowel disease; Infliximab; Biological treatment; Crohn's disease; Ulcerative colitis

Core tip: Data on the use of biosimilars among paediatric patients are limited. Nevertheless, several original papers support adult findings that biosimilars are as effective and safe as the reference infliximab in this population.

Sieczkowska-Golub J, Jarzebicka D, Oracz G, Kierkus J. Biosimilars

in paediatric inflammatory bowel disease. *World J Gastroenterol* 2018; 24(35): 4021-4027 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4021.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4021

INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease (CD), ulcerative colitis (UC), and unclassified colitis. These diseases are characterised mainly by gastrointestinal symptoms, although extra-intestinal symptoms including general complications like mature or pubertal relapse and malnutrition can also occur. Therefore, it is very important to initiate effective treatment promptly. The introduction of biological therapies has dramatically changed treatment approaches and outcomes for patients with IBD. Biologics are medicinal products derived from living cell lines using recombinant DNA technology. At the beginning, biologics were reserved only for the most severe disease. However, the good safety profile of these agents has increasingly resulted in introduction shortly after diagnosis, especially in patients with poor prognostic factors. Early-onset IBD can have a more aggressive disease course[1,2]. Moreover, an increase in the incidence of paediatric IBD is being observed[3]. The first biologicals introduced to treat IBD patients were anti-tumour necrosis factor (TNF) inhibitors. TNFα is an inflammatory cytokine produced by immune cells. The anti-TNF-reference molecules available to treat children with IBD are adalimumab (Humira, AbbVie) and infliximab (Remicade, Janssen). Infliximab was introduced for adult patients with IBD in 1998, and was the first biological molecule used to treat this disease. In 2007, Hyams *et al*[4] reported high efficacy and safety for infliximab among paediatric CD patients. In 2012, this was also documented in children with UC[5]. The safety and efficacy of adalimumab for children was also proven prospectively by Hyams *et al*[6] Similar results have been presented in other retrospective analyses[7].

BIOSIMILARS: SIMILAR BUT NOT IDENTICAL

Biosimilars are biological products that are highly similar to the reference drugs. Their similarity needs to be proven in terms of their characteristics, biological activity, immunogenicity, efficacy and safety. Biosimilars cannot be viewed as generics because generics must be identical to the reference products. In 2013, after the licences for infliximab had expired, the first biosimilar for IBD approved by the European Medicinal Agency (EMA) was biosimilar infliximab under the brand names Remsima (Celltrion, Inc, Incheon, South Korea) and Inflectra (Pfizer, New York, NY, United States). In April 2016, the Food and Drug Administration (FDA) also approved the use of biosimilars. To be approved, all new biologics require physicochemical analyses, animal studies, clinical evaluations and clinical trials for each proposed indication. The approval pathway is concerned mostly about clinical trials to confirm safety and efficacy. For approval of biosimilars, structural, analytical and *in vitro* similarity must be shown. A clinical trial is sufficient to prove conformity for only one indication. If equivalence is revealed, this indication can be extrapolated for all indications involving the reference drug[8]. Indeed, approval to use the biosimilar infliximab in IBD patients has been based on extrapolation. The clinical testing of biosimilar infliximab has been performed in rheumatologic diseases. A multicentre, double-blind, randomised phase Ⅰ study (PLANETAS) compared the pharmacokinetics, safety and efficacy of the reference infliximab and the biosimilar infliximab (CT-P13) in 250 anti-TNF-naive ankylosing-spondylitis patients[9]. The pharmacokinetics of both infliximab molecules were equivalent. Further, the efficacy and safety profiles were both highly similar. "PLANETRA" was a multicentre, double-blind, randomised phase Ⅲ study conducted among patients with rheumatoid arthritis[10]. The patients had concomitant therapy with methotrexate. The authors ascertained that the efficacy, safety and immunogenicity of both molecules were similar. Approval by extrapolation met with deep concern among gastroenterologists, and with reluctance to initiate use. This was reflected in the first European Crohn's and Colitis Organisation (ECCO) recommendations[11]. Similar results for rheumatology were not considered sufficiently conclusive to ensure the safety and effectiveness of biosimilars in IBD patients. There was a suspicion that the different mechanisms of anti-TNF action, and especially the concomitant therapy used for rheumatic disease, might change the appearance of antibodies. Thus, the work undertaken in rheumatological conditions would not be suitable for proving the safety and efficacy of new biosimilars in IBD, especially for children. Non-clinical *in vitro* studies on CT-P13 highlighted the differences in FcgRⅢa-receptor binding, and in antibody-dependent cell-mediated cytotoxicity from the reference infliximab molecule[12]. Although the differences were considered to be clinically insignificant in IBD patients, the problem was widely discussed in the context of patient safety and treatment efficacy[13,14]. An interesting study describing biological activities of CT-P13 and the reference infliximab has been published recently. Lim *et al*[15] used especially produced intestinal cells stimulated by a mixture of cytokines to start the inflammatory process to determine whether both drugs had similar functions *in vitro*. The research design included varying evaluations of the supposed anti-TNF action. Firstly, the suppression of pro-inflammatory cytokine secretion was detected. TNFα mobilised immune cells to the inflammatory site, which induced the extrication of inflammatory cytokines and mediators from epithelial and immune cells. Infliximab neutralised and inhibited soluble-TNFα, which had the effect of diminishing the expression of mediators[16]. Lim *et al*[15] detected that inhibition of the secretion of the pro-inflammatory cytokines, interleukin (IL)-6 and IL-8,

Table 1 Treatment of Crohn's disease paediatric patients with biosimilar infliximab

Study	Number of patients	PCDAI before treatment	Time of assessment after induction	Remission (%)
Richmond *et al*[21]	29	27.5 (7.5-55)	12 wk	67
Chanchlani *et al*[19]	29	28 (20, 40)	3 mo	79
Sieczkowska-Golub *et al*[20]	36	52.5	14 wk	67

PCDAI: Paediatric Crohn's Disease Activity Index.

was similar with both infliximab forms. This research group also evaluated how neutralisation of soluble TNFα induced apoptosis of intestinal epithelial cells. The induction of apoptosis in monocytes and lymphocytes by infliximab is a significant action because of the diminished cytokine release, which leads to blockade of the inflammatory response[17]. The action of infliximab in neutralising soluble TNFα suppresses intestinal epithelial apoptosis. For this, both infliximab forms were shown to work similarly. Another comparison aim was examination of apoptosis and cytokine suppression, which is induced by reverse signalling when infliximab molecules bind to transmembrane TNFα. The authors detected that the infliximabs had similar ability to induce apoptosis, and both molecules demonstrated congruous dose-dependent cytokine suppression. Other tests revealed similar results for both infliximab molecules in the case of the promotion of regulatory macrophages. Based on their analyses, the authors verified an insignificant difference in antibody-dependent cell-mediated cytotoxicity[15].

A STATEMENT OF BIOSIMILAR USE IN PAEDIATRICS

Shortly after biosimilars became available on the market, a statement was released about their use by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the Paediatric IBD Porto Group. The paper was an expert opinion directed at paediatric gastroenterologists taking care of children with IBD. Because of the lack of clinical trials among IBD patients, the paper could not be regarded as containing strict recommendations, and was summarised in three propositions. Firstly, the authors advocated giving high priority to conducting paediatric trials with long-term follow-up, to support EMA decisions on biosimilar approvals for paediatric IBD. Secondly, the experts did not recommend switching patients to a biosimilar during sustained remission until clinical trials verifying the safety and efficacy of biosimilars in IBD were available. Thirdly, all participants agreed that post-marketing surveillance programmes measuring the efficacy, safety and immunogenicity in children with IBD, should be a mandatory requirement for the marketing of biologics and biosimilars for specific indications[18].

INFLIXIMAB BIOSIMILAR EXPERIENCES

Induction and maintenance therapy with biosimilar infliximab

Regardless of the above-mentioned statement, in some centres biosimilars were used in paediatric IBD patients, mainly due to the unavailability of the originator molecule. Nevertheless, there are only a few studies reporting the use of biosimilars in children. A recently published multi-centre study involved 278 paediatric patients, who started infliximab-reference therapy (n = 175), infliximab biosimilars (n = 82) or adalimumab (n = 21). Unfortunately, in assessing infliximab efficacy with the Paediatric Crohn's Disease Activity Index (PCDAI) score, only 24% (42/175) of the reference infliximab patients were assessed at baseline along with 35% (29/82) of the biosimilar infliximab group. At the 3-mo follow-up, the PCDAI scores were known only for 11% (19/175) and 18% (15/82) of the reference and biosimilar groups, respectively. Most of the reference infliximab (28/33 *i.e.*, 85%) and biosimilar (19/22 *i.e.*, 86%) groups presented with a response. Remission was achieved in 25/37 (68%) and 19/24 (79%), respectively. Some of the patients had their disease severity assessed by Physician's Global Assessment (PGA), with an improvement also observed. Among this cohort no unexpected adverse events occurred, but six of the 121 (5%) patients assessed at the 3-mo follow-up experienced various conditions including a rash (2 patients), fever (2 patients), blood abnormality (1 patient) and difficulty in breathing (1 patient)[19] (Table 1). A study conducted among Polish paediatric patients assessed the induction efficacy of the biosimilar infliximab. The assessment involved 36 patients from three hospitals. Three induction doses were administered to 34/36 (94.4%) patients. Fourteen weeks after the first biosimilar dose was given, a clinical response was achieved in 31/36 (86%) patients, and remission in 24/36 (67%). Only one allergic reaction was reported during the drug infusion. Mild adverse events occurred, mainly upper-respiratory tract infections. No serious adverse events were observed[20] (Table 1). Two of the above-mentioned studies compared their results to historical work in similar patient cohorts treated with the reference infliximab, with similar findings. The authors reported a similar efficacy and safety profile among both cohorts, treated with biosimilar and reference infliximabs. A study from the United Kingdom used 40 paediatric patients, most of whom were naïve for anti-TNF treatment. The cohort consisted of 29 CD and 11 UC patients, with almost all (95%) receiving concomitant therapy. The biosimilar infliximab induced remission in 14/21 (67%) patients. Significant decreases in PCDAI were observed. One patient presented with an infusion-associated allergic reaction[21] (Table 1). As with the Polish cohort patients with allergic reactions, their reactions had been affected by prior exposure to originator infliximab.

Table 2 Switching experiences among paediatric inflammatory bowel disease patients

Study	Number of patients	Time of follow up	Remission (%)
Kang *et al*[25]	32 CD and 6 UC	1 yr	78.9
Sieczkowska *et al*[24]	32 CD and 7 UC	8 ± 2.6 mo (range 2-11) for CD	88
		5 ± 3.6 mo (range 0-9) for UC	57

CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Biosimilars approved by the European Medicinal Agency in use among inflammatory bowel disease patients[38-40]

Name of biosimilar	Manufacturer	Date of approval
Infliximab		
Flixabi	Samsung Bioepis	May-16
Inflextra	Hospira	Sep-13
Remsima	Celtrion	Sep-13
Adalimumab		
Amgevita	Amgen	Jan-17
Cyltezo	Boehringer Ingelheim	Sep-17
Hyrimoz	Sandoz	Jun-18
Imraldi	Samsung Bioepis	June 20016
Solymbic	Amgen	Jan-17

The paediatric data are supported by studies in adults. To date, studies assessing the efficacy and safety of biosimilars have primarily been conducted among adult patients. Komaki *et al*[22] presented a systematic review of 829 IBD patients from 11 observational studies. The patients either received biosimilar therapy from the beginning or were switched from the reference infliximab. The authors concluded that both infliximab molecules were highly similar in terms of safety and efficacy. Another systematic review, which aggregated data from 11 observational studies, was carried out by Radin *et al*[23]. A total of 1007 patients were included. The authors did not observe any significant difference in efficacy or safety between the reference infliximab and the biosimilar CT-P13. There are no data on use of the new biosimilar infliximab Flixabi (Samsung Bioepis, United Kingdom) among children.

Switching

Only two studies in paediatric patients reported their experiments involving changing the infliximab molecule during the same course of therapy (Table 2). The first study, conducted in Poland, concerned 39 patients, 32 of whom had CD while 7 had UC. All the patients who were over 16 years of age, and all parents needed to consent to continuation of therapy with biosimilars, due to the unavailability of the reference molecule. The young people had the drug change at different times in the maintenance phase, with none presenting during follow-up with disease exacerbation after biosimilar introduction. No serious adverse events occurred, and the incidence of mild adverse events did not differ before and after drug change[24] (Table 2). The second study, conducted in Korea, was a comparison of patients after the switch to biosimilars with those remaining on the reference infliximab therapy. The 74 patients were divided into two groups (the reference and biosimilar groups) who were followed-up for one year after therapy. Decisions on the treatment types were made by the patients and their guardians. The reference infliximab group comprised 36 patients (28 with CD and 8 with UC), while 38 (32 with CD and 6 with UC) elected to switch to CT-P13. Maintenance therapy of one-year duration was continued by 86.1% of the patients with the reference infliximab, and 92.1% with the biosimilar. Sustained remission was attained in 28/36 (77.8%) of the patients on the reference infliximab and 30/38 (78.9%) of the patients in the CT-P13-switch group. Eight of the patients taking part in the study did not finish the year of follow-up. Three achieved total remission and did not wish to continue with further therapy, three needed to change to adalimumab due to loss of response, and two were lost to follow-up. No serious adverse events or infusion-related reactions were observed[25] (Table 2). Several studies of adults assessed patients around the time of switching. Most of them aimed at assessing disease activity, safety and immunogenicity. None of the adult studies reported worsening disease after switching[26-31].

BIOSIMILAR ADALIMUMAB

The first biosimilar adalimumab appeared in India and was named ZRC-3197 (Exemptia - Zydus Cadila Healthcare Ltd.). A prospective, randomised, double-blind, multi-centre, parallel-group, active, controlled study among rheumatoid arthritis patients confirmed that the biosimilar adalimumab was similarly effective and tolerated as the reference molecule[32]. The licence for adalimumab in Europe expired. The currently available biosimilar substances approved by the EMA and FDA are presented in Tables 3 and 4. Many new biosimilars are in the pipeline[33]. To date, there appears to be no paper published on the use of the biosimilar adalimumab for IBD.

CHANGES IN KNOWLEDGE OF BIOSIMILARS

One year after the biosimilar infliximab became available clinically, Danese *et al*[34] conducted an anonymous survey of gastroenterologists to assess their knowledge and perception about the biosimilars that were emer-

Table 4 Biosimilars approved by the Food and Drug Administration in use among inflammatory bowel disease patients[41]

Name	Brand name	Date of licensure
Infliximab		
Infliximab-abda	Renflexis	Apr-17
Infliximab-dyyb	Inflectra	Apr-16
Infliximab-qbtx	Ixifi	Dec-17
Adalimumab		
Adalimumab-adbm	Cyltezo	Aug-17
Adalimumab-atto	Amjevita	Sep-16

ging. Within the time frame from the appearance of the biosimilars to the analysis, there was a change in opinion as ascertained by observational studies and personal experience. Among all the participants, 83% had access to biosimilars. One year after the introduction of biosimilars, only 19.5% of the respondents felt no confidence in them. This reflected a substantial drop compared with the 63% reported in 2013. There were no significant changes in the granting of permission for automatic substitution (89.8% *vs* 84.8% in 2013). The change of mind was about interchangeability. Only 5.9% of respondents in 2013 thought that the two infliximab forms were interchangeable compared with 44.4% in the survey. In 2013, 72.3% would not switch during maintenance therapy. Currently, 39.9% would not switch, due to concerns about insufficient safety data[34]. Apprehension about interchangeability is still high, especially as more new biosimilar molecules might soon be available.

COST SAVINGS

The high efficacy and safety of biologics makes them the preferred therapy type. The main limitation of their use is high cost. Because of the expense of therapy, biologics are usually used in the most severe disease forms. Furthermore, therapy is often discontinued too early due to cost limitations. The introduction of biosimilars raised great expectations regarding reductions in therapy costs[35-37]. Cost reductions bring enormous benefits through making treatment available to a greater number of patients, and increasing the possibility of a long maintenance phase. In two paediatric studies, calculations of cost reductions were done. In a Scottish paediatric study, the average cost reduction was around 38%[21]. A recently published United Kingdom study confirmed savings during one-year of therapy of around 10%-30%[19].

CONCLUSION

To date, published data on paediatric IBD remain limited. Nevertheless, the above-mentioned studies show that the efficacy and safety of biosimilars and the originator infliximab are similar. The results are comparable to data on adults.

REFERENCES

1 **Pigneur B**, Seksik P, Viola S, Viala J, Beaugerie L, Girardet JP, Ruemmele FM, Cosnes J. Natural history of Crohn's disease: comparison between childhood- and adult-onset disease. *Inflamm Bowel Dis* 2010; **16**: 953-961 [PMID: 19834970 DOI: 10.1002/ibd.21152]

2 **Van Limbergen J**, Russell RK, Drummond HE, Aldhous MC, Round NK, Nimmo ER, Smith L, Gillett PM, McGrogan P, Weaver LT, Bisset WM, Mahdi G, Arnott ID, Satsangi J, Wilson DC. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. *Gastroenterology* 2008; **135**: 1114-1122 [PMID: 18725221 DOI: 10.1053/j.gastro.2008.06.081]

3 **Benchimol EI**, Fortinsky KJ, Gozdyra P, Van den Heuvel M, Van Limbergen J, Griffiths AM. Epidemiology of pediatric inflammatory bowel disease: a systematic review of international trends. *Inflamm Bowel Dis* 2011; **17**: 423-439 [PMID: 20564651 DOI: 10.1002/ibd.21349]

4 **Hyams J**, Crandall W, Kugathasan S, Griffiths A, Olson A, Johanns J, Liu G, Travers S, Heuschkel R, Markowitz J, Cohen S, Winter H, Veereman-Wauters G, Ferry G, Baldassano R; REACH Study Group. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. *Gastroenterology* 2007; **132**: 863-873; quiz 1165-1166 [PMID: 17324398 DOI: 10.1053/j.gastro.2006.12.003]

5 **Hyams J**, Damaraju L, Blank M, Johanns J, Guzzo C, Winter HS, Kugathasan S, Cohen S, Markowitz J, Escher JC, Veereman-Wauters G, Crandall W, Baldassano R, Griffiths A; T72 Study Group. Induction and maintenance therapy with infliximab for children with moderate to severe ulcerative colitis. *Clin Gastroenterol Hepatol* 2012; **10**: 391-399.e1 [PMID: 22155755 DOI: 10.1016/j.cgh.2011.11.026]

6 **Hyams JS**, Griffiths A, Markowitz J, Baldassano RN, Faubion WA Jr, Colletti RB, Dubinsky M, Kierkus J, Rosh J, Wang Y, Huang B, Bittle B, Marshall M, Lazar A. Safety and efficacy of adalimumab for moderate to severe Crohn's disease in children. *Gastroenterology* 2012; **143**: 365-374.e2 [PMID: 22562021 DOI: 10.1053/j.gastro.2012.04.046]

7 **Russell RK**, Wilson ML, Loganathan S, Bourke B, Kiparissi F, Mahdi G, Torrente F, Rodrigues A, Davies I, Thomas A, Akobeng AK, Fagbemi A, Hyer W, Spray C, Vaish S, Rogers P, McGrogan P, Heuschkel RB, Ayub N, Fell JM, Afzal NA, Green M, Murphy MS, Rao P, Shah N, Ho GT, Naik S, Wilson DC. A British Society of Paediatric Gastroenterology, Hepatology and Nutrition survey of the effectiveness and safety of adalimumab in children with inflammatory bowel disease. *Aliment Pharmacol Ther* 2011; **33**: 946-953 [PMID: 21342211 DOI: 10.1111/j.1365-2036.2011.04603.x]

8 **Chang S**, Hanauer S. Extrapolation and Interchangeability of Infliximab and Adalimumab in Inflammatory Bowel Disease. *Curr Treat Options Gastroenterol* 2017; **15**: 53-70 [PMID: 28164249 DOI: 10.1007/s11938-017-0122-6]

9 **Park W**, Hrycaj P, Jeka S, Kovalenko V, Lysenko G, Miranda P, Mikazane H, Gutierrez-Ureña S, Lim M, Lee YA, Lee SJ, Kim H, Yoo DH, Braun J. A randomised, double-blind, multicentre, parallel-group, prospective study comparing the pharmacokinetics, safety, and efficacy of CT-P13 and innovator infliximab in patients with ankylosing spondylitis: the PLANETAS study. *Ann Rheum Dis* 2013; **72**: 1605-1612 [PMID: 23687259 DOI: 10.1136/annrheumdis-2012-203091]

10 **Yoo DH**, Hrycaj P, Miranda P, Ramiterre E, Piotrowski M, Shevchuk S, Kovalenko V, Prodanovic N, Abello-Banfi M, Gutierrez-Ureña S, Morales-Olazabal L, Tee M, Jimenez R, Zamani O, Lee SJ, Kim H, Park W, Müller-Ladner U. A randomised, double-blind, parallel-group study to demonstrate equivalence in efficacy and safety of CT-P13 compared with innovator infliximab when coadministered with methotrexate in patients with active rheumatoid arthritis: the PLANETRA study. *Ann Rheum Dis* 2013; **72**: 1613-1620 [PMID: 23687260 DOI: 10.1136/annrheumdis-2012-203090]

11 **Danese S**, Gomollon F; Governing Board and Operational Board of ECCO. ECCO position statement: the use of biosimilar medicines in the treatment of inflammatory bowel disease (IBD). *J Crohns Colitis* 2013; **7**: 586-589 [PMID: 23623738 DOI: 10.1016/j.crohns.2013.03.011]

12 **Jung SK**, Lee KH, Jeon JW, Lee JW, Kwon BO, Kim YJ, Bae JS, Kim DI, Lee SY, Chang SJ. Physicochemical characterization of Remsima. *MAbs* 2014; **6**: 1163-1177 [PMID: 25517302 DOI: 10.4161/mabs.32221]

13 **Russell RK,** Irving P, Probert C. Biosimilars: what's around the corner? *Frontline Gastroenterol* 2015; **6**: 262-263 [PMID: 28839821 DOI: 10.1136/flgastro-2015-100587]

14 **Ha CY**, Kornbluth A. A Critical Review of Biosimilars in IBD: The Confluence of Biologic Drug Development, Regulatory Requirements, Clinical Outcomes, and Big Business. *Inflamm Bowel Dis* 2016; **22**: 2513-2526 [PMID: 27564646 DOI: 10.1097/MIB.0000000000000886]

15 **Lim KJ**, Lee SJ, Kim S, Lee SY, Lee MS, Park YA, Choi EJ, Lee EB, Jun HK, Cho JM, Lee S, Kwon KS, Lim BP, Jeon MS, Shin EC, Choi YS, Fudim E, Picard O, Yavzori M, Ben-Horin S, Chang SJ. Comparable Immune Function Inhibition by the Infliximab Biosimilar CT-P13: Implications for Treatment of Inflammatory Bowel Disease. *J Crohns Colitis* 2017; **11**: 593-602 [PMID: 28453766 DOI: 10.1093/ecco-jcc/jjw183]

16 **Guo Y**, Lu N, Bai A. Clinical use and mechanisms of infliximab treatment on inflammatory bowel disease: a recent update. *Biomed Res Int* 2013; **2013**: 581631 [PMID: 23484133 DOI: 10.1155/2013/581631]

17 **Zeissig S**, Bojarski C, Buergel N, Mankertz J, Zeitz M, Fromm M, Schulzke JD. Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor alpha antibody treatment. *Gut* 2004; **53**: 1295-1302 [PMID: 15306588 DOI: 10.1136/gut.2003.036632]

18 **de Ridder L**, Waterman M, Turner D, Bronsky J, Hauer AC, Dias JA, Strisciuglio C, Ruemmele FM, Levine A, Lionetti P; ESPGHAN Paediatric IBD Porto Group. Use of Biosimilars in Paediatric Inflammatory Bowel Disease: A Position Statement of the ESPGHAN Paediatric IBD Porto Group. *J Pediatr Gastroenterol Nutr* 2015; **61**: 503-508 [PMID: 26154031 DOI: 10.1097/MPG.0000000000000903]

19 **Chanchlani N**, Mortier K, Williams LJ, Muhammed R, Auth MKH, Cosgrove M, Fagbemi A, Fell J, Chong S, Zamvar V, Hyer W, Bisset WM, Morris MA, Rodrigues A, Mitton SG, Bunn S, Beattie RM, Willmott A, Wilson DC, Russell RK. Use of Infliximab Biosimilar Versus Originator in a Paediatric United Kingdom Inflammatory Bowel Disease Induction Cohort. *J Pediatr Gastroenterol Nutr* 2018 [PMID: 29697550 DOI: 10.1097/MPG.0000000000002011]

20 **Sieczkowska-Golub J**, Meglicka M, Plocek A, Banaszkiewicz A, Jarzębicka D, Toporowska-Kowalska E, Gawronska A, Oracz G, Kierkus J. Induction Therapy With Biosimilar Infliximab in Children With Crohn Disease. *J Pediatr Gastroenterol Nutr* 2017; **65**: 285-288 [PMID: 28542043 DOI: 10.1097/MPG.0000000000001643]

21 **Richmond L**, Curtis L, Garrick V, Rogers P, Wilson M, Tayler R, Henderson P, Hansen R, Wilson DC, Russell RK. Biosimilar infliximab use in paediatric IBD. *Arch Dis Child* 2018; **103**: 89-91 [PMID: 28988215 DOI: 10.1136/archdischild-2017-313404]

22 **Komaki Y**, Yamada A, Komaki F, Micic D, Ido A, Sakuraba A. Systematic review with meta-analysis: the efficacy and safety of CT-P13, a biosimilar of anti-tumour necrosis factor-α agent (infliximab), in inflammatory bowel diseases. *Aliment Pharmacol Ther* 2017; **45**: 1043-1057 [PMID: 28239873 DOI: 10.1111/apt.13990]

23 **Radin M**, Sciascia S, Roccatello D, Cuadrado MJ. Infliximab Biosimilars in the Treatment of Inflammatory Bowel Diseases: A Systematic Review. *BioDrugs* 2017; **31**: 37-49 [PMID: 28035633 DOI: 10.1007/s40259-016-0206-1]

24 **Sieczkowska J**, Jarzębicka D, Banaszkiewicz A, Plocek A, Gawronska A, Toporowska-Kowalska E, Oracz G, Meglicka M, Kierkus J. Switching Between Infliximab Originator and Biosimilar in Paediatric Patients with Inflammatory Bowel Disease. Preliminary Observations. *J Crohns Colitis* 2016; **10**: 127-132 [PMID: 26721942 DOI: 10.1093/ecco-jcc/jjv233]

25 **Kang B**, Lee Y, Lee K, Choi YO, Choe YH. Long-term Outcomes After Switching to CT-P13 in Pediatric-Onset Inflammatory Bowel Disease: A Single-Center Prospective Observational Study. *Inflamm Bowel Dis* 2018; **24**: 607-616 [PMID: 29390113 DOI: 10.1093/ibd/izx047]

26 **Razanskaite V**, Bettey M, Downey L, Wright J, Callaghan J, Rush M, Whiteoak S, Ker S, Perry K, Underhill C, Efrem E, Ahmed I, Cummings F. Biosimilar Infliximab in Inflammatory Bowel Disease: Outcomes of a Managed Switching Programme. *J Crohns Colitis* 2017; **11**: 690-696 [PMID: 28130330 DOI: 10.1093/ecco-jcc/jjw216]

27 **Smits LJ**, Derikx LA, de Jong DJ, Boshuizen RS, van Esch AA, Drenth JP, Hoentjen F. Clinical Outcomes Following a Switch from Remicade® to the Biosimilar CT-P13 in Inflammatory Bowel Disease Patients: A Prospective Observational Cohort Study. *J Crohns Colitis* 2016; **10**: 1287-1293 [PMID: 27095751 DOI: 10.1093/ecco-jcc/jjw087]

28 **Smits LJT**, Grelack A, Derikx LAAP, de Jong DJ, van Esch AAJ, Boshuizen RS, Drenth JPH, Hoentjen F. Long-Term Clinical Outcomes After Switching from Remicade® to Biosimilar CT-P13 in Inflammatory Bowel Disease. *Dig Dis Sci* 2017; **62**: 3117-3122 [PMID: 28667429 DOI: 10.1007/s10620-017-4661-4]

29 **Argüelles-Arias F**, Guerra Veloz MF, Perea Amarillo R, Vilches-Arenas A, Castro Laria L, Maldonado Pérez B, Chaaro Benallal D, Benítez Roldán A, Merino V, Ramirez G, Calleja-Hernández MA, Caunedo Álvarez A, Romero Gómez M. Switching from reference infliximab to CT-P13 in patients with inflammatory bowel disease: 12 months results. *Eur J Gastroenterol Hepatol* 2017; **29**: 1290-1295 [PMID: 28902041 DOI: 10.1097/MEG.0000000000000953]

30 **Buer LC**, Moum BA, Cvancarova M, Warren DJ, Medhus AW, Høivik ML. Switching from Remicade® to Remsima® is well Tolerated and Feasible: A Prospective, Open-label Study. *J Crohns Colitis* 2017; **11**: 297-304 [PMID: 27660339 DOI: 10.1093/ecco-jcc/jjw166]

31 **Jørgensen KK**, Olsen IC, Goll GL, Lorentzen M, Bolstad N, Haavardsholm EA, Lundin KEA, Mørk C, Jahnsen J, Kvien TK; NOR-SWITCH study group. Switching from originator infliximab to biosimilar CT-P13 compared with maintained treatment with originator infliximab (NOR-SWITCH): a 52-wk, randomised, double-blind, non-inferiority trial. *Lancet* 2017; **389**: 2304-2316 [PMID: 28502609 DOI: 10.1016/S0140-6736(17)30068-5]

32 **Jani RH**, Gupta R, Bhatia G, Rathi G, Ashok Kumar P, Sharma R, Kumar U, Gauri LA, Jadhav P, Bartakke G, Haridas V, Jain D, Mendiratta SK. A prospective, randomized, double-blind, multicentre, parallel-group, active controlled study to compare efficacy and safety of biosimilar adalimumab (Exemptia; ZRC-3197) and adalimumab (Humira) in patients with rheumatoid arthritis. *Int J Rheum Dis* 2016; **19**: 1157-1168 [PMID: 26176644 DOI: 10.1111/1756-185X.12711]

33 **Rawla P**, Sunkara T, Raj JP. Role of biologics and biosimilars in inflammatory bowel disease: current trends and future perspectives. *J Inflamm Res* 2018; **11**: 215-226 [PMID: 29844695 DOI: 10.2147/JIR.S165330]

34 **Danese S**, Fiorino G, Michetti P. Changes in Biosimilar Knowledge among European Crohn's Colitis Organization [ECCO] Members: An Updated Survey. *J Crohns Colitis* 2016; **10**: 1362-1365 [PMID: 27112706 DOI: 10.1093/ecco-jcc/jjw090]

35 **Brodszky V**, Rencz F, Péntek M, Baji P, Lakatos PL, Gulácsi L. A budget impact model for biosimilar infliximab in Crohn's disease in Bulgaria, the Czech Republic, Hungary, Poland, Romania, and Slovakia. *Expert Rev Pharmacoecon Outcomes Res* 2016; **16**: 119-125 [PMID: 26162458 DOI: 10.1586/14737167.2015.1067142]

36 **Rencz F**, Gulácsi L, Péntek M, Gecse KB, Dignass A, Halfvarson J, Gomollón F, Baji P, Peyrin-Biroulet L, Lakatos PL, Brodszky V. Cost-utility of biological treatment sequences for luminal Crohn's disease in Europe. *Expert Rev Pharmacoecon Outcomes Res* 2017; **17**: 597-606 [PMID: 28434387 DOI: 10.1080/14737167.2017.1322509]

37 **Jha A**, Upton A, Dunlop WC, Akehurst R. The Budget Impact of Biosimilar Infliximab (Remsima®) for the Treatment of Autoimmune Diseases in Five European Countries. *Adv Ther* 2015; **32**: 742-756 [PMID: 26343027 DOI: 10.1007/s12325-015-0233-1]
38 Biosimilars of Infliximab. Accessed July 20, 2018 Available form: URL: http://gabionline.net/Biosimilars/General/Biosimilars-of-infliximab
39 Biosimilars of Adalimumab. Accessed June 29, 2018 Available from: URL: http://gabionline.net/Biosimilars/General/Biosimilars-of-adalimumab
40 EMA Approves Adalimumab and Trastuzumab Biosimilars. Accessed June 8, 2018 Available from: URL: http://www.gabionline.net/Biosimilars/News/EMA-approves-adalimumab-and-trastuzumab-biosimilars
41 CDER List of Licensed Biological Products with (1) Reference Product Exclusivity and (2) Biosimilarity or Interchangeability Evaluations to Date. Accessed August 8, 2018 Available from: URL: https://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/UCM560162.pdf

P- Reviewer: Triantafillidis JK **S- Editor**: Gong ZM **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4028-4035

DOI: 10.3748/wjg.v24.i35.4028

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

ORIGINAL ARTICLE

Basic Study

Adiponectin affects the mechanical responses in strips from the mouse gastric fundus

Eglantina Idrizaj, Rachele Garella, Giovanni Castellini, Hermine Mohr, Natalia S Pellegata, Fabio Francini, Valdo Ricca, Roberta Squecco, Maria Caterina Baccari

Eglantina Idrizaj, Rachele Garella, Fabio Francini, Roberta Squecco, Maria Caterina Baccari, Department of Experimental and Clinical Medicine, Section of Physiological Sciences, University of Florence, Florence 50134, Italy

Giovanni Castellini, Valdo Ricca, Psychiatry Unit, Department of Health Sciences, University of Florence, Florence 50134, Italy

Hermine Mohr, Natalia S Pellegata, Institute for Diabetes and Cancer, Helmholtz Zentrum München, Neuherberg 85764, Germany

ORCID number: Eglantina Idrizaj (0000-0002-2756-6552); Rachele Garella (0000-0003-3194-7603); Giovanni Castellini (0000-0003-1265-491X); Hermine Mohr (0000-0002-3061-1166); Natalia S Pellegata (0000-0002-8000-7784); Fabio Francini (0000-0002-9255-1824); Valdo Ricca (0000-0002-9291-2124); Roberta Squecco (0000-0002-6534-3675); Maria Caterina Baccari (0000-0003-4665-1426).

Author contributions: Idrizaj E and Garella R contributed equally to this work; Idrizaj E and Garella R performed the functional experiments; Idrizaj E and Mohr H performed the PCR analysis; Castellini G, Ricca V, Squecco R and Baccari MC designed the research study; Pellegata NS and Francini F contributed to design the research study; Idrizaj E, Garella R and Baccari MC analyzed the data; Baccari MC wrote the paper; Idrizaj E, Garella R, Castellini G, Mohr H, Pellegata NS, Francini F, Ricca V, Squecco R and Baccari MC critically revised the manuscript.

Supported by the Florence University (No. RTD CO 090101010107; RICATEN14) and Fondazione CRF (No. 2017.0777).

Institutional animal care and use committee statement: The experimental protocol was designed in compliance with the guidelines of the European Communities Council Directive 2010/63/UE and the recommendations for the care and use of laboratory animals approved by the Animal Care Committee of the University of Florence, Italy, with authorization from the Italian Ministry of Health nr. 787/2016-PR.

Conflict-of-interest statement: No conflicts of interest, financial or otherwise, are declared by the authors.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Maria Caterina Baccari, PhD, Professor, Department of Experimental and Clinical Medicine, Section of Physiological Sciences, University of Florence, Viale G.B. Morgagni 63, Florence 50134, Italy. mcaterina.baccari@unifi.it
Telephone: +39-55-2751600
Fax: +39-55-4379506

Received: June 8, 2018
Peer-review started: June 8, 2018
First decision: July 3, 2018
Revised: July 12, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: September 21, 2018

Abstract

AIM

To investigate whether the adipocytes derived hormone adiponectin (ADPN) affects the mechanical responses in strips from the mouse gastric fundus.

METHODS

For functional experiments, gastric strips from the fundal

DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4028

region were cut in the direction of the longitudinal muscle layer and placed in organ baths containing Krebs-Henseleit solution. Mechanical responses were recorded *via* force-displacement transducers, which were coupled to a polygraph for continuous recording of isometric tension. Electrical field stimulation (EFS) was applied *via* two platinum wire rings through which the preparation was threaded. The effects of ADPN were investigated on the neurally-induced contractile and relaxant responses elicited by EFS. The expression of ADPN receptors, Adipo-R1 and Adipo-R2, was also evaluated by touchdown-PCR analysis.

RESULTS

In the functional experiments, EFS (4-16 Hz) elicited tetrodotoxin (TTX)-sensitive contractile responses. Addition of ADPN to the bath medium caused a reduction in amplitude of the neurally-induced contractile responses ($P < 0.05$). The effects of ADPN were no longer observed in the presence of the nitric oxide (NO) synthesis inhibitor L-N^{G}-nitro arginine (L-NNA) ($P > 0.05$). The direct smooth muscle response to methacholine was not influenced by ADPN ($P > 0.05$). In carbachol precontracted strips and in the presence of guanethidine, EFS induced relaxant responses. Addition of ADPN to the bath medium, other than causing a slight and progressive decay of the basal tension, increased the amplitude of the neurally-induced relaxant responses ($P < 0.05$). Touchdown-PCR analysis revealed the expression of both Adipo-R1 and Adipo-R2 in the gastric fundus.

CONCLUSION

The results indicate for the first time that ADPN is able to influence the mechanical responses in strips from the mouse gastric fundus.

Key words: Adiponectin; Adiponectin receptors; Gastric motility; Nitric oxide; Non-adrenergic, non-cholinergic neurotransmission

Core tip: Evidence exists that some white adipose-tissue derived hormones that are involved in the regulation of food intake also influence the motor responses of the gastrointestinal tract. In this view, adiponectin (ADPN) too has been reported to influence food intake but no data concerning its effects on the gastric mechanical activity are available. The present results indicate for the first time that ADPN is able to influence the mechanical responses in strips from the mouse gastric fundus. It could be speculated that these peripheral effects on motor phenomena might represent an additional mechanism engaged by the hormone to control food intake.

Idrizaj E, Garella R, Castellini G, Mohr H, Pellegata NS, Francini F, Ricca V, Squecco R, Baccari MC. Adiponectin affects the mechanical responses in strips from the mouse gastric fundus. *World J Gastroenterol* 2018; 24(35): 4028-4035 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4028.htm

INTRODUCTION

White adipose tissue is recognized as an endocrine organ[1], being able to secrete many substances, known as adipokines, that are involved in a wide range of functions[1,2]. Among them, accumulating evidence suggests that adipose-tissue derived hormones, such as leptin and adiponectin (ADPN), play an important role in the systemic metabolic homeostasis, also acting as key neuromodulators of food intake[1-6]. ADPN is a peptide hormone mainly produced by white adipose tissue although its expression has been reported also in other organs of different animal species[7], including the mouse stomach[8]. ADPN exerts its effects mainly through the activation of two seven transmembrane domain receptors, Adipo-R1 and Adipo-R2[9], whose expression has been revealed, other than in the brain, in a variety of mammalian peripheral tissues[7]. Particularly, Adipo-R2 mRNA expression has been reported in the gastrointestinal tract of rats[10].

Interestingly, many substances that act centrally to modulate food intake also influence the activity of gastrointestinal smooth muscle[11-14], whose motor phenomena represent a source of peripheral signals involved in the control of feeding behavior thought the gut-brain axis[15].

All the above knowledge are consistent with the possibility that ADPN too could affect gastrointestinal motor responses, although no data are present in the literature concerning these effects.

On this ground, in the present study we investigated whether ADPN influences the mechanical responses in strips from the mouse gastric fundus. The expression of both the ADPN receptors was also evaluated by touchdown-PCR analysis.

MATERIALS AND METHODS

Animals

Experiments were carried out on 8-12 wk old C57BL/6 female mice (Charles River, Lecco, Italy). The animals were fed standard laboratory chow and water, and were housed under a 12-h light/12-h dark (12 L : 12 D) photoperiod and controlled temperature (21 ℃± 1 ℃). The experimental protocol was designed in compliance with the guidelines of the European Communities Council Directive 2010/63/UE and the recommendations for the care and use of laboratory animals approved by the Animal Care Committee of the University of Florence, Italy, with authorization from the Italian Ministry of Health nr. 787/2016-PR. The mice were killed by prompt cervical dislocation to minimize animal suffering.

Mechanical experiments

As previously published by our research group[16,17], the

Table 1 PCR components

Reagents	Volume
Forward primer (0.01 pmol/L)	1 μL
Reverse primer (0.01 pmol/L)	1 μL
GOTaq colorless Master Mix sample (Promega®)	12.5 μL
H_2O	9.5 μL
cDNA template	1 μL

Table 2 Touchdown-PCR setup

Step	Temperature (℃)	Time	Note
1	94	3 min	-
2	94	20 s	-
3	64	30 s	-0.5 ℃ per cycle
4	72	35 s	Repeat steps 2-4 for 12 cycles
5	94	20 s	-
6	58	30 s	-
7	72	35 s	Repeat steps 5-7 for 25 cycles
8	72	2 min	-
9	4		Hold

stomach was rapidly dissected from the abdomen and two full-thickness strips (2 mm × 10 mm) were cut in the direction of the longitudinal muscle layer from each fundal region. One end of each strip was tied to a platinum rod while the other was connected to a force displacement transducer (Grass model FT03) by a silk thread for continuous recording of isometric tension. The transducer was coupled to a polygraph (7K; Grass Instrument). Muscle strips were mounted in 5 mL double-jacketed organ baths containing Krebs-Henseleit solution, which consisted of 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, and 10 mmol/L glucose pH 7.4, and bubbled with 95% O_2/5% CO_2. Prewarmed water (37 ℃) was circulated through the outer jacket of the tissue bath *via* a constant-temperature circulator pump. The temperature of the Krebs-Henseleit solution in the organ bath was maintained within ± 0.5 ℃.

Electrical field stimulation (EFS) was applied *via* two platinum wire rings (2 mm diameter, 5 mm apart) through which the preparation was threaded. Electrical impulses (rectangular waves, 80 V, 4-16 Hz, 0.5 ms, for 15 s) were provided by a Grass model S8 stimulator. Strips were allowed to equilibrate for 1 h under an initial load of 0.8 g. During this period, repeated and prolonged washes of the preparations with Krebs-Henseleit solution were done to avoid accumulation of metabolites in the organ baths.

Drugs

The following drugs were used: acetyl-β-methylcholine chloride (methacholine), recombinant full-length mouse adiponectin (ADPN), atropine sulphate, carbachol (CCh), guanethidine sulphate, L-N^G-nitro arginine (L-NNA), tetrodotoxin (TTX). All drugs were obtained from Sigma Chemical (St. Louis, MO, United States). Solutions were prepared on the day of the experiment, except for TTX and ADPN, for which stock solutions were kept stored at -20 ℃.

Drug concentrations are referred as final bath concentrations. For ADPN we started from 20 pmol/L, *i.e.*, the dose previously reported to be effective in *in vitro* gastric preparations[8]. Direct smooth muscle contractions were obtained by addition of methacholine to the bath medium. The interval between two subsequent applications of methacholine was no less than 30 min, during which repeated and prolonged washes of the preparations with Krebs-Henseleit solution were performed. In order to investigate the effects of ADPN in non-adrenergic, non-cholinergic (NANC) condition, guanethidine and CCh were added to the bath medium, to rule out the adrenergic and the cholinergic influences, respectively. When contraction elicited by CCh reached a stable plateau phase, EFS or drugs were applied.

Polymerase chain reaction for ADPN receptors

Aiming to assess the presence of mRNA for ADPN receptors in gastric fundal tissue, mouse tissues were snap-frozen in liquid nitrogen and stored at -80 ℃ until use.

Then, from frozen tissue sections, RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed for AdipoR1 and AdipoR2 receptors and the following primers were used:

AdipoR1 forward 5'-CAG AGA AGC TGA CAC AGT GGA G-3' and reverse 5'-GTC CCT CCC AGA CCT TAT ACA C-3'; AdipoR2 forward 5'-GGA CTC CAG AGC CAG ATA TAC G-3' and reverse 5'-ACT CTT CCA TTC GTT CCA TAG C-3'.

The mixtures listed in Table 1 were prepared in thin walled 0.2 mL tubes and analysed by Biometra PCR cycler. PCRs were run with the program reported in Table 2.

Three gastric fundus were evaluated and a negative control without cDNA template as well as a positive control containing cDNA of inguinal fat pad were analyzed in parallel. The positive control was chosen since both Adipo-R1 and Adipo-R2 are well expressed in adipocytes which are the most relevant cell types for these receptors[18].

Data collection

Amplitude of contractile responses is expressed as percentage of the muscular contraction evoked by 2 μmol/L methacholine, assumed as 100% or as absolute values (g). Amplitude of contractile responses to methacholine was measured 30 s after a stable plateau phase was reached. Relaxant responses are expressed as percentage decrease relative to the muscular tension induced by 1 μmol/L CCh just before obtaining relaxations. Amplitude values of EFS-induced relaxations refer to the maximal peak obtained during the stimulation period.

Statistical analysis

Statistical analysis was performed by means of

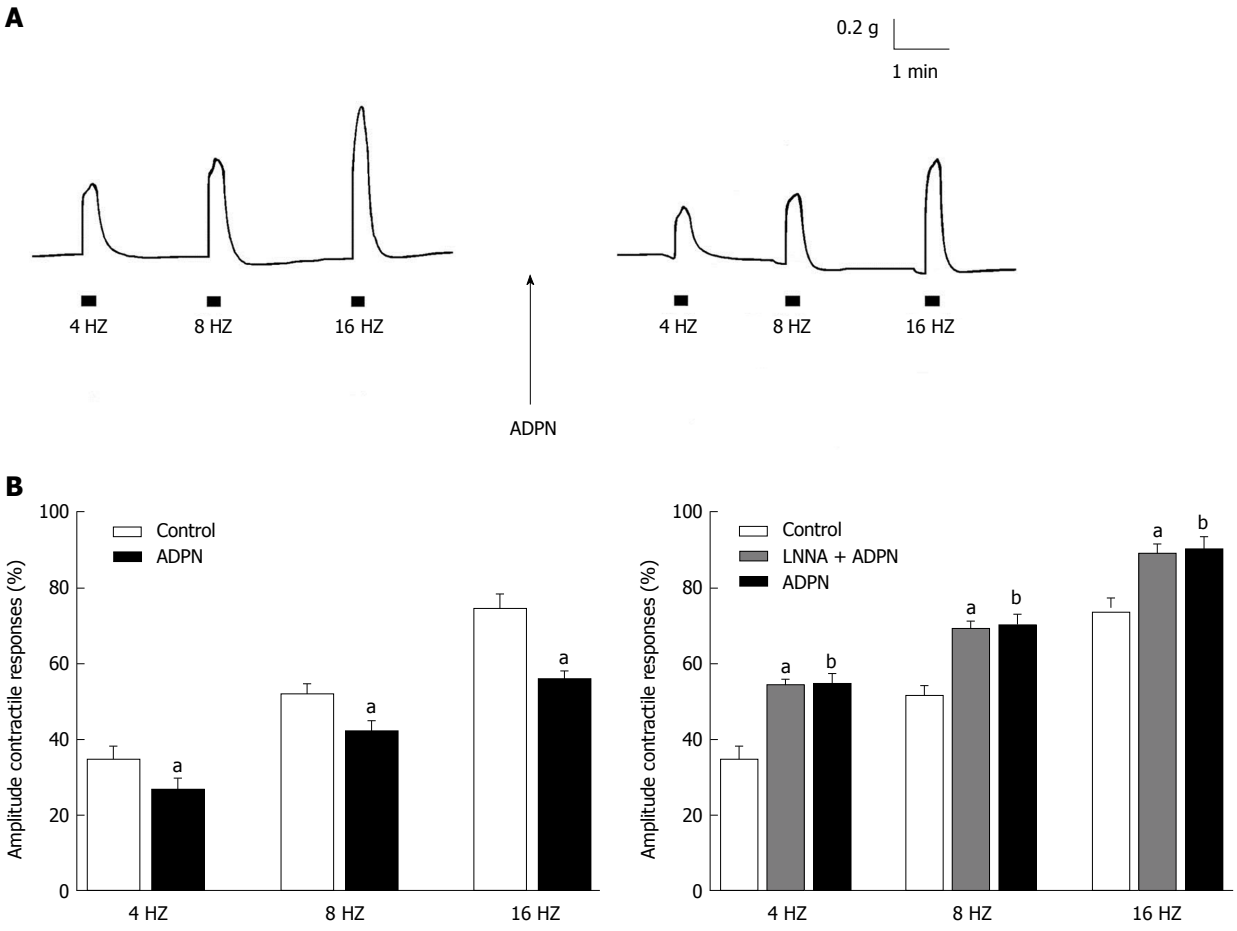


Figure 1 Effects of adiponectin on the neurally-induced contractile responses in strips from the mouse gastric fundus. A: Typical tracing showing the contractile responses to EFS (left hand panel). ADPN (20 nmol/L) decreases the amplitude of the neurally-induced contractile responses (right hand panel); B: Bar charts showing the influence of ADPN (20 nmol/L) on the mean amplitude of the EFS-induced contractile responses in the absence (left hand panel) and in the presence (right hand panel) of L-NNA (200 μmol/L). Note that, in the presence of L-NNA, ADPN no longer decreases the amplitude of the neurally-induced excitatory responses in the whole range of stimulation frequency employed. Amplitude of contractile responses is expressed as percentage of the muscular contraction evoked by 2 μmol/L methacholine, assumed as 100%. All values are means ± SE of 6 strips from 3 mice. $^{a}P < 0.05$ *vs* the control; $^{b}P < 0.05$ and $P > 0.05$ *vs* the control and *vs* L-NNA, respectively (Student's *t*-test plus ANOVA and Newman-Keuls post-test). ADPN: Adiponectin; L-NNA: L-N^{G}-nitro arginine.

Student's *t*-test to compare two experimental groups or one-way ANOVA followed by Newman-Keuls posttest when more than two groups were compared. Values were considered significantly different with $P < 0.05$. Results are given as means ± SE. The number of muscle strip preparations is designated by n in the results.

RESULTS

Mechanical experiments

At basal tension EFS (4-16 Hz) induced (n = 22) contractile responses, whose amplitude increased by increasing the stimulation frequency (Figure 1A). As previously observed in the mouse gastric fundus[16,17], the EFS-evoked contractile responses were abolished by TTX (1 μmol/L) ($P < 0.05$) or atropine (1 μmol/L) ($P < 0.05$), thus indicating that they were neurally-induced and cholinergic in nature.

Addition of ADPN to the bath medium caused, at 20 nmol/L (n = 12), a statistically significant reduction ($P < 0.05$) in amplitude of the neurally-induced excitatory responses in the whole range of stimulation frequency employed (Figure 1A and B). The effects of ADPN were already appreciable 10 min after its addition to the bath medium and persisted up to 60 min (longer time not observed). Addition of the nitric oxide (NO) synthesis inhibitor L-NNA (200 μmol/L) to the bath medium (n = 6) caused an increase in amplitude of the EFS-induced contractile responses (Figure 1B). In the presence of L-NNA, the depressant effects of ADPN on the neurally-induced contractile responses were no longer observed (Figure 1B).

Addition of the muscarinic receptors agonist methacholine (2 μmol/L) to the bath medium (n = 4) caused, after 10-15 s of contact time, a sustained contracture which reached a plateau phase (mean amplitude 0.99 g ± 0.2 g) that persisted until washout. The amplitude of the direct muscular response elicited by methacholine was not influenced by 20 nmol/L ADPN (mean amplitude 0.98 ± 0.3; $P > 0.05$).

The effects of ADPN on the fundal strips were also tested in NANC conditions. As previously observed[16],

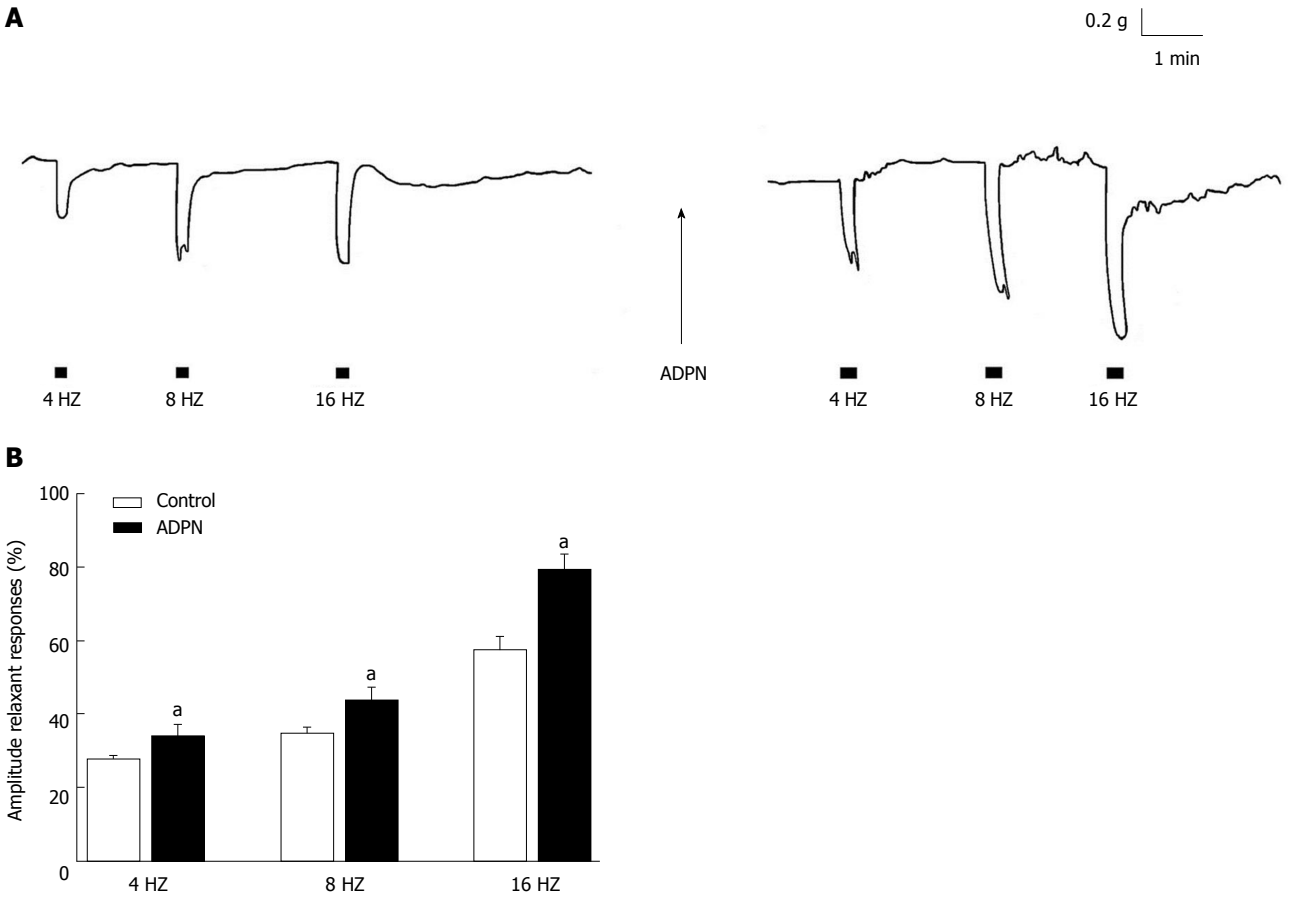


Figure 2 Effects of adiponectin on the neurally-induced relaxant responses in strips from the mouse gastric fundus. A: Typical tracing showing the relaxant responses to EFS (left hand panel). ADPN (20 nmol/L) increases the amplitude of the neurally-induced relaxant responses (right hand panel); B: Bar chart showing the influence of ADPN on the mean amplitude of the EFS-induced relaxant responses. Note that ADPN increases the amplitude of the neurally-induced relaxation in the whole range of stimulation frequency employed. Relaxant responses are expressed as percentage decrease relative to the muscular tension induced by 1 μmol/L CCh just before obtaining relaxations. All values are means ± SE of 6 strips from 3 mice. [a]*P* < 0.05 *vs* the control (Student's *t*-test). ADPN: Adiponectin.

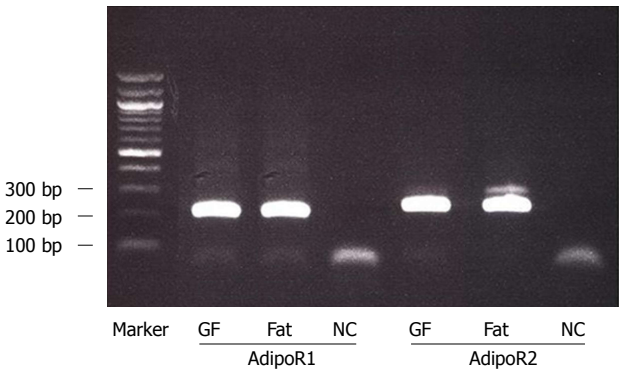


Figure 3 Expression of adiponectin receptors, Adipo-R1 and Adipo-R2, in murine gastric fundus. Typical PCR analysis recorded in the same GF revealed the expression of both AdipoR1 (left hand) and AdipoR2 (right hand) receptors. Positive controls derived from inguinal fat pad cDNA (Fat) and NC without cDNA template (water). Marker: 100 bp marker (NEB). GF: Gastric fundus; NC: Negative control.

CCh (1 μmol/L) induced (*n* = 18) a contractile response (mean amplitude 1.1 g ± 0.3 g) that reached a plateau phase which persisted until washout. Addition of ADPN (20 nmol/L) to the bath medium caused, in 14 out of the 18 strips examined, a slight (mean amplitude 0.15 g ± 0.02 g) and progressive decay of the basal tension that was not influenced by 1 μmol/L TTX (*P* > 0.05). In CCh precontracted strips, EFS (4-16 Hz) elicited (*n* = 16), during the stimulation period, relaxant responses (Figure 2A) whose amplitudes were increased following the addition of ADPN (20 nmol/L) to the bath medium (Figure 2A and B).

Touchdown-PCR analysis

By touchdown-PCR analysis ADPN receptors expression, analysed using specific primer pairs for Adipo-R1 and Adipo-R2, has been detected in cDNA in three murine gastric fundus. Two bands corresponding to Adipo-R1 and Adipo-R2 were identified in agarose gel electrophoresis. The positive controls were derived from inguinal fat pad cDNA. A typical experiment from a gastric fundus is shown in Figure 3.

DISCUSSION

The results of the present study indicate for the first time that ADPN is able to influence the motor responses

in strips from the mouse gastric fundus in which we revealed the expression of both Adipo-R1 and Adipo-R2. Particularly, the ability of ADPN to reduce the amplitude of the neurally-induced contractile responses, without affecting the direct muscular response to methacholine, indicates that the hormone exerts a neuromodulatory action. Furthermore, the lack of effects of ADPN on the contraction to methacholine, other than excluding non-specific effects, demonstrated that muscle responsiveness was not compromised.

It is known that gastric motor responses are the result of a balance between nervous excitatory and inhibitory influences on the smooth muscle and that during EFS both excitatory cholinergic and NANC inhibitory nervous fibres are activated. Thus, the reduction in amplitude of the neurally-induced contractile responses by ADPN, observed in the present experiments, may be ascribable to either a minor activation of the excitatory component or to a major nervous inhibitory influence exerted on the smooth muscle. In this view, NO is considered the main NANC inhibitory neurotransmitter released during EFS to cause gastrointestinal relaxation[19,20]. Actually, the increase in amplitude of the EFS-induced contractile responses by the NO synthesis inhibitor L-NNA supports the removal of a nitrergic inhibitory nervous influence. Notably, the observation that ADPN, in the presence of L-NNA, is no longer able to depress the amplitude of the neurally-induced excitatory responses strongly indicates that the nitrergic neurotransmission is involved in the depressant effects of the hormone. This is further confirmed by the ability of ADPN to increase the amplitude of the EFS-induced relaxant responses (elicited by EFS during the stimulation period) whose nitrergic nature has been reported in the mouse gastric fundus[16,21]. Actually, NO appears to be a shared target pathway in the hormonal control of gastrointestinal motility[16,21-24] and thus ADPN effects too may occur, at least in part, through NO. In this view, the ability of the hormone to increase NO synthase expression has been reported in vascular tissues[25,26].

However, in the present study, the observation that ADPN is also able to cause a TTX-insensitive decay of the basal tension indicates that the hormone, in addition to its neuromodulatory effects, exerts a direct muscular action. Experiments are in progress to better clarify these direct effects of ADPN on gastric smooth muscle.

From a physiological point of view, the ability of ADPN to decrease the amplitude of the neurally-induced contractile responses and to enhance that of the relaxant ones, coupled to a decay of the basal tension, suggests that the hormone effects are directed to facilitate gastric relaxation, so increasing the distension of the gastric wall and in turn the organ capacity. Thus, it could be speculated that the effects of ADPN represent a peripheral satiety signal that might contribute to the anorexigenic effects of the hormone[4,27]. In this view, the importance of the gastrointestinal tract in controlling food intake is well recognized[28] and the ability of signals generated in this apparatus to influence food intake through the vagal afferent fibers is well established[29,30]. In this regard, the presence of ADPN receptors in both mucosal and muscular vagal afferents in the gastric antrum of mice has been reported[8], suggesting a stomach-vagal-brain pathway for ADPN to modulate satiety signals. Thus, the observed effects of ADPN on gastric motor responses might be regarded as a reinforcing peripheral mechanism engaged by ADPN to regulate food intake.

In conclusion, the present study demonstrates for the first time that ADPN, likely involving the NO pathway, is able to affect the mechanical responses in strips from the mouse gastric fundus. This may furnish the basis to better explore the link between the peripheral and the central effects of ADPN in sending satiety signals and, thus, in the regulation of food intake[31]. Interestingly, clinical evidence of an increased NO production in patients affected by eating disorders, such as anorexia nervosa and bulimia nervosa, has been reported[32]. Thus, even if caution is needed when transferring data obtained in animal to human, the present results might represent a contribution to consider ADPN as a potential candidate for both novel therapeutic strategies in eating disorders and diagnostic tools.

ARTICLE HIGHLIGHTS

Research background

The adipose-tissue derived hormone adiponectin (ADPN) has been reported to have a role as a key neuromodulator of food intake. It is known that gastrointestinal motor phenomena represent a source of peripheral signals involved in the control of feeding behavior. However, no data are available concerning on the effects of ADPN on gastrointestinal motility. This novel information could highlight an additional mechanism engaged by the hormone in the control of food intake.

Research motivation

The finding that ADPN could influence gastric motility might represent an initial step to regard the peripheral effects of the hormone as possible signals involved in the control of food intake. This aspect, that certainly deserves to be deeper investigated, could lead to consider ADPN as a potential candidate for both novel therapeutic strategies in eating disorders and diagnostic tools.

Research objectives

The main objective of the present study was to investigate the influence of ADPN on the mechanical responses and the expression of its receptors in the mouse gastric fundus.

Research methods

For the mechanical experiments, longitudinal muscle strips from the mouse gastric fundus were connected to force displacement transducers for continuous recording of isometric tension. The expression of ADPN receptors in gastric fundal tissue was revealed by touchdown- polymerase chain reaction (PCR) analysis.

Research results

The present results highlight that, in the mouse gastric strips, ADPN induces inhibitory effects by decreasing the amplitude of the neurally-induced contractile responses, enhancing that of the relaxant ones and causing a decay of the basal tension. Some of these actions appear to be mediated, at least in part, by nitric oxide although further studies are needed to better clarify the mechanism through which the hormone exerts its effects.

Research conclusions

The results of the present study indicate for the first time that ADPN is able to exert inhibitory effects on the mechanical responses of the mouse gastric

fundus in which we revealed the expression of ADPN receptors. It could be hypothesized that the hormone effects may be directed to facilitate muscle relaxation, so increasing the distension of the gastric wall that represents a peripheral satiety signal. In this view, the results of the present study may furnish the basis to better explore the link between peripheral and central effects of ADPN in the regulation of food intake.

Research perspectives

The results of the present basic research might furnish a contribution to consider ADPN as a potential candidate for both novel therapeutic strategies in eating disorders and diagnostic tools.

ACKNOWLEDGMENTS

We thank Mr. Adrio Vannucchi for preparation of the Figures 1 and 2.

REFERENCES

1 **Harwood HJ Jr**. The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. *Neuropharmacology* 2012; **63**: 57-75 [PMID: 22200617 DOI: 10.1016/j.neuropharm.2011.12.010]

2 **Fasshauer M**, Blüher M. Adipokines in health and disease. *Trends Pharmacol Sci* 2015; **36**: 461-470 [PMID: 26022934 DOI: 10.1016/j.tips.2015.04.014]

3 **Blüher M**, Mantzoros CS. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. *Metabolism* 2015; **64**: 131-145 [PMID: 25497344 DOI: 10.1016/j.metabol.2014.10.016]

4 **Coope A**, Milanski M, Araújo EP, Tambascia M, Saad MJ, Geloneze B, Velloso LA. AdipoR1 mediates the anorexigenic and insulin/leptin-like actions of adiponectin in the hypothalamus. *FEBS Lett* 2008; **582**: 1471-1476 [PMID: 18394428 DOI: 10.1016/j.febslet.2008.03.037]

5 **Stern JH**, Rutkowski JM, Scherer PE. Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk. *Cell Metab* 2016; **23**: 770-784 [PMID: 27166942 DOI: 10.1016/j.cmet.2016.04.011]

6 **Wang ZV**, Scherer PE. Adiponectin, the past two decades. *J Mol Cell Biol* 2016; **8**: 93-100 [PMID: 26993047 DOI: 10.1093/jmcb/mjw011]

7 **Thundyil J**, Pavlovski D, Sobey CG, Arumugam TV. Adiponectin receptor signalling in the brain. *Br J Pharmacol* 2012; **165**: 313-327 [PMID: 21718299 DOI: 10.1111/j.1476-5381.2011.01560.x]

8 **Kentish SJ**, Ratcliff K, Li H, Wittert GA, Page AJ. High fat diet induced changes in gastric vagal afferent response to adiponectin. *Physiol Behav* 2015; **152**: 354-362 [PMID: 26074203 DOI: 10.1016/j.physbeh.2015.06.016]

9 **Yamauchi T**, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003; **423**: 762-769 [PMID: 12802337 DOI: 10.1038/nature01705]

10 **González CR**, Caminos JE, Gallego R, Tovar S, Vázquez MJ, Garcés MF, Lopez M, García-Caballero T, Tena-Sempere M, Nogueiras R, Diéguez C. Adiponectin receptor 2 is regulated by nutritional status, leptin and pregnancy in a tissue-specific manner. *Physiol Behav* 2010; **99**: 91-99 [PMID: 19887079 DOI: 10.1016/j.physbeh.2009.10.015]

11 **Florian V**, Caroline F, Francis C, Camille S, Fabielle A. Leptin modulates enteric neurotransmission in the rat proximal colon: an in vitro study. *Regul Pept* 2013; **185**: 73-78 [PMID: 23816465 DOI: 10.1016/j.regpep.2013.06.010]

12 **Okumura T**, Nozu T. Role of brain orexin in the pathophysiology of functional gastrointestinal disorders. *J Gastroenterol Hepatol* 2011; **26** Suppl 3: 61-66 [PMID: 21443712 DOI: 10.1111/j.1440-1746.2011.06626.x]

13 **Squecco R**, Garella R, Luciani G, Francini F, Baccari MC. Muscular effects of orexin A on the mouse duodenum: mechanical and electrophysiological studies. *J Physiol* 2011; **589**: 5231-5246 [PMID: 21911618 DOI: 10.1113/jphysiol.2011.214940]

14 **Yarandi SS**, Hebbar G, Sauer CG, Cole CR, Ziegler TR. Diverse roles of leptin in the gastrointestinal tract: modulation of motility, absorption, growth, and inflammation. *Nutrition* 2011; **27**: 269-275 [PMID: 20947298 DOI: 10.1016/j.nut.2010.07.004]

15 **Camilleri M**. Peripheral mechanisms in appetite regulation. *Gastroenterology* 2015; **148**: 1219-1233 [PMID: 25241326 DOI: 10.1053/j.gastro.2014.09.016]

16 **Garella R**, Idrizaj E, Traini C, Squecco R, Vannucchi MG, Baccari MC. Glucagon-like peptide-2 modulates the nitrergic neurotransmission in strips from the mouse gastric fundus. *World J Gastroenterol* 2017; **23**: 7211-7220 [PMID: 29142468 DOI: 10.3748/wjg.v23.i40.7211]

17 **Squecco R**, Garella R, Francini F, Baccari MC. Influence of obestatin on the gastric longitudinal smooth muscle from mice: mechanical and electrophysiological studies. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G628-G637 [PMID: 23989009 DOI: 10.1152/ajpgi.00059.2013]

18 **Blüher M**, Fasshauer M, Kralisch S, Schön MR, Krohn K, Paschke R. Regulation of adiponectin receptor R1 and R2 gene expression in adipocytes of C57BL/6 mice. *Biochem Biophys Res Commun* 2005; **329**: 1127-1132 [PMID: 15752770 DOI: 10.1016/j.bbrc.2005.02.081]

19 **Min YW**, Hong YS, Ko EJ, Lee JY, Ahn KD, Bae JM, Rhee PL. Nitrergic Pathway Is the Main Contributing Mechanism in the Human Gastric Fundus Relaxation: An In Vitro Study. *PLoS One* 2016; **11**: e0162146 [PMID: 27589594 DOI: 10.1371/journal.pone.0162146]

20 **Rand MJ**. Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clin Exp Pharmacol Physiol* 1992; **19**: 147-169 [PMID: 1325878 DOI: 10.1111/j.1440-1681.1992.tb00433.x]

21 **Vannucchi MG**, Garella R, Cipriani G, Baccari MC. Relaxin counteracts the altered gastric motility of dystrophic (mdx) mice: functional and immunohistochemical evidence for the involvement of nitric oxide. *Am J Physiol Endocrinol Metab* 2011; **300**: E380-E391 [PMID: 21081707 DOI: 10.1152/ajpendo.00375.2010]

22 **Abot A**, Lucas A, Bautzova T, Bessac A, Fournel A, Le-Gonidec S, Valet P, Moro C, Cani PD, Knauf C. Galanin enhances systemic glucose metabolism through enteric Nitric Oxide Synthase-expressed neurons. *Mol Metab* 2018; **10**: 100-108 [PMID: 29428595 DOI: 10.1016/j.molmet.2018.01.020]

23 **Idrizaj E**, Garella R, Francini F, Squecco R, Baccari MC. Relaxin influences ileal muscular activity through a dual signaling pathway in mice. *World J Gastroenterol* 2018; **24**: 882-893 [PMID: 29491682 DOI: 10.3748/wjg.v24.i8.882]

24 **Squecco R**, Garella R, Idrizaj E, Nistri S, Francini F, Baccari MC. Relaxin Affects Smooth Muscle Biophysical Properties and Mechanical Activity of the Female Mouse Colon. *Endocrinology* 2015; **156**: 4398-4410 [PMID: 26360621 DOI: 10.1210/en.2015-1428]

25 **Chen H**, Montagnani M, Funahashi T, Shimomura I, Quon MJ. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J Biol Chem* 2003; **278**: 45021-45026 [PMID: 12944390 DOI: 10.1074/jbc.M307878200]

26 **Nour-Eldine W**, Ghantous CM, Zibara K, Dib L, Issaa H, Itani HA, El-Zein N, Zeidan A. Adiponectin Attenuates Angiotensin II-Induced Vascular Smooth Muscle Cell Remodeling through Nitric Oxide and the RhoA/ROCK Pathway. *Front Pharmacol* 2016; **7**: 86 [PMID: 27092079 DOI: 10.3389/fphar.2016.00086]

27 **Qi Y**, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE, Ahima RS. Adiponectin acts in the brain to decrease body weight. *Nat Med* 2004; **10**: 524-529 [PMID: 15077108 DOI: 10.1038/nm1029]

28 **Monteiro MP**, Batterham RL. The Importance of the Gastrointestinal Tract in Controlling Food Intake and Regulating

Energy Balance. *Gastroenterology* 2017; **152**: 1707-1717.e2 [PMID: 28193513 DOI: 10.1053/j.gastro.2017.01.053]
29 **Bray GA**. Afferent signals regulating food intake. *Proc Nutr Soc* 2000; **59**: 373-384 [PMID: 10997653 DOI: 10.1017/S0029665100000422]
30 **Cummings DE**, Overduin J. Gastrointestinal regulation of food intake. *J Clin Invest* 2007; **117**: 13-23 [PMID: 17200702 DOI: 10.1172/JCI30227]
31 **Wilson JL**, Enriori PJ. A talk between fat tissue, gut, pancreas and brain to control body weight. *Mol Cell Endocrinol* 2015; **418** Pt 2: 108-119 [PMID: 26316427 DOI: 10.1016/j.mce.2015.08.022]
32 **Vannacci A**, Ravaldi C, Giannini L, Rotella CM, Masini E, Faravelli C, Ricca V. Increased nitric oxide production in eating disorders. *Neurosci Lett* 2006; **399**: 230-233 [PMID: 16495002 DOI: 10.1016/j.neulet.2006.01.060]

P- Reviewer: Beales ILP **S- Editor**: Gong ZM **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4036-4053

DOI: 10.3748/wjg.v24.i35.4036

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

ORIGINAL ARTICLE

Basic Study

Daikenchuto (Da-Jian-Zhong-Tang) ameliorates intestinal fibrosis by activating myofibroblast transient receptor potential ankyrin 1 channel

Keizo Hiraishi, Lin-Hai Kurahara, Miho Sumiyoshi, Yao-Peng Hu, Kaori Koga, Miki Onitsuka, Daibo Kojima, Lixia Yue, Hidetoshi Takedatsu, Yu-Wen Jian, Ryuji Inoue

Keizo Hiraishi, Lin-Hai Kurahara, Miho Sumiyoshi, Yao-Peng Hu, Ryuji Inoue, Department of Physiology, Graduate School of Medical Sciences, Fukuoka University, Fukuoka 8140180, Japan

Kaori Koga, Miki Onitsuka, Department of Pathology, Faculty of Medicine, Fukuoka University, Fukuoka 8140180, Japan

Daibo Kojima, Department of Gastroenterological Surgery, Faculty of Medicine, Fukuoka University, Fukuoka 8140180, Japan

Lixia Yue, Department of Cell Biology, University of Connecticut Health Center, Farmington, CT 06030, United States

Hidetoshi Takedatsu, Department of Gastroenterology and Medicine, Faculty of Medicine, Fukuoka University, Fukuoka 8140180, Japan

Yu-Wen Jian, College of Letters and Science, University of California, Davis, CA 95616, United States

ORCID number: Keizo Hiraishi (0000-0002-2050-1721); Lin-Hai Kurahara (0000-0001-7425-5768); Miho Sumiyoshi (0000-0002-0264-3689); Yao-Peng Hu (0000-0002-0893-2705); Kaori Koga (0000-0001-7103-242X); Miki Onitsuka (0000-0002-2546-7091); Daibo Kojima (0000-0001-5388-8320); Lixia Yue (0000-0002-3558-091X); Hidetoshi Takedatsu (0000-0002-0318-0129); Yu-Wen Jian (0000-0003-4080-2879); Ryuji Inoue (0000-0002-8494-4900).

Author contributions: Kurahara LH designed the experiments and wrote the paper, Hiraishi K, Kurahara LH and Sumiyoshi M performed most of the experiments and analyzed the data; Hu YP conducted patch-clamp experiments and analyzed the obtained data; Koga K and Onitsuka M performed the pathological analysis; Yue L provided comments; Kojima D and Takedatsu H gave clinical advice, recruited study participants, and performed the biopsies and surgical resections of tissue; Jian YW created the graphical abstract; and Inoue R supervised the study and assisted in the preparation of the manuscript.

Supported by MEXT, KAKENHI, No. 15K08978, No. 22790677 and No. 25860571; a MEXT-Supported Program supporting research activities of female researchers; the Clinical Research Foundation; and the Central Research Institute of Fukuoka University, No. 151045 and No. 147104.

Institutional review board statement: The study was reviewed and approved by the Clinical Research Ethics Committee of Fukuoka University, No. 15-10-04.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Animal experiment ethics committee of Fukuoka University, No. 1709099; and the Genetic Modification Experiment Safety Commission of Fukuoka University approved experiments on genetically modified animals, No. 167.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: Readers can request the data of this paper by contacting us *via* hailin@fukuoka-u.ac.jp.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Lin-Hai Kurahara, PhD, Lecturer, Department of Physiology, Fukuoka University School of Medicine, Fukuoka 8140180, Japan. hailin@fukuoka-u.ac.jp

Telephone: +81-92-8011011-3236
Fax: +81-92-8656032

Received: May 16, 2018
Peer-review started: May 16, 2018
First decision: June 13, 2018
Revised: July 6, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: September 21, 2018

Abstract

AIM

To investigate the anti-fibrotic effects of the traditional oriental herbal medicine Daikenchuto (DKT) associated with transient receptor potential ankyrin 1 (TRPA1) channels in intestinal myofibroblasts.

METHODS

Inflammatory and fibrotic changes were detected in a 2,4,6-trinitrobenzenesulfonic acid (TNBS) chronic colitis model of wild-type and TRPA1-knockout (TRPA1-KO) mice *via* pathological staining and immunoblotting analysis. Ca^{2+} imaging experiments examined the effects of DKT and its components/ingredients on intestinal myofibroblast (InMyoFib) cell TRPA1 channel function. Profibrotic factors and transforming growth factor (TGF)-β1-associated signaling were tested in an InMyoFib cell line by qPCR and immunoblotting experiments. Samples from non-stenotic and stenotic regions of the intestines of patients with Crohn's disease (CD) were used for pathological analysis.

RESULTS

Chronic treatment with TNBS caused more severe inflammation and fibrotic changes in TRPA1-KO than in wild-type mice. A one-week enema administration of DKT reduced fibrotic lesions in wild-type but not in TRPA1-KO mice. The active ingredients of DKT, *i.e.*, hydroxy α-sanshool and 6-shogaol, induced Ca^{2+} influxes in InMyoFib, and this was antagonized by co-treatment with a selective TRPA1 channel blocker, HC-030031. DKT counteracted TGF-β1-induced expression of Type Ⅰ collagen and α-smooth muscle actin (α-SMA), which were accompanied by a reduction in the phosphorylation of Smad-2 and p38-mitogen-activated protein kinase (p38-MAPK) and the expression of myocardin. Importantly, 24-h incubation with a DKT active component Japanese Pepper increased the mRNA and protein expression levels of TRPA1 in InMyoFibs, which in turn negatively regulated collagen synthesis. In the stenotic regions of the intestines of CD patients, TRPA1 expression was significantly enhanced.

CONCLUSION

The effects of DKT on the expression and activation of the TRPA1 channel could be advantageous for suppressing intestinal fibrosis, and benefit inflammatory bowel disease treatment.

Key words: Intestinal fibrosis; Myofibroblast; Transient receptor potential ankyrin 1; Crohn's disease; Collagen; α-smooth muscle actin

Core tip: Active ingredients of the famous Chinese medicine Da-Jian-Zhong-Tang (Daikenchuto; DKT), *i.e.*, hydroxy α-sanshool and 6-shogaol, induced Ca^{2+} influxes in intestinal myofibroblast (InMyoFib), which were antagonized by co-treatment with a selective transient receptor potential ankyrin 1 (TRPA1) channel blocker HC-030031. DKT counteracted the transforming growth factor (TGF)-β1-induced expression of Type Ⅰ collagen, α-smooth muscle actin (α-SMA), and this was accompanied by a reduction in fibrosis signaling downstream of the TGF-β1 receptor. Importantly, a 24-h incubation with another DKT active ingredient of Japanese Pepper increased mRNA and protein expression in TRPA1, which in turn negatively regulated collagen synthesis in InMyoFibs. In stenotic regions of the intestines of patients with Crohn's disease, TRPA1 expression was significantly increased.

Hiraishi K, Kurahara LH, Sumiyoshi M, Hu YP, Koga K, Onitsuka M, Kojima D, Yue L, Takedatsu H, Jian YW, Inoue R. Daikenchuto (Da-Jian-Zhong-Tang) ameliorates intestinal fibrosis by activating myofibroblast transient receptor potential ankyrin 1 channel. *World J Gastroenterol* 2018; 24(35): 4036-4053 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4036.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4036

INTRODUCTION

Daikenchuto (DKT; Da-Jian-Zhong-Tang in Chinese) is a traditional herbal medicine, originally described in *Jin Gui Yao Lue* ("Essential Prescriptions from the Golden Cabinet"), a classic book of Chinese medicine published more than 1800 years ago. DKT is often used for postoperative ileus and constipation, and is composed of several crude components, *Zingiberis rhizoma* (Ginger), *Panax ginseng* (Ginseng; Ginseng radix), *Zanthoxyli fructus* (Japanese pepper), and malt sugar. DKT generally accelerates gastrointestinal motility; it increases intestinal blood flow and gastrointestinal hormone secretion[1-3]. Numerous basic studies have demonstrated the effects of DKT on vasodilation, inflammation, and bacterial translocation[4-9]. Ginger contains several active ingredients, such as gingerols and shogaols (6-, 8-, and 10- isomers), which have anti-inflammatory and vasoprotective effects *via* modulating the activities of mitogen-activated protein kinase (MAPK), protein kinase B (Akt), and NF-κB[10-12]. Japanese pepper contains hydroxy-α and hydroxy-β-sanshools that alter intestinal blood flow, motility, and barrier function by inducing the

synthesis/secretion of adrenomedullin and calcitonin gene-related peptides[1,13]. DKT accelerates the recovery of gastrointestinal function in patients undergoing an open colectomy for colon cancer[14,15]. However, the essential pharmacological mechanism(s) underlying these effects remains largely unclear.

Myofibroblasts are crucial for the pathogenesis of tissue fibrosis. The formation of stress fibers in activated myofibroblasts results in the release of a myocardin-related transcription factor, a transcriptional coactivator of serum response factor. The major pro-fibrotic factor, transforming growth factor (TGF)-β, secreted from many types of cells, induces or augments myofibroblast functions, *i.e.*, transformation, proliferation, invasion, migration, stress fiber formation, and collagen synthesis[16,17]. Previously, we used an intestinal myofibroblast (InMyoFib) cell line, stimulated by TGF-β1, to produce a pathological fibrosis model[18]. TGF-β1 treatment induced dramatic morphological changes in myofibroblasts, including an enlarged shape and a filamentous microstructure characteristic in transformed cells[19]. The activated TGF-β receptor 1 (TGFR) complex phosphorylates the transcription factors Smad-2 and Smad-3, which in turn promote collagen synthesis[20]. TGF-β can also signal through non-canonical pathways involving extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases, and p38-MAPK. Both pathways are implicated in myofibroblast-mediated cytokine production and fibrosis in the gut[21,22]. Levels of TGF-β were found to be elevated in the inflamed intestines of patients with Crohn's disease (CD) and ulcerative colitis. TGF-β1 is also essential for anti-inflammatory responses. Thus, there is a caveat against the use of a TGF-β1-neutralizing strategy for anti-fibrotic treatment in clinical practice, as TGF-β1-neutralizing antibodies might exacerbate the progression of CD by attenuating the anti-inflammatory actions of TGF-β1. Further, abnormal TGF-β signaling was reported to impair intestinal immune tolerance and tissue repair[23].

There is mounting evidence for the role of transient receptor potential (TRP) channels in a variety of cellular remodeling processes[24-27]. For instance, TRPA1 is expressed not only in peripheral sensory neurons, but also in intestinal epithelial cells[7,28]. The TRPA1 agonist allyl isothiocyanate (AITC) exhibits anti-fibrogenic effects in hepatic stellate cells, and another TRPA1 agonist allicin prevents fibrotic changes in the oral submucosa and heart[29,30]. Our recent study also demonstrated that TRPA1 mediates the anti-fibrotic effects of steroids and pirfenidone in the mouse TNBS-colitis model, and TRPA1 expression is significantly increased in the intestinal stenotic regions of CD patients[18]. Moreover, TRPA1 activity has been ascribed to anti-inflammatory responses in dextran sulfate sodium-induced chronic colitis, where TRPA1 regulates the inflammatory potential of T cells[31]. A later study based on interleukin knockout and T-cell-adoptive transfer colitis models suggested that TRPA1 likely counteracted the transient receptor potential vanilloid type 1 (TRPV1)-mediated differentiation of $CD4^+$-T cells into Th1-effector cells[32]. Finally, TRPA1 activation by cannabichromene was found to reduce the severity of dinitrobenzene sulfonic acid (DNBS)-induced colitis[33].

Hydroxy-α-sanshool and 6-shogaol are the active ingredients of DKT components ginger and Japanese pepper. Both are capable of activating TRPA1 and TRPV1 channels[34,35]. Activation by DKT of the TRPA1 channel endogenously expressed in intestinal epithelial cells improves gastrointestinal microcirculation *via* adrenomedullin release[28], and facilitates intestinal transit in a murine model of postoperative ileus[36].

Currently, surgical operation is the only available option that prevents the fibrotic complications of CD, but it often only has temporary benefits. It is, therefore, crucial to develop new alternative treatments for fibrotic stenosis. Recently, DKT has often been used to mitigate inflammatory bowel disease (IBD)-associated fibrosis and the resulting stenosis and strictures, common and severe complications in CD patients. However, the pathogenesis of CD-related fibrosis and how DKT exerts its therapeutic effects are poorly understood[37].

In this study, we tested whether DKT can activate myofibroblast TRPA1 channels to reduce TGF-β1-induced fibrotic events. To this end, we examined the effects of DKT and its ingredients on TRPA1 channel activity and the expression of pro-fibrotic factors downstream of the TGF-β1 signaling pathway. Moreover, surgical samples from stenotic and non-stenotic regions of the intestines of patients with CD were used to confirm the validity of the observations in cell experiments for human pathogenesis.

MATERIALS AND METHODS

Materials

DKT is an aqueous extract containing processed ginger, ginseng (ginseng radix), and Japanese pepper at a ratio of 5:3:2. The dried powdered extract forms of DKT (TU-100: without maltose syrup), ginger, ginseng, and Japanese pepper were obtained from Tsumura Co. (Tokyo, Japan). These extracted powders were dissolved in ethanol by sonication and purified by filtering. Ginsenoside (Wako, Japan), 6-shogaol (Wako, Japan), hydroxy-α-sanshool (Adipogen Life Science), AITC, and HC-030031 (Sigma-Aldrich, St Louis, MO, United States) were also used in this study. Recombinant human TGF-β1 (Wako) and Type Ⅰ collagen (IFP, Higashine, Japan) were added to cultured cells, as indicated by the manufacturer's instructions. Human stealth siRNAs for TRPA1: TRPA1HSS113276 (5′-GGAGCAAUUGCUGUUUACUUCUAUU-3′ and 5′-AAUAGAAGUAAACAGCAAUUGCUCC-3′) were obtained from Invitrogen (Carlsbad, CA, United States) and used for gene silencing, according to the manufacturer's instructions. Other TRPA1-siRNAs, TRPA1HSS113277 and TRPA1HSS189723, were also evaluated, but the silencing efficacy was inferior to that of TRPA1HSS113276 for InMyoFibs. Antibodies against TRPA1 (mouse; Sigma-

Aldrich), α-SMA (Abcam, Cambridge, MA, United States), β-actin (Abcam, Cambridge, MA, United States), Collagen Ⅰ (Abcam, Cambridge, MA, United States), Smad-2/3, phospho-Smad-2, and p38-MAPK (Cell Signaling Technology, Beverly, MA, United States) were used for immunoblotting and immunostaining experiments. An ELISA Kit was used to measure the Type Ⅰ collagen levels in the conditioned medium.

Trinitrobenzenesulfonic acid chronic colitis model

Chronic TNBS-associated colitis was induced as previously reported[18]. TNBS intracolonic administration for mice (8-9 wk old, n = 8) was achieved after 24-h fasting by inserting a polyethylene catheter 4 cm into the rectum under pentobarbital (40 mg/kg injected *i.p.* with saline) anesthesia. TNBS solution in 30% ethanol/phosphate-buffered saline (PBS) (10 mg/mL; 50 μL) and sonicated was weekly injected for six weeks, as illustrated in Figure 1A. The vehicle control group received 30% ethanol/PBS (50 μL). DKT was administered daily (5 mg/kg/d; anesthetized by isoflurane) by enema for one week after the last TNBS treatment. Colonic tissues were excised from the anus to the caecum at week six after cervical dislocation. All animal experiments were conducted in accordance with the guidelines of the Animal Center of Fukuoka University.

Histological evaluation

The mouse distal colon and patient samples were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections for routine hematoxylin-eosin (HE) and Masson's trichrome (MT) staining. Stained colon tissues were micro-photographed at 200 × magnification on six randomly chosen locations of each tissue, and analyzed by a pathologist (Koga K) in a blinded manner. For all mice, the fibrosis score was measured at the tissue located 1 cm from the anus, where the most obvious fibrosis was observed. The optical density of the blue-stained collagen fibers was measured and expressed as a fibrosis score on a scale of 0-3: (0) No fibrosis wild-type vehicle control mouse; (1) mild fibrosis; (2) moderate fibrosis; and (3) severe fibrosis as described previously[18].

Cell culture

Normal human intestinal myofibroblasts (InMyoFibs) were purchased from Lonza (CC-2902; Basel, Switzerland) and grown in smooth muscle basal medium (SmBM), supplemented with 5% fetal bovine serum (FBS), antibiotics (gentamicin/amphotericin-B), and growth factors (insulin, human epidermal growth factor-β, and human fibroblastic growth factor). InMyoFibs were passaged 10-19 times. TGF-β1 (5 ng/mL) was used under low serum (1% FBS) conditions.

Electrophysiological study

Whole-cell voltage clamp recording was implemented by an EPC-10 patch-clamp amplifier (HEKA Electronics, Lambrecht, Pfalz, Germany). The recording electrodes (patch pipettes), with resistances of 2-4 MΩ, were fabricated from borosilicate glass capillaries. An Ag-AgCl wire was used as a grounding electrode. Capacitive currents were electronically compensated, and the linear leak and residual capacitance were subtracted. More than 70% of series resistance was compensated to minimize voltage errors.

Data analysis and illustration were performed offline using the versatile analysis software programs Clampfit v.9.2 (Axon Instruments, Foster City, CA, United States) and KaleidaGraph v.4.0 (Hulinks, Tokyo, Japan). Long-term recordings were performed at a sampling rate of 100 Hz in conjunction with an A/D, D/A-converter PowerLab/400 (ADInstruments, Australia) and analyzed by the accessory software LabChart v.8.1.5. A solenoid valve-driven fast solution change device similar to the "Y-tube" system was used to rapidly apply drugs onto cells as previously described[38]. The density of the membrane current (pA/pF) was calculated by normalizing its amplitude by the cell capacitance to minimize cell size-dependent variations. To prevent the quick Ca^{2+}-dependent inactivation of TRPA1 currents, a Ca^{2+} chelator BAPTA was added to both the bath and pipette solutions[39].

The bath solution contained 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 5 mmol/L EGTA, 10 mmol/L HEPES, and 10 mmol/L glucose (pH = 7.4, adjusted with Tris base). K^+ currents were eliminated by including Cs^+ and TEA in the patch pipette solution. Patch pipettes were filled with 130 mmol/L Cs-aspartate, 10 mmol/L TEA-Cl, 5 mmol/L BAPTA, 1.374 mmol/L Ca-gluconate, 1 mmol/L $MgCl_2$, 2 mmol/L $MgSO_4$, 2 mmol/L Na_2ATP, and 10 mmol/L HEPES (pH = 7.2, adjusted with Tris base).

Measurement of $[Ca^{2+}]_i$

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of myofibroblasts was monitored using a digital fluorescence imaging technique with Fura-2. Briefly, InMyoFibs were enzymatically dispersed and lodged on a poly-L-lysine-coated (Sigma-Aldrich, United States) glass chamber placed on the stage of an inverted fluorescent microscope (DMI600B; Leica, Germany). InMyoFibs cells were then loaded with 5 μmol/L Fura-2/AM in the dark at 20-25 ℃ for 30 min. The intensities of Fura-2 emissions at 510 nm (± 10 nm) resulting from excitation at 340 or 380 nm were measured through a fluorescent microscope (DMI600B) and a Cascade EMCCD camera (Nippon Roper, Tokyo, Japan). Data acquisition and analysis were performed using SlideBook v.4.2 software (Intelligent Imaging Innovation Inc., Denver, Colorado, United States). Fluorescence intensities were corrected for background fluorescence, and changes in $[Ca^{2+}]_i$ were defined as the ratio of corrected fluorescence intensities at 340 and 380 nm excitations (F_{340}/F_{380}). The normal external solution for Ca^{2+}-imaging experiments contained 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L

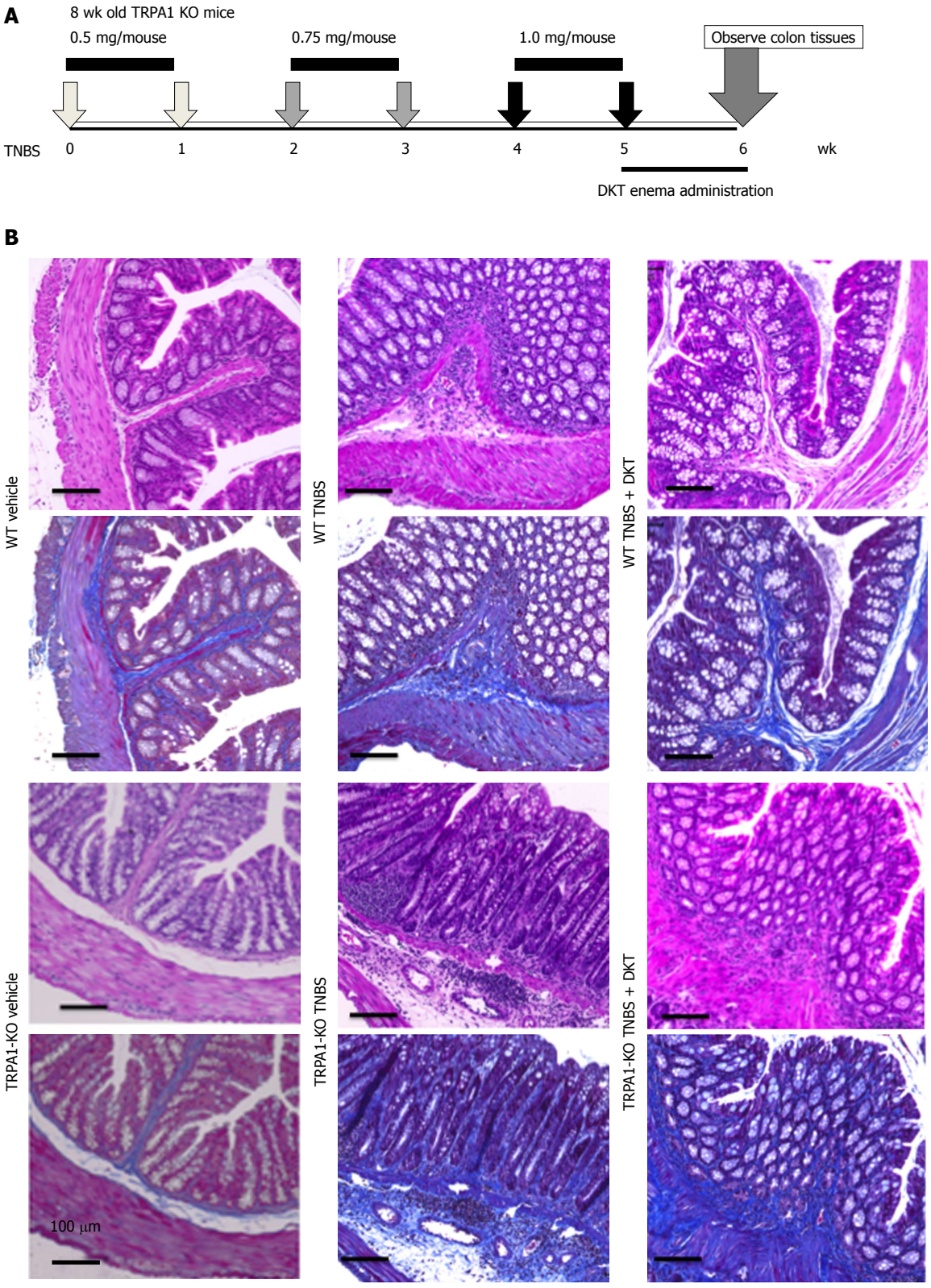


Figure 1 Chronic 2, 4, 6-trinitrobenzenesulfonic acid-induced colitis models of wild-type and transient receptor potential ankyrin 1-knockout mice. Chronic colitis was induced by weekly intrarectal injection of TNBS for six weeks. Immediately after the final TNBS treatment, a DKT enema was administered daily for one week. A: schematic presentation of TNBS injection and DKT enema; B: Hematoxylin–eosin (HE)- and Masson's trichrome (MT)-stained tissues from the vehicle control, TNBS and TNBS + DKT groups of WT and TRPA1-/- mice. TNBS: 2, 4, 6-trinitrobenzenesulfonic acid; WT: Wild-type; TRPA1: Transient receptor potential ankyrin 1; TRPA1-/-: Transient receptor potential ankyrin 1-knockout; DKT: Daikenchuto; HE: Hematoxylin-eosin; MT: Masson's trichrome.

glucose (pH = 7.4, adjusted with Tris base).

Immunostaining

InMyoFibs cells were fixed in 4% formaldehyde for 15 min and permeabilized with 0.3% Triton X-100 in 5% normal goat serum (Wako). Cells were then incubated overnight with primary antibodies (1:200 dilution) against α-SMA at 4 ℃, washed with PBS, and incubated

with an Alexa Fluor-conjugated secondary antibody (Life Technologies, Carlsbad, California, United States, 1:200 dilution) for an additional 1 h. Immunostained cells were analyzed using a Zeiss LSM 710 Confocal Microscope (Oberkochen, Germany). Images were taken through a 40 × objective lens using the default settings for pinhole width, laser intensity, and detector gain.

Real-time RT-PCR

Total RNA was extracted from InMyoFibs using the RNeasy RNA Extraction Kit (Qiagen, Venlo, Netherlands). To quantify mRNA expression levels, real-time PCR was performed using a BioMark™ HD System (Fluidigm, South San Francisco, CA, United States). Thermocycling was performed using an initial denaturation at a hot start phase of 60 s at 95 ℃. This was followed by 35 cycles of 5 s at 96 ℃ and 20 s at 60 ℃. The following TaqMan® Gene Expression Assay kits from Life Technologies were used for real-time PCR reactions: *acta2* (α-SMA; Hs00426835_g1), *myocd* (myocardin; Hs00538071_m1), and *col1a1* (Hs00164004_m1).

Immunoblotting analysis

Immunoblotting experiments were performed to examine the protein levels of Type Ⅰ collagen, α-SMA, β-actin, Smad-2/3, phospho-Smad-2, and p38-MAPK in InMyoFibs, as described previously[19]. Total cell lysates were homogenized in radioimmunoprecipitation assay buffer with protease inhibitors, and prepared in a sample buffer and diluted in 5% (v/v) 2-mercaptoethanol and 1% (w/v) bromophenol blue prior to electrophoresis. Proteins were resolved using 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinyldifluoridine (PVDF) membranes. The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated overnight (at 4 ℃) with the appropriate primary antibodies. The protein levels were detected by incubating the PVDF membranes with appropriate species-specific, horseradish peroxidase-conjugated secondary antibodies (20-25 ℃, 45 min), and visualized using the ECL Western Blotting Detection System (GE Healthcare, Little Chalfont, United Kingdom).

Patient samples

We obtained surgical samples from three male CD patients with colonic lesions with a median age of 47 years (range: 40-51 years) and a median history of CD 22 years (range: 15-26 years). All patients had suffered, in addition to coexisting stenosis, an intra-abdominal or perianal fistula and/or abscess, during the course of the disease. All patients received an anti-TNF agent. The Fukuoka University Hospital Ethics Committee approved the protocol, and written informed consent was obtained from all patients.

Statistical analysis

Results are expressed as means ± SEM. Experimental protocols were repeated with at least four different batches of cells under each condition, and pooled data were averaged and subjected to statistical analyses. Statistically significant differences among groups were evaluated by analysis of variance (ANOVA), and Dunnett's test was employed for multiple comparisons. *P*-values < 0.01 or 0.05 were considered statistically significant.

RESULTS

Chronic TNBS fibrosis model of TRPA1-KO mice

To better visualize the time course of chronic colitis, we adopted the TNBS-induced colitis model, as it is known to allow a more moderate and slower progression of gut inflammation and fibrosis in mice than the other colitis models.

At the sixth week of TNBS treatment, TRPA1-KO mice showed more severe signs of inflammation/fibrosis than did wild-type mice, where prominent cell infiltration occurred in some mucosal and submucosal layers. The co-administration of DKT reduced these pathological changes in wild-type (WT), but not in TRPA1-KO mice (Figure 1B). Concomitantly, the Type Ⅰ collagen protein expression and fibrosis score, which were remarkably enhanced and aggravated by TNBS treatment, respectively, were significantly reduced in WT but not in TRPA1-KO mice (Figures 1B, 2A and B). Double immunostaining experiments indicated a high co-incidence of immunoreactivities against TRPA1 and heat shock protein (HSP) 47, an endoplasmic reticulum-resident molecule essential for correct procollagen folding[18]. The number of TRPA1/HSP47-double-positive cells per unit area greatly increased in the TNBS-treated groups compared to in the vehicle control group (Figure 2C).

DKT activates TRPA1 channel and antagonizes fibrotic changes in InMyoFibs

We next examined the ability of DKT to induce TRPA1 activity in InMyoFib. The workflow for ethanol extraction of crude components from DKT, *i.e.*, ginger, ginseng radix, and Japanese pepper, are shown in Figure 3A. In the whole-cell mode of patch-clamp recording (holding potential: -60 mV), a potent TRPA1 agonist, AITC (10 μmol/L), induced a robust non-selective cation current. This current was strongly inhibited by a TRPA1-selective antagonist, HC-030031 (10 μmol/L) (Figure 3B). The same concentration of AITC also evoked a prominent rise in $[Ca^{2+}]_i$, which was completely inhibited by the application of HC-030031 (10 μmol/L)[18]. A similar-magnitude rise in HC-030031-sensitive $[Ca^{2+}]_i$ was also induced by DKT (0.1% EtOH extract) (Figure 3C). These results suggest that InMyoFibs express functional TRPA1 channels with Ca^{2+} permeability, which can be activated by DKT.

We then examined how DKT affects the fibrotic changes induced by TGF-β1 treatment. As shown in Figure 3D, the 24-h treatment of InMyoFibs with 5 ng/mL TGF-β1 facilitated stress fiber formation and protein

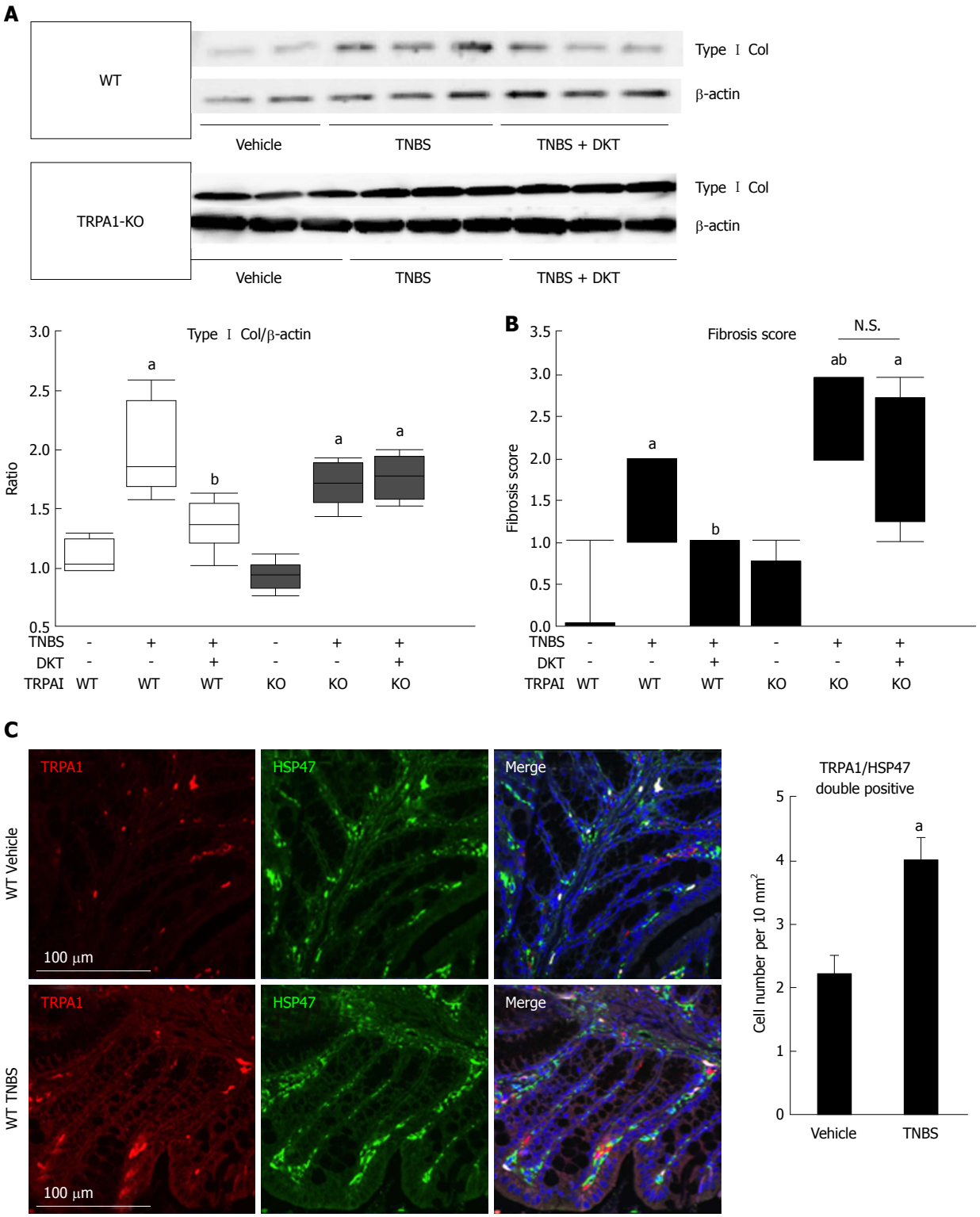


Figure 2 Fibrotic changes in wild-type and transient receptor potential ankyrin 1-knockout CRISPR mice treated with 2, 4, 6-trinitrobenzenesulfonic acid and/or Daikenchuto. A: Type Ⅰ collagen expression was examined by western blotting. The ratio of changes in each experimental condition is shown in the middle lower-left graph. [a]*P* < 0.01 *vs* vehicle control, [b]*P* < 0.01 *vs* TNBS (*n* = 6); B: Fibrosis scores based on histological examination ranging from 0 (no fibrosis) to 3 (severe fibrosis) are shown as the dot-plot graph. The leftmost circles (*n* = 8) show absolutely zero values. Values are the means ± SD; C: Co-localization of TRPA1 and HSP47 proteins in the mouse intestine. WT vehicle control, WT TNBS: Immunostaining images of mouse intestine stained with anti-TRPA1 (red) antibody, anti-HSP47 (green), and DAPI (blue). Cell number per 10 mm^2 is summarized in right graph, [a]*P* < 0.05 *vs* vehicle control (*n* = 6). TNBS: 2, 4, 6-trinitrobenzenesulfonic acid; WT: Wild-type; HSP47: Heat shock protein 47; TRPA1: Transient receptor potential ankyrin 1; DAPI: 4',6-diamidino-2-phenylindole.

expression of α-SMA, which was effectively suppressed by co-treatment with 0.1% EtOH extract of DKT. Concomitantly, the 24-h treatment of InMyoFibs with 5 ng/mL TGF-β1 enhanced the mRNA expression levels

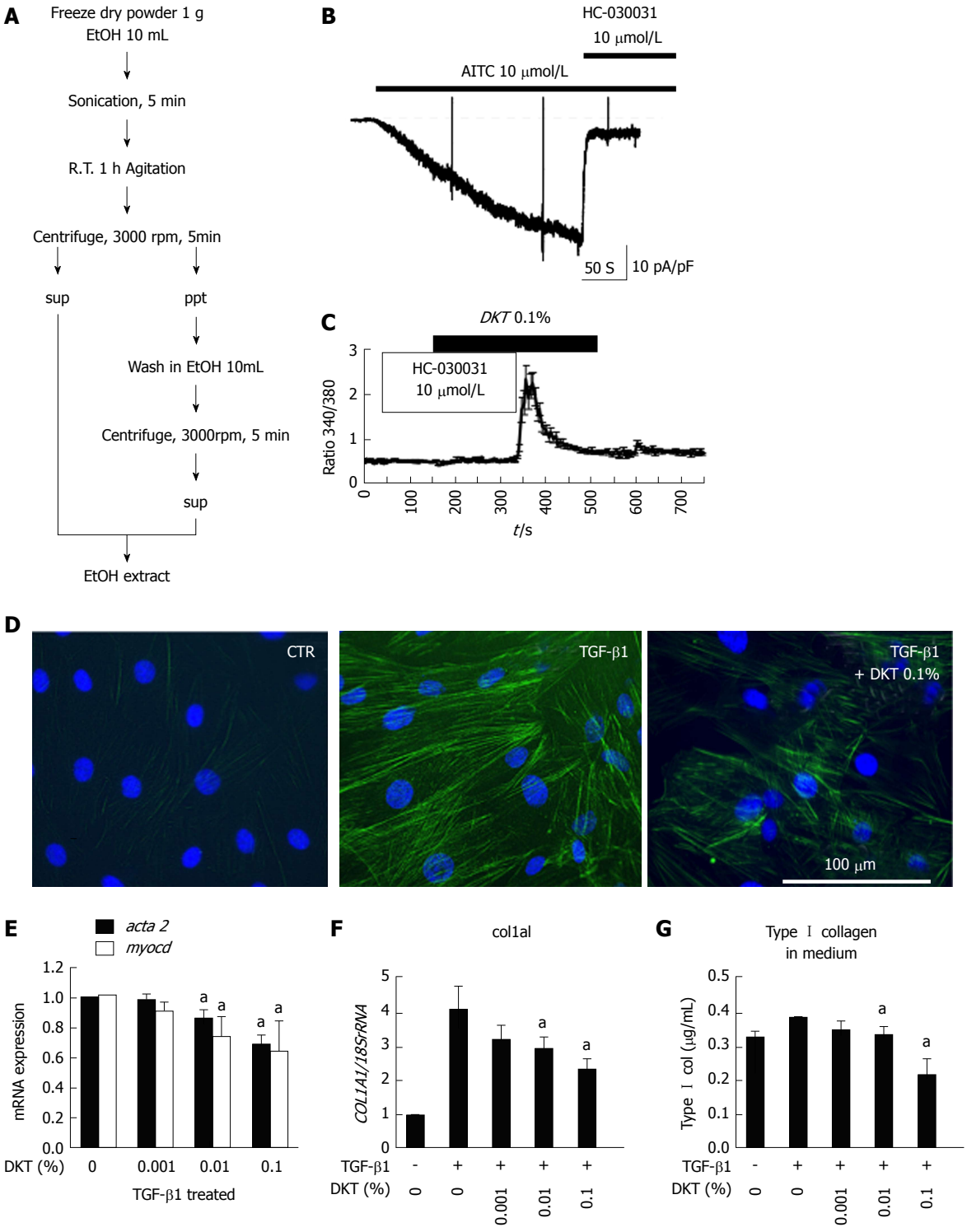


Figure 3 Daikenchuto-induced Ca^{2+} influx *via* transient receptor potential ankyrin 1 and anti-fibrosis effects in intestinal myofibroblasts. A: Method for ethanol-extraction of DKT and its components, *i.e.*, ginger, Japanese pepper, and ginseng; B: Representative traces of whole-cell currents recorded from InMyoFibs cells at a holding potential of -60 mV. TRPA1 nonselective cation currents are activated by AITC (10 μmol/L) and inhibited by HC-030031 (10 μmol/L); C: Representative $[Ca^{2+}]_i$ response in InMyoFibs cells. Cells were exposed to DKT extracts (0.1%), which evoked prominent increases in $[Ca^{2+}]_i$. The TRPA1 antagonist HC-030031 (10 μmol/L) was added to examine whether the $[Ca^{2+}]_i$ response involved the activation of TRPA1 channels. Respective data points represent means ± SEM from > 30 cells; D: Immunostaining images of InMyoFibs with anti-α-SMA (green) antibody and DAPI (blue) in untreated controls, 24 h TGF-β1 (5 ng/mL) treatment, or 24 h TGF-β1 (5 ng/mL) + DKT extracts (0.1%) treatment; E and F: DKT extracts (0.001%, 0.01%, and 0.1%) were co-administered with TGF-β1, and acta2 (α-SMA), myocd (myocardin), and col1a1 mRNA expression levels were quantified by real-time PCR; G: Concentrations of Type Ⅰ collagen in the culture medium were measured by ELISA from the control and TGF-β1-treated cells. $^aP < 0.05$ *vs* TGF-β1-treated cells ($n = 4$). DKT extracts (0.001%, 0.01%, and 0.1%) were co-administered with TGF-β1. DKT: Daikenchuto; InMyoFibs: Intestinal myofibroblasts; TRPA1: Transient receptor potential ankyrin 1; AITC: Allyl isothiocyanate; α-SMA: α-Smooth muscle actin; DAPI: 4',6-diamidino-2-phenylindole; TGF-β1: Transforming growth factor-β1.

of the master transcription regulator *MYOCD*, α-SMA (*ACTA2*), collagen Type Ⅰ alpha 1 (*COL1A1*), and Type Ⅰ collagen released in the culture medium. DKT (0.01%, 0.1% EtOH extract) effectively suppressed these mRNA upregulations in a dose-dependent manner (Figure 3E-G). In addition, DKT (0.001%, 0.01%, 0.1% EtOH extract) did not affect the mRNA levels of *COL1A1* in the absence of TGF-β1 stimulation, but 100 μg/mL DKT suppressed Type III collagen gene (*COL3A1*) expression, irrespective of TGF-β1 treatment (data not shown).

Active components and ingredients of DKT activate TRPA1 and show anti-fibrotic effects

We next examined the efficacies of the active components and ingredients contained in DKT. Among the three active components of DKT (ginger, ginseng, Japanese pepper), ginger was the most effective, showing a similar efficacy to DKT (Figure 4A). In contrast, Japanese pepper was less efficacious at activating Ca^{2+} influx, and ginseng had almost no effect (Figure 4C and 4E, respectively). The Ca^{2+} mobilizing effects of ginger and Japanese pepper were almost completely antagonized by the prior application of a selective TRPA1 channel blocker HC-030031, suggesting the involvement of TRPA1 activation (Figure 4A and C). The active ingredients of the respective DKT components were further examined by the same Ca^{2+}-imaging protocol as used above. The active ingredient of ginger, 6-shogaol (100 μmol/L), induced a comparable Ca^{2+} influx to that of AITC (10 μmol/L), while that of Japanese pepper, hydroxy α-sanshool (1 μmol/L), induced a much milder influx. The active ingredient of ginseng radix, ginsenoside Rb1, was virtually ineffective (Figure 4B, D and F).

We then explored the inhibitory effects of ginger and Japanese pepper on the fibrotic changes induced by 24-h treatment with 5 ng/mL TGF-β1. Co-treatment with ginger or Japanese pepper significantly suppressed TGF-β1-induced stress (α-SMA) fiber formation in InMyoFibs (Figure 4G). In accord with these immunostaining results, ginger, Japanese pepper, and AITC all significantly inhibited the TGF-β1-induced mRNA transcriptions of α-SMA (*ACTA2*) and Type Ⅰ collagen (*COL1A1*) (Figure 5A and 5B).

The active ingredient of ginger, 6-shogaol, and that of Japanese pepper, hydroxy α-sanshool, also significantly suppressed the TGF-β1-induced increase in *COL1A1* mRNA expression, with a similar efficacy to that of AITC (Figure 5B, C and F). The 6-shogaol dose-dependently suppressed the Smad-2 phosphorylation and α-SMA mRNA expression (*ACTA2*) induced by TGF-β1 (Figure 5D and F). Although ineffective at evoking a rise in TRPA1-mediated $[Ca^{2+}]_i$, ginsenoside inhibited the TGF-β1-induced increase in *COL1A1* mRNA expression.

DKT upregulates TRPA1 channel expression

In another series of experiments, we found that in addition to activating TRPA1 channels, DKT also enhanced TRPA1 expression in InMyoFibs. As shown in Figure 6A, 24-h incubation with DKT dose-dependently increased TRPA1 mRNA expression in InMyoFibs. The TRPA1-upregulating effects were also observed for the DKT components Japanese pepper and ginseng radix, but not ginger, and this was particularly prominent when TGF-β1 coexisted (Figure 6B). Notably, treatment with TNFα or TGF-β1 themselves enhanced TRPA1 expression in InMyoFibs: when treated with both, they seemingly acted additively or synergistically (Figure 6C). In parallel to the upregulation of TRPA1 expression, the key downstream events of TGF-β1 signaling, phosphorylations of Smad-2 and p38-MAPK, were also dose-dependently inhibited by the presence of DKT (Figure 6D).

TRPA1 is a negative feedback regulator of collagen synthesis/secretion

We previously found that extracellular applied collagen downregulated many pro-fibrotic gene transcripts involved in TGF-β signaling in InMyoFibs, including *CDH2*, *ACTA2*, and *COL1A1*[19]. We therefore examined whether a major extracellular matrix collagen also regulated TRPA1 expression. When Type Ⅰ collagen was added into the culture medium, the expression of TRPA1 was enhanced more than two-fold, resulting in the decreased endogenous production of collagen (Figure 7A and B). Concomitantly, the expression levels of *COL1A1*, *ACTA2*, and *CDH2* mRNAs in InMyoFibs were downregulated by extracellular applied Type Ⅰ collagen, regardless of the presence of TGF-β1. Moreover, extracellular applied collagen caused a significant decrease in TGF-β1-induced *MYOCD* expression (Figure 7B-D). Strikingly, following siRNA knockdown of TRPA1 expression, which exacerbated the fibrogenic effects of TGF-β1[18], extracellular applied collagen was no longer effective in suppressing the mRNA expression of fibrotic factors *COL1A1*, *MYOCD*, and *ACTA2* (Figure 7E). Taken together, these results indicate that TRPA1 activity may be commonly involved in anti-fibrotic processes in InMyoFibs, which are activated by DKT and extracellular applied collagen.

Enhanced TRPA1 expression in stenotic tissues of CD patients

Because intestinal stricture formation in CD is driven by a local excessive accumulation of myofibroblasts, we examined whether TRPA1 is upregulated in highly fibrotic areas of human CD patients. Surgical samples obtained from the patient's intestines were subjected to histological examination and immunostaining. HE and MT staining revealed a much denser deposition of collagen fibers in the mucosal layer of the fibrotic stenotic areas than those of the non-stenotic areas. Moreover, TRPA1 immunoreactivity detected by diaminobenzidine (DAB) staining was more diffusely distributed in the mucosal and submucosal layers of the stenotic regions in the intestines of CD patients (Figure 8).

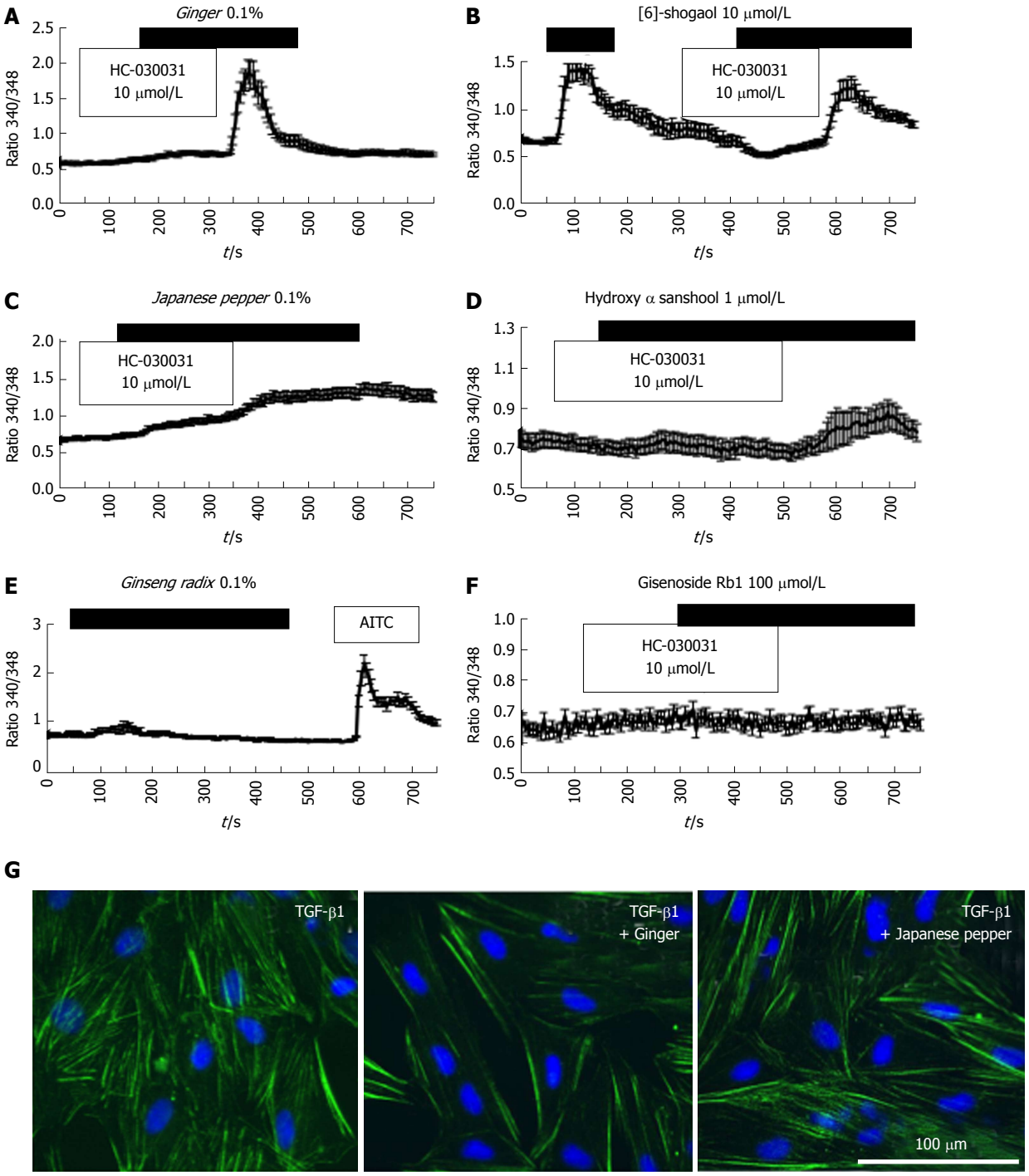


Figure 4 Crude components/ingredient of Daikenchuto induce Ca^{2+} responses *via* the transient receptor potential ankyrin 1 channel with anti-fibrotic effects in intestinal myofibroblasts. A-D: Representative $[Ca^{2+}]_i$ responses in InMyoFibs cells. Cells were exposed to ginger (0.1%), Japanese pepper (0.1%), 6-shogaol (10 μmol/L), or hydroxy-α-sanshool (1 μmol/L), which evoked measurable increases in $[Ca^{2+}]_i$; E and F: Cells were exposed to ginseng radix (0.1%), ginsenoside Rb1 (100 μmol/L), or AITC (10 μmol/L). Only AITC evoked a measurable increase in $[Ca^{2+}]_i$ in InMyoFib. The vertical axis indicates the 340/380 fluorescence ratio. Respective data points represent means ± SEM from > 30 cells; G: Ginger (0.1%) or Japanese pepper (0.1%) suppressed TGF-β1-induced α-SMA expression in InMyoFibs. Immunostaining images of InMyoFibs with anti-α-SMA (green) antibody and DAPI (blue), and no treatment or 24 h TGF-β1 (5 ng/mL) treatment. AITC: Allyl isothiocyanate; InMyoFib: Intestinal myofibroblast; α-SMA: α-Smooth muscle actin; TGF-β1: Transforming growth factor-β1.

DISCUSSION

Daikenchuto (DKT), a traditional oriental herbal medicine, is widely used for the treatment of gastrointestinal disorders. DKT is known to improve post-operative complications and is frequently prescribed for ileus, abdominal bloating, and cold sensation[40]. Several clinical meta-analyses have shown, for instance, that the perioperative administration of DKT effectively relieves the symptoms of post-operative ileus in patients undergoing gastrointestinal cancer surgery[41]. These beneficial effects of DKT are in part ascribed to its multiple actions th-

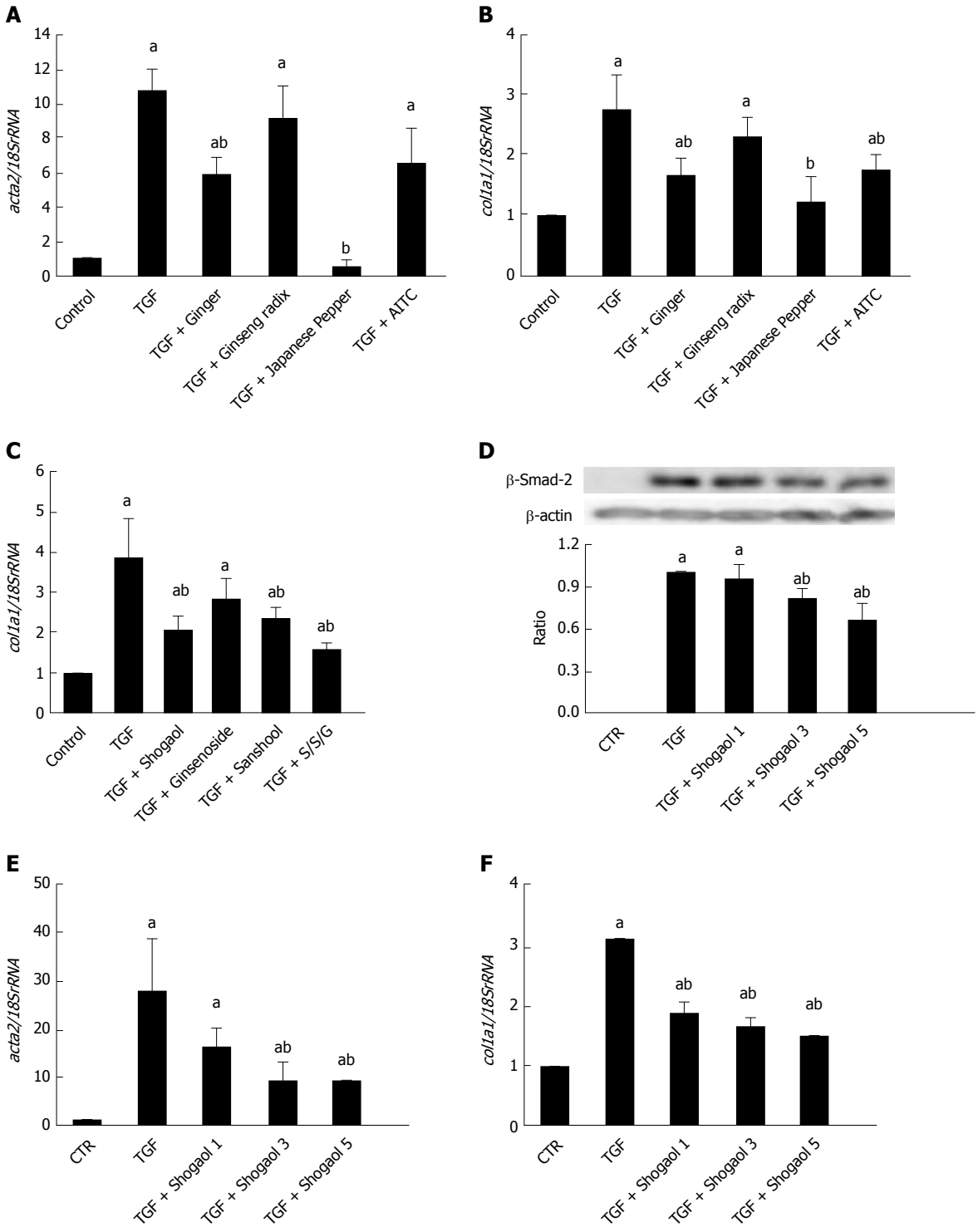


Figure 5 Daikenchuto components/ingredients suppress increased expression of α-smooth muscle actin and collagen by transforming growth factor-β1. ACTA2 and COL1A1 mRNAs were quantified by real-time PCR in control and TGF-β1-treated (5 ng/mL, 24 h) InMyoFibs. A-C: DKT components Ginger (0.1%), Japanese pepper (0.1%) and Ginseng radix (0.1%) (A, B), and DKT ingredients 6-shogaol (10 μmol/L), hydroxy-α-sanshool (1 μmol/L) and ginsenoside Rb1 (100 μmol/L) (C) were co-administered with TGF-β1. AITC (10 μmol/L) was used as the reference of full TRPA1 activation; D: Phosphorylation of Smad-2 was measured by western blot analysis. 1, 3, or 5 μmol/L 6-shogaol was co-administered with TGF-β1. Relative expression of Smad-2 protein (normalized to β-actin) in TGF-β1-treated cells is shown as the ratio of the two in the histogram; E and F: COL1A1 and ACTA2 mRNAs were quantified by RT-PCR in TGF-β1 treated InMyoFibs and normalized to 18S rRNA. 1, 3 or 5 μmol/L 6-shogaol was co-administered with TGF-β1. [a]$P < 0.05$ *vs* control cells; [b]$P < 0.05$ *vs* TGF-β1-treated cells ($n = 4$). TGF-β1: Transforming growth factor-β1; InMyoFibs: Intestinal myofibroblasts; DKT: Daikenchuto; AITC: Allyl isothiocyanate; TRPA1: Transient receptor potential ankyrin 1.

rough differential pharmacokinetics of respective DKT compounds on a number of ion channels (TRPA1, TRPV1, two-pore-domain KCNK channels) distributed throughout the enteric/myenteric and sensory nerves and intestinal epithelial cells. The activation or inhibition of these channels are thought to cause the release of

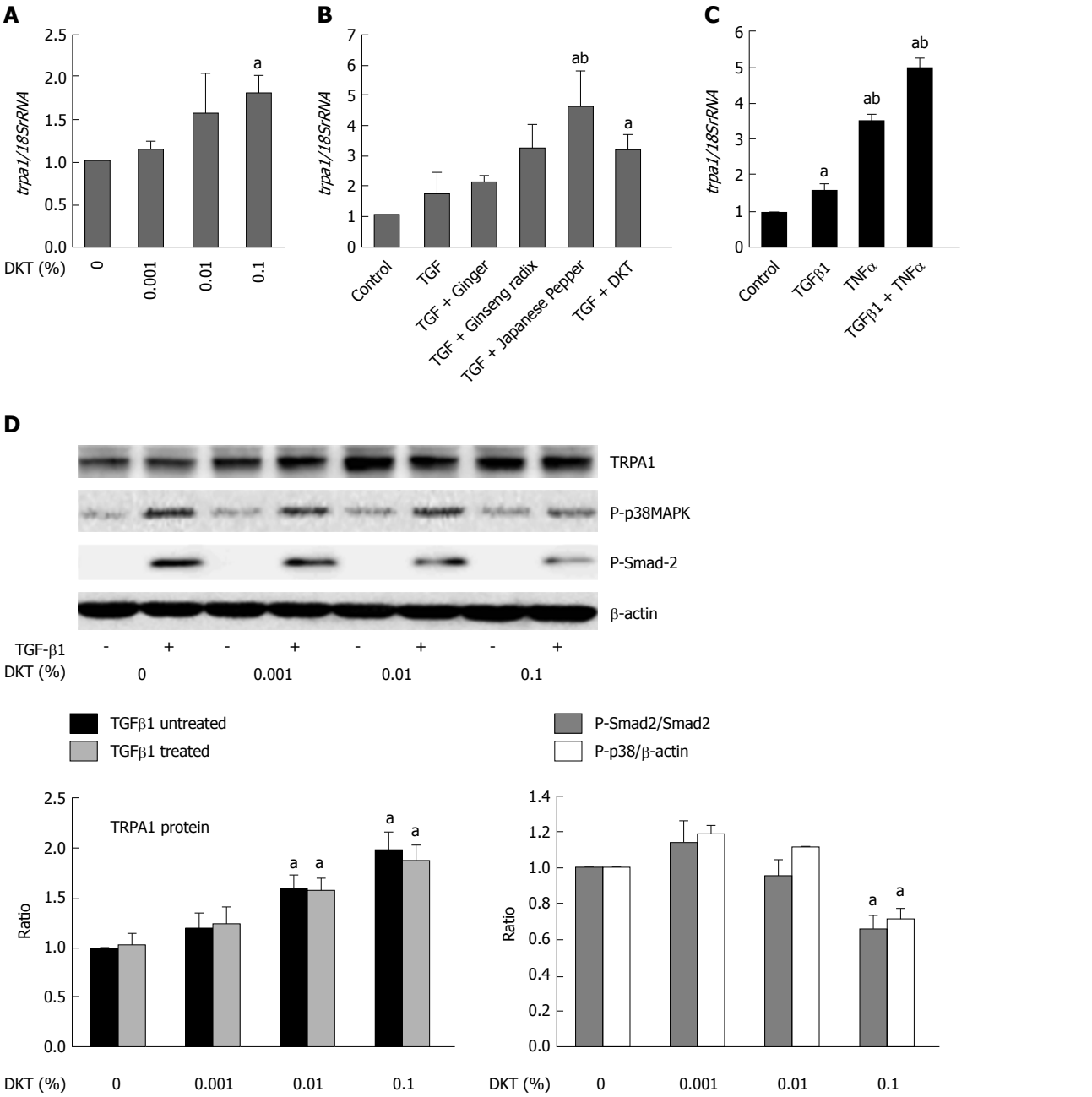


Figure 6 Daikenchuto upregulates transient receptor potential ankyrin 1 expression and suppresses phosphorylation events downstream of transforming growth factor-β1 signaling. A: *trpa1* mRNA expression quantified by real-time PCR in control and TGF-β1-treated (5 ng/mL, 24 h) InMyoFibs (normalized to 18S rRNA). DKT extracts (0.001%, 0.01%, 0.1%) were applied for 24 h; B: Relative mRNA expression of trpa1 with ginger (0.1%), ginseng radix (0.1%), Japanese pepper (0.1%), or DKT (0.1%) plus co-administered TGF-β1; C: *trpa1* mRNA expression was upregulated by TGF-β1, TNF-α, or both; D: TRPA1 protein expression and phosphorylation of Smad-2 or p38-MAPK with and without TGF-β1 treatment at various concentrations of DKT extracts (0.001%, 0.01%, and 0.1%). The upper panel shows actual immunoblots. Graphs show relative expression level of TRPA1 (normalized to β-actin) and the relative ratios of phosphorylated/unphosphorylated Smad-2 and p38-MAPK (normalized to β-actin) after 1 h TGF-β1 treatment at various DKT concentrations. $^{a}P < 0.05$ *vs* control cells, $^{b}P < 0.05$ *vs* TGF-β1-treated cells ($n = 4$). TGF-β1: Transforming growth factor-β1; InMyoFibs: Intestinal myofibroblasts; DKT: Daikenchuto; TRPA1: Transient receptor potential ankyrin 1; p38-MAPK: p38-mitogen-activated protein kinase.

two vasoactive/anti-inflammatory peptides, CGRP and adrenomeduline, or enhance the excitability of smooth muscle and neurons in the intestine, which improve intestinal microcirculation and motility, and induce anti-inflammatory responses[40]. However, although a few studies using chronic inflammation models have already reported the anti-fibrotic effects of DKT[3,42,43], the critical involvement of intestinal myoblasts in fibrogenesis and the therapeutic potential of the myofibroblast TRPA1 channel against it have not yet been addressed[18].

In this respect, the present study has provided several novel findings. First, our TNBS colitis model with the comparative use of wild-type and TRPA1-KO mice successfully demonstrated the anti-fibrotic actions of DKT *via* TRPA1 channel activation (Figure 1).

Specifically, the fibrosis score exacerbated by TNBS

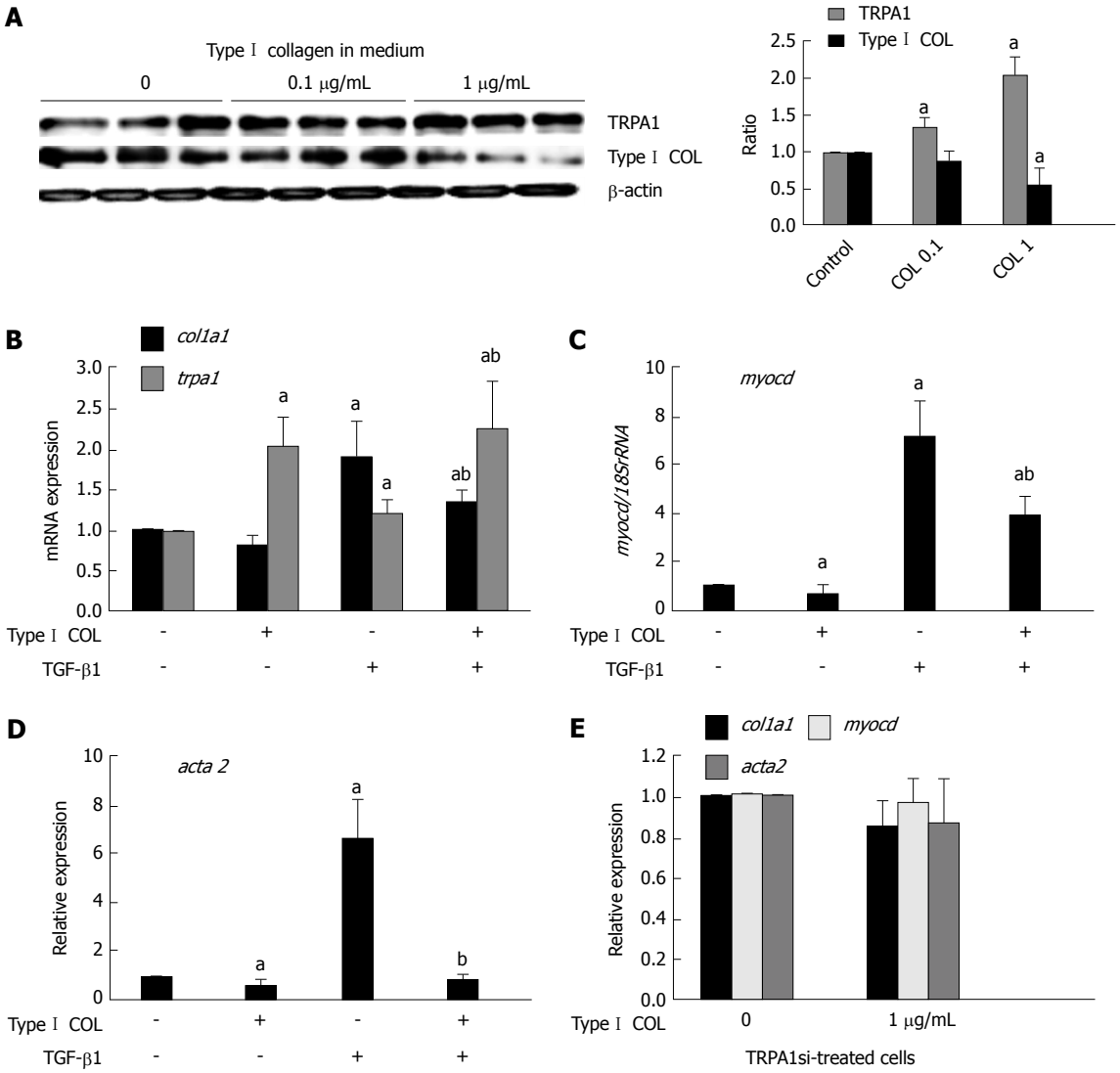


Figure 7 Extracellular collagen upregulates transient receptor potential ankyrin 1 expression to exert anti-fibrotic effects. A: Type Ⅰ collagen solution (0, 0.1, or 1 μg/mL) was added into the culture medium, and immunoblotting was performed to detect TRPA1 and Type Ⅰ collagen expression in InMyoFibs. The rightmost graph shows the summary (n = 4) B-E: Relative expression levels (shown as the post/pre ratio) of col1a1, trpa1, myocd, and acta2 mRNAs quantified by RT-PCR in Type Ⅰ collagen- or TGF-β1-treated InMyoFibs. In E, InMyoFibs were pre-treated with TRPA1 siRNA. [a]P < 0.05 *vs* control cells; [b]P < 0.05 *vs* TGF-β1-treated cells (n = 4). TRPA1: Transient receptor potential ankyrin 1; InMyoFibs: Intestinal myofibroblasts; TGF-β1: Transforming growth factor-β1.

treatment was significantly improved by enema administration of DKT during the chronic phase of inflammation (*i.e.*, at five weeks after TNBS), which was abrogated by the deletion of the *trpa1* gene (Figure 2)[18]. Second, our *in vitro* experiments with a human myofibroblast cell line clearly indicated that myofibroblast TRPA1 channel activation is a requisite for the downregulation of fibrotic factors/activities by DKT. Additionally, among its components, ginger and Japanese pepper, both being able to activate the TRPA1 channel in the expression system, showed comparable anti-fibrotic effects. Further investigation of active DKT ingredients suggested that 6-shogaol is likely the most effective ingredient, and that hydroxy α-sanshool may also mediate some part of DKT's anti-fibrotic actions (Figures 3-5). Intriguingly, DKT has an additional action of enhancing TRPA1 expression *per se*, which appears to be exerted by its Japanese pepper component. Finally, in support of these findings, histological and immunohistochemical examinations of human intestinal samples showed that myofibroblasts expressing TRPA1, as well as the fibrosis biomarker HSP47, were coincidentally accumulated in the stenosis areas of CD patients (Figure 8)[18], as observed in the mice TNBS-colitis model (Figure 2C).

Taken together, these findings can be interpreted to indicate that in chronically inflamed intestines, TRPA1 channel may be antagonistically upregulated in both expression and activity against the growing fibrogenic drive, which could further be potentiated by TRPA1-stimulating actions of DKT. As we discussed in a recent paper[18], this view could be extended to various tissues and organs undergoing chronic inflammation where therapeutic activation TRPA1 channels *in situ* may work beneficially to decelerate locally enhanced fibrogenic processes. In fact, there are several lines of evidence that TRPA1 channels in both neuronal and non-neuronal

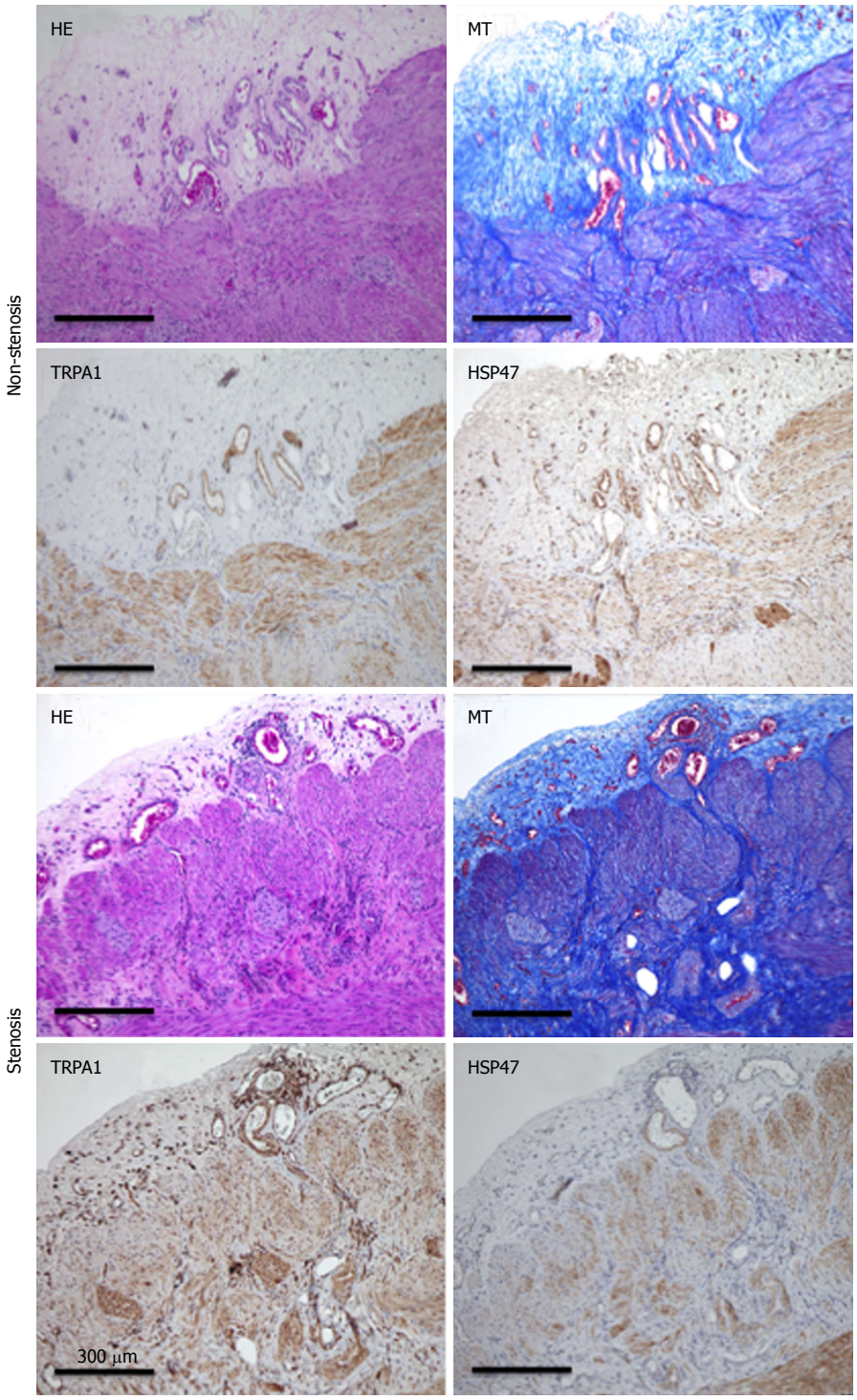


Figure 8 Hematoxylin-eosin, Masson's trichrome staining. TRPA1-DAB, and HSP47-DAB-stained non-stenosis/stenosis surgical tissue from the transverse colon of a CD patient. TRPA1: Transient receptor potential ankyrin 1; DAB: Diaminobenzidine; HSP47: Heat shock protein 47; CD: Crohn's disease.

cells modulate local tissue inflammation accompanying fibrotic changes, as exemplified by the animal models of acute pulmonary inflammation induced by acrolein and cigarette smoke, experimental colitis, or carrageenan-induced paw edema[7,44,45].

In addition to the above findings, we also found that extracellular applied collagen can induce the expression of TRPA1, with downregulation of the mRNAs of fibrotic factors *MYOCD*, *COL1A1*, and *ACTA2* (Figure 9). It has been reported that cell contact with collagen reduces cadherin expression, which in turn eliminates the mechanotransduction between fibroblasts[46]; therefore, extracellular collagen might modulate the expression of cadherin and the dynamics of cytoskeleton stress fiber formation *via* an as-yet-unknown membrane sensor(s), which is crucial for the modulation of fibrogenesis signaling. In our study, extracellular collagen application downregulated the expression levels of intrinsic collagen,

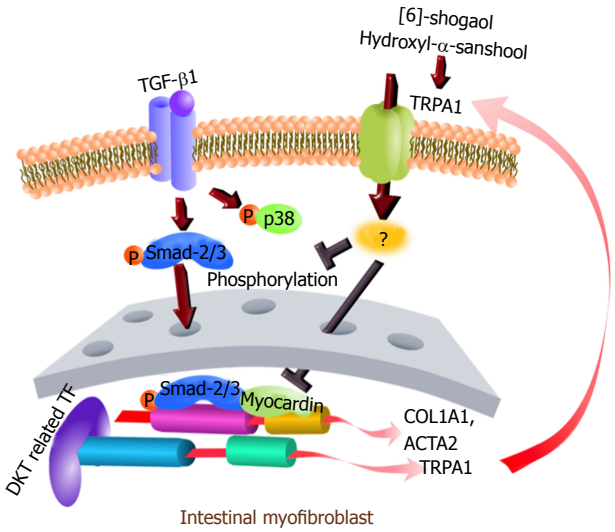


Figure 9 Graphical abstract.

α-SMA, and myocardin, but these changes were not observed in TRPA1-siRNA-treated InMyoFibs. These data strongly support the idea that TRPA1 channel activity is negatively correlated with the collagen synthesis of intestinal myofibroblasts. In the context of the present study, this would again represent another type of TRPA1-mediated negative feedback regulation that resists against a heightening fibrogenic potential.

In a few clinical studies, TGF-β1 mRNA levels were found elevated in the affected intestinal mucosa from the patients with CD and ulcerative colitis, particularly in the regions confined to the lamina propria where immune cells, as well as myofibroblasts, preferentially reside[23]. Combining this fact with our own finding from human surgical samples (*i.e.*, the co-accumulation of TRPA1-/HSP47-double positive myofibroblasts in the stenotic regions), it seems plausible to assume that in an interwoven network of TGF-β-mediated signaling, TRPA1 in myofibroblasts may operate as a crucial anti-fibrotic negative feedback to lower the pathologically increased fibrogenic potential. Thus, targeting the myofibroblast TRPA1 signaling axis could be a tenable therapeutic strategy to reinforce the anti-fibrotic potential in chronic fibrotic diseases such as CD.

The higher prevalence of fibrosis in CD is thought to be the complex consequences of transmural bowel inflammation that exposes all mesenchymal cells producing extracellular matrix to fibrotic mediators[47]. Therefore, anti-TNF (*i.e.*, anti-inflammatory) treatment is generally recommended as the first-line therapy for CD patients with poor prognoses who have severe complications or bowel damages[48]. However, anti-TNF treatment has an increased risk of worsening stenotic fibrosis[49], and stenotic lesions can be present in variable pathological states, such as inflammatory, fibrogenic, and neoplastic, or a combination of these states. Therefore, a therapeutic strategy that can distinguish between these different states might be more desirable than the currently available anti-inflammatory approaches. In this respect, the present finding, that the activation of myofibroblast TRPA1 attenuates intestinal fibrosis, may provide great therapeutic benefits, and could presumably be generalized to other fibrotic lesions such as those in the skin, lungs, and liver, where several TRP channels, including TRPA1, are highly expressed.

Finally, we should point out several limitations of this study. First, the method adopted in this study (*i.e.*, TNBS treatment) could not create a severe fibrotic stenosis model, which only allowed us to see rather mild fibrotic changes. Second, all biopsy samples were obtained from IBD patients but not from healthy donors. If this had been done, the results of this study might clinically be more attractive. Third, other Chinese medicines that are potentially capable of activating TRPA1 channel were not tested in this study. This would somewhat compromise the first priority of DKT for anti-fibrotic treatment. In addition, DKT used in this study was obtained from a single source (*i.e.*, Tsumura Co.); therefore, the possibility cannot be entirely excluded that the observed effects in this study might differ in details from those obtained with the same drug of a different origin.

In summary, the present study revealed a new role for myofibroblast TRPA1 channels in intestinal fibrosis, *i.e.*, its negative regulation, and showed that DKT upregulates this channel protein in both activity and expression to exert its anti-fibrotic actions. These findings not only provide a novel molecular target for the anti-fibrotic therapy of IBDs in the future, but also a framework to reinterpret the unique theories of traditional Oriental medicine.

ARTICLE HIGHLIGHTS

Research background

Daikenchuto (DKT) is a traditional oriental herbal medicine, widely used to mitigate post-operative ileus and constipation. The mechanism of its actions remains less understood, in particular regarding its molecular target(s). However, an important hint to addressing this issue has come from our previous findings that the transient receptor potential ankyrin 1 (TRPA1) is highly expressed in intestinal myofibroblasts and mediates the antifibrotic effects of steroid and pirfenidone.

Research motivation

We hypothesized that DKT may activate TRPA1 channels in myofibroblasts to directly counteract the fibrotic changes of inflamed intestines. Although DKT has been shown to be beneficial for gut motility, intestinal blood flow and gastrointestinal hormone secretion, its anti-fibrotic actions through TRPA1 channel activation will be an entirely new finding and of considerable clinical significance.

Research objectives

We aimed at investigating at both *in vitro* and *in vivo* levels whether DKT targets TRPA1 channels expressed in intestinal myofibroblasts to exert its anti-fibrotic actions. The results would not only disclose a novel molecular target of anti-fibrotic therapy for inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis, but also promote our general understanding about the complex actions of herbal medicines.

Research methods

A trinitrobenzene sulfonic acid (TNBS) chronic colitis model was established

in both wild-type and TRPA1 knock out mice, in which the impact of DKT administration were evaluated by pathological and biochemical analyses. An intestinal myofibroblast cell line (InMyoFibs) stimulated by transforming growth factor-β1 (TGF-β1) was used as a cellular model of intestinal fibrosis, where expression of TRPA1 and pro-fibrotic factors and related intracellular signaling (in particular TGF-β-mediated one) were examined in detail. The counteracting effects of DKT on these changes were also evaluated. Biopsy samples from non-stenotic and stenotic regions of CD patient's intestines were subjected to histological examination and immunoblot analysis with particular respect to the altered expression pattern of TRPA1 channel and fibrotic factors. As compared with preceding works on a similar topic, the methodological approach applied to this study is more comprehensive covering a broad range of DKT's actions that include cellular and animal models and human patients.

Research results

In TNBS chronic colitis model mice, the extents of inflammation and fibrotic changes were more prominent in TRPA1-/- knockout than in wild-type mice. One-week enema administration of DKT suppressed fibrotic lesions in wild-type mice, but not in TRPA1 knockout mice. Active ingredients of DKT, induced Ca^{2+} influxes in InMyoFib, which were antagonized by TRPA1 channel blocker HC-030031. DKT counteracted TGF-β1-induced expression of Type 1 collagen, α-SMA, and this was accompanied by attenuated fibrosis-associated signaling. A DKT's active ingredient Japanese Pepper increased the mRNA and protein expressions of TRPA1, which in turn negatively regulated collagen synthesis in InMyoFibs. In stenotic regions of CD patient's intestines, TRPA1 expression was significantly increased. However, several limitations remain in this study that may compromise the value and relevance of this study, which include the inability of TNBS to create a severe phenotype of chronic colitis, the lack of control data from human healthy donors, and no comparative data from other Chinese (herbal) medicines that may have similar actions.

Research conclusions

DKT-induced expression and activation of TRPA1 could be important mechanisms for suppressing intestinal fibrosis, which would in part account for the reported beneficial actions of DKT on inflamed intestines. Thus, targeting myofibroblast TRPA1 may serve as a new and promising therapeutic strategy for fibrotic stenotic changes occurring in incurable chronic inflammatory bowel diseases such as CD and ulcerative colitis.

Research perspectives

Our findings not only provide a novel molecular target for the anti-fibrotic therapy of inflammatory bowel diseases in the future, but also a framework to reinterpret the unique theories of traditional Oriental medicine.

REFERENCES

1 **Kono T**, Omiya Y, Hira Y, Kaneko A, Chiba S, Suzuki T, Noguchi M, Watanabe T. Daikenchuto (TU-100) ameliorates colon microvascular dysfunction *via* endogenous adrenomedullin in Crohn's disease rat model. *J Gastroenterol* 2011; **46**: 1187-1196 [PMID: 21808981 DOI: 10.1007/s00535-011-0438-2]

2 **Endo M**, Hori M, Ozaki H, Oikawa T, Hanawa T. Daikenchuto, a traditional Japanese herbal medicine, ameliorates postoperative ileus by anti-inflammatory action through nicotinic acetylcholine receptors. *J Gastroenterol* 2014; **49**: 1026-1039 [PMID: 23846546 DOI: 10.1007/s00535-013-0854-6]

3 **Kitamura M**, Nishino T, Obata Y, Oka S, Abe S, Muta K, Ozono Y, Koji T, Kohno S. The kampo medicine Daikenchuto inhibits peritoneal fibrosis in mice. *Biol Pharm Bull* 2015; **38**: 193-200 [PMID: 25747978 DOI: 10.1248/bpb.b14-00469]

4 **Ogasawara T**, Morine Y, Ikemoto T, Imura S, Fujii M, Soejima Y, Shimada M. Influence of Dai-kenchu-to (DKT) on human portal blood flow. *Hepatogastroenterology* 2008; **55**: 574-577 [PMID: 18613410]

5 **Pan MH**, Hsieh MC, Hsu PC, Ho SY, Lai CS, Wu H, Sang S, Ho CT. 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *Mol Nutr Food Res* 2008; **52**: 1467-1477 [PMID: 18683823 DOI: 10.1002/mnfr.200700515]

6 **Penuelas A**, Tashima K, Tsuchiya S, Matsumoto K, Nakamura T, Horie S, Yano S. Contractile effect of TRPA1 receptor agonists in the isolated mouse intestine. *Eur J Pharmacol* 2007; **576**: 143-150 [PMID: 17825279 DOI: 10.1016/j.ejphar.2007.08.015]

7 **Poole DP**, Pelayo JC, Cattaruzza F, Kuo YM, Gai G, Chiu JV, Bron R, Furness JB, Grady EF, Bunnett NW. Transient receptor potential ankyrin 1 is expressed by inhibitory motoneurons of the mouse intestine. *Gastroenterology* 2011; **141**: 565-575, 575.e1-575.e4 [PMID: 21689654 DOI: 10.1053/j.gastro.2011.04.049]

8 **Satoh K**, Hayakawa T, Kase Y, Ishige A, Sasaki H, Nishikawa S, Kurosawa S, Yakabi K, Nakamura T. Mechanisms for contractile effect of Dai-kenchu-to in isolated guinea pig ileum. *Dig Dis Sci* 2001; **46**: 250-256 [PMID: 11281170 DOI: 10.1023/A:1005636412287]

9 **Tokita Y**, Yamamoto M, Satoh K, Nishiyama M, Iizuka S, Imamura S, Kase Y. Possible involvement of the transient receptor potential vanilloid type 1 channel in postoperative adhesive obstruction and its prevention by a kampo (traditional Japanese) medicine, daikenchuto. *J Pharmacol Sci* 2011; **115**: 75-83 [PMID: 21186335 DOI: 10.1254/jphs.10226FP]

10 **Kim MO**, Lee MH, Oi N, Kim SH, Bae KB, Huang Z, Kim DJ, Reddy K, Lee SY, Park SJ, Kim JY, Xie H, Kundu JK, Ryoo ZY, Bode AM, Surh YJ, Dong Z. [6]-shogaol inhibits growth and induces apoptosis of non-small cell lung cancer cells by directly regulating Akt1/2. *Carcinogenesis* 2014; **35**: 683-691 [PMID: 24282290 DOI: 10.1093/carcin/bgt365]

11 **Radhakrishnan EK**, Bava SV, Narayanan SS, Nath LR, Thulasidasan AK, Soniya EV, Anto RJ. [6]-Gingerol induces caspase-dependent apoptosis and prevents PMA-induced proliferation in colon cancer cells by inhibiting MAPK/AP-1 signaling. *PLoS One* 2014; **9**: e104401 [PMID: 25157570 DOI: 10.1371/journal.pone.0104401]

12 **Dubowski KM**, Luke JL. Measurement of carboxyhemoglobin and carbon monoxide in blood. *Ann Clin Lab Sci* 1973; **3**: 53-65 [PMID: 4691500 DOI: 10.1158/1940-6207.CAPR-13-0420]

13 **Kono T**, Kaneko A, Hira Y, Suzuki T, Chisato N, Ohtake N, Miura N, Watanabe T. Anti-colitis and -adhesion effects of daikenchuto *via* endogenous adrenomedullin enhancement in Crohn's disease mouse model. *J Crohns Colitis* 2010; **4**: 161-170 [PMID: 21122500 DOI: 10.1016/j.crohns.2009.09.006]

14 **Katsuno H,** Maeda K, Kaiho T, Kunieda K, Funahashi K, Sakamoto J, Kono T, Hasegawa H, Furukawa Y, Imazu Y, Morita S, Watanabe M: Clinical efficacy of Daikenchuto for gastrointestinal dysfunction following colon surgery: a randomized, double-blind, multicenter, placebo-controlled study (JFMC39-0902). *Jpn J Clin Oncol* 2015; **45**: 650-656. [PMID: 25972515 DOI: 10.1093/jjco/hyv056]

15 **Katsuno H**, Maeda K, Ohya M, Yoshioka K, Tsunoda A, Koda K, Matsuoka H, Ohge H, Morita S, Saji S, Kanematsu T, Kitajima M. Clinical pharmacology of daikenchuto assessed by transit analysis using radiopaque markers in patients with colon cancer undergoing open surgery: a multicenter double-blind randomized placebo-controlled study (JFMC39-0902 additional study). *J Gastroenterol* 2016; **51**: 222-229 [PMID: 26162646 DOI: 10.1007/s00535-015-1100-1]

16 **Rieder F**, Brenmoehl J, Leeb S, Schölmerich J, Rogler G. Wound healing and fibrosis in intestinal disease. *Gut* 2007; **56**: 130-139 [PMID: 17172588 DOI: 10.1136/gut.2006.090456]

17 **Hinz B**. Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 2007; **127**: 526-537 [PMID: 17299435 DOI: 10.1038/sj.jid.5700613]

18 **Kurahara LH**, Hiraishi K, Hu Y, Koga K, Onitsuka M, Doi M, Aoyagi K, Takedatsu H, Kojima D, Fujihara Y, Jian Y, Inoue R. Activation of Myofibroblast TRPA1 by Steroids and Pirfenidone Ameliorates Fibrosis in Experimental Crohn's Disease. *Cell Mol Gastroenterol Hepatol* 2017; **5**: 299-318 [PMID: 29552620 DOI: 10.1016/j.jcmgh.2017.12.005]

19 **Kurahara LH**, Sumiyoshi M, Aoyagi K, Hiraishi K, Nakajima K, Nakagawa M, Hu Y, Inoue R. Intestinal myofibroblast TRPC6 channel may contribute to stenotic fibrosis in Crohn's disease.

Inflamm Bowel Dis 2015; **21**: 496-506 [PMID: 25647156 DOI: 10.1097/MIB.0000000000000295]

20 **Medina C**, Santos-Martinez MJ, Santana A, Paz-Cabrera MC, Johnston MJ, Mourelle M, Salas A, Guarner F. Transforming growth factor-beta type 1 receptor (ALK5) and Smad proteins mediate TIMP-1 and collagen synthesis in experimental intestinal fibrosis. *J Pathol* 2011; **224**: 461-472 [PMID: 21465486 DOI: 10.1002/path.2870]

21 **Biancheri P**, Giuffrida P, Docena GH, MacDonald TT, Corazza GR, Di Sabatino A. The role of transforming growth factor (TGF)-β in modulating the immune response and fibrogenesis in the gut. *Cytokine Growth Factor Rev* 2014; **25**: 45-55 [PMID: 24332927 DOI: 10.1016/j.cytogfr.2013.11.001]

22 **Leask A**. Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circ Res* 2010; **106**: 1675-1680 [PMID: 20538689 DOI: 10.1161/CIRCRESAHA.110.217737]

23 **Babyatsky MW**, Rossiter G, Podolsky DK. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. *Gastroenterology* 1996; **110**: 975-984 [PMID: 8613031 DOI: 10.1053/gast.1996.v110.pm8613031]

24 **Inoue R**, Jensen LJ, Shi J, Morita H, Nishida M, Honda A, Ito Y. Transient receptor potential channels in cardiovascular function and disease. *Circ Res* 2006; **99**: 119-131 [PMID: 16857972 DOI: 10.1161/01.RES.0000233356.10630.8a]

25 **Nishida M**, Onohara N, Sato Y, Suda R, Ogushi M, Tanabe S, Inoue R, Mori Y, Kurose H. Galpha12/13-mediated up-regulation of TRPC6 negatively regulates endothelin-1-induced cardiac myofibroblast formation and collagen synthesis through nuclear factor of activated T cells activation. *J Biol Chem* 2007; **282**: 23117-23128 [PMID: 17533154 DOI: 10.1074/jbc.M611780200]

26 **Onohara N**, Nishida M, Inoue R, Kobayashi H, Sumimoto H, Sato Y, Mori Y, Nagao T, Kurose H. TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *EMBO J* 2006; **25**: 5305-5316 [PMID: 17082763 DOI: 10.1038/sj.emboj.7601417]

27 **Du J**, Xie J, Zhang Z, Tsujikawa H, Fusco D, Silverman D, Liang B, Yue L. TRPM7-mediated Ca2+ signals confer fibrogenesis in human atrial fibrillation. *Circ Res* 2010; **106**: 992-1003 [PMID: 20075334 DOI: 10.1161/CIRCRESAHA.109.206771]

28 **Kono T**, Kaneko A, Omiya Y, Ohbuchi K, Ohno N, Yamamoto M. Epithelial transient receptor potential ankyrin 1 (TRPA1)-dependent adrenomedullin upregulates blood flow in rat small intestine. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G428-G436 [PMID: 23275609 DOI: 10.1152/ajpgi.00356.2012]

29 **Lee BH**, Hsu WH, Hsu YW, Pan TM. Suppression of dimerumic acid on hepatic fibrosis caused from carboxymethyl-lysine (CML) by attenuating oxidative stress depends on Nrf2 activation in hepatic stellate cells (HSCs). *Food Chem Toxicol* 2013; **62**: 413-419 [PMID: 24036144 DOI: 10.1016/j.fct.2013.09.007]

30 **Jiang X**, Zhang Y, Li F, Zhu Y, Chen Y, Yang S, Sun G. Allicin as a possible adjunctive therapeutic drug for stage II oral submucous fibrosis: a preliminary clinical trial in a Chinese cohort. *Int J Oral Maxillofac Surg* 2015; **44**: 1540-1546 [PMID: 26165773 DOI: 10.1016/j.ijom.2015.06.015]

31 **Kun J**, Szitter I, Kemény A, Perkecz A, Kereskai L, Pohóczky K, Vincze A, Gódi S, Szabó I, Szolcsányi J, Pintér E, Helyes Z. Upregulation of the transient receptor potential ankyrin 1 ion channel in the inflamed human and mouse colon and its protective roles. *PLoS One* 2014; **9**: e108164 [PMID: 25265225 DOI: 10.1371/journal.pone.0108164]

32 **Bertin S**, Aoki-Nonaka Y, Lee J, de Jong PR, Kim P, Han T, Yu T, To K, Takahashi N, Boland BS, Chang JT, Ho SB, Herdman S, Corr M, Franco A, Sharma S, Dong H, Akopian AN, Raz E. The TRPA1 ion channel is expressed in CD4+ T cells and restrains T-cell-mediated colitis through inhibition of TRPV1. *Gut* 2017; **66**: 1584-1596 [PMID: 27325418 DOI: 10.1136/gutjnl-2015-310710]

33 **Romano B**, Borrelli F, Fasolino I, Capasso R, Piscitelli F, Cascio M, Pertwee R, Coppola D, Vassallo L, Orlando P, Di Marzo V, Izzo A. The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis. *Br J Pharmacol* 2013; **169**: 213-229 [PMID: 23373571 DOI: 10.1111/bph.12120]

34 **Riera CE**, Menozzi-Smarrito C, Affolter M, Michlig S, Munari C, Robert F, Vogel H, Simon SA, le Coutre J. Compounds from Sichuan and Melegueta peppers activate, covalently and non-covalently, TRPA1 and TRPV1 channels. *Br J Pharmacol* 2009; **157**: 1398-1409 [PMID: 19594761 DOI: 10.1111/j.1476-5381.2009.00307.x]

35 **Koo JY**, Jang Y, Cho H, Lee CH, Jang KH, Chang YH, Shin J, Oh U. Hydroxy-alpha-sanshool activates TRPV1 and TRPA1 in sensory neurons. *Eur J Neurosci* 2007; **26**: 1139-1147 [PMID: 17767493 DOI: 10.1111/j.1460-9568.2007.05743.x]

36 **Tsuchiya K**, Kubota K, Ohbuchi K, Kaneko A, Ohno N, Mase A, Matsushima H, Yamamoto M, Miyano K, Uezono Y, Kono T. Transient receptor potential ankyrin 1 agonists improve intestinal transit in a murine model of postoperative ileus. *Neurogastroenterol Motil* 2016; **28**: 1792-1805 [PMID: 27284001 DOI: 10.1111/nmo.12877]

37 **Furio L**, Pampalakis G, Michael IP, Nagy A, Sotiropoulou G, Hovnanian A. KLK5 Inactivation Reverses Cutaneous Hallmarks of Netherton Syndrome. *PLoS Genet* 2015; **11**: e1005389 [PMID: 26390218 DOI: 10.1371/journal.pgen.1005389]

38 **Shi J**, Mori E, Mori Y, Mori M, Li J, Ito Y, Inoue R. Multiple regulation by calcium of murine homologues of transient receptor potential proteins TRPC6 and TRPC7 expressed in HEK293 cells. *J Physiol* 2004; **561**: 415-432 [PMID: 15579537 DOI: 10.1113/jphysiol.2004.075051]

39 **Takahashi N**, Kuwaki T, Kiyonaka S, Numata T, Kozai D, Mizuno Y, Yamamoto S, Naito S, Knevels E, Carmeliet P, Oga T, Kaneko S, Suga S, Nokami T, Yoshida J, Mori Y. TRPA1 underlies a sensing mechanism for O2. *Nat Chem Biol* 2011; **7**: 701-711 [PMID: 21873995 DOI: 10.1038/nchembio.640]

40 **Kono T**, Shimada M, Yamamoto M, Kaneko A, Oomiya Y, Kubota K, Kase Y, Lee K, Uezono Y. Complementary and synergistic therapeutic effects of compounds found in Kampo medicine: analysis of daikenchuto. *Front Pharmacol* 2015; **6**: 159 [PMID: 26300774 DOI: 10.3389/fphar.2015.00159]

41 **Ishizuka M**, Shibuya N, Nagata H, Takagi K, Iwasaki Y, Hachiya H, Aoki T, Kubota K. Perioperative Administration of Traditional Japanese Herbal Medicine Daikenchuto Relieves Postoperative Ileus in Patients Undergoing Surgery for Gastrointestinal Cancer: A Systematic Review and Meta-analysis. *Anticancer Res* 2017; **37**: 5967-5974 [PMID: 29061775]

42 **Yada K**, Ishibashi H, Mori H, Morine Y, Zhu C, Feng R, Kono T, Shimada M. The Kampo medicine "Daikenchuto (TU-100)" prevents bacterial translocation and hepatic fibrosis in a rat model of biliary atresia. *Surgery* 2016; **159**: 1600-1611 [PMID: 26994485 DOI: 10.1016/j.surg.2016.02.002]

43 **Inoue K**, Naito Y, Takagi T, Hayashi N, Hirai Y, Mizushima K, Horie R, Fukumoto K, Yamada S, Harusato A, Hirata I, Omatsu T, Yoshida N, Uchiyama K, Ishikawa T, Handa O, Konishi H, Wakabayashi N, Yagi N, Ichikawa H, Kokura S, Yoshikawa T. Daikenchuto, a Kampo medicine, regulates intestinal fibrosis associated with decreasing expression of heat shock protein 47 and collagen content in a rat colitis model. *Biol Pharm Bull* 2011; **34**: 1659-1665 [PMID: 22040876 DOI: 10.1248/bpb.34.1659]

44 **Nassini R**, Pedretti P, Moretto N, Fusi C, Carnini C, Facchinetti F, Viscomi AR, Pisano AR, Stokesberry S, Brunmark C, Svitacheva N, McGarvey L, Patacchini R, Damholt AB, Geppetti P, Materazzi S. Transient receptor potential ankyrin 1 channel localized to non-neuronal airway cells promotes non-neurogenic inflammation. *PLoS One* 2012; **7**: e42454 [PMID: 22905134 DOI: 10.1371/journal.pone.0042454]

45 **Moilanen LJ**, Laavola M, Kukkonen M, Korhonen R, Leppänen T, Högestätt ED, Zygmunt PM, Nieminen RM, Moilanen E. TRPA1 contributes to the acute inflammatory response and mediates carrageenan-induced paw edema in the mouse. *Sci Rep* 2012; **2**: 380 [PMID: 22532928 DOI: 10.1038/srep00380]

46 **Ehrlich HP**, Allison GM, Leggett M. The myofibroblast, cadherin, alpha smooth muscle actin and the collagen effect. *Cell Biochem Funct* 2006; **24**: 63-70 [PMID: 15584087 DOI: 10.1002/cbf.1188]

47 **Latella G**, Sferra R, Speca S, Vetuschi A, Gaudio E. Can we prevent, reduce or reverse intestinal fibrosis in IBD? *Eur Rev Med Pharmacol Sci* 2013; **17**: 1283-1304 [PMID: 23740440]
48 **Peyrin-Biroulet L**, Fiorino G, Buisson A, Danese S. First-line therapy in adult Crohn's disease: who should receive anti-TNF agents? *Nat Rev Gastroenterol Hepatol* 2013; **10**: 345-351 [PMID: 23458890 DOI: 10.1038/nrgastro.2013.31]
49 **Condino G**, Calabrese E, Zorzi F, Onali S, Lolli E, De Biasio F, Ascolani M, Pallone F, Biancone L. Anti-TNF-alpha treatments and obstructive symptoms in Crohn's disease: a prospective study. *Dig Liver Dis* 2013; **45**: 258-262 [PMID: 23195667 DOI: 10.1016/j.dld.2012.10.009]

P- Reviewer: Masia DO, Naser SA, Vasudevan A
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4054-4060

DOI: 10.3748/wjg.v24.i35.4054

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Portosplenomesenteric vein thrombosis in patients with early-stage severe acute pancreatitis

Ling Ding, Feng Deng, Chen Yu, Wen-Hua He, Liang Xia, Mi Zhou, Xin Huang, Yu-Peng Lei, Xiao-Jiang Zhou, Yin Zhu, Nong-Hua Lu

Ling Ding, Feng Deng, Mi Zhou, Medical College of Nanchang University, Nanchang 330006, Jiangxi Province, China

Chen Yu, Department of medical image, The First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

Wen-Hua He, Liang Xia, Xin Huang, Yu-Peng Lei, Xiao-Jiang Zhou, Yin Zhu, Nong-Hua Lu, Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

ORCID number: Ling Ding (0000-0002-2655-5616); Feng Deng (0000-0001-9463-5661); Chen Yu (0000-0002-6426-6698); Wen-Hua He (0000-0001-5499-1346); Liang Xia (0000-0001-7209-9407); Mi Zhou (0000-0002-7326-3747); Xin Huang (0000-0001-5031-5527); Yu-Peng Lei (0000-0003-0504-1090); Xiao-Jiang Zhou (0000-0003-4622-6040); Yin Zhu (0000-0002-4791-6126); Nong-Hua Lu (0000-0003-4373-551X).

Author contributions: Ding L and Deng F designed the study and wrote the manuscript; Yu C reassessed and reviewed patients' cross-sectional images; Zhou M collected data; He WH, Xia L and Zhou XJ made critical revisions; Huang X and Lei YP interpreted and analyzed data; Zhu Y and Lu NH made substantial contributions to conception, design and coordination of the study and gave final approval of the version to be published. All authors read and approved the final manuscript.

Supported by the National Natural Science Foundation of China, No. 81760120.

Institutional review board statement: This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Informed consent statement: All involved persons gave their informed consent (written or verbal) prior to study inclusion.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

STROBE statement: The authors have read the STROBE Statemen-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Manuscript source: Unsolicited manuscript

Correspondence to: Yin Zhu, PhD, Professor of Medicine, Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, 17 YongWaiZheng Street, Nanchang 330006, Jiangxi Province, China. zhuyin27@sina.com
Telephone: +86-791-88692540
Fax: +86-791-86292217

Received: May 3, 2018
Peer-review started: May 4, 2018
First decision: June 6, 2018
Revised: June 17, 2018
Accepted: June 25, 2018
Article in press: June 25, 2018
Published online: September 21, 2018

Abstract

AIM

To investigate the incidence and risk factors of portosplenomesenteric vein thrombosis (PSMVT) in the early stage of severe acute pancreatitis (SAP).

METHODS

Patients with SAP in a tertiary care setting from January 2014 to December 2016 were retrospectively reviewed.

All contrast-enhanced computed tomography (CT) studies were reassessed and reviewed. Clinical outcome measures were compared between SAP patients with and without PSMVT in the early stage of the disease. Univariate and multivariate logistic regression analyses were sequentially performed to assess potential risk factors for the development of PSMVT in SAP patients. A receiver operating characteristic (ROC) curve was generated for the qualifying independent risk factors.

RESULTS

Twenty-five of the one hundred and forty (17.86%) SAP patients developed PSMVT 6.19 ± 2.43 d after acute pancreatitis (AP) onset. PSMVT was confirmed by contrast-enhanced CT. Multivariate stepwise logistic regression analyses showed that Balthazar's CT severity index (CTSI) scores [odds ratio (OR): 2.742; 95% confidence interval (CI): 1.664-4.519; P = 0.000], hypoalbuminemia (serum albumin level < 25 g/L) (OR: 32.573; 95%CI: 2.711-391.353; P = 0.006) and gastrointestinal wall thickening (OR: 4.367, 95%CI: 1.218-15.658; P = 0.024) were independent risk factors for PSMVT developed in patients with SAP. The area under the ROC curve for Balthazar's CTSI scores was 0.777 (P = 0.000), the sensitivity was 52%, and the specificity was 93% at a cut-off value of 5.5.

CONCLUSION

High Balthazar's CTSI scores, hypoalbuminemia and gastrointestinal wall thickening are independent risk factors for PSMVT developed in the early stage of SAP.

Key words: Vascular complication; Portosplenomesenteric vein thrombosis; Severe acute pancreatitis; Early stage; Risk factors; Contrast-enhanced computed tomography

Core tip: Studies on portosplenomesenteric vein thrombosis (PSMVT) occurring in the early stage of acute pancreatitis (AP) were rare. We found that 17.86% of severe AP patients developed PSMVT 6.19 d after AP onset. High Balthazar's computed tomography severity index (CTSI) scores, hypoalbuminemia and gastrointestinal wall thickening are independent risk factors for the development of PSMVT, and Balthazar's CTSI scores can predict the occurrence of PSMVT with a high degree of specificity which indicated that a low Balthazar's CTSI score could be a good indicator that PSMVT would not occur.

Ding L, Deng F, Yu C, He WH, Xia L, Zhou M, Huang X, Lei YP, Zhou XJ, Zhu Y, Lu NH. Portosplenomesenteric vein thrombosis in patients with early-stage severe acute pancreatitis. *World J Gastroenterol* 2018; 24(35): 4054-4060 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4054.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4054

INTRODUCTION

Portosplenomesenteric vein thrombosis (PSMVT) involves the portal vein (PV), splenic vein and superior mesenteric vein (SMV), either separately or in combination. In most cases, PSMVT does not cause any additional symptoms, but may sometimes generate localized portal hypertension, leading to gastrointestinal and intra-abdominal hemorrhage, ascites, splenomegaly, and splenic infarction, among other problems[1,2]. The previous studies reported that PSMVT mostly occurs late during acute pancreatitis (AP)[3-5], and is usually detected incidentally on radiological imaging performed to evaluate the severity of AP[6].

Until now, the detailed natural history of PSMVT has remained unknown, and the pathogenesis underlying PSMVT in patients with AP has remained unclear and may involve many clinical factors. There is little data regarding the risk factors for this complication in the early stage of AP. The results about the risk factors of PSMVT in AP patients vary among studies[3,5,7-10]. Therefore, in this study, we analyzed the data to explore the incidence of PSMVT in the early stage of severe AP (SAP), the risk factors for the development of PSMVT, and the clinical outcomes of PSMVT.

MATERIALS AND METHODS

Patients

Consecutive adult patients (age ≥ 18 years old) with SAP who were admitted to the First Affiliated Hospital of Nanchang University between January 2014 and December 2016 were enrolled in this study. Exclusion criteria include the following: (1) Admission > 6 d after AP onset; (2) history of AP, chronic pancreatitis, pancreatic malignancy or cysts; (3) no contrast-enhanced CT examination ≤ 10 d after AP onset; (4) pregnancy; and (5) cirrhosis or coagulopathy disorder diseases or other systemic tumors.

Data collection

The following data were collected: (1) General characteristics, including age, gender, etiology of AP, modified Marshall score, systemic inflammatory response syndrome score, acute physiology and chronic health evaluation Ⅱ score, and Balthazar's computer tomography severity index (CTSI)[11] at admission; (2) clinical outcomes, including microbiological outcomes, ascites, and intra-abdominal pressure measured with a catheter inserted into the bladder according to the guidelines[12], complications including gastrointestinal hemorrhage, intra-abdominal hemorrhage, multiple organ dysfunction syndrome, and infected pancreatic necrosis, and clinical treatments including minimally invasive and surgical interventions and hospital and intensive care unit lengths of stay and mortality; and (3) laboratory markers,

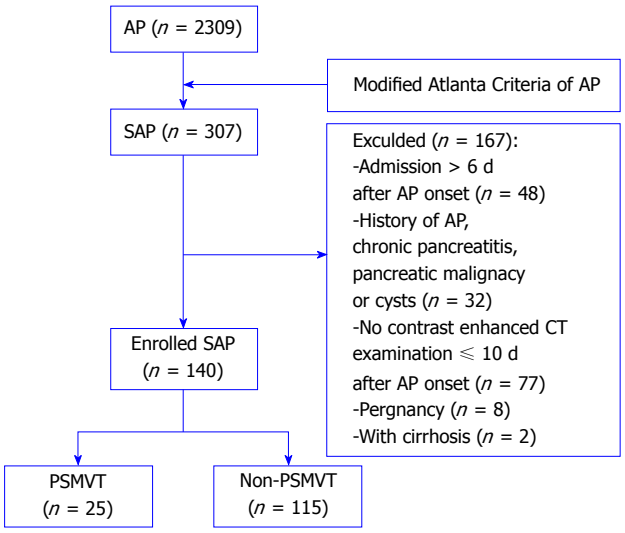


Figure 1 Flow chart of patients with or without portosplenomesenteric vein thrombosis secondary to severe acute pancreatitis in the early stage. AP: Acute pancreatitis; SAP: Severe acute pancreatitis; CT: Computed tomography; PSMVT: Portosplenomesenteric vein thrombosis.

including hypoalbuminemia (serum albumin level < 25 g/L), hematocrit, cholesterol, triglycerides, inflammation markers including leucocytes, C-reactive protein and procalcitonin, coagulation tests including platelets, prothrombin time, activated partial thromboplastin time, fibrinogen and D-dimer during the 24 h following admission. Anticoagulation therapy was not included in the manage protocol of PSMVT at early stage (within 10 d after AP onset).

Imaging protocols

All contrast-enhanced CT studies were reassessed and reviewed by a radiologist who specialized in abdominal imaging and was blinded to the clinical data. Contrast-enhanced CT was generally performed approximately 3-10 d after AP onset, with an average of 7 d. Thus, PSMVT, which occurred within 10 d after AP onset, was detected. All contrast-enhanced CT examinations were performed using a 64-channel multidetector CT scanner (Somatom Definition AS+, Siemens Medical Systems, Erlangen, Germany). After noncontrast scans were obtained, 1.3 mL/kg iopromide (Ultravist 370; Bayer Schering Pharma, Berlin, Germany) was intravenously injected at a rate of 3 mL/s with a high-pressure injector. Then, contrasted arterial and portal phase scans were obtained when the attenuation of the aorta at the thoracolumbar junction had reached 180 HU with a fixed 60 s delay. The scanned area extended from the diaphragmatic domes to the pubic symphysis.

Definitions

The diagnosis of PSMVT was based on the results of the contrasted-enhanced CT. Thrombosis was defined as a filling defect within the lumen of the vessel seen on contrast-enhanced CT images. SAP was defined by persistent organ failure, that was, organ failure for longer than 48 h[13]. Extrapancreatic necrosis alone was defined as extrapancreatic morphological changes exceeding fat stranding with complete enhancement of the pancreatic parenchyma without signs of focal or diffuse nonenhancement that could be determined on contrast-enhanced CT images[13]. Pancreatic parenchymal necrosis was defined as focal or diffuse nonenhancement of the pancreatic gland. When pancreatic necrosis was present, its location (head, neck, body, tail) and amount (< 30%, 30%-50%, > 50%) were noted. Acute peripancreatic fluid collections (APFCs) were defined as peripancreatic fluid collections with homogeneous liquefied components and without well-defined walls. The anatomical locations of APFCs included the anterior and posterior renal spaces, perirenal space, great and lesser omentum, paracolic sulci, mesenteric root, and transverse mesocolon. APFCs were recorded when the diameter was > 1 cm. Gastrointestinal wall thickening was defined as the thickening and edema of the gastrointestinal wall, with wall thickness greater than 4 mm, and as robust enhancement of the mucosa with reduced enhancement of the submucosa[13,14].

Statistical analysis

Continuous variables are described as the mean ± SD, and categorical variables are described as the absolute numbers and percentages. Continuous variables were compared using *t*-tests, and categorical data were analyzed with the chi-squared test. Variables found to be statistically significant in the univariate logistic regression analysis were introduced into a multivariate logistic analytic model (stepwise regression) to identify independent risk factors with odds ratios (ORs) and 95% confidence intervals (CIs). Furthermore, receiver operating characteristic (ROC) curves were generated for each of the qualified independent risk factors. A *P*-value < 0.05 was considered statistically significant. Data were analyzed using SPSS software (v17.0; SPSS Inc., Chicago, IL, United States).

RESULTS

As shown in the flow chart in Figure 1, 140 eligible patients were enrolled in this study. Twenty-five patients developed PSMVT (17.86%), and all the PSMVT cases were confirmed 6.19 ± 2.43 d after AP onset by contrast-enhanced CT. Supplementary Table 1 shows the demographic characteristics of all patients. The average age of all patients was 54.59 ± 15.90 years, and the study included 67 males (47.85%) and 73 females (52.14%). The PSMVT and non-PSMVT groups were matched by age, sex, AP etiology, Marshall score, systemic inflammatory response syndrome score, and acute physiology and chronic health evaluation Ⅱ score. Balthazar's CTSI score was higher in the PSMVT group than in the non-PSMVT group (7.20 ± 2.65 *vs* 4.63 ± 1.45, *P* = 0.000).

The most commonly involved vessel was the splenic

Table 1 Results of univariate logistic regression analyses

Variables	OR	95%CI	*P* value
Age	0.974	0.948-1.002	0.065
Sex	0.828	0.347-1.976	0.670
Etiology	0.775	0.405-1.483	0.441
APACHEII score	1.03	0.924-1.148	0.590
Modified Marshall score	1.06	0.816-1.377	0.660
SIRS score	1.369	0.873-2.147	0.171
Balthazar's CTSI score	1.78	1.419-2.232	0.000
[1]Hypoalbuminemia	21.143	2.25-198.68	0.008
Hematocrit	1.027	0.974-1.083	0.331
Leucocyte	1.021	0.955-1.092	0.539
C-reactive protein	1.001	0.997-1.005	0.596
Procalcitonin	0.988	0.955-1.022	0.485
Platelet	1	0.995-1.006	0.928
Prothrombin time	0.973	0.875-1.081	0.606
APTT	1.01	0.981-1.040	0.504
Fibrinogen	0.984	0.865-1.120	0.806
D-dimer	1.008	0.954-1.064	0.782
Cholesterol	1.034	0.893-1.198	0.654
Triglyceride	1.006	0.952-1.063	0.831
[2]Average of IAP	1.161	0.947-1.423	0.151
[2]Highest value of IAP	1.174	0.985-1.400	0.074
[3]Culture positive of drainage fluid	3.97	1.566-10.067	0.004
Culture positive of blood	1.889	0.766-4.655	0.167
APFC on Mesenteric root	2.765	1.126-6.791	0.026
Extrapancreatic necrosis alone	0.314	0.125-0.787	0.013
Pancreatic parenchymal necrosis	0	-	0.999
Extrapancreatic and parenchymal necrosis	6.021	2.357-15.379	0.000
Location of necrosis			
Head	4.58	1.514-13.855	0.007
Neck	4.413	1.624-11.997	0.004
Body	6.431	2.536-16.306	0.000
Tail	8.5	3.209-22.514	0.000
Amount of necrosis			
< 30%	0.722	0.226-2.304	0.582
30%-50%	3	0.668-13.482	0.152
> 50%	24.889	6.148-100.750	0.000
Gastrointestinal wall thickening	4.25	1.725-10.474	0.002

[1]Hypoalbuminemia was defined as serum albumin level < 25 g/L; [2]IAP was measured in the first three days from admission and the average and highest value of IAP was noted; [3]The fluid was obtained by percutaneous paracentesis or drainage pancreatic necrosis. OR: Odds ratio; CI: Confidence interval; APACHE: Acute Physiology, Age, and Chronic Health Evaluation; SIRS: Systemic inflammatory response syndrome; CTSI: Computed tomography severity index; APTT: Activated partial thromboplastin time; IAP: Intra-abdominal pressure; APFC: Acute peripancreatic fluid collection.

vein, which was detected in ten patients, followed by the SMV in five patients, the PV in four patients, the splenic vein and PV in three patients, the splenic vein and SMV in one patient, and the splenic vein, SMV and PV in two patients (Figure 2). In addition, we further detected other vascular complications and local complications, which are shown in Supplementary Table 2. There were pseudoaneurysms (Figure 3) in two patients, and CT revealed a hematoma in one of them, who was subsequently discharged without any treatment.

Supplementary Table 3 shows the relationship of a variety of clinical variables with the clinical outcomes. Twenty-one of the one hundred and forty patients (15%) died during hospitalization. Three patients experienced gastrointestinal hemorrhage. In one patient, digital subtraction angiography detected that the gastrointestinal hemorrhage was the result of the duodenal artery branch breaking; in the second patient, gastroscopy detected an acute gastric mucosal injury; and in the third patient, no reason was found. Intra-abdominal hemorrhage was detected in eight patients by abdominal CT, digital subtraction angiography, or during invasive interventions and surgical processes. Patients with PSMVT were more severely ill, as evidenced by their greater need for minimally invasive interventions, higher rates of multiple organ dysfunction syndrome, infected pancreatic necrosis, and longer durations of hospital and intensive care unit stays.

Table 1 shows the results of the univariate regression analysis of PSMVT. The following were potential risk factors: Higher Balthazar's CTSI score (OR: 1.780, $P = 0.000$); hypoalbuminemia (OR: 0.047, $P < 0.01$); culture-positive drainage fluid (OR: 3.97, $P < 0.01$); the APFC at the mesenteric root (OR: 2.765, $P < 0.05$); extrapancreatic necrosis alone (OR: 0.314, $P < 0.05$); peripancreatic and pancreatic parenchymal necrosis (OR: 6.021, $P = 0.000$); necrosis located in the head (OR: 4.580, $P < 0.01$), neck (OR: 4.413, $P < 0.01$), body (OR:

Table 2 Results of multivariate logistic regression analyses

Variables	OR	95%CI	*P* value
Balthazar's CTSI score	2.742	1.664-4.519	0.000
hypoalbuminemia	32.573	2.711-391.353	0.006
Gastrointestinal wall thickening	4.367	1.218-15.658	0.024

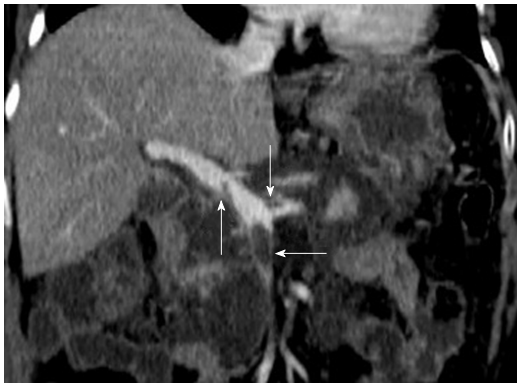


Figure 2 Portosplenomesenteric vein thrombosis. Contrast-enhanced CT image in a 70-year-old female patient with severe acute biliary pancreatitis shows a filling defect within the lumen of the portal vein (up arrow), splenic vein (down arrow) and superior mesenteric vein (left arrow). CT: Computed tomography

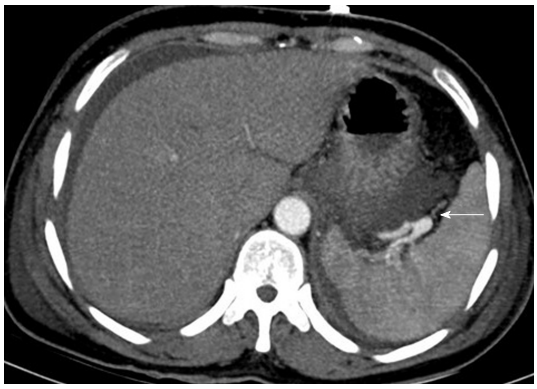


Figure 3 Pseudoaneurysm. Axial contrast-enhanced CT image in a 42-year-old female patient with severe acute biliary pancreatitis shows a pseudoaneurysm of the splenic artery (arrow), and the patient was discharged without gastrointestinal hemorrhage or intra-abdominal hemorrhage.

6.431, *P* = 0.000), or tail (OR: 8.500, *P* = 0.000) of the pancreas; an extent of pancreatic necrosis > 50% (OR: 24.889, *P* = 0.000); and gastrointestinal wall thickening (OR: 4.053, *P* = 0.000).

Multivariate stepwise logistic regression analyses showed that higher Balthazar's CTSI score (OR: 2.742; 95%CI: 1.664-4.519; *P* = 0.000), hypoalbuminemia (serum albumin level < 25 g/L) (OR:32.573; 95%CI: 2.711-391.353; *P* = 0.006) and gastrointestinal wall thickening (OR: 4.367, 95%CI: 1.218-15.658; *P* = 0.024) were independent risk factors for PSMVT in patients with SAP (Table 2). The area under the ROC curve for Balthazar's CTSI score was 0.777 (*P* = 0.000), the sensitivity was 52%, and the specificity was 93% at a cut-off value of 5.5 (Figure 4).

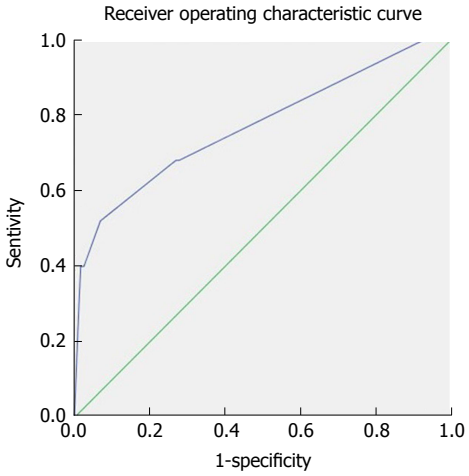


Figure 4 The receiver operating characteristic curve for Balthazar's computed tomography severity index score in predicting PSMVT secondary to severe acute pancreatitis in the early stage.

DISCUSSION

There is a lack of data on the prevalence and natural course of PSMVT in AP. It has been reported that the incidence of vascular abnormalities varies from 1.8% to 57% because of the different vascular abnormalities described, and differences in the severity of AP, and time or method of detecting vascular abnormalities[3-5,10,15-18]. In the present study, PSMVT developed in 25 of 140 (17.86%) patients with early-stage SAP. It has been reported that most PSMVTs have been clinically confirmed in the late stage of AP onset[3-5,10]. This study is the first report to focus on PSMVT occurring as early as within 10 d (average 6 d) from AP onset and to indicate that high Balthazar's CTSI, hypoalbuminemia and gastrointestinal wall thickening are independent prognostic factors of PSMVT in the early stage of the onset of SAP.

The development of PSMVT is associated with the presence, location, and extent of pancreatic necrosis[5,19]. In contrast, some papers showed a weaker association between Balthazar's CTSI and superficial venous thrombosis (SVT) or PSMVT in AP[3,15]. In our study, Balthazar's CTSI was an independent risk factor of PSMVT and the area under the ROC curve for Balthazar's CTSI score was 0.777 at a cut-off value of 5.5. The high specificity indicated that a low Balthazar's CTSI score could be a good indicator that PSMVT would not occur. The splenic vein runs behind the tail and the body of the

pancreas. Behind the neck of the pancreas, the splenic vein joins the SMV to form the hepatic PV. Necrosis could directly impact the vascular system, resulting in the formation of PSMVT. In addition, pancreatic necrosis is thought to be associated with a severe local inflammatory response and may surround the vein directly, causing impaired vasomotor function, reduced capillary perfusion, and enhanced thrombosis. However, the appropriate time for drainage is controversial, and it is recommended that drainage should be delayed until the fluid is well encapsulated or there is necrosis[20]. In our study, we found that PSMVT could form in the early stage of AP and hence, for these patients, early drainage might alleviate local inflammation and lower the risk of PSMVT.

Low albumin status reflects poor nutritional status. Low albumin levels are thought to be associated with increased venous thromboembolism in head and neck surgery patients[21], hepatocellular carcinoma patients[22], colorectal surgery patients[23], and thoracolumbar surgery patients[24]. A prospective study determined that lower serum albumin levels were more accurate than clinical scores for diagnosing DVT in patients with several comorbidities[25]. In our study, hypoalbuminemia with albumin levels $\leqslant$ 25 g/L was a good predictor of PSMVT, with a very high OR (32.573). Thus, we speculate that correcting hypoalbuminemia during the early stage of AP may help prevent the occurrence of PSMVT. Low albumin levels may lead to peripheral edema and possibly to increased venous stasis. However, the exact mechanism needs further study.

This study revealed that gastrointestinal wall thickening was an independent risk factor for PSMVT. Gastrointestinal wall thickening is represented by the thickening and edema of the gastrointestinal wall, which means that the intestinal barrier is more vulnerable. There is speculation that intestinal bacteria and their products can infiltrate the blood circulation through the damaged intestinal barrier[26], causing severe local inflammation, which is related to venous thrombosis. The level of D-dimer is mostly used as an effective diagnostic tool to rule out deep vein thrombosis (DVT) and pulmonary embolism[27], and it has been reported to have great predictive power in the early phase of AP[28]. However, our study showed that coagulation tests including platelets, prothrombin time, activated partial thromboplastin time, fibrinogen and D-dimer were not independent risk factors in the logistic regression analysis, suggesting that coagulative disturbance may not be a direct cause of PSMVT, as reported before[3].

There were several limitations of our study. First, it was a retrospective study with a limited sample size. Second, we focused on the vascular complications during the early stage of AP; thus, whether more patients experienced PSMVT in later stages was unknown. Third, anticoagulation therapy was not included in the management protocol of PSMVT at the early stage (within 10 d after AP onset). However, when and whether anticoagulants and other treatments were given 10 d after AP onset depended on the decisions made by the attending doctors. Whether these interventions could have affected the long-term prognosis is unclear.

In conclusion, we demonstrated that the occurrence rate of PSMVT is high in SAP patients in the early stage, and high Balthazar's CTSI scores, hypoalbuminemia and gastrointestinal wall thickening were independent prognostic factors.

ARTICLE HIGHLIGHTS

Research background

The natural history of portosplenomesenteric vein thrombosis (PSMVT) was mostly reported to occur late during acute pancreatitis (AP). There is little data regarding risk factors for this complication in the early stage of severe acute pancreatitis (SAP).

Research motivation

We wanted to determine the potential risk factors for the development of PSMVT in SAP patients and further qualify independent risk factors.

Research objectives

To investigate the incidence and risk factors of PSMVT in the early stage of SAP.

Research methods

Clinical outcomes and imaging measures were compared between 25 patients with PSMVT developed in the early stage of SAP and 115 patients without PSMVT. Independent risk factors were assessed by logistic regression analyses.

Research results

Twenty-five of the one hundred and forty (17.86%) SAP patients developed PSMVT 6.19 $\pm$ 2.43 d after AP onset. Balthazar's computed tomography (CT) severity index scores, hypoalbuminemia (serum albumin level < 25 g/L) and gastrointestinal wall thickening were independent risk factors for PSMVT developed in patients with SAP. The area under the receiver operating characteristic (ROC) curve for Balthazar's CT severity index scores was 0.777 with high specificity at a cut-off value of 5.5.

Research conclusions

High Balthazar's CT severity index scores, hypoalbuminemia and gastrointestinal wall thickening are independent risk factors for PSMVT developed in the early stage of SAP.

Research perspectives

High Balthazar's CT severity index scores, hypoalbuminemia and gastrointestinal wall thickening may be good forecasting indictors for PSMVT at the early stage of SAP. We hope that a future prospective, multicenter study will study the prevention and therapy of PSMVT at the early stage of AP.

REFERENCES

1 **Heider TR**, Azeem S, Galanko JA, Behrns KE. The natural history of pancreatitis-induced splenic vein thrombosis. *Ann Surg* 2004; **239**: 876-880; discussion 880-882 [PMID: 15166967 DOI: 10.1097/01.sla.0000128685.74686.1e]

2 **Zhou J**, Ke L, Yang D, Chen Y, Li G, Tong Z, Li W, Li J. Predicting the clinical manifestations in necrotizing acute pancreatitis patients with splanchnic vein thrombosis. *Pancreatology* 2016; **16**: 973-978 [PMID: 27727096 DOI: 10.1016/j.pan.2016.10.001]

3 **Zhou J**, Ke L, Tong Z, Li G, Li W, Li N, Li J. Risk factors and outcome of splanchnic venous thrombosis in patients with necrotizing acute pancreatitis. *Thromb Res* 2015; **135**: 68-72 [PMID:

25466845 DOI: 10.1016/j.thromres.2014.10.021]
4 **Harris S**, Nadkarni NA, Naina HV, Vege SS. Splanchnic vein thrombosis in acute pancreatitis: a single-center experience. *Pancreas* 2013; **42**: 1251-1254 [PMID: 24152951 DOI: 10.1097/MPA.0b013e3182968ff5]
5 **Easler J**, Muddana V, Furlan A, Dasyam A, Vipperla K, Slivka A, Whitcomb DC, Papachristou GI, Yadav D. Portosplenomesenteric venous thrombosis in patients with acute pancreatitis is associated with pancreatic necrosis and usually has a benign course. *Clin Gastroenterol Hepatol* 2014; **12**: 854-862 [PMID: 24161350 DOI: 10.1016/j.cgh.2013.09.068]
6 **Nadkarni NA**, Khanna S, Vege SS. Splanchnic venous thrombosis and pancreatitis. *Pancreas* 2013; **42**: 924-931 [PMID: 23587854 DOI: 10.1097/MPA.0b013e318287cd3d]
7 **Fei Y**, Gao K, Hu J, Tu J, Li WQ, Wang W, Zong GQ. Predicting the incidence of portosplenomesenteric vein thrombosis in patients with acute pancreatitis using classification and regression tree algorithm. *J Crit Care* 2017; **39**: 124-130 [PMID: 28254727 DOI: 10.1016/j.jcrc.2017.02.019]
8 **Fei Y**, Hu J, Gao K, Tu J, Wang W, Li WQ. Risk Prediction for Portal Vein Thrombosis in Acute Pancreatitis Using Radial Basis Function. *Ann Vasc Surg* 2018; **47**: 78-84 [PMID: 28943487 DOI: 10.1016/j.avsg.2017.09.004]
9 **Tripodi A**, Legnani C, Palareti G. The risk of a first and a recurrent venous thrombosis associated with an elevated D-dimer level and an elevated thrombin potential: results of the THE-VTE study: comment. *J Thromb Haemost* 2015; **13**: 2283-2286 [PMID: 26391712 DOI: 10.1111/jth.13151]
10 **Toqué L**, Hamy A, Hamel JF, Cesbron E, Hulo P, Robert S, Aube C, Lermite E, Venara A. Predictive factors of splanchnic vein thrombosis in acute pancreatitis: A 6-year single-center experience. *J Dig Dis* 2015; **16**: 734-740 [PMID: 26513113 DOI: 10.1111/1751-2980.12298]
11 **Balthazar EJ**, Robinson DL, Megibow AJ, Ranson JH. Acute pancreatitis: value of CT in establishing prognosis. *Radiology* 1990; **174**: 331-336 [PMID: 2296641 DOI: 10.1148/radiology.174.2.2296641]
12 **Reintam Blaser A**, Malbrain ML, Starkopf J, Fruhwald S, Jakob SM, De Waele J, Braun JP, Poeze M, Spies C. Gastrointestinal function in intensive care patients: terminology, definitions and management. Recommendations of the ESICM Working Group on Abdominal Problems. *Intensive Care Med* 2012; **38**: 384-394 [PMID: 22310869 DOI: 10.1007/s00134-011-2459-y]
13 **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS; Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]
14 **Vikram R**, Balachandran A, Bhosale PR, Tamm EP, Marcal LP, Charnsangavej C. Pancreas: peritoneal reflections, ligamentous connections, and pathways of disease spread. *Radiographics* 2009; **29**: e34 [PMID: 19168761 DOI: 10.1148/rg.e34]
15 **Mortelé KJ**, Mergo PJ, Taylor HM, Wiesner W, Cantisani V, Ernst MD, Kalantari BN, Ros PR. Peripancreatic vascular abnormalities complicating acute pancreatitis: contrast-enhanced helical CT findings. *Eur J Radiol* 2004; **52**: 67-72 [PMID: 15380848 DOI: 10.1016/j.ejrad.2003.10.006]
16 **Dörffel T**, Wruck T, Rückert RI, Romaniuk P, Dörffel Q, Wermke W. Vascular complications in acute pancreatitis assessed by color duplex ultrasonography. *Pancreas* 2000; **21**: 126-133 [PMID: 10975705 DOI: 10.1097/00006676-200008000-00004]
17 **Butler JR**, Eckert GJ, Zyromski NJ, Leonardi MJ, Lillemoe KD, Howard TJ. Natural history of pancreatitis-induced splenic vein thrombosis: a systematic review and meta-analysis of its incidence and rate of gastrointestinal bleeding. *HPB* (Oxford) 2011; **13**: 839-845 [PMID: 22081918 DOI: 10.1111/j.1477-2574.2011.00375.x]
18 **Gonzelez HJ**, Sahay SJ, Samadi B, Davidson BR, Rahman SH. Splanchnic vein thrombosis in severe acute pancreatitis: a 2-year, single-institution experience. *HPB* (Oxford) 2011; **13**: 860-864 [PMID: 22081920 DOI: 10.1111/j.1477-2574.2011.00392.x]
19 **Park DE**, Chae KM. Chylous ascites caused by acute pancreatitis with portal vein thrombosis. *J Korean Surg Soc* 2011; **81** Suppl 1: S64-S68 [PMID: 22319743 DOI: 10.4174/jkss.2011.81.Suppl1.S64]
20 **Tenner S**, Baillie J, DeWitt J, Vege SS; American College of Gastroenterology. American College of Gastroenterology guideline: management of acute pancreatitis. *Am J Gastroenterol* 2013; **108**: 1400-14115; 1416 [PMID: 23896955 DOI: 10.1038/ajg.2013.218]
21 **Mowery A**, Light T, Clayburgh D. Venous thromboembolism incidence in head and neck surgery patients: Analysis of the Veterans Affairs Surgical Quality Improvement Program (VASQIP) database. *Oral Oncol* 2018; **77**: 22-28 [PMID: 29362122 DOI: 10.1016/j.oraloncology.2017.12.002]
22 **Carr BI**, Guerra V. Serum albumin levels in relation to tumor parameters in hepatocellular carcinoma patients. *Int J Biol Markers* 2017; **32**: e391-e396 [PMID: 28862714 DOI: 10.5301/ijbm.5000300]
23 **Moghadamyeghaneh Z**, Hanna MH, Carmichael JC, Nguyen NT, Stamos MJ. A nationwide analysis of postoperative deep vein thrombosis and pulmonary embolism in colon and rectal surgery. *J Gastrointest Surg* 2014; **18**: 2169-2177 [PMID: 25213583 DOI: 10.1007/s11605-014-2647-5]
24 **Sebastian AS**, Currier BL, Kakar S, Nguyen EC, Wagie AE, Habermann ES, Nassr A. Risk Factors for Venous Thromboembolism following Thoracolumbar Surgery: Analysis of 43,777 Patients from the American College of Surgeons National Surgical Quality Improvement Program 2005 to 2012. *Global Spine J* 2016; **6**: 738-743 [PMID: 27853656 DOI: 10.1055/s-0036-1579553]
25 **Malatino L**, Cardella AM, Puccia G, Cilia C, Terranova V, Cataudella E, Buonacera A, Tripepi G, Di Marca S, Mastrosimone G, Pisano M, Giordano M, Stancanelli B. Testing Clinical Scores to Diagnose Incident Deep Vein Thrombosis in Patients Hospitalized in a Department of Medicine: Can Biomarkers Improve Accuracy? *Angiology* 2016; **67**: 245-251 [PMID: 25991607 DOI: 10.1177/0003319715586289]
26 **Cicalese L**, Sahai A, Sileri P, Rastellini C, Subbotin V, Ford H, Lee K. Acute pancreatitis and bacterial translocation. *Dig Dis Sci* 2001; **46**: 1127-1132 [PMID: 11341659 DOI: 10.1023/A:1010786701289]
27 **Wells PS**, Anderson DR, Rodger M, Forgie M, Kearon C, Dreyer J, Kovacs G, Mitchell M, Lewandowski B, Kovacs MJ. Evaluation of D-dimer in the diagnosis of suspected deep-vein thrombosis. *N Engl J Med* 2003; **349**: 1227-1235 [PMID: 14507948 DOI: 10.1056/NEJMoa023153]
28 **Salomone T**, Tosi P, Palareti G, Tomassetti P, Migliori M, Guariento A, Saieva C, Raiti C, Romboli M, Gullo L. Coagulative disorders in human acute pancreatitis: role for the D-dimer. *Pancreas* 2003; **26**: 111-116 [PMID: 12604906 DOI: 10.1097/00006676-200303000-00003]

P- Reviewer: Kitamura K, Sharma V, Wang W, Wilcox CM
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4061-4068

DOI: 10.3748/wjg.v24.i35.4061

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

ORIGINAL ARTICLE

Retrospective Study

Serum anti-*Helicobacter pylori* antibody titer and its association with gastric nodularity, atrophy, and age: A cross-sectional study

Osamu Toyoshima, Toshihiro Nishizawa, Kosuke Sakitani, Tadahiro Yamakawa, Yoshiyuki Takahashi, Nobutake Yamamichi, Keisuke Hata, Yasuyuki Seto, Kazuhiko Koike, Hidenobu Watanabe, Hidekazu Suzuki

Osamu Toyoshima, Toshihiro Nishizawa, Kosuke Sakitani, Tadahiro Yamakawa, Yoshiyuki Takahashi, Gastroenterology, Toyoshima Endoscopy Clinic, Tokyo 157-0066, Japan

Nobutake Yamamichi, Kazuhiko Koike, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Keisuke Hata, Department of Surgical Oncology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Yasuyuki Seto, Department of Gastrointestinal Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Hidenobu Watanabe, Pathology and Cytology Laboratory Japan, Tokyo 166-0003, Japan

Hidekazu Suzuki, Medical Education Center, Keio University School of Medicine, Tokyo 160-8582, Japan

ORCID number: Osamu Toyoshima (0000-0002-6953-6079); Toshihiro Nishizawa (0000-0003-4876-3384); Kosuke Sakitani (0000-0002-4537-6023); Tadahiro Yamakawa (0000-0002-7352-6375); Yoshiyuki Takahashi (0000-0002-6724-8057); Nobutake Yamamichi (0000-0002-5741-9887); Keisuke Hata (0000-0003-4064-8701); Yasuyuki Seto (0000-0002-6953-8752); Kazuhiko Koike (0000-0002-9739-9243); Hidenobu Watanabe (0000-0002-7871-4738); Hidekazu Suzuki (0000-0002-3855-3140).

Author contributions: All authors were involved in designing the study; Toyoshima O, Nishizawa T, Sakitani K, and Suzuki H prepared the manuscript; Toyoshima O was involved with statistical analysis.

Institutional review board statement: This retrospective study was approved by the Ethical Review Committee of Hattori Clinic on September 7, 2017.

Informed consent statement: Written informed consent was obtained from all participants.

Conflict-of-interest statement: During the last five years, Yamamichi N received funds for the research from Denka Seiken Co., Ltd.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Osamu Toyoshima, MD, Director, Gastroenterology, Toyoshima Endoscopy Clinic, 6-17-5 Seijo, Setagaya-ku, Tokyo 157-0066, Japan. t@ichou.com
Telephone: +81-3-54299555
Fax: +81-3-54299511

Received: July 17, 2018
Peer-review started: July 17, 2018
First decision: July 31, 2018
Revised: August 2, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 21, 2018

Abstract

AIM

To clarify the role of serum anti-*Helicobacter pylori* (*H. pylori*) antibody titers in gastric cancer.

METHODS

In this cross-sectional study, the effect of patients' baseline characteristics and endoscopic findings on their serum antibody titers were assessed. We evaluated consecutive patients who underwent esophagogastroduodenoscopy and their first evaluation for *H. pylori* infection using a serum antibody test. We excluded patients with a history of eradication therapy. The participants were divided into four groups according to their E-plate serum antibody titer. Patients with serum antibody titers < 3, 3-9.9, 10-49.9, and ⩾ 50 U/mL were classified into groups A, B, C, and D, respectively.

RESULTS

In total, 874 participants were analyzed with 70%, 16%, 8.7%, and 5.1% of them in the groups A, B, C, and D, respectively. Patients in group C were older than patients in groups A and B. Gastric open-type atrophy, intestinal metaplasia, enlarged folds, diffuse redness, and duodenal ulcers were associated with a high titer. Regular arrangements of collecting venules, fundic gland polyps, superficial gastritis, and gastroesophageal reflux disease were related to a low titer. Multivariate analysis revealed that nodularity (P = 0.0094), atrophy (P = 0.0076), and age 40-59 years (*vs* age ⩾ 60 years, P = 0.0090) were correlated with a high serum antibody titer in *H. pylori*-infected patients. Intestinal metaplasia and atrophy were related to age ⩾ 60 years in group C and D.

CONCLUSION

Serum antibody titer changes with age, reflects gastric mucosal inflammation, and is useful in predicting the risk of gastric cancer.

Key words: Antibody; *Helicobacter pylori*; Gastritis; Gastric cancer; Endoscopy

Core tip: A positive-low serum anti-*Helicobacter pylori* (*H. pylori*) antibody titer (E-plate Eiken) (10-49.9 U/mL) and a negative-high titer (3-9.9 U/mL) are associated with intestinal-type gastric cancer. A positive-high titer (⩾ 50 U/mL) correlates with diffuse-type gastric cancer. Few studies have reported on the relationship between the serum antibody titer and endoscopic findings. In *H. pylori*-infected patients, a high titer of serum antibody was associated with gastric nodularity and atrophy. In *H. pylori*-infected patients, the antibody titer decreased in patients aged 60 years. Intestinal metaplasia and gastric atrophy were related to age ⩾ 60 years in patients with positive titers.

Toyoshima O, Nishizawa T, Sakitani K, Yamakawa T, Takahashi Y, Yamamichi N, Hata K, Seto Y, Koike K, Watanabe H, Suzuki H. Serum anti-*Helicobacter pylori* antibody titer and its association with gastric nodularity, atrophy, and age: A cross-sectional study. *World J Gastroenterol* 2018; 24(35): 4061-4068 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4061.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4061

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is the main carcinogenic entity in the stomach, and eradicating it reduces the incidence of gastric cancer[1-5]. The ^{13}C-urea breath test (UBT), serum immunoglobulin G anti-*H. pylori* antibodies, stool antigen test, esophagogastroduodenoscopy (EGD), rapid urease test, culture, and pathology are used in routine practice to diagnose *H. pylori* infection. UBT is the gold standard for diagnosing *H. pylori* infection because its accuracy is the best of all these tests[2,6]. However, UBT requires the patients to stop using proton pump inhibitors or antibiotics.

Other than UBT, measuring the serum antibody titer is useful because serum antibody testing is easy, inexpensive, and hardly affected by changes in the stomach[7]. Some serological tests are of high-quality, and measuring the antibody titer once in adults makes it possible to observe subsequent changes in it with time and diagnose *H. pylori* infection[8-11]. Serum antibody titer is useful to evaluate both new-onset and successful eradication of the disease[12]. Furthermore, serum antibody titer is associated with the risk of gastric cancer. For example, a high titer correlates with diffuse-type of gastric cancer according to Lauren's classification. A positive-low titer and negative-high titer are associated with intestinal-type cancer[10,13-15]. An E-plate (Eiken Chemical, Tokyo, Japan) is frequently used for commercial serological examination in routine clinical practice in Japan. The E-plate is a direct enzyme immunoassay test designed to identify the Japanese strain of *H. pylori* and has been widely applied in large-scale studies in Japanese participants[14,16]. The manufacturer defined the cutoff E-plate titer as 10 U/mL and reported that its accuracy, sensitivity, specificity, positive predictive value, and negative predictive value were 94.0%, 95.2%, 92.6%, 93.8%, and 94.3%, respectively[17].

EGD is another diagnostic tool for *H. pylori* infection because it is able to not only accurately diagnose gastric malignancies, but also stratify the risk of gastric cancer by evaluating gastritis[3,18]. In Japan, EGD is performed to diagnose *H. pylori* infection in routine clinical practice. We previously reported that the endoscopic Kyoto classification of gastritis is associated not only with gastric cancer, but also with *H. pylori* infection[11].

Few studies have described the relationship between the serum anti-*H. pylori* antibody titers and endoscopic findings. We conducted this cross-sectional study to investigate the association between patients' baseline characteristics and endoscopic findings, and the serum antibody titer; subsequently, we determined the role of

serum antibody titers in these patients.

MATERIALS AND METHODS

Ethics

This retrospective study was approved by the ethical review committee of Hattori Clinic on September 7, 2017. Written informed consent was obtained from the participants. All clinical investigations were conducted according to the ethical guidelines of the Declaration of Helsinki.

Patients

We enrolled consecutive patients who underwent EGD and serum antibody testing at Toyoshima Endoscopy Clinic, which is an endoscopy specialty clinic, between September 2016 and August 2017. We included patients who were evaluated for *H. pylori* infection for the first time. The indications for EGD were the symptoms, abnormal findings on upper gastrointestinal radiography, screening, or surveillance for upper gastrointestinal diseases. The serum antibody titer was measured at the time of EGD. We excluded patients with a history of *H. pylori* eradication therapy, gastric cancer, or gastrectomy.

We grouped the subjects based on their serum antibody titers. Data on the patients' baseline characteristics, including age, sex, and indication for EGD, were collected.

The serum anti-H. pylori antibody

The serum antibody titer was measured using the following enzyme-linked immunoassay kit using antigens derived from Japanese individuals: E-plate Eiken H. pylori antibody Ⅱ kit (Eiken Chemical, Tokyo, Japan). The measurable titers were ≥ 3 U/mL and < 100 U/mL. The manufacturer recommended a cut-off value of 10 U/mL for *H. pylori* positivity. We previously reported that a titer of 3-9.9 U/mL had a lower negative predictive value than a total titer of < 10 U/mL did (83.1% *vs* 94.3%)[11]. A titer of 3-9.9 U/mL was also reported to indicate the risk of intestinal gastric cancer[15]. The previous study set the cut-off point as 50 U/mL to differentiate the low and high serum antibody titer groups, ensuring that the ratio of patients in both groups was approximately half of all *H. pylori*-seropositive subjects. A titer of 10-49.9 U/mL was reported to indicate the risk of intestinal gastric cancer, and a titer ≥ 50 U/mL was considered to indicate the risk of diffuse cancer[10].

In the present study, we divided the subjects into four groups according to their serum antibody titer as follows: group A: Titer < 3 U/mL (negative-low), group B: 3-9.9 U/mL (negative-high), Group C: 10-49.9 U/mL (positive-low), and group D: ≥ 50 U/mL (positive-high).

EGD

EGD was performed using the Olympus Evis Lucera Elite system with GIF-HQ290 or GIF-H290Z endoscope (Olympus Corporation, Tokyo, Japan) by expert physicians who jointly met and discussed the endoscopic images before this study. We performed EGD with the patient under conscious sedation with midazolam and/or pethidine hydrochloride. Other expert physicians retrospectively reviewed the EGD images. Discrepancies in diagnoses between the two sets of physicians were resolved through a discussion between them.

We evaluated the Kyoto classification of gastritis score[19]. The Kyoto classification of gastritis is based on the sum of the scores of the following five endoscopic findings, which are scored on a scale from 0-8: Atrophy, intestinal metaplasia (IM), enlarged folds, nodularity, and redness. A high score represents an increased risk of gastric cancer[15,20]. Gastric atrophy was classified according to the extent of mucosal atrophy, as described by Kimura and Takemoto[21]. Classifications of C-Ⅱ and C-Ⅲ were scored as 1, while those of O-Ⅰ to O-Ⅲ were scored as 2. IM is typically observed as grayish-white and slightly opalescent patches. IM within the antrum was scored as 1 and IM extending into the corpus was scored as 2. Enlarged folds greater than 5 mm were scored as 1. Nodularity is characterized by the appearance of multiple whitish elevated lesions, mainly in the pyloric gland mucosa. Nodularity was scored as 1. Diffuse redness refers to uniform redness involving the entire fundic gland mucosa. Redness with regular arrangements of collecting venules (RAC) was scored as 1 and that without RAC was scored as 2.

We also investigated gastric and duodenal ulcers. Ulcer scars were considered positive findings. RAC, fundic gland polyps, superficial gastritis, gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), and hiatal hernia were considered *H. pylori* infection-negative endoscopic findings[19]. We defined the three endoscopic findings of hematin (bleeding spots), red streaks (linear erythema), and raised erosion as superficial gastritis. We defined grade A (one or more mucosal breaks of ≤ 5 mm in length) or worse of the Los Angeles classification of GERD as positive. Short-segment BE was classified as BE.

Statistical analysis

First, we evaluated the association between the serum antibody titer and age, sex, indication, and endoscopic findings using Kruskal-Wallis test for continuous variables and Cochran-Armitage test, chi-squared test, or polytomous logistic regression analysis for categorical variables in the four groups. Simultaneously, in comparisons between two groups, Steel-Dwass test was used for continuous variables. Second, we compared patients with *H. pylori* infection (groups C and D) based on age (< 40 years, 40-59 years, and ≥ 60 years) using the Cochran-Armitage test. Subsequently, we conducted a subgroup analysis of *H. pylori*-infected patients (groups C and D). We evaluated the effect of age, sex, indication, and endoscopic findings on the serum antibody titer in univariate analysis using the Mann-Whitney *U* test

Table 1 Characteristics and endoscopic findings of the serum antibody titers of the 874 study participants *n* (%)

Group	Total	A	B	C	D	*P* value
Serum antibody titer (U/mL)		< 3	3-9.9	10-49.9	⩾ 50	
Number	874	612 (70.0)	141 (16.1)	76 (8.7)	45 (5.1)	
Male	336 (38.4)	241 (39.4)	48 (34.0)	33 (43.4)	14 (31.1)	0.36
Age (yr), continuous, mean ± SD	48.3 ± 13.8	47.8 ± 13.1	47.1 ± 14.9	52.5 ± 15.8	50.9 ± 14.3	0.010
Age, categorical						0.002
< 40 yr	233	167 (71.7)	43 (18.5)	15 (6.4)	8 (3.4)	
40-59 yr	473	342 (72.3)	70 (14.8)	33 (7.0)	28 (5.9)	
⩾ 60 yr	168	103 (61.3)	28 (16.7)	28 (16.7)	9 (5.4)	
Indication						0.52
Symptoms	415	295	65	36	19	
Abnormal upper gastrointestinal radiography findings	93	60	14	12	7	
Screening	227	165	39	14	9	
Surveillance for upper gastrointestinal diseases	139	92	23	14	10	
Kyoto classification score, mean ± SD	0.43 ± 1.09	0.11 ± 0.57	0.43 ± 0.95	1.92 ± 1.70	2.33 ± 1.45	< 0.001
Open-type atrophy	65 (7.4)	11 (1.8)	11 (7.8)	21 (27.6)	22 (48.9)	< 0.001
Intestinal metaplasia	46 (5.3)	8 (1.3)	7 (5.0)	19 (25.0)	12 (26.7)	< 0.001
Enlarged folds	25 (2.9)	6 (1.0)	1 (0.7)	12 (15.8)	6 (13.3)	< 0.001
Nodularity	15 (1.7)	1 (0.2)	1 (0.7)	3 (3.9)	10 (22.2)	< 0.001
Diffuse redness	31 (3.5)	4 (0.7)	3 (2.1)	15 (19.7)	9 (20.0)	< 0.001
Gastric ulcer	14 (1.6)	9 (1.5)	0 (0)	5 (6.6)	0 (0.0)	0.43
Duodenal ulcer	19 (2.2)	3 (0.5)	2 (1.4)	10 (13.2)	4 (8.9)	< 0.001
Regular arrangement of collecting venules	470 (53.8)	376 (61.4)	75 (53.2)	16 (21.1)	3 (6.7)	< 0.001
Fundic gland polyps	311 (35.6)	259 (42.3)	48 (34.0)	4 (5.3)	0 (0.0)	< 0.001
Superficial gastritis	390 (44.6)	314 (51.3)	53 (37.6)	16 (21.1)	7 (15.6)	< 0.001
Gastroesophageal reflux disease	108 (12.4)	84 (13.7)	16 (11.3)	3 (3.9)	5 (11.1)	0.047
Barrett's esophagus	250 (28.6)	175(28.6)	41 (29.1)	23 (30.3)	11 (24.4)	0.95
Hiatal hernia	105 (12.0)	75 (12.3)	17 (12.1)	10 (13.2)	3 (6.7)	0.66

P-values were calculated by comparing groups A, B, C, and D using the Kruskal-Wallis test, Cochran-Armitage test, chi-squared test, or a polytomous logistic regression analysis, as appropriate. The Kyoto classification of gastritis score was estimated based on gastric atrophy, intestinal metaplasia, enlarged folds, nodularity, and redness[19]. SD: Standard deviation.

for continuous variables and Fisher's exact test or a binominal logistic regression analysis for categorical variables. We considered age 40-59 years as the reference. Finally, we performed multivariate analysis to identify the factors that were independently associated with the serum antibody titer using a binominal logistic regression analysis of the variables with a *P*-value less than 0.1 in the univariate analysis. A two-sided *P*-value less than 0.05 was considered statistically significant. The data were analyzed using Ekuseru-Toukei 2015 software (Social Survey Research Information, Tokyo, Japan).

RESULTS

A total of 919 patients were enrolled. We excluded 37 patients with a history of eradication therapy, four with a history of gastric cancer, and four with a history of gastrectomy. Finally, 874 patients were analyzed. The age of the study participants ranged between 16-95 years (mean: 48.3, standard deviation: 13.8). Thirty-eight percent of the patients were men. The mean Kyoto classification score was 0.43 (standard deviation: 1.09). There were 612 (70%), 141 (16%), 76 (8.7%), and 45 (5.1%) patients in groups A, B, C, and D, respectively (Table 1).

In our analysis of the association between serum antibody titer and baseline characteristics and endoscopic findings in all participants, we found that those in group C were significantly older than those in groups A and B (P = 0.018 and 0.025, respectively, Table 1). There was no difference in sex and indication between the patients in the four groups. We found that the Kyoto classification of gastritis score in group B was higher than that in group A, and those in groups C and D were higher than that in group B (mean score of groups A, B, C, and D: 0.11, 0.43, 1.92, and 2.33, respectively; group A *vs* B, group B *vs* C, and group B *vs* D: $P < 0.001$; group C *vs* D: $P = 0.32$, Table 1). Open-type atrophy (according to the Kimura-Takemoto classification), IM, enlarged folds, nodularity, diffuse redness, and duodenal ulcers occurred more frequently in patients with a higher titer than in those with a lower titer. However, the frequencies of RAC, fundic gland polyps, superficial gastritis, and GERD were lower in patients with a higher titer (Table 1). Representative endoscopic images are shown in Figure 1A-F. The proportion of patients in group C and D who were *H. pylori*-infected increased with age (< 40 years: 9.9%; 40-59 years: 12.9%; ⩾ 60 years: 22.0%; $P <$ 0.001, Figure 2A).

We conducted a sub-analysis of groups C and D to determine the difference between low-positive and high-positive serum antibody titers in *H. pylori*-infected patients. We compared the proportion of patients in groups C and D, stratified by age, as shown in Figure 2B. The proportion of patients in group D among those aged ⩾ 60 years was lower than that in patients aged 40-59

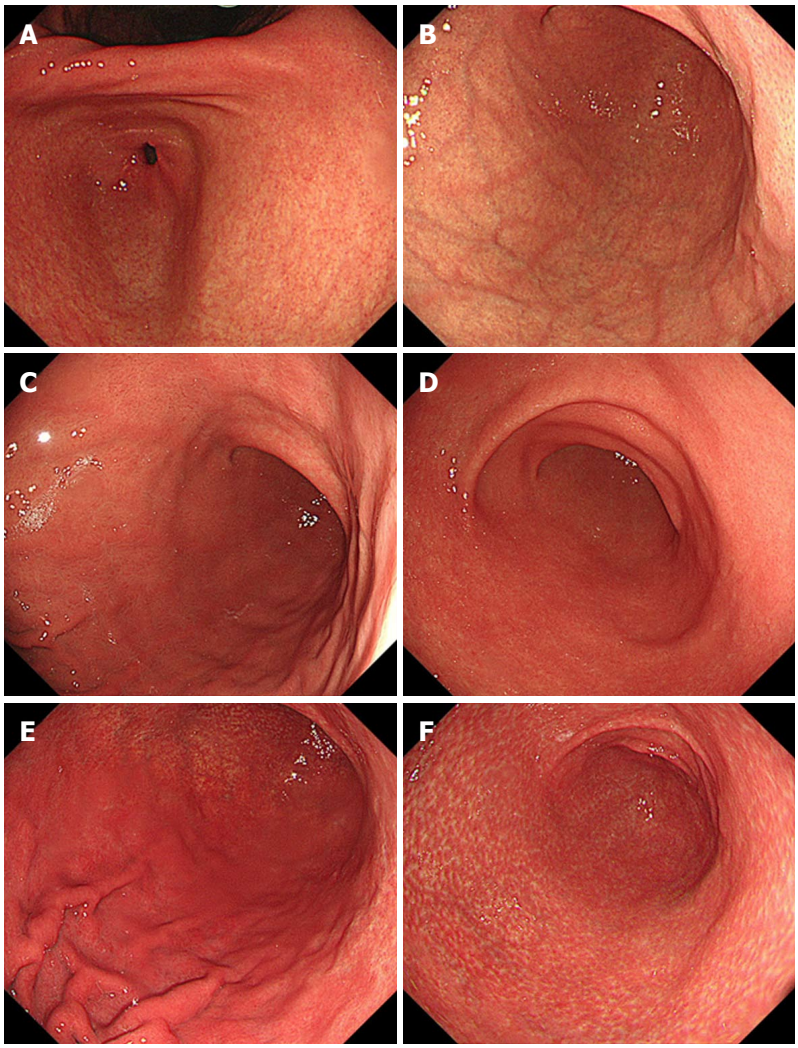


Figure 1 Representative endoscopic images of patients in each of the groups. A and B: Group A (serum antibody titer < 3 U/mL). A 20-year-old woman with a serum antibody titer < 3 U/mL and Kyoto classification score of 0. Atrophy was absent and RAC and superficial gastritis were present; C and D: Group C (serum antibody titer of 10-49.9 U/mL). A 36-year-old woman with a serum antibody titer of 25.5 U/mL and Kyoto classification score of 1. Closed-type atrophy was present and enlarged folds, nodularity, diffuse redness, and RAC were absent; E and F: Group D (serum antibody titer ⩾ 50 U/mL). A 50-year-old woman with a serum antibody titer ⩾ 100 U/mL and Kyoto classification score of 7. Open-type atrophy, enlarged folds, nodularity, and diffuse redness were present, and RAC was absent. RAC: Regular arrangement of collecting venules.

years [odds ratio (OR): 0.38, 95% confidence interval (CI): 0.15-0.94, P = 0.035]. There was no difference between patients aged < 40 and those between 40-59 years in terms of the proportion of patients in group D (OR: 0.63, 95%CI: 0.23-1.7, P = 0.36). In our comparison of the endoscopic findings of patients in groups C and D, we found that the frequencies of nodularity (P = 0.0042) and open-type atrophy (P = 0.018) were higher in group D than those in group C. The frequency of RAC was lower in group D than that in group C (P = 0.040). We evaluated the effect of patients' baseline characteristics and endoscopic findings on the serum antibody titer using multivariate analysis. Age ⩾ 60 years (OR: 0.24, 95%CI: 0.083-0.70, P = 0.0090), nodularity (OR: 7.1, 95%CI: 1.6-31, P = 0.0094), and open-type atrophy (OR: 3.9, 95%CI: 1.4-10, P = 0.0076) were independently associated with the serum antibody titer in *H. pylori*-infected patients (Table 2).

We compared the endoscopic findings of *H. pylori*-infected patients aged < 60 years and those ⩾ 60 years, as shown in Table 3. Intestinal metaplasia and gastric atrophy were related to age ⩾ 60 years in group C and D.

DISCUSSION

In this study, we investigated the association between patients' baseline characteristics and endoscopic findings and the serum antibody titer, and we clarified the role of serum antibody titers in patients with *H. pylori* infection. We found that nodularity and endoscopic open-type atrophy correlated with a high serum antibody titer in *H. pylori*-infected patients. In patients aged > 60 years, the serum antibody titer tended to decrease and IM tended to be more prevalent. Higher bacterial counts induce intense immune responses, resulting in higher

Table 2 Multivariate analysis of the effect of patients' characteristics and endoscopic findings on the serum antibody titer in *Helicobacter pylori*-infected patients

	Odds ratio	95%CI	*P*-value
Age < 40 yr[1]	0.69	0.21-2.2	0.54
Age ⩾ 60 yr[1]	0.24	0.083-0.70	0.0090
Open-type atrophy	3.9	1.4-10	0.0076
Nodularity	7.1	1.6-31	0.0094
Regular arrangement of collecting venules	0.36	0.088-1.5	0.16

[1]The odds ratios were calculated by considering age 40-59 years as reference. *P*-values were calculated using a binominal logistic regression analysis. CI: Confidence interval.

Table 3 Comparison of the endoscopic findings of *Helicobacter pylori*-infected patients aged < 60 years and those ⩾ 60 years

	Group C		*P* value	Group D		*P* value
	Age < 60 yr	Age ⩾ 60 yr		Age < 60 yr	Age ⩾ 60 yr	
Number	48	28		36	9	
Kyoto classification score, mean ± SD	1.52 ± 1.56	2.61 ± 1.75	0.0068	2.19 ± 1.53	2.89 ± 0.928	0.084
Open-type atrophy	7	14	0.0014	14	8	0.0098
Intestinal metaplasia	4	15	< 0.001	6	6	0.0062
Enlarged folds	8	4	1.0	3	3	0.084
Nodularity	2	1	1.0	10	0	0.17
Diffuse redness	9	6	0.77	9	0	0.17
Gastric ulcer	3	2	1.0	0	0	1.0
Duodenal ulcer	4	6	0.16	4	0	0.57
Regular arrangement of collecting venules	12	4	0.38	3	0	1.0
Fundic gland polyps	4	0	0.29	0	0	1.0
Superficial gastritis	11	5	0.77	7	0	0.31
Gastroesophageal reflux disease	2	1	1.0	3	2	0.26
Barrett's esophagus	13	10	0.45	9	2	1.0
Hiatal hernia	5	5	0.48	3	0	1.0

P-values were calculated with Mann-Whitney *U* test or Fisher's exact test, as appropriate. SD: Standard deviation.

serum antibody titers, while genetic differences among human hosts may affect the antibody levels in response to pathogens[4,22]. Tatemichi *et al*[14,23] demonstrated that a low-positive serum antibody titer was associated with intestinal-type gastric cancer and a high-positive titer was considered to indicate a high risk of diffuse-type cancer. The intestinal type of cancer develops through a sequence in which atrophy progresses and IM appears as a person ages, while the diffuse type of cancer is associated with high mucosal inflammation, particularly in young patients and those with nodularity[2,4,6,24-26]. In this study, we elucidated that the natural history of *H. pylori* infection is as follows: 40-59-year-old *H. pylori*-infected patients develop high inflammatory gastritis, frequently with nodularity and a high serum antibody titer, and they have a higher risk of developing diffuse-type gastric cancer. Subsequently, some *H. pylori*-infected patients older than 60 years of age have less gastric mucosal inflammation, progression of gastric atrophy, IM, decreased serum antibody titers, and the risk of developing intestinal-type gastric cancer.

In our investigation of all subjects, endoscopic findings indicating *H. pylori* infection were associated not only with positive or negative serum antibodies, but also with the serum antibody value. Regardless of whether they have an *H. pylori* infection, patients with a high serum antibody titer are more likely to have *H. pylori* infection-related endoscopic findings and are less likely to have *H. pylori* infection-negative endoscopic findings than are those with a lower serum antibody titer. Endoscopic findings are related to the risk of gastric cancer; therefore, these results confirmed that the serum antibody titer is associated with the risk of gastric cancer[2,6].

The *H. pylori* infection rate is declining in Japan; however, our study indicated that a large number of people have negative antibodies, but high titers. Since some previous studies have demonstrated that patients with negative-high serum antibody titers, especially those with *H. pylori* infection, were at a risk of developing intestinal gastric cancer, these results should be considered in clinical practice[10,11,14,15].

We previously reported that the *H. pylori* infection rate was 17% in group B[11]. The infection rate at the time serum antibodies were measured was 13% in people < 40 years old, 15% for those aged 40-59 years, and 25% for those ⩾ 60 years. Ueda *et al*[27] described that the prevalence of *H. pylori* infection in Japan was the highest in patients born between 1940-1949 and then decreased in those born in the ensuing years. Our results were concordant with those of their reports and might indicate the current infection rate in urban areas in Japan.

This study has some limitations. The subjects

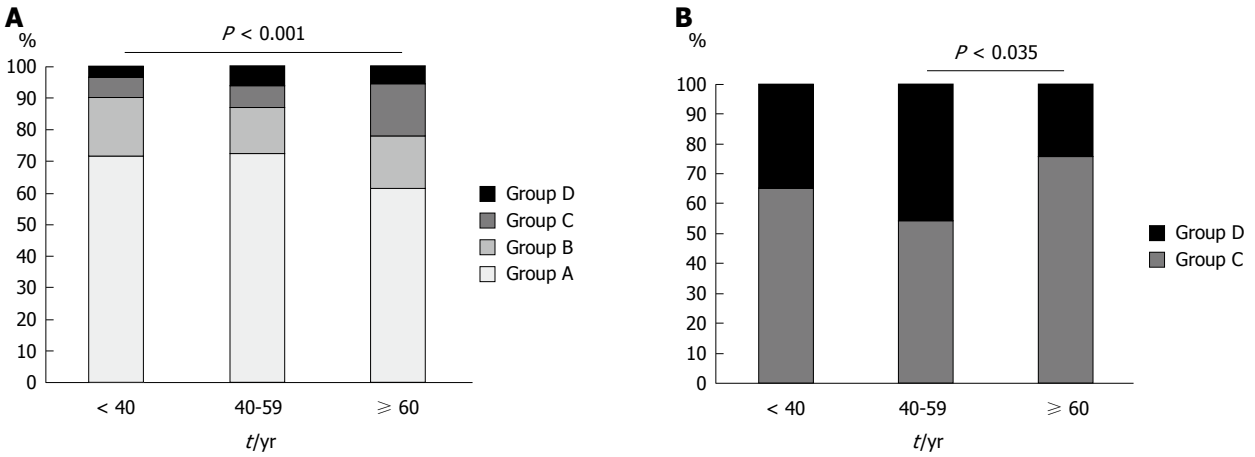


Figure 2 Proportion of *Helicobacter pylori*-infected patients. A: The proportion of patients in each of the groups, stratified by age. B: The proportion of patients in groups C and D, stratified by age.

included in the study were limited to outpatients. In the future, population-based research is expected. We did not diagnose *H. pylori* infection accurately because this study based on serum antibodies for diagnostic method. Investigating *H. pylori* infection with UBT is needed to determine the fluctuations in serum antibody titers in a time series in *H. pylori*-infected patients. Cytotoxin-associated gene A (CagA), which is a virulent form of *H. pylori,* was also not evaluated because 95% of Japanese patients with *H. pylori* infection have East Asian-type CagA[28]. Further investigations in those who do not have East Asian-type CagA-positive *H. pylori* infection is necessary.

In *H. pylori*-infected patients, high titers of serum anti-*H. pylori* antibodies were associated with gastric nodularity and atrophy, and the serum antibody titer tended to decrease in 60-year-old patients. Serum antibody titer reflects gastric mucosal inflammation; therefore, patient with high antibody titer may be at risk for diffuse gastric cancer and should be carefully screened in clinical practice.

ARTICLE HIGHLIGHTS

Research background

Serum anti-*Helicobacter pylori* (*H. pylori*) antibody titer and endoscopic findings are associated with the risk of gastric cancer.

Research motivation

Few studies have reported on the relationship between the serum antibody titer and endoscopic findings.

Research objectives

To clarify the role of serum anti-*H. pylori* antibody titers in gastric cancer.

Research methods

A cross-sectional study was conducted to investigate the effect of patients' baseline characteristics and endoscopic findings on their serum antibody titers. We excluded patients with a history of eradication therapy.

Research results

Gastric nodularity, atrophy, and age 40-59 years (*vs* age ⩾ 60 years) were correlated with a high serum antibody titer in *H. pylori*-infected patients.

Research conclusions

Serum antibody titer reflects gastric mucosal inflammation

Research perspective

In the future, population-based research is expected.

ACKNOWLEDGMENTS

We would like to thank Kanazawa T, Matsumoto S, Yoshida S, Isomura Y, Arano T, Kinoshita H, Kataoka Y, Ohki D, Fukagawa K, and Sekiba K for performing esophagogastroduodenoscopy.

REFERENCES

1 **Ford AC**, Forman D, Hunt RH, Yuan Y, Moayyedi P. Helicobacter pylori eradication therapy to prevent gastric cancer in healthy asymptomatic infected individuals: systematic review and meta-analysis of randomised controlled trials. *BMJ* 2014; **348**: g3174 [PMID: 24846275 DOI: 10.1136/bmj.g3174]

2 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]

3 **Toyoshima O,** Yamaji Y, Yoshida S, Matsumoto S, Yamashita H, Kanazawa T, Hata K. Endoscopic gastric atrophy is strongly associated with gastric cancer development after Helicobacter pylori eradication. *Surg Endosc* 2017; **31**: 2140-2148 [PMID: 27604367 DOI: 10.1007/s00464-016-5211-4]

4 **Toyoshima O,** Tanikawa C, Yamamoto R, Watanabe H, Yamashita H, Sakitani K, Yoshida S, Kubo M, Matsuo K, Ito H, Koike K, Seto Y, Matsuda K. Decrease in *PSCA* expression caused by *Helicobacter pylori* infection may promote progression to severe gastritis. *Oncotarget* 2017; **9**: 3936-3945 [PMID: 29423095 DOI: 10.18632/oncotarget.23278]

5 **Sakitani K,** Nishizawa T, Arita M, Yoshida S, Kataoka Y, Ohki D, Yamashita H, Isomura Y, Toyoshima A, Watanabe H, Iizuka T, Saito Y, Fujisaki J, Yahagi N, Koike K, Toyoshima O. Early detection of gastric cancer after Helicobacter pylori eradication due to endoscopic surveillance. *Helicobacter* 2018; **23**: e12503 [PMID:

29924436 DOI: 10.1111/hel.12503]
6 **Dinis-Ribeiro M**, Areia M, de Vries AC, Marcos-Pinto R, Monteiro-Soares M, O'Connor A, Pereira C, Pimentel-Nunes P, Correia R, Ensari A, Dumonceau JM, Machado JC, Macedo G, Malfertheiner P, Matysiak-Budnik T, Megraud F, Miki K, O' Morain C, Peek RM, Ponchon T, Ristimaki A, Rembacken B, Carneiro F, Kuipers EJ; European Society of Gastrointestinal Endoscopy; European Helicobacter Study Group; European Society of Pathology; Sociedade Portuguesa de Endoscopia Digestiva. Management of precancerous conditions and lesions in the stomach (MAPS): guideline from the European Society of Gastrointestinal Endoscopy (ESGE), European Helicobacter Study Group (EHSG), European Society of Pathology (ESP), and the Sociedade Portuguesa de Endoscopia Digestiva (SPED). *Endoscopy* 2012; **44**: 74-94 [PMID: 22198778 DOI: 10.1055/s-0031-1291491]
7 **Tonkic A**, Tonkic M, Lehours P, Mégraud F. Epidemiology and diagnosis of Helicobacter pylori infection. *Helicobacter* 2012; **17** Suppl 1: 1-8 [PMID: 22958148 DOI: 10.1111/j.1523-5378.2012.00975.x]
8 **Burucoa C**, Delchier JC, Courillon-Mallet A, de Korwin JD, Mégraud F, Zerbib F, Raymond J, Fauchère JL. Comparative evaluation of 29 commercial Helicobacter pylori serological kits. *Helicobacter* 2013; **18**: 169-179 [PMID: 23316886 DOI: 10.1111/hel.12030]
9 **Ueda J,** Okuda M, Nishiyama T, Lin Y, Fukuda Y, Kikuchi S. Diagnostic accuracy of the E-plate serum antibody test kit in detecting Helicobacter pylori infection among Japanese children. *J Epidemiol* 2014; **24**: 47-51 [PMID: 24240631]
10 **Kishikawa H**, Kimura K, Takarabe S, Kaida S, Nishida J. Helicobacter pylori Antibody Titer and Gastric Cancer Screening. *Dis Markers* 2015; **2015**: 156719 [PMID: 26494936 DOI: 10.1155/2015/156719]
11 **Toyoshima O**, Nishizawa T, Arita M, Kataoka Y, Sakitani K, Yoshida S, Yamashita H, Hata K, Watanabe H, Suzuki H. *Helicobacter pylori* infection in subjects negative for high titer serum antibody. *World J Gastroenterol* 2018; **24**: 1419-1428 [PMID: 29632423 DOI: 10.3748/wjg.v24.i13.1419]
12 **Marchildon P**, Balaban DH, Sue M, Charles C, Doobay R, Passaretti N, Peacock J, Marshall BJ, Peura DA. Usefulness of serological IgG antibody determinations for confirming eradication of Helicobacter pylori infection. *Am J Gastroenterol* 1999; **94**: 2105-2108 [PMID: 10445535 DOI: 10.1111/j.1572-0241.1999.01285.x]
13 **Yamaji Y**, Mitsushima T, Ikuma H, Okamoto M, Yoshida H, Kawabe T, Shiratori Y, Saito K, Yokouchi K, Omata M. Weak response of Helicobacter pylori antibody is high risk for gastric cancer: a cross-sectional study of 10,234 endoscoped Japanese. *Scand J Gastroenterol* 2002; **37**: 148-153 [PMID: 11843049]
14 **Tatemichi M**, Sasazuki S, Inoue M, Tsugane S; JPHC Study Group. Clinical significance of IgG antibody titer against Helicobacter pylori. *Helicobacter* 2009; **14**: 231-236 [PMID: 19702853 DOI: 10.1111/j.1523-5378.2009.00681.x]
15 **Kiso M**, Yoshihara M, Ito M, Inoue K, Kato K, Nakajima S, Mabe K, Kobayashi M, Uemura N, Yada T, Oka M, Kawai T, Boda T, Kotachi T, Masuda K, Tanaka S, Chayama K. Characteristics of gastric cancer in negative test of serum anti-Helicobacter pylori antibody and pepsinogen test: a multicenter study. *Gastric Cancer* 2017; **20**: 764-771 [PMID: 28025702 DOI: 10.1007/s10120-016-0682-5]
16 **Matsuo T**, Ito M, Takata S, Tanaka S, Yoshihara M, Chayama K. Low prevalence of Helicobacter pylori-negative gastric cancer among Japanese. *Helicobacter* 2011; **16**: 415-419 [PMID: 22059391 DOI: 10.1111/j.1523-5378.2011.00889.x]
17 **Chemical E.** E-plate Eiken H. pylori antibody Ⅱ. 2011. Accessed May 26, 2018 Available from: URL: http://www.info.pmda.go.jp/downfiles/ivd/PDF/170005_22200AMX00935000_A_01_02.pdf
18 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297 DOI: 10.1056/NEJMoa001999]
19 **Kato M.** Endoscopic Findings of H. pylori Infection. In: Suzuki H, Warren R, Marshall B, editors. Helicobacter pylori. Tokyo: Springer Japan, 2016: 157-167
20 **Shichijo S**, Hirata Y, Niikura R, Hayakawa Y, Yamada A, Koike K. Association between gastric cancer and the Kyoto classification of gastritis. *J Gastroenterol Hepatol* 2017; **32**: 1581-1586 [PMID: 28217843 DOI: 10.1111/jgh.13764]
21 **Kimura K,** Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. *Endoscopy* 1969; **3**: 87-97 [DOI: 10.1055/s-0028-1098086]
22 **Rubicz R,** Leach CT, Kraig E, Dhurandhar NV, Duggirala R, Blangero J, Yolken R, Göring HH. Genetic factors influence serological measures of common infections. *Hum Hered* 2011; **72**: 133-141 [PMID: 21996708 DOI: 10.1159/000331220]
23 **Tatemichi M**, Sasazuki S, Inoue M, Tsugane S; Japan Public Health Center Study Group. Different etiological role of Helicobacter pylori (Hp) infection in carcinogenesis between differentiated and undifferentiated gastric cancers: a nested case-control study using IgG titer against Hp surface antigen. *Acta Oncol* 2008; **47**: 360-365 [PMID: 18347999 DOI: 10.1080/02841860701843035]
24 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740 [PMID: 1458460]
25 **Kamada T**, Sugiu K, Hata J, Kusunoki H, Hamada H, Kido S, Nagashima Y, Kawamura Y, Tanaka S, Chayama K, Haruma K. Evaluation of endoscopic and histological findings in Helicobacter pylori-positive Japanese young adults. *J Gastroenterol Hepatol* 2006; **21**: 258-261 [PMID: 16460483 DOI: 10.1111/j.1440-1746.2006.04128.x]
26 **Yoshida T**, Kato J, Inoue I, Yoshimura N, Deguchi H, Mukoubayashi C, Oka M, Watanabe M, Enomoto S, Niwa T, Maekita T, Iguchi M, Tamai H, Utsunomiya H, Yamamichi N, Fujishiro M, Iwane M, Takeshita T, Ushijima T, Ichinose M. Cancer development based on chronic active gastritis and resulting gastric atrophy as assessed by serum levels of pepsinogen and Helicobacter pylori antibody titer. *Int J Cancer* 2014; **134**: 1445-1457 [PMID: 24009139 DOI: 10.1002/ijc.28470]
27 **Ueda J**, Gosho M, Inui Y, Matsuda T, Sakakibara M, Mabe K, Nakajima S, Shimoyama T, Yasuda M, Kawai T, Murakami K, Kamada T, Mizuno M, Kikuchi S, Lin Y, Kato M. Prevalence of Helicobacter pylori infection by birth year and geographic area in Japan. *Helicobacter* 2014; **19**: 105-110 [PMID: 24506211 DOI: 10.1111/hel.12110]
28 **Yamaoka Y**. Mechanisms of disease: Helicobacter pylori virulence factors. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 629-641 [PMID: 20938460 DOI: 10.1038/nrgastro.2010.154]

P- Reviewer: Chiba T, Kishikawa H, Romano M **S- Editor**: Ma RY
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21 24(35): 4069-4076

DOI: 10.3748/wjg.v24.i35.4069

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

ORIGINAL ARTICLE

Observational Study

Real-life chromoendoscopy for dysplasia surveillance in ulcerative colitis

Pasquale Klepp, Anita Tollisen, Arne Røseth, Milada Cvancarova Småstuen, Solveig N Andersen, Morten Vatn, Bjørn A Moum, Stephan Brackmann

Pasquale Klepp, Anita Tollisen, Arne Røseth, Unger-Vetlesen Institute, Department of Internal Medicine, Lovisenberg Diaconal Hospital, Oslo 0456, Norway

Pasquale Klepp, Milada Cvancarova Småstuen, Morten Vatn, Bjørn A Moum, Stephan Brackmann, Institute of Clinical Medicine, University of Oslo, Oslo 0317, Norway

Solveig N Andersen, Department of Pathology, Akershus University Hospital, Lørenskog 1474, Norway

Bjørn A Moum, Department of Gastroenterology, Oslo University Hospital, Oslo 0450, Norway

Stephan Brackmann, Department of Gastroenterology, Akershus University Hospital, Lørenskog 1474, Norway

ORCID number: Pasquale Klepp (0000-0002-0477-691X); Anita Tollisen (0000-0002-4776-9808); Arne Røseth (0000-0003-0916-5111); Milada Cvancarova Småstuen (0000-0001-8947-8649); Solveig N Andersen (0000-0002-4814-8894); Morten Vatn (0000-0002-1179-4550); Bjørn A Moum (0000-0002-5884-4543); Stephan Brackmann (0000-0001-6434-0571).

Author contributions: Klepp P contributed to acquisition, analysis and interpretation of the data, and drafting and revision of the manuscript; Tollisen A contributed to planning and collection of the data; Røseth A contributed to conception and design of the study, and performed critical revision of the manuscript for important intellectual content; Cvancarova Småstuen M contributed to the statistical analysis and critical revision of the manuscript for important intellectual content; Andersen SN provided technical support and contributed to revision of the histopathological material; Vatn M provided critical revision of the manuscript for important intellectual content and performed study supervision; Moum BA and Brackmann S contributed to conception and design of the study, and performed critical revision of the manuscript for important intellectual content and study supervision; all authors have approved the final draft.

Supported by the Unger-Vetlesen Institute, Department of Internal Medicine, Lovisenberg Hospital.

Institutional review board statement: The study protocol was designed according to the combined knowledge and expertise of Assistant Professor Stephan Brackmann (Akershus University Hospital), Professor Bjørn A Moum (Oslo University Hospital) and Professor Morten Vatn (University of Oslo). The study protocol was approved by the Regional Committee for Medical and Health Research Ethics (REC Project NO. 2010/1093).

Informed consent statement: Written informed consent was collected from subjects prior to study inclusion.

Conflict-of-interest statement: The authors have no conflicts of interest.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement, and the manuscript was prepared and revised according to the STROBE Statement.

Manuscript source: Unsolicited manuscript

Correspondence to: Pasquale Klepp, MD, Attending Doctor, Department of Internal Medicine, Lovisenberg Hospital, Lovisenberggt.17, Oslo 0456, Norway. pasklepp@gmail.com
Telephone: +47-23225000

Received: May 30, 2018
Peer-review started: May 30, 2018
First decision: July 4, 2018
Revised: August 6, 2018
Accepted: August 24, 2018

Article in press: August 24, 2018
Published online: September 21, 2018

Abstract

AIM

To evaluate the use of chromoendoscopy for surveillance of ulcerative colitis in a real-life community hospital setting.

METHODS

Patients with extensive ulcerative colitis, having disease duration of more than 8 years and who presented between the years of 1999 to 2013, were offered enrolment in this single cohort prospective study. All participants underwent standard bowel preparation with sodium phosphate and chromoendoscopy. Two expert endoscopists, novice to chromoendoscopy, evaluated each segment of the colon with standard-definition colonoscopes after spray application of 0.4% indigo carmine. All observed lesions were recorded and evaluated before being removed and/or biopsied. In addition, nontargeted biopsies were taken from each segment of the colon. The dysplasia detection rate and dysplasia detection yield were ascertained.

RESULTS

A total of 21 neoplastic lesions (2 carcinomas, 4 of high-grade dysplasia and 15 of low-grade dysplasia) and 27 nondysplastic lesions were detected in 16 of the total 67 patients (70% male; median disease duration: 17 years; median age at diagnosis: 25 years; 92% aminosalicylate-treated). The dysplasia detection rate was 10.5% (7/67 patients). The dysplasia detection yield was 20.8% (10/48) for targeted biopsies and 3.5% (11/318) for nontargeted biopsies. The sensitivity and specificity for the macroscopic evaluation of neoplasia using chromoendoscopy were 48% [95% confidence interval (CI): 26%-70%] and 96% (95%CI: 93%-98%), respectively. The positive predictive and negative predictive values were 42% (95%CI: 27%-59%) and 97% (95%CI: 95%-98%), respectively. A total of 19/21 dysplastic lesions were detected in mucosa with histologic inflammation.

CONCLUSION

Chromoendoscopy seems to be of value for dysplasia surveillance of ulcerative colitis in a community hospital setting. The yield of non-targeted biopsies is negligible.

Key words: Colorectal cancer; Dysplasia; Ulcerative colitis; Surveillance; Chromoendoscopy

Core tip: Patients with longstanding and extensive ulcerative colitis are at increased risk of developing colonic neoplasia and are advised to undergo regular colonoscopic surveillance. Current clinical guidelines favour chromoendoscopy with targeted biopsies, as it detects dysplasia more accurately and thus requires fewer biopsies than white-light endoscopy. However, these recommendations are based on studies performed in advanced endoscopic units and chromoendoscopy is not routinely applied in everyday clinical practice. This prospective cohort study suggests that, although novice to chromoendoscopy, endoscopists can accurately evaluate the absence of neoplasia. The yield of nontargeted biopsies was also found to be negligible.

Klepp P, Tollisen A, Røseth A, Cvancarova Småstuen M, Andersen SN, Vatn M, Moum BA, Brackmann S. Real-life chromoendoscopy for dysplasia surveillance in ulcerative colitis. *World J Gastroenterol* 2018; 24(35): 4069-4076 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4069.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4069

INTRODUCTION

Patients with extensive and long-standing ulcerative colitis (UC) carry an increased risk of developing colonic neoplastic lesions[1]. Carcinoma in UC is thought to develop through a stepwise progression from inflammation to low-grade dysplasia and finally to carcinoma[2]. Patients are therefore advised to undergo periodic colonoscopic surveillance, so as to detect neoplasia at an early stage[3]. Visualization of dysplastic lesions in UC represents a challenge as they may be flat or obscured by inflammatory changes and/or pseudopolyps[4]. Dysplasia surveillance using white-light endoscopy relies on random 4-quadrant biopsies taken every 10 cm, being a laborious and costly method[5]. Under-sampling is common with that technique and even if the recommended 30-40 biopsies are harvested, only a fraction of the entire mucosal surface of the colon is examined[6].

Chromoendoscopy (CE), on the other hand, uses a topical dye, which highlights mucosal abnormalities and allows for more precise biopsies[7]. Targeted biopsies are considered superior to random biopsies of apparently unaffected mucosa, as the latter are of little additional value since they have poor diagnostic yield[8]. Thus, recommendations for surveillance using CE are based on the assumption that CE requires fewer biopsies and is more cost effective than standard white-light endoscopy[7,9-11]. European clinical guidelines recommend CE with targeted biopsies as the favoured technique for dysplasia surveillance[3]. It is important to note that these guidelines are based on studies that were performed in endoscopic units with highly advanced expertise. However, in many countries, such as Norway, CE is not routinely applied for surveillance of UC patients. A retrospective multicentre study conducted over a 14-year period of CE implementation also did not show significant increase in the detection of

Table 1 Patient demographics and clinical features

Feature	Data
Study patients	67
Age (yr)	40 (27-73)
Male sex	46 (70)
Disease duration (yr)	17 (8-51)
Age at diagnosis (yr)	25 (12-59)
Colitis activity index score[2]	0 (0-8)
Primary sclerosing cholangitis	3 (5), missing n = 2
Colorectal cancer in first degree relative	3 (5), missing n = 5
Previous dysplasia in colon	3 (5), missing n = 3
Treatment	
Aminosalicylate[1]	58 (92), missing n = 3
Steroids[1]	12 (18), missing n = 4
Azathioprine[1]	4 (6)
Antitumour necrosis factor[1]	4 (6), missing n = 1

[1]At time of surveillance colonoscopy and/or during past two years; [2]Simple clinical colitis activity index. Data are presented as n (%) or median (interquartile range).

dysplastic lesions[12].

The aim of this study was to assess the macroscopic and histologic evaluation of CE when implemented in real-life surveillance of patients with long-standing UC in a community hospital in Norway.

MATERIALS AND METHODS

Ethical considerations

The study protocol was designed according to the combined knowledge and expertise of Assistant Professor Stephan Brackmann (Akershus University Hospital), Professor Bjørn A Moum (Oslo University Hospital) and Professor Morten Vatn (University of Oslo). The study protocol was approved by the Regional Committee for Medical and Health Research Ethics (Project NO. 2010/1093). Written informed consent was collected from all subjects prior to study inclusion.

Recruitment of participants

Patients registered in the database of Lovisenberg Hospital from 1999-2013 were invited to participate in the present study if they had (1) extensive UC, documented by endoscopy at any time during the course of disease, and (2) disease duration of 8 years or more. Exclusion criteria included colectomy at any time during follow-up and poor bowel preparation (Figure 1). Demographic and clinical data were extracted from digital medical journals and by interview of the patients (Table 1).

CE

Patients underwent standard bowel preparation with sodium phosphate. CE was performed with standard definition endoscope (CF190 colonoscope; Olympus, Tokyo, Japan) and only carried out when the quality of bowel preparation was adequate (n = 67/68). The colonoscope was advanced to the cecum and during the extubation each segment (cecum, ascending colon, transverse colon, descending sigmoid and rectum) was scrutinised for lesions after the spray catheter application of 0.3% indigo-carmine. Extensive colitis was defined as endoscopic inflammation proximal to the splenic flexure. Endoscopic degree of inflammation was classified according to the Mayo endoscopic score for UC[13].

Biopsies

The location and size of all lesions identified after spray catheter application of 0.3% indigo-carmine dye were reported. Also the appearance of the lesions was classified according to terminology adapted from the Scenic Consensus[14] as either nonpolypoid flat or elevated or polypoid pedunculated or nonpedunculated before the lesions were biopsied or removed.

In addition, after spray catheter application of 0.3% indigo-carmine dye, a minimum of one nontargeted biopsy was taken from each of the six segments (cecum, ascending, transverse and descending colon, sigmoid and rectum) to determine the extent of disease and grade of inflammation.

Independent and blind analyses of the formalin-fixed paraffin-embedded biopsies were performed by two expert gastropathologists. The histologic degree of inflammation was graded based on the histological activity index[15]. Mucosal biopsies were classified as either negative for neoplasia, indefinite for dysplasia, or positive for low-grade dysplasia (LGD), high-grade dysplasia (HGD) or adenocarcinoma[16]. Neoplasia was considered proximal or distal according to its anatomic location to the splenic flexure. The dysplasia detection yield was defined as the proportion of bioptic sites/lesions containing dysplasia or invasive colorectal cancer (CRC) in relation to the total number of bioptic sites/lesions. The dysplasia detection rate was defined as the proportion of patients who had at least one dysplastic lesion or invasive CRC in relation to the total number of screened patients.

Statistical analysis

The statistical methods of this study were reviewed by Milada Cvancarova Småstuen (Institute of Clinical Medicine, University of Oslo, PO Box 1122 Blinderen, 0317 Oslo, Norway). All statistical analyses were carried out with the SPSS software, version 24 (IBM Corp, Armonk, NY, United States). Patient demographic characteristics were summarized as median (interquartile range) for continuous variables and as percentage of subgroup totals for categorical variables. Sensitivity and specificity were estimated with corresponding 95% confidence intervals (CIs) that were calculated using the exact binomial distribution.

RESULTS

CE was performed in 67 patients with extensive UC. A

Table 2 Histologic diagnosis of the seven neoplastic lesions detected among the 67 total patients with long-standing and extensive ulcerative colitis

Sex	Age (yr)	LGD		HGD	Adenocarcinoma	Total
		Targeted biopsy	Nontargeted biopsy	Targeted biopsy	Targeted biopsy	
Male	34	1	7	4	1[1]	13
Male	67				1[2]	1
Male	49	1	1			2
Male	33	1				1
Female	36	1				1
Male	44		1			1
Female	52		2			2
Total		4	11	4	2	21

[1]Adenocarcinoma stage T3N1MO in patient with primary sclerosing cholangitis; [2]Adenocarcinoma stage T2N0M0. No neoplasia was found in biopsies despite high suspicion of malignancy at endoscopy. Adenocarcinoma was subsequently verified in the resected surgical specimen. LGD: Low-grade dysplasia; HGD: High-grade dysplasia.

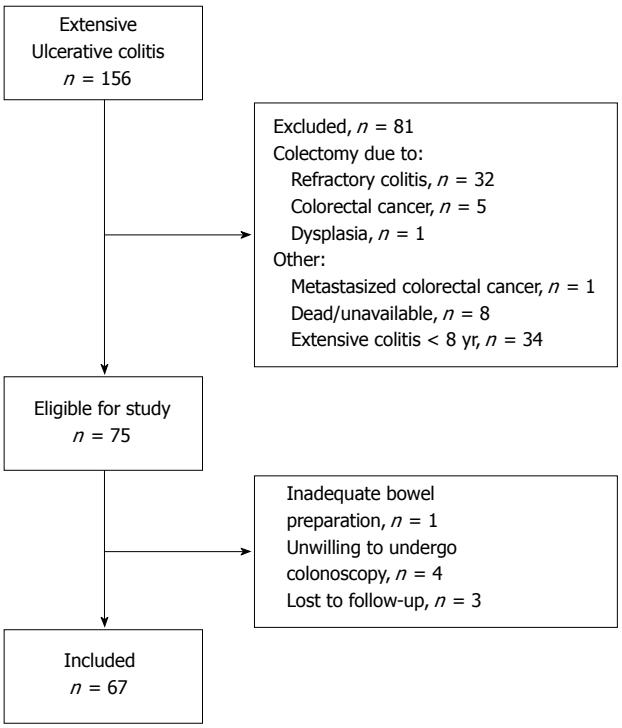


Figure 1 Flowchart of patient inclusion for this study.

total of 21 neoplastic lesions were detected in 7 of the 67 patients, including 10 (comprised of 2 CRC, 4 HGD and 4 LGD) identified by targeted biopsies and 11 (all LGD) by nontargeted biopsies.

Dysplasia detection rate

Neoplasia was detected in 7 of the total 67 patients, giving a dysplasia detection rate of 10.5%. In 4, neoplasia was detected from both targeted and non-targeted biopsies. In 2, dysplasia was detected by nontargeted biopsies alone. In 1, adenocarcinoma was diagnosed after partial colonic resection.

Dysplasia detection yield

A total of 48 lesions were visualised in 16 of the total 67 patients. The median number of lesions per patient was 1 (range: 0-6). The distribution and findings from endoscopic and histologic evaluations of the lesions are described in detail in Figure 2 and Tables 2 and 3. Ten of the visualised lesions harboured neoplasia, resulting in a dysplasia detection yield of 20.8% for targeted biopsies. Among the 318 nontargeted biopsies, 11 harboured LGD, resulting in a dysplasia detection yield of 3.5% for nontargeted biopsies.

Macroscopic evaluation

Correct classification by the endoscopists was achieved for 307 of the 345 nondysplastic sites/lesions and 10 of the 21 dysplastic sites/lesions. On the other hand, 11 of the 21 dysplastic lesions were assessed as nondysplasia, whereas 38 of the 345 nondysplastic sites /lesions were assessed as dysplasia. As a result, the sensitivity and specificity for the macroscopic evaluation of neoplasia using CE were 48% (95%CI: 26%-70%) and 96% (95%CI: 93%-98%), respectively. The positive predictive value was 42% (95%CI: 27%-59%) and the negative predictive value (NPV) was 97% (95%CI: 95%-98%).

Follow-up of patients with colonic neoplasia

In 2 of the 7 patients with LGD, the dysplasia was detected solely by nontargeted biopsies taken during a CE described as macroscopically normal. Follow-up with colonoscopy neither confirmed nor revealed any further dysplasia for either patient. In another 2 of the 7 patients, the LGD was detected solely by targeted biopsies. In yet another 2 of the 7 patients, nontargeted biopsies confirmed a field effect by detecting LGD when dysplastic lesions were identified elsewhere in the colorectum by targeted biopsies; during intensified follow-up colonoscopy, no further dysplasia was detected after 2.5 years follow-up (range: 2-5 years) in these patients.

In 1 of the 7 patients with neoplasia, nontargeted biopsies showed multifocal fields of LGD synchronous with targeted biopsies that showed multifocal lesions with HGD and adenocarcinoma. The patient had primary sclerosing cholangitis and proctocolectomy

Table 3 Morphology and histology findings for the 16 bioptic sites of the 67 total patients with long-standing and extensive ulcerative colitis

Morphology	Histology	*n*
Targeted biopsy		
Nonpolypoid		
Flat lesion	No neoplasia	26
	LGD	2
	HGD	1[1]
Superficial elevated	No neoplasia	10
	HGD	1[1]
	Adenocarcinoma	1
Polypoid		
Pedunculated	No neoplasia	1
	LGD	1
Nonpedunculated	LGD	1
Sessile with central ulceration	HGD	2[1]
Sessile	Adenocarcinoma	1[1]
	IND	1
Nontargeted biopsy		
	No neoplasia	307
	LGD	11
Total		366

[1]Concomitant primary sclerosing cholangitis. HGD: High-grade dysplasia; IND: Indefinite dysplasia; LGD: Low-grade dysplasia.

was performed. Finally, adenocarcinoma was detected after partial resection of the colon in 1 of the 7 patients for who endoscopy had raised suspicion of malignancy, whereas targeted biopsies of this area were normal on two consecutive colonoscopies. After surgery, no further neoplasia was detected during 3 years of intensified follow-up colonoscopies.

Mucosal inflammation

No signs of histologic inflammation were recorded in 93 of the 366 bioptic sites. A total of 237 had Mayo grade 0-1 and 39 had Mayo grade 2-3. The presence of neoplasia in relation to histologic inflammatory changes is described in detail in Table 4.

Schedule of surveillance colonoscopy

The median time from the prior "prestudy" surveillance colonoscopy (n = 61/67) until the next scheduled colonoscopy was 24 mo (range: 0-96 mo). The median time from the prior "prestudy" surveillance colonoscopy (n = 66/67) until the study CE was 26 mo (range: 1-105 mo).

DISCUSSION

This prospective cohort study performed in a community hospital suggests that, although novice to CE, endoscopists were able to accurately evaluate the absence of neoplasia during real-life surveillance of patients with UC. Neoplasia was detected by targeted biopsies in 5 of the 67 total patients, of whom 2 had a field effect confirmed by nontargeted biopsies. Two additional patients were diagnosed with LGD in the colon by nontargeted biopsies alone. The neoplasia detection rate for the 7 of the 67 total patients in the present study is similar to that found in studies performed in tertiary referral centres (11.2%). It is important to note, however, that the present study was conducted in a community hospital in which patients may present with a less aggressive UC than seen in advanced units.

The neoplasia detection yield of the present study was 20.8%, which is lower than the average rate of 14% found by Mooiweer *et al*[12] in several prior randomized trials. The endoscopists in our study were able to accurately rule-out neoplasia (NPV = 97%); thus, when the endoscopists evaluated the lesion as benign, the probability of dysplasia was minimal (3%). These results are in line with a recent prospective multicentre cohort study in which both CE novice endoscopists and CE expert endoscopists evaluated lesions, and had a high NPV[17]. That same study found a sensitivity of 70% for CE, which is lower than the pooled sensitivity of 91% reported from a recent meta-analysis[17,18]. In the present study, the sensitivity for the detection of LGD by CE was modest, which could be related to the endoscopists' lack of prior CE experience.

Alternatively, these results support the presence of "invisible" dysplasia. In our cohort, "invisible" dysplasia was rare in the absence of dysplastic lesions elsewhere in the colon, similar to findings reported by Matsumoto *et al*[9,19] and underlining the concept of field cancerization in UC[9,19]. The results must, however, be evaluated with caution due to the size of the study sample and the low observed rate of cases.

The clinical importance of dysplasia detected through random biopsies is debatable. In the present study, the dysplasia detection yield was 3.5% for nontargeted biopsies compared to a 20.8% for targeted biopsies. The nontargeted biopsies were primarily taken not to detect neoplasia but to evaluate the grade of mucosal inflammation. In line with previous studies, the low dysplasia yield of nontargeted biopsies leads to questions about their clinical value[7,8,17,20,21]. Also, the follow-up of the patients in which LGD was detected by nontargeted biopsy alone did not reveal any further dysplasia. However, a recent study has suggested that despite a low bioptic neoplasia yield, nontargeted biopsies are advisable in patients with inflammatory bowel disease and related high risk of CRC[21].

Clinical guidelines recommend the first surveillance colonoscopy to be performed between 8-10 years after the diagnosis of UC, with ensuing colonoscopies based on individual risk. In our cohort, the median time until the next scheduled colonoscopy was 24 mo, which is in accordance with guidelines[3].

The visualisation of small lesions harbouring dysplasia may have been hampered by inflammation surrounding multifocal lesions. However, the minimal level of inflammatory changes in those patients in who dysplasia was diagnosed by nontargeted biopsies only did not likely impede the detection.

Table 4 Histologic grade of inflammation in the biopsies taken from all 67 patients

	Grade of inflammation					
	Mayo 0[1]	Mayo 1	Mayo 2	Mayo 3	No signs of previous inflammation	Total
No dysplasia/IND	133	83	33	3	93	345
LGD	3	12	0	0	0	15
HGD	0	2	2	0	0	4
CRC	0	1	1	0	0	2
Total	136	98	36	3	93	366

[1]Inactive colitis. CRC: Colorectal cancer; HGD: High-grade dysplasia; IND: Indefinite dysplasia; LGD: Low-grade dysplasia.

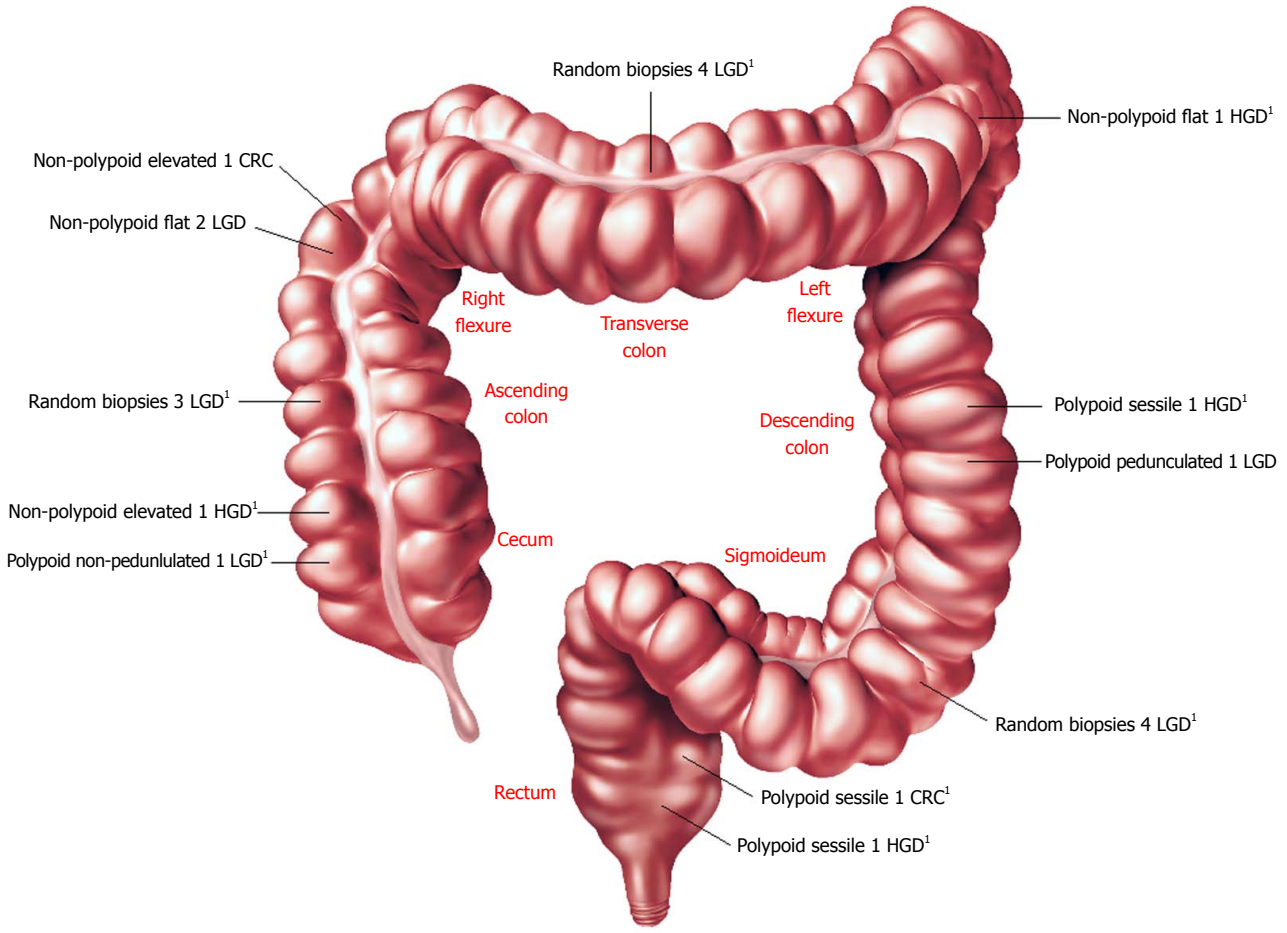


Figure 2 Distribution and findings of endoscopic and histologic evaluation of the 21 total neoplastic lesions found in this study. [1]Lesions detected in patient with know primary sclerosing cholangits.

In the present study, current or previous inflammatory changes were found to be present around all dysplastic lesions, including for nontargeted biopsies harbouring lesions with LGD. These results support the findings of Watanabe *et al*[22], who suggested that random biopsies may be omitted in the absence of previous or current inflammation.

We recognize that the present study has several limitations. The adenoma detection rate of the endoscopists is not currently implemented in Norway and therefore not available for the endoscopists in the present study. Both endoscopists had however substantial experience in non-CE endoscopy. The present study reflects the everyday clinical world in this location. Each CE was performed with standard and not high-definition endoscopes, which may have affected the detection of lesions. The prospective cohort design of the study minimizes patient selection bias; however, the sample size is moderate, although considerable effort was spent on the inclusion of patients. Patients may have been reluctant to undergo colonoscopy. Low compliance rates for surveillance endoscopy have previously been described, although the reasons remain unclear[23]. Additionally, although all the patients in the sample carry an increased risk of colonic neoplasia, the low observed rate of neoplasia resulted in wide CIs around sensitivity estimates for the detection of

dysplastic lesions.

Despite the limitations outlined above, the results of the present study are thought to reflect the real-life scenario of dysplasia surveillance in UC in a community-based hospital in Norway, which could represent the typical setting in similar hospitals in Europe where CE is not routinely applied.

In conclusion, the present study suggests that, although lacking in previous CE expertise, the endoscopists were able to accurately evaluate the absence of neoplasia. The yield of nontargeted biopsies with LGD was negligible, and LGD appears to be present in mucosa with signs of histologic inflammation. Finally, dysplasia in endoscopically unsuspicious appearing mucosa seems to occur mostly when visible neoplasia is diagnosed elsewhere in the colon. Although further larger studies are needed, CE seems to be of value for surveillance of neoplasia in UC in a community hospital setting.

ARTICLE HIGHLIGHTS

Research background

Patients with longstanding and extensive ulcerative colitis carry an increased risk of developing colonic neoplasia and are advised to undergo regular colonoscopic surveillance.

Research motivation

The current clinical guidelines favour chromoendoscopy with targeted biopsies for dysplasia surveillance of ulcerative colitis. These recommendations, however, are based on studies performed in advanced endoscopic units and chromoendoscopy is not routinely applied in everyday clinical practice.

Research objectives

Our aim was to evaluate chromoendoscopy for real-life dysplasia surveillance for cases of long-standing ulcerative colitis in a community hospital.

Research methods

Patients with extensive ulcerative colitis, with disease duration of more than 8 years, were prospectively included in this single cohort study. The chromoendoscopies were performed by two expert endoscopists novice to the method. Lesions were evaluated macroscopically and removed and/or biopsied. Nontargeted biopsies were also taken from each segment of the colon.

Research results

A total of 21 neoplastic lesions (consisting of 2 carcinomas, 4 high-grade dysplasias and 15 low-grade dysplasias) and 27 nondysplastic lesions were detected in 16 of the 67 total patients included in the study. The dysplasia detection rate was 10.5% (for 7 of the 67 patients). The dysplasia detection yield was 20.8% (10/48) for targeted biopsies and 3.5% (11/318) for nontargeted biopsies. The endoscopists accurately evaluated the absence of neoplasia (specificity of 96%, with 95% confidence interval of 93-98; and a negative predictive value of 97%, with 95% confidence interval of 95%-98%).

Research conclusions

Although novice to chromoendoscopy, the endoscopists in this Norwegian community hospital accurately evaluated the absence of neoplasia. In addition, the yield of nontargeted biopsies was negligible.

Research perspectives

Chromoendoscopy appears to be of value for dysplasia surveillance for cases of long-standing ulcerative colitis who are treated in a community hospital setting in which endoscopists are novice to the technique.

ACKNOWLEDGMENTS

The authors thank the staff at the Unger-Vetlesen Research Institute, Lovisenberg Diaconal Hospital and Lars Gustav Lyckander at the Department of Pathology, Akershus University Hospital.

REFERENCES

1 **Rubin DT**, Huo D, Kinnucan JA, Sedrak MS, McCullom NE, Bunnag AP, Raun-Royer EP, Cohen RD, Hanauer SB, Hart J, Turner JR. Inflammation is an independent risk factor for colonic neoplasia in patients with ulcerative colitis: a case-control study. *Clin Gastroenterol Hepatol* 2013; **11**: 1601-1608.e1-e4 [PMID: 23872237 DOI: 10.1016/j.cgh.2013.06.023]

2 **Ullman TA**, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology* 2011; **140**: 1807-1816 [PMID: 21530747 DOI: 10.1053/j.gastro.2011.01.057]

3 **Magro F**, Gionchetti P, Eliakim R, Ardizzone S, Armuzzi A, Barreiro-de Acosta M, Burisch J, Gecse KB, Hart AL, Hindryckx P, Langner C, Limdi JK, Pellino G, Zagórowicz E, Raine T, Harbord M, Rieder F; European Crohn's and Colitis Organisation [ECCO]. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 1: Definitions, Diagnosis, Extra-intestinal Manifestations, Pregnancy, Cancer Surveillance, Surgery, and Ileo-anal Pouch Disorders. *J Crohns Colitis* 2017; **11**: 649-670 [PMID: 28158501 DOI: 10.1093/ecco-jcc/jjx008]

4 **Velayos FS**, Loftus EV Jr, Jess T, Harmsen WS, Bida J, Zinsmeister AR, Tremaine WJ, Sandborn WJ. Predictive and protective factors associated with colorectal cancer in ulcerative colitis: A case-control study. *Gastroenterology* 2006; **130**: 1941-1949 [PMID: 16762617 DOI: 10.1053/j.gastro.2006.03.028]

5 **Annese V**, Beaugerie L, Egan L, Biancone L, Bolling C, Brandts C, Dierickx D, Dummer R, Fiorino G, Gornet JM, Higgins P, Katsanos KH, Nissen L, Pellino G, Rogler G, Scaldaferri F, Szymanska E, Eliakim R; ECCO. European Evidence-based Consensus: Inflammatory Bowel Disease and Malignancies. *J Crohns Colitis* 2015; **9**: 945-965 [PMID: 26294789 DOI: 10.1093/ecco-jcc/jjv141]

6 **Eaden JA**, Ward BA, Mayberry JF. How gastroenterologists screen for colonic cancer in ulcerative colitis: an analysis of performance. *Gastrointest Endosc* 2000; **51**: 123-128 [PMID: 10650251]

7 **Rutter MD**, Saunders BP, Schofield G, Forbes A, Price AB, Talbot IC. Pancolonic indigo carmine dye spraying for the detection of dysplasia in ulcerative colitis. *Gut* 2004; **53**: 256-260 [PMID: 14724160 DOI: 10.1136/gut.2003.016386]

8 **van den Broek FJ**, Stokkers PC, Reitsma JB, Boltjes RP, Ponsioen CY, Fockens P, Dekker E. Random biopsies taken during colonoscopic surveillance of patients with longstanding ulcerative colitis: low yield and absence of clinical consequences. *Am J Gastroenterol* 2014; **109**: 715-722 [PMID: 21427710 DOI: 10.1038/ajg.2011.93]

9 **Matsumoto T**, Nakamura S, Jo Y, Yao T, Iida M. Chromoscopy might improve diagnostic accuracy in cancer surveillance for ulcerative colitis. *Am J Gastroenterol* 2003; **98**: 1827-1833 [PMID: 12907339 DOI: 10.1111/j.1572-0241.2003.07580.x]

10 **Marion JF**, Waye JD, Israel Y, Present DH, Suprun M, Bodian C, Harpaz N, Chapman M, Itzkowitz S, Abreu MT, Ullman TA, McBride RB, Aisenberg J, Mayer L; Chromoendoscopy Study Group at Mount Sinai School of Medicine. Chromoendoscopy Is More Effective Than Standard Colonoscopy in Detecting Dysplasia During Long-term Surveillance of Patients With Colitis. *Clin Gastroenterol Hepatol* 2016; **14**: 713-719 [PMID: 26656297 DOI: 10.1016/j.cgh.2015.11.011]

11 **Konijeti GG**, Shrime MG, Ananthakrishnan AN, Chan AT. Cost-effectiveness analysis of chromoendoscopy for colorectal cancer surveillance in patients with ulcerative colitis. *Gastrointest Endosc* 2014; **79**: 455-465 [PMID: 24262637 DOI: 10.1016/j.gie.2013.10.026]

12 **Mooiweer E**, van der Meulen-de Jong AE, Ponsioen CY, Fidder HH, Siersema PD, Dekker E, Oldenburg B. Chromoendoscopy for Surveillance in Inflammatory Bowel Disease Does Not Increase Neoplasia Detection Compared With Conventional Colonoscopy With Random Biopsies: Results From a Large Retrospective Study. *Am J Gastroenterol* 2015; **110**: 1014-1021 [PMID: 25823770 DOI: 10.1038/ajg.2015.63]

13 **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629 [PMID: 3317057 DOI: 10.1056/NEJM198712243172603]

14 **Laine L**, Kaltenbach T, Barkun A, McQuaid KR, Subramanian V, Soetikno R; SCENIC Guideline Development Panel. SCENIC international consensus statement on surveillance and management of dysplasia in inflammatory bowel disease. *Gastrointest Endosc* 2015; **81**: 489-501.e26 [PMID: 25708752 DOI: 10.1016/j.gie.2014.12.009]

15 **Gupta RB**, Harpaz N, Itzkowitz S, Hossain S, Matula S, Kornbluth A, Bodian C, Ullman T. Histologic inflammation is a risk factor for progression to colorectal neoplasia in ulcerative colitis: a cohort study. *Gastroenterology* 2007; **133**: 1099-1105; quiz 1340-1341 [PMID: 17919486 DOI: 10.1053/j.gastro.2007.08.001]

16 **Riddell RH**, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum Pathol* 1983; **14**: 931-968 [PMID: 6629368 DOI: 10.1016/S0046-8177(83)80175-0]

17 **Carballal S**, Maisterra S, López-Serrano A, Gimeno-García AZ, Vera MI, Marín-Garbriel JC, Díaz-Tasende J, Márquez L, Álvarez MA, Hernández L, De Castro L, Gordillo J, Puig I, Vega P, Bustamante-Balén M, Acevedo J, Peñas B, López-Cerón M, Ricart E, Cuatrecasas M, Jimeno M, Pellisé M; EndoCAR group of the Spanish Gastroenterological Association and Spanish Digestive Endoscopy Society. Real-life chromoendoscopy for neoplasia detection and characterisation in long-standing IBD. *Gut* 2018; **67**: 70-78 [PMID: 27612488 DOI: 10.1136/gutjnl-2016-312332]

18 **Wu L**, Li P, Wu J, Cao Y, Gao F. The diagnostic accuracy of chromoendoscopy for dysplasia in ulcerative colitis: meta-analysis of six randomized controlled trials. *Colorectal Dis* 2012; **14**: 416-420 [PMID: 21073646 DOI: 10.1111/j.1463-1318.2010.02505.x]

19 **Leedham SJ**, Graham TA, Oukrif D, McDonald SA, Rodriguez-Justo M, Harrison RF, Shepherd NA, Novelli MR, Jankowski JA, Wright NA. Clonality, founder mutations, and field cancerization in human ulcerative colitis-associated neoplasia. *Gastroenterology* 2009; **136**: 542-550.e6 [PMID: 19103203 DOI: 10.1053/j.gastro.2008.10.086]

20 **Marion JF**, Waye JD, Present DH, Israel Y, Bodian C, Harpaz N, Chapman M, Itzkowitz S, Steinlauf AF, Abreu MT, Ullman TA, Aisenberg J, Mayer L; Chromoendoscopy Study Group at Mount Sinai School of Medicine. Chromoendoscopy-targeted biopsies are superior to standard colonoscopic surveillance for detecting dysplasia in inflammatory bowel disease patients: a prospective endoscopic trial. *Am J Gastroenterol* 2008; **103**: 2342-2349 [PMID: 18844620 DOI: 10.1111/j.1572-0241.2008.01934.x]

21 **Moussata D**, Allez M, Cazals-Hatem D, Treton X, Laharie D, Reimund JM, Bertheau P, Bourreille A, Lavergne-Slove A, Brixi H, Branche J, Gornet JM, Stefanescu C, Moreau J, Marteau P, Pelletier AL, Carbonnel F, Seksik P, Simon M, Fléjou JF, Colombel JF, Charlois AL, Roblin X, Nancey S, Bouhnik Y, Berger F, Flourié B; the GETAID. Are random biopsies still useful for the detection of neoplasia in patients with IBD undergoing surveillance colonoscopy with chromoendoscopy? *Gut* 2018; **67**: 616-624 [PMID: 28115492 DOI: 10.1136/gutjnl-2016-311892]

22 **Watanabe T**, Ajioka Y, Mitsuyama K, Watanabe K, Hanai H, Nakase H, Kunisaki R, Matsuda K, Iwakiri R, Hida N, Tanaka S, Takeuchi Y, Ohtsuka K, Murakami K, Kobayashi K, Iwao Y, Nagahori M, Iizuka B, Hata K, Igarashi M, Hirata I, Kudo SE, Matsumoto T, Ueno F, Watanabe G, Ikegami M, Ito Y, Oba K, Inoue E, Tomotsugu N, Takebayashi T, Sugihara K, Suzuki Y, Watanabe M, Hibi T. Comparison of Targeted vs Random Biopsies for Surveillance of Ulcerative Colitis-Associated Colorectal Cancer. *Gastroenterology* 2016; **151**: 1122-1130 [PMID: 27523980 DOI: 10.1053/j.gastro.2016.08.002]

23 **Vienne A**, Simon T, Cosnes J, Baudry C, Bouhnik Y, Soulé JC, Chaussade S, Marteau P, Jian R, Delchier JC, Coffin B, Admane H, Carrat F, Drouet E, Beaugerie L. Low prevalence of colonoscopic surveillance of inflammatory bowel disease patients with longstanding extensive colitis: a clinical practice survey nested in the CESAME cohort. *Aliment Pharmacol Ther* 2011; **34**: 188-195 [PMID: 21615760 DOI: 10.1111/j.1365-2036.2011.04711]

P- Reviewer: Hiraoka S, Luo H, Tandon RK, Tang ZP
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4077-4085

DOI: 10.3748/wjg.v24.i35.4077

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

ORIGINAL ARTICLE

Randomized Clinical Trial

Usefulness of the clip-flap method of endoscopic submucosal dissection: A randomized controlled trial

Hiromitsu Ban, Mitsushige Sugimoto, Taketo Otsuka, Masaki Murata, Toshiro Nakata, Hiroshi Hasegawa, Osamu Inatomi, Shigeki Bamba, Akira Andoh

Hiromitsu Ban, Mitsushige Sugimoto, Taketo Otsuka, Division of Digestive Endoscopy, Shiga University of Medical Science Hospital, Shiga 520-2192, Japan

Masaki Murata, Toshiro Nakata, Hiroshi Hasegawa, Osamu Inatomi, Akira Andoh, Department of Gastroenterology, Shiga University of Medical Science Hospital, Shiga 520-2192, Japan

Shigeki Bamba, Division of Clinical Nutrition, Shiga University of Medical Science Hospital, Shiga 520-2192, Japan

ORCID number: Hiromitsu Ban (0000-0002-5782-9210); Mitsushige Sugimoto (0000-0002-9184-7392); Taketo Otsuka (0000-0001-5023-9771); Masaki Murata (0000-0002-4951-0584); Toshiro Nakata (0000-0001-6644-3347); Hiroshi Hasegawa (0000-0001-9590-5408); Osamu Inatomi (0000-0002-5837-6575); Shigeki Bamba (0000-0002-4108-5894); Akira Andoh (0000-0001-8533-2669).

Author contributions: Ban H, Sugimoto M, Otsuka T, Murata M, Nakata T, Hasegawa H, Inatomi O, Bamba S and Andoh A designed this study; Ban H was the clinical investigator with more patients recruited and treated; Hasegawa H, Inatomi O, Bamba S and Andoh A, took part in trial coordination and monitoring; Ban H, Sugimoto M, Otsuka T, Murata M, Nakata T, Hasegawa H collected the data and their management; Ban H, Sugimoto M contributed to the statistical analysis; Ban H, Sugimoto M analyzed and interpreted the data; Ban H, Sugimoto M drafted the manuscript and made the final approval of the version to the published.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Shiga University of Medical Science.

Clinical trial registration statement: This study is registered on Universal hospital Medical Information Network (UMIN000018199).

Informed consent statement: All study participants provided written informed consent prior to study enrolment.

Conflict-of-interest statement: None of the authors have any conflicts of interest related to this study.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The CONSORT 2010 Statement have been adopted.

Manuscript source: Invited manuscript

Correspondence to: Mitsushige Sugimoto, MD, PhD, Associate Professor, Division of Digestive Endoscopy, Shiga University of Medical Science Hospital, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan. sugimo@belle.shiga-med.ac.jp
Telephone: +81-77-5482618
Fax: +81-77-5482618

Received: June 21, 2018
Peer-review started: June 21, 2018
First decision: July 31, 2018
Revised: August 5, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 21, 2018

Abstract

AIM

To prospectively investigate the efficacy and safety of clip-flap assisted endoscopic submucosal dissection (ESD) for gastric tumors.

METHODS

From May 2015 to October 2016, we enrolled 104 patients with gastric cancer or adenoma scheduled for ESD at Shiga University of Medical Science Hospital. We randomized patients into two subgroups using the minimization method based on location of the tumor (upper, middle or lower third of the stomach), tumor size (< 20 mm or > 20 mm) and ulcer status: ESD using an endoclip (the clip-flap group) and ESD without an endoclip (the conventional group). Therapeutic efficacy (procedure time) and safety (complication: Gastrointestinal bleeding and perforation) were assessed.

RESULTS

En bloc resection was performed in all patients. Four patients had delayed bleeding (3.8%) and two had perforation (1.9%). No significant differences in *en bloc* resection rate (conventional group: 100%, clip flap group: 100%), curative endoscopic resection rate (conventional group: 90.9%, clip flap group: 89.8%, P = 0.85), procedure time (conventional group: 70.8 ± 46.2 min, clip flap group: 74.7 ± 53.3 min, P = 0.69), area of resected specimen (conventional group: 884.6 ± 792.1 mm^2, clip flap group: 1006.4 ± 1004.8 mm^2, P = 0.49), delayed bleeding rate (conventional group: 5.5%, clip flap group: 2.0%, P = 0.49), or perforation rate (conventional group: 1.8%, clip flap group: 2.0%, P = 0.93) were found between the two groups. Less-experienced endoscopists did not show any differences in procedure time between the two groups.

CONCLUSION

For patients with early-stage gastric tumors, the clip-flap method has no advantage in efficacy or safety compared with the conventional method.

Key words: Gastric cancer; Clip flap method; Endoscopic submucosal dissection; Procedure time; Complication; Randomized clinical trial

Core tip: We conducted a prospective study to investigate efficacy of the clip-flap method of endoscopic submucosal dissection (ESD) for early-stage gastric tumor. Recently, although the efficacy of the clip-flap method for ESD of large colorectal tumors is shown, we failed to show advantage of clip-flap method in efficacy or safety compared with the conventional method. Efficacy of clip-flap method-assisted ESD for gastric tumors may be limited, especially in cases with large size of tumor and with difficulty to make mucosal flap.

Ban H, Sugimoto M, Otsuka T, Murata M, Nakata T, Hasegawa H, Inatomi O, Bamba S, Andoh A. Usefulness of the clip-flap method of endoscopic submucosal dissection: A randomized controlled trial. *World J Gastroenterol* 2018; 24(35): 4077-4085 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4077.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4077

INTRODUCTION

Endoscopic submucosal dissection (ESD) is a procedure that enables *en bloc* resection of gastric neoplastic lesions that are difficult to resect *via* conventional endoscopic mucosal resection (EMR)[1,2]. ESD is first-line treatment for early-stage gastrointestinal cancer[3-5]. Treatment of relatively large lesions and lesions with peptic ulcers, ulcer scars, or fibrosis increases operating time, and increases the risk of adverse events such as perforation and bleeding from the artificial ulcer produced[6-10]. Poor visualization in the resection area also results in longer procedure times and their associated adverse events. Poor visualization may be associated with lesion size, histological type, location, ulcer status, condition of the gastric mucosa, and the degree of operator experience[11,12]. Although precise visualization is important to perform ESD safely, the gold standardized method for resection by ESD for all of patients with early-stage gastrointestinal cancer has not been established.

To create a mucosal flap at the early phase after starting ESD procedure is important to prevent complications[13]. The efficacy and safety of several traction systems, such as sinker assistance[14], magnetic anchor guidance[15], use of a clip with a line[16], use of a spring-action clip[17], the clip-band technique[18], and the double-channel scope method[19]. These traction methods are complicated to perform safety and correctly. Recently, Yamamoto *et al*[20-22] reported on the efficacy of the clip-flap method, in which an endoclip is used to substitute for the mucosal flap until it is formed, for ESD of large colorectal tumors. This method is simple and effective in most cases with colorectal tumors, even in the presence of submucosal fibrosis or with a vertical approach. However, it is unknown whether the clip-flap method is appropriate for patients with early-stage gastric tumors. Because the clip-flap techniques differ between ESD of colorectal tumors and gastric tumors, we wished to assess the efficacy of the clip-flap method for gastric tumor ESD.

MATERIALS AND METHODS

We prospectively compared the efficacy (*i.e.*, procedure time) and safety (*i.e.*, incidence of complications) of the clip-flap method in ESD of tumors in different locations (upper, middle or lower third of the stomach), sizes (< 20 mm or > 20 mm), ulcer status (positive or negative), Kyoto classification of gastric mucosa, *Helicobacter pylori* (*H. pylori*) infection status, and operator experience.

Patients

We enrolled 104 patients who were scheduled to undergo ESD for gastric cancers or gastric adenomas at Shiga University of Medical Science Hospital from May 2015 to October 2016 (Figure 1). Inclusion criteria were age > 20 years and the diagnosis of gastric adenoma or clinical early-stage gastric cancer, irrespective of *H. pylori* infection. Early-stage gastric cancers were clinically

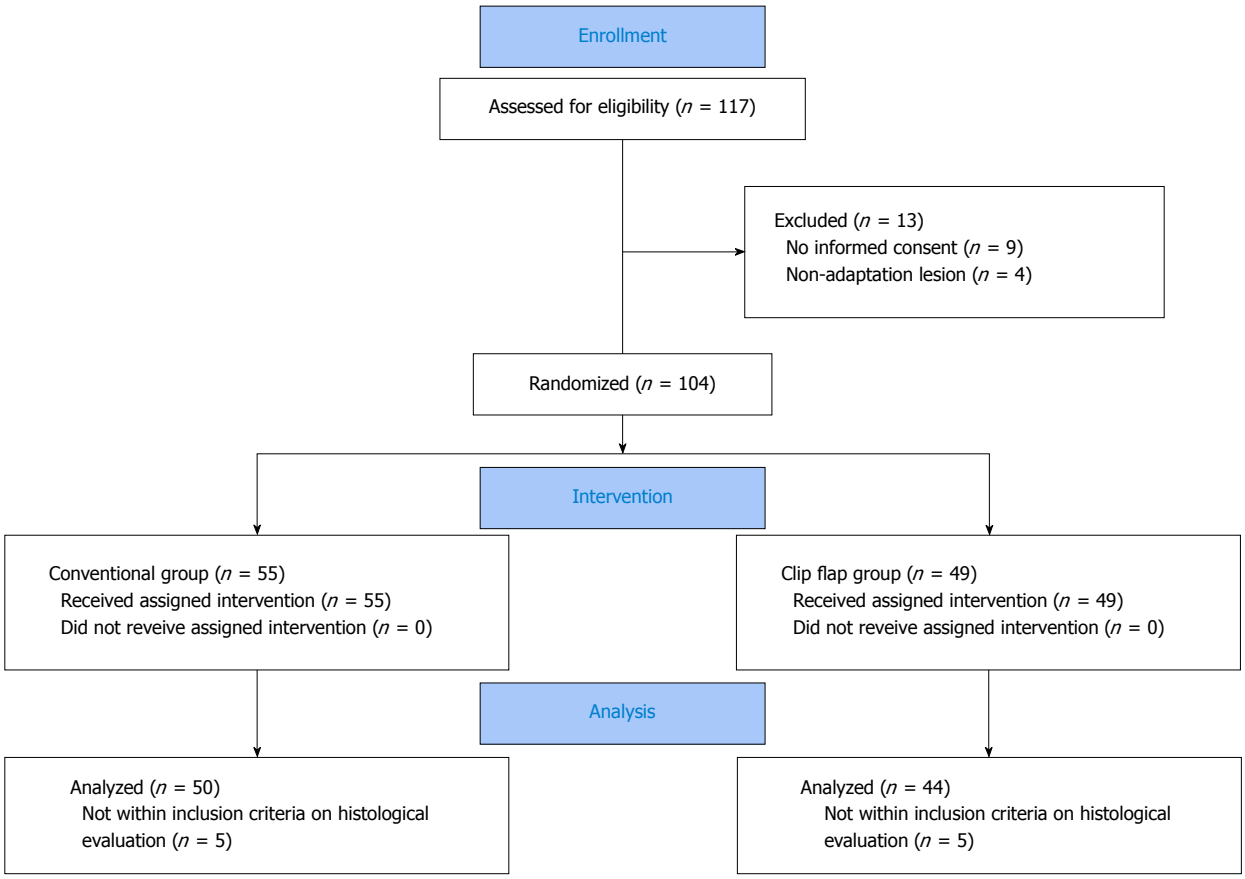


Figure 1 Flow diagram of this study. We enrolled 117 patients who were scheduled to undergo ESD for gastric tumors from May 2015 to October 2016. A total of 104 patients were randomized to the conventional and the clip-flap groups. After ESD, ten patients had a lesion outside the inclusion criteria of early-stage gastric cancer. ESD: Endoscopic submucosal dissection.

diagnosed using endoscopy, endoscopic ultrasonography, histopathology, and computed tomography. The criteria of early-stage gastric cancer were: (1) An intramucosal intestinal-type cancer without ulcerative lesion, regardless of tumor size; (2) intramucosal intestinal-type cancer with ulcerative lesion, ⩽ 3 cm in size; and (3) intramucosal diffuse-type cancer ⩽ 2 cm in size without ulcerative lesion. Exclusion criteria were advanced-stage gastric cancer and lack of informed consent. Written informed consent was obtained from all other patients, and approval for the study protocol was given in advance by the Institutional Review Board of the Shiga University of Medicine Science (Number: 26-207). This trial was registered in the University Hospital Medical Information Network, UMIN 000018199.

Study design

This study was a prospective randomized trial to assess the efficacy of clip-flap-assisted ESD with regard to operation time and incidence rates of ESD-induced complications in relation to endoscopist experience, characteristics of gastric tumor (*i.e.*, size, differentiation and location), and Kyoto classification of gastric mucosa. Using the minimization method based on location of the tumor (upper, middle or lower third of the stomach), tumor size (< 20 mm or > 20 mm) and ulcer status (positive or negative), we randomized patients with early-stage gastric tumor into two groups: ESD using an endoclip (EZCLIP, HX-610-135; Olympus, Tokyo, Japan) to make the mucosal flap (clip-flap group) (*n* = 49) and ESD without an endoclip (conventional group) (*n* = 55) (Figure 1). Procedure time was calculated as the time from the beginning of submucosal injection to the end of submucosal dissection. We performed ESD for patients receiving anti-thrombotic drugs according to the guideline for endoscopic procedures in antithrombotic drug-users from Japan Gastroenterological Endoscopy Society on July 2012.

Curative endoscopic resection rate was decided as lesion within criteria of early-stage gastric cancer. Delayed bleeding was defined as postprocedural bleeding with hematemesis or melena requiring endoscopic hemostasis, decrease in the hemoglobin level by > 2 g/dL.

Primary endpoint of this study was to clarify the reduction effects of procedure time in the clip-flap-assisted ESD for early-stage gastric cancers compared with the conventional ESD. Secondary endpoint were to compare with incident rates of ESD-associated complications, such as bleeding and perforation, between two kinds of treatment methods, and to clarify efficacy of the clip-flap-assisted ESD for *en bloc* resection rate of gastric neoplastic lesions.

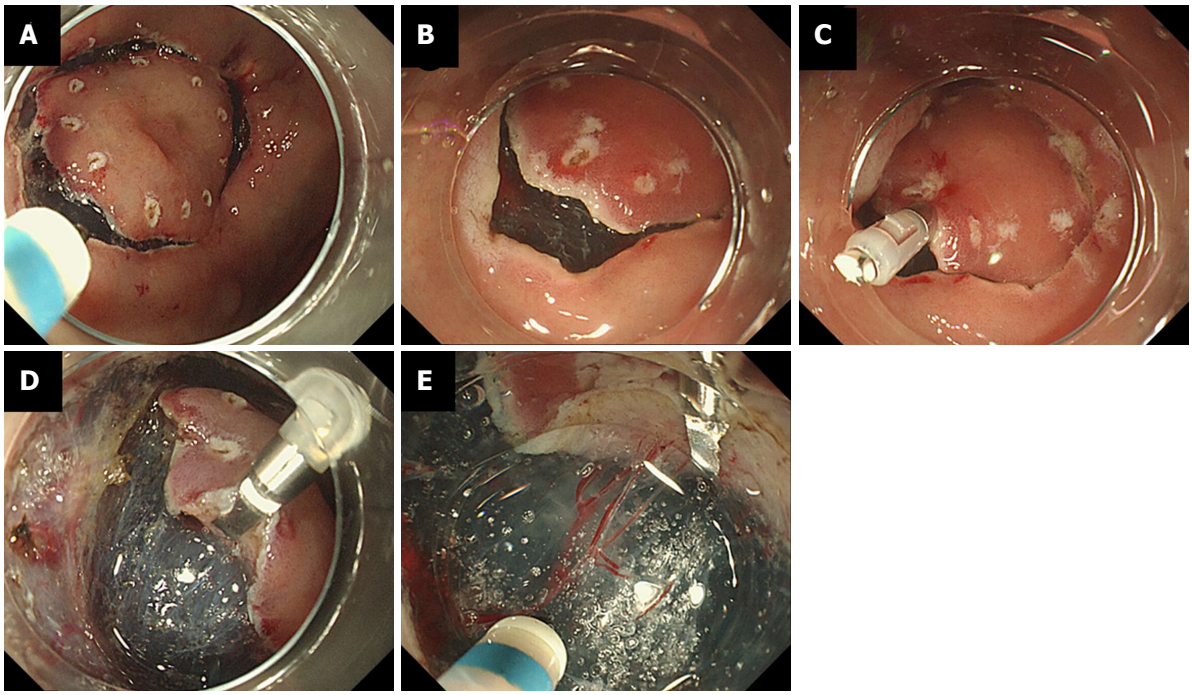


Figure 2 Clip-flap methods. A: The mucosal circumference incision of gastric tumor is performed in the conventional manner; B: A deeper cut is made at the point attached the endoclip; C: The endoclip is attached to the exfoliated mucosa. The head of the endoclip falls slightly toward the gastric lumen, allowing the attachment to be easily inserted under the endoclip; D: The attachment is inserted under the endoclip, and then the mucosa and submucosal layer are elevated by the endoclip; E: The gastric tumor is dissected with the endoknife under direct vision.

After pathological evaluation of ESD sample, we excluded patients with gastric tumor penetrating > 500 μm from the muscularis mucosa into the submucosa.

ESD procedure

ESD was carried out with a single channel endoscope (GIF-H290Z; Olympus, Tokyo, Japan). We used a dual knife (KD-650; Olympus, Tokyo, Japan) as the cutting device, and an electrical current was applied using an electrosurgical generator (VIO300D; ERBE Elektromedizin GmbH, Tubingen, Germany). Visible vessels were heat-coagulated using hemostatic forceps (Coagrasper G; FD-412LR, Olympus, Tokyo, Japan).

In the clip-flap group, after the mucosal circumference of the tumor was incised in the conventional manner, an edge of the exfoliated mucosa was grasped with an endoclip (Figure 2). The endoscope attachment was slipped under the endoclip, and the submucosal layer was then dissected with the endoknife. After creating the mucosal flap, ESD was performed in the conventional manner. We divided endoscopists into two groups; experts (higher that 50 procedure experiences, n = 2) and beginner (less than 50 procedure experiences, n = 3).

H. pylori infection

H. pylori infection status was determined with an anti-*H. pylori* IgG serological test (E plate Eiken *H. pylori* antibody®; Eiken Chemical Co. Ltd., Tochigi, Japan).

Evaluation of endoscopic gastric mucosa

The endoscopic severity of *H. pylori*-associated gastritis was characterized by the Kyoto classification[23,24]. According to the Kyoto classification of gastritis, patients are scored according to atrophy (none: A0, atrophic patterns with a margin between the non-atrophic fundic mucosa and atrophic mucosa located in the lesser curvature of the stomach: A1, and atrophic patterns whose margin does not cross the lesser curvature: A2), intestinal metaplasia (none: IM0, within antrum: IM1, and up to corpus: IM2), hypertrophy of gastric folds (negative: H0, positive: H1), and diffuse erythema (negative: DR0, mild: DR1, severe: DR2)[23,24].

Statistical analysis

Age, body weight, body mass index, and ESD procedure time are expressed as mean ± standard deviation (SD). Statistically significant differences in these parameters between the clip-flap group and the conventional group were determined using one-way ANOVA and Fisher's exact tests. All P values were two-sided, and $P < 0.05$ was considered statistically significant. Calculations were conducted using commercial software (SPSS version 20, IBM Inc; Armonk NY, United States).

The sample size and the study power were calculated by using our previous data of procedure time of conventional ESD method for gastric tumor (80 min). We chose an unmatched case-control study design (assuming 1 conventional ESD per clip-flap-assisted ESD) and hypothesized that clip-flap-assisted ESD reduced 25% of procedure time compared with conventional ESD method. For the desired power of our study of 80% with a significance level of 0.05 in a two-sided test, at

Table 1 Characteristics of patients scheduled to undergo endoscopic submucosal dissection between those with or without endoclip

	Total	Conventional group	Clip-flap group	*P* value
Number (*n*)	104	55	49	
Age (yr)	70.1 ± 8.3	69.0 ± 9.5	71.2 ± 6.5	0.17
Sex (male : female)	80:24	42:13	38:11	0.89
Body weight (kg)	59.0 ± 11.2	58.0 ± 11.0	60.2 ± 11.5	0.34
BMI (kg/m^2)	22.2 ± 3.1	21.7 ± 2.9	22.8 ± 3.3	0.08
Drugs, *n* (%)				
Anticoagulants	19 (18.3)	11(20.0)	8 (16.3)	0.63
Antihypertensive dugs	56 (53.8)	25 (45.5)	31 (63.3)	0.07
Oral hypoglycemics	16 (15.4)	8 (14.5)	8 (16.3)	0.80
Cholesterol-lowering agents	25 (24.0)	13 (23.6)	12 (24.5)	0.91
H. pylori infection (positive)	36 (34.6)	16 (29.1)	20 (40.8)	0.21
Hemodialysis, *n* (%)	0 (0)	0 (0)	0 (0)	-
Kyoto classification of gastric mucosa				
Atrophy (A0:A1:A2)	3:7:94	2:4:49	1:3:45	0.86
Intestinal metaplasia (IM0:IM1:IM2)	8:36:60	5:15:35	3:21:25	0.24
Diffuse redness (DR0:DR1 :DR2)	47:24:33	26:13:16	21:11:17	0.83
Tumor				
Histological diagnosis (adenoma/cancer)	9/95	6/49	3/46	0.39
Differentiation (tub1 + tub2/por + sig)	92/3	48/1	44/2	0.52
Depth (m/sm)	82/13	42/7	40/6	0.86
Location (upper third/middle/lower)	10/37/57	7/20/28	3/17/29	0.47
Major axis of tumor (mm)	18.5 ± 13.4	17.5 ± 11.8	19.7 ± 15.1	0.39
ESD				
Procedure time (min)	72.6 ± 49.5	70.8 ± 46.2	74.7 ± 53.3	0.69
Area of resected specimen (mm^2)	962 1 ± 896.2	884.6 ± 792.1	1006.4 ± 1004.8	0.49
En bloc resection rate (*n*, %)	104 (100)	55 (100)	49 (100)	-
Curative endoscopic resection rate (*n*, %)	94 (90.4)	50 (90.9)	44 (89.8)	0.85
Coagulation of vessels at 2nd look (*n*, %)	28 (26.9)	16 (29.6)	12 (25.0)	0.60
Delayed bleeding (*n*, %)	4 (3.8)	3 (5.5)	1 (2.0)	0.37
Perforation (*n*, %)	2 (1.9)	1 (1.8)	1 (2.0)	0.93
Operator				
Procedure times (< 50 cases)	83	43	40	0.66

A0: No atrophic pattern; A1: Atrophic patterns with a margin between the non-atrophic fundic mucosa and atrophic mucosa located in the lesser curvature of the stomach; A2: Atrophic patterns; whose margin does not cross the lesser curvature; BMI: Body mass index; DR0: No diffuse erythema; DR1: Mild diffuse erythema; DR2: Severe diffuse erythema; IM0: No intestinal metaplasia; IM1: Intestinal metaplasia within antrum; IM2: Intestinal metaplasia up to corpus; lower: Lower third of the stomach; m: mucosal layer; middle: Middle third of the stomach; por: Poorly differentiated adenocarcinoma; sig: Signet ring cell carcinoma; sm: Submucosal layer; tub1: Well differentiated adenocarcinoma; tub2: Moderately differentiated adenocarcinoma; upper: Upper third of the stomach; ESD: Endoscopic submucosal dissection; *H. pylori*: *Helicobacter pylori*.

least 100 patients by conventional ESD method and 100 patients by clip-flap-assisted ESD were required. At first, we decided to conduct an intermediate analysis when total patients for gastric tumor reached 100, half of required total patients. If there was no significant difference of efficacy between both regimens at an intermediate analysis, we decided to stop the examination.

RESULTS

At an intermediate analysis after enrolling half of required patients, because the reduction effect of procedure time as primary endpoint of this study was similar between two kinds of methods, we decided to stop the examination as initial protocol.

Characteristics of patients with gastric tumors

Of 117 patients undergoing ESD from May 2015 to October 2016, 104 patients were randomized into two groups: The conventional group (*n* = 55) and the clip-flap group (*n* = 49) (Figure 1). Thirteen patients were excluded due to the withholding of informed consent (*n* = 9) and non-adaptation lesion (*n* = 4). There were no significant differences in demographic characteristics (age, sex, body weight, BMI, received drugs, *H. pylori* infection rate), Kyoto classification, or clinical characteristics of gastric neoplasms (*i.e.*, histological diagnosis, depth, location and size) between the conventional group and the clip-flap group (Table 1).

ESD procedure

All patients underwent *en bloc* resection. The curative endoscopic resection rate within the criteria of early-stage gastric cancer was 90.4% (94/104). In histopathological evaluation after ESD, lesions of nine patients did not meet the inclusion criteria of clinical early-stage gastric cancer. Five patients had tumor > 500 μm from the muscularis mucosa, 2 had tumors > 3 cm in size with submucosal layer invasion, 2 had diffuse-type adenocarcinoma > 2 cm in size, and 1 had a tumor > 3 cm in size with ulceration (Table 1).

The mean area of resected specimens was 962.1 ± 896.2 mm^2 and mean procedure time was 72.6 min ± 49.5 min. ESD-related adverse events included delayed bleeding in 4 patients (3.8%) and perforation in 2 (1.9%)

Table 2 Efficacy by procedure time of endoscopic submucosal dissection for gastric tumors within inclusion criteria

		Conventional group (n = 50)	Clip-flap group (n = 44)	P value
Procedure time (min)		67.3 ± 44.9	67.6 ± 48.4	0.98
H. pylori infection	Positive	64.1 ± 27.8 (n = 15)	65.6 ± 53.1 (n = 19)	0.92
	Negative	68.6 ± 50.7 (n = 35)	69.1 ± 45.5 (n = 25)	0.98
Kyoto classification of gastric mucosa				
Atrophy	A2	68.7 ± 45.2 (n = 44)	70.3 ± 49.7 (n = 40)	0.87
	A0 + 1	57.2 ± 44.8 (n = 6)	40.3 ± 17.6 (n = 4)	0.50
Intestinal metaplasia	IM2	71.6 ± 45.8 (n = 31)	65.2 ± 45.2 (n = 22)	0.62
	IM0 + IM1	60.2 ± 43.6 (n = 19)	70.0 ± 52.3 (n = 22)	0.53
Tumor				
Histological diagnosis	Adenoma	36.5 ± 11.1 (n = 6)	58.0 ± 40.2 (n = 3)	0.24
	Cancer	71.5 ± 46.1 (n = 44)	68.3 ± 49.3 (n = 41)	0.76
Differentiation	tub1 + tub2	71.1 ± 46.6 (n = 43)	69.0 ± 49.7 (n = 40)	0.85
	Por + sig	90 (n = 1)	40 (n = 1)	-
Depth	m	71.4 ± 47.2 (n = 42)	62.7 ± 40.1 (n = 38)	0.38
	sm	74.0 ± 1.4 (n = 2)	139.6 ± 97.1 (n = 3)	0.43
Location	Upper	119.8 ± 60.9 (n=6)	158.5 ± 101.1 (n=2)	0.52
	Middle	74.8 ± 37.3 (n = 19)	91.4 ± 52.1 (n = 14)	0.29
	Lower	49.0 ± 34.7 (n = 25)	49.2 ± 28.0 (n = 28)	0.98
Major axis of tumor (mm)	< 10	50.0 ± 35.9 (n = 24)	46.6 ± 31.0 (n = 20)	0.74
	10 <, < 20	72.3 ± 46.2 (n = 16)	59.8 ± 23.7 (n = 14)	0.37
	20 <	100.8 ± 44.9 (n = 10)	120.5 ± 64.5 (n = 10)	0.44
ESD				
Area of specimen (mm^2)	< 500	33.8 ± 14.0 (n = 22)	53.2 ± 51.7 (n = 15)	0.17
	500 <, < 1000	72.6 ± 38.4 (n = 16)	71.1 ± 29.3 (n = 23)	0.89
	1000 <	86.0 ± 32.1 (n = 12)	138.5 ± 79.3 (n = 9)	0.06
Coagulation of vessels at 2^{nd} look	Done	69.5 ± 38.4 (n = 13)	67.4 ± 28.8 (n = 12)	0.89
	No	66.5 ± 47.4 (n = 37)	67.6 ± 52.6 (n = 35)	0.92
Delayed bleeding (n, %)		105.7 ± 23.7 (n = 3)	80 (n = 1)	-
Perforation (n, %)		71 (n = 1)	234 (n = 1)	-
Operator				
Procedure times	< 50 times	61.1 ± 37.1 (n = 39)	58.8 ± 32.1 (n = 36)	0.78
	> 50 times	89.3 ± 62.9 (n = 11)	107.0 ± 83.9 (n = 8)	0.60

A0: No atrophic pattern; A1: Atrophic patterns with a margin between the non-atrophic fundic mucosa and atrophic mucosa located in the lesser curvature of the stomach; A2: Atrophic patterns; whose margin does not cross the lesser curvature; BMI: Body mass index; DR0: No diffuse erythema; DR1: Mild diffuse erythema; DR2: Severe diffuse erythema; IM0: No intestinal metaplasia; IM1: Intestinal metaplasia within antrum; IM2: Intestinal metaplasia up to corpus; lower: Lower third of the stomach; m: mucosal layer; middle: Middle third of the stomach; por: Poorly differentiated adenocarcinoma; sig: Signet ring cell carcinoma; sm: Submucosal layer; tub1: Well differentiated adenocarcinoma; tub2: Moderately differentiated adenocarcinoma; upper: Upper third of the stomach; ESD: Endoscopic submucosal dissection; *H. pylori*: *Helicobacter pylori*.

(Table 1). There were no significant differences in Kyoto classification, background of tumor, *en bloc* resection rate, curative endoscopic resection rate, procedure time, area of resected specimens, or complication rate between the two groups.

In analysis of efficacy for treatment methods in patients within inclusion criteria, all parameters, such as *H. pylori* infection, background of gastric mucosa, characteristics of tumor, and ESD-related factors, procedure time of ESD were similar between two groups (Table 2). In addition, the clip-flap method had no effect on procedure time, regardless of operator experience.

As shown in previous reports, there were significance in the difference of procedure time between tumor size (< 20 mm or > 20 mm) and location of tumor (upper and middle or lower), whereas the clip-flap method was selected or not.

Risk factors for prolonged procedure time in conventional and clip-flap assisted ESD

Factors associated with prolonged procedure time were the same in both groups (Table 3).

DISCUSSION

We wished to clarify the efficacy and safety of the clip-flap assisted ESD for patients with early-stage gastric tumors, looking at the effect of operator experience as well as other factors. We however failed to show any benefits of the clip-flap method (*en bloc* resection, procedure time and complication). Although this method is efficacious for patients with colorectal tumors, our observations suggest that it does not improve rate of *en bloc* resection, procedure time of ESD and safety (bleeding and perforation) in patients with gastric tumors.

Clip-flap method-assisted ESD

To prevent ESD-associated complications, such as perforation and unexpected bleeding, it is crucial to ensure good visualization of the submucosal layer by creating a mucosal flap[13-19]. When the clip-flap method was selected as treatment for patients with

Table 3 Univariate analysis of parameters for delayed procedure time of endoscopic submucosal dissection

	Parameters	Conventional group			Clip-flap group		
		Odds ratio	95%CI	*P* value	Odds ratio	95%CI	*P* value
Age (yr)	> 70	0.67	0.22-2.10	0.49	0.79	0.22-2.86	0.72
Sex	Male	2.70	0.63-11.55	0.18	-	-	-
H. pylori infection	Positive	1.31	0.39-4.44	0.66	0.69	0.20-2.43	0.57
Kyoto classification of gastric mucosa							
Atrophy (*vs* A0 + A1)	A2	1.52	0.25-9.19.57	0.65	-	-	-
Intestinal metaplasia (*vs* IM0 + IM1)	IM2	2.03	0.61-6.72	0.25	1.00	0.29-3.42	1.00
Tumor							
Depth (*vs* m)	sm	-	-	-	3.85	0.31-46.49	0.29
Location (*vs* Lower)	Upper/middle	10.12	2.42-42.41	0.02	4.75	1.41-16.05	< 0.01
Major axis of tumor (mm) (*vs* < 10)	10 <, < 20	3.00	0.78-11.54	0.11	3.15	0.61-16.29	0.17
	20 <	7.00	1.36-36.01	0.02	22.67	3.14-163.63	< 0.01
Ulceration	UL+	1.42	0.18-10.99	0.74	1.80	0.104-30.89	0.69
ESD							
Area of specimen (mm^2) (*vs* < 500)	500 <	3.29	0.28-39.14	0.35	-	-	-
Operator							
Procedure experience	< 50 times	0.32	0.08-1.29	0.11	0.26	0.05-1.30	0.10

A0: No atrophic pattern; A1: Atrophic patterns with a margin between the non-atrophic fundic mucosa and atrophic mucosa located in the lesser curvature of the stomach; A2: Atrophic patterns; whose margin does not cross the lesser curvature; BMI: Body mass index; DR0: No diffuse erythema; DR1: Mild diffuse erythema; DR2: Severe diffuse erythema; IM0: No intestinal metaplasia; IM1: Intestinal metaplasia within antrum; IM2: Intestinal metaplasia up to corpus; lower: Lower third of the stomach; m: mucosal layer; middle: Middle third of the stomach; por: Poorly differentiated adenocarcinoma; sig: Signet ring cell carcinoma; sm: Submucosal layer; tub1: Well differentiated adenocarcinoma; tub2: Moderately differentiated adenocarcinoma; upper: Upper third of the stomach; ESD: Endoscopic submucosal dissection; *H. pylori*: *Helicobacter pylori*.

early-stage gastrointestinal tumors, it was important that the endoclip securely clipped the edge of the exfoliated mucosa on the oral or anal side of the tumor, excluding the deep layer of the submucosa, after the mucosal circumference incision (Figure 2)[20-22]. Then the endoscope attachment is slipped under the endoclip. If this technique is performed, the endoclip attached to the exfoliated mucosa acts like a surgical hook to widen the cutting area when lifted with the endoscope attachment, with resulting good visualization of the cutting area and countertraction against the submucosal layer until the mucosal flap is created[20-22]. In this study, there was no benefit of the clip-flap method for gastric tumors, irrespective of different background of gastric mucosa (severity of gastric atrophic atrophy and inflammation), histological diagnosis, depth, location, or size. There were no significant differences in the results between the conventional group and the clip-flap group between operators of different experience. Importantly, although operators scored superiority of the clip-flap method in 30% of ESD procedures in the visual analogue scale, in 25% of cases the operators evaluated the clip-flap method as inferior, usually because the head of the endoclip interfered with the cutting edge, making ESD more difficult. In our experience, the best way to do this method is to property place the clip on the lesion. Yamamoto *et al*[20-22] recommended the use of one endoclip for each tumor, adding additional endoclips (*i.e.*, a single endoclip at a different point or a cross pattern of endoclips at same point) as needed. The endoclip cross pattern further stabilized the visual field by providing good countertraction when the attachment was slipped under the endoclip. In this study, however, we used one endoclip according to first protocol. The characteristics of tumors and required techniques of ESD differ between colorectal and gastric tumors. Although fine visualization is required to perform ESD, because ESD for patients with gastric tumors is easy compared with that for colorectal tumors, any merit of the clip-flap method for gastric tumors may be minor. When considering clip-flap-assisted ESD, endoscopists should select patients, especially cases in which it is difficult to ensure a fine visualization, and should apply the endoclip carefully so that the head of the endoclip pushes downward.

Limitation

There were any limitations in this study, as below. First, this study is single-center study and non double-blinded study. Second, sample power is insufficient. Although 200 patients with gastric tumors were required for the desired power of 80% with a significance level of 0.05 in a two-sided test, because there was no significant difference of efficacy between both regimens at an intermediate analysis, we decided to stop the examination according to initial protocol. Because the power of conclusions are insufficient, we think that it will be required to plan further study that investigate efficacy of the clip-flap assisted ESD by multi-center study enrolled many patients.

Conclusions

We conducted a prospective randomized study to investigate the efficacy and safety of the clip-flap method-assisted ESD for patients with early-stage gastric tumors. We demonstrated similar resection times between the conventional group and the clip-flap group and failed to show any benefits of the clip-flap method for patients with early-stage gastric tumors. However, the clip-flap

method prevented poor visualization in the cutting area and also saved procedure time. Although, compared with the conventional method without traction, the clip-flap method has been proven to be advantageous for patients with early-stage colorectal tumors, the superiority of clip-flap method-assisted ESD in the stomach is unproven.

ARTICLE HIGHLIGHTS

Research background

The endoscopic submucosal dissection (ESD) for early-stage gastric cancer is first-line endoscopic therapy in Japan, because of *en bloc* resection and a lower local recurrence rate of gastric cancer. However, ESD often causes development of adverse events, such as gastric bleeding and perforation. When ESD is performed for gastric cancer, poor visualization in the resection area during ESD procedure results in longer procedure times and their associated development of above adverse events. Now, the gold standardized method for resection by ESD for all of patients with early-stage gastric cancer and adenoma has not been established in point of continuous clear visualization in the resection area.

Research motivation

To keep clear visualization at early-phase after starting ESD procedure, endoscopists are required to create a mucosal flap. Of several traction systems to create mucosal flap, recently, the clip-flap method is focused, because of safety and correctly compared with other methods. However, it is unknown whether the clip-flap method is appropriate for patients with early-stage gastric tumors.

Research objectives

The main objective was to investigate prospectively the efficacy (the rate of en bloc resection and procedure time of ESD) and safety (gastric bleeding and perforation) of clip-flap assisted ESD for gastric cancer and adenoma.

Research methods

We enrolled 104 patients with gastric cancer or adenoma scheduled for ESD. Inclusion criteria were age > 20 years and the diagnosis of gastric adenoma or clinical early-stage gastric cancer. Early-stage gastric cancers were clinically diagnosed using endoscopy, endoscopic ultrasonography, histopathology, and computed tomography. We randomized patients into two subgroups using the minimization method based on location of the tumor, tumor size and ulcer status: ESD using an endoclip (the clip-flap group) and ESD without an endoclip (the conventional group). Therapeutic efficacy and safety were assessed.

Research results

No significant differences in *en bloc* resection rate ($P = 1.00$), curative endoscopic resection rate ($P = 0.85$), procedure time ($P = 0.69$), area of resected specimen ($P = 0.49$), delayed bleeding rate ($P = 0.49$), or perforation rate ($P = 0.93$) were found between the clip-flap group and the conventional group.

Research conclusions

For patients with early-stage gastric cancer and adenoma, the clip-flap method has no advantage in efficacy or safety compared with the conventional method. Although operators scored superiority of the clip-flap method in 30% of ESD procedures, in 25% of cases the operators evaluated the clip-flap method as inferior, usually because the head of the endoclip interfered with the cutting edge, making ESD more difficult. Therefore, the best way to do this method is to property place the clip on the lesion.

Research perspectives

Although the clip-flap method has been proven to be advantageous for patients with early-stage colorectal tumors compared with the conventional method, the superiority of clip-flap method-assisted ESD in the stomach is unproven. When considering clip-flap-assisted ESD, endoscopists should select patients, especially cases in which it is difficult to ensure a fine visualization, and should apply the endoclip carefully so that the head of the endoclip pushes downward.

REFERENCES

1 **Fujishiro M**. Endoscopic submucosal dissection for stomach neoplasms. *World J Gastroenterol* 2006; **12**: 5108-5112 [PMID: 16937520 DOI: 10.3748/wjg.v12.i32.5108]

2 **Sugimoto M**, Jang JS, Yoshizawa Y, Osawa S, Sugimoto K, Sato Y, Furuta T. Proton Pump Inhibitor Therapy before and after Endoscopic Submucosal Dissection: A Review. *Diagn Ther Endosc* 2012; **2012**: 791873 [PMID: 22851882 DOI: 10.1155/2012/791873]

3 **Gotoda T**, Yamamoto H, Soetikno RM. Endoscopic submucosal dissection of early gastric cancer. *J Gastroenterol* 2006; **41**: 929-942 [PMID: 17096062 DOI: 10.1007/s00535-006-1954-3]

4 **Ono H**, Yao K, Fujishiro M, Oda I, Nimura S, Yahagi N, Iishi H, Oka M, Ajioka Y, Ichinose M, Matsui T. Guidelines for endoscopic submucosal dissection and endoscopic mucosal resection for early gastric cancer. *Dig Endosc* 2016; **28**: 3-15 [PMID: 26234303 DOI: 10.1111/den.12518]

5 **Hatta W**, Gotoda T, Oyama T, Kawata N, Takahashi A, Yoshifuku Y, Hoteya S, Nakamura K, Hirano M, Esaki M, Matsuda M, Ohnita K, Shimoda R, Yoshida M, Dohi O, Takada J, Tanaka K, Yamada S, Tsuji T, Ito H, Hayashi Y, Nakamura T, Shimosegawa T. Is radical surgery necessary in all patients who do not meet the curative criteria for endoscopic submucosal dissection in early gastric cancer? A multi-center retrospective study in Japan. *J Gastroenterol* 2017; **52**: 175-184 [PMID: 27098174 DOI: 10.1007/s00535-016-1210-4]

6 **Fujishiro M**, Yahagi N, Kakushima N, Kodashima S, Muraki Y, Ono S, Kobayashi K, Hashimoto T, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Successful nonsurgical management of perforation complicating endoscopic submucosal dissection of gastrointestinal epithelial neoplasms. *Endoscopy* 2006; **38**: 1001-1006 [PMID: 17058165 DOI: 10.1055/s-2006-944775]

7 **Tanaka M**, Ono H, Hasuike N, Takizawa K. Endoscopic submucosal dissection of early gastric cancer. *Digestion* 2008; **77** Suppl 1: 23-28 [PMID: 18204258 DOI: 10.1159/000111484]

8 **Cao Y**, Liao C, Tan A, Gao Y, Mo Z, Gao F. Meta-analysis of endoscopic submucosal dissection versus endoscopic mucosal resection for tumors of the gastrointestinal tract. *Endoscopy* 2009; **41**: 751-757 [PMID: 19693750 DOI: 10.1055/s-0029-1215053]

9 **Chung IK**, Lee JH, Lee SH, Kim SJ, Cho JY, Cho WY, Hwangbo Y, Keum BR, Park JJ, Chun HJ, Kim HJ, Kim JJ, Ji SR, Seol SY. Therapeutic outcomes in 1000 cases of endoscopic submucosal dissection for early gastric neoplasms: Korean ESD Study Group multicenter study. *Gastrointest Endosc* 2009; **69**: 1228-1235 [PMID: 19249769 DOI: 10.1016/j.gie.2008.09.027]

10 **Ban H**, Sugimoto M, Otsuka T, Murata M, Nakata T, Hasegawa H, Fukuda M, Inatomi O, Bamba S, Kushima R, Andoh A. Letter: a potassium-competitive acid blocker vs a proton pump inhibitor for healing endoscopic submucosal dissection-induced artificial ulcers after treatment of gastric neoplasms. *Aliment Pharmacol Ther* 2017; **46**: 564-565 [PMID: 28776744 DOI: 10.1111/apt.14202]

11 **Lim JH**, Kim SG, Choi J, Im JP, Kim JS, Jung HC. Risk factors of delayed ulcer healing after gastric endoscopic submucosal dissection. *Surg Endosc* 2015; **29**: 3666-3673 [PMID: 25740642 DOI: 10.1007/s00464-015-4123-z]

12 **Yoshizawa Y**, Sugimoto M, Sato Y, Sahara S, Ichikawa H, Kagami T, Hosoda Y, Kimata M, Tamura S, Kobayashi Y, Osawa S, Sugimoto K, Miyajima H, Furuta T. Factors associated with healing of artificial ulcer after endoscopic submucosal dissection with reference to Helicobacter pylori infection, CYP2C19 genotype, and tumor location: Multicenter randomized trial. *Dig Endosc* 2016; **28**: 162-172 [PMID: 26331711 DOI: 10.1111/den.12544]

13 **Toyonaga T**, Nishino E, Man-I M, East JE, Azuma T. Principles of quality controlled endoscopic submucosal dissection with appropriate dissection level and high quality resected specimen. *Clin Endosc* 2012; **45**: 362-374 [PMID: 23251883 DOI: 10.5946/ce.2012.45.4.362]

14 **Saito Y**, Emura F, Matsuda T, Uraoka T, Nakajima T, Ikematsu

H, Gotoda T, Saito D, Fujii T. A new sinker-assisted endoscopic submucosal dissection for colorectal cancer. *Gastrointest Endosc* 2005; **62**: 297-301 [PMID: 16046999 DOI: 10.1016/S0016-5107(05)00546-8]

15 **Gotoda T**, Oda I, Tamakawa K, Ueda H, Kobayashi T, Kakizoe T. Prospective clinical trial of magnetic-anchor-guided endoscopic submucosal dissection for large early gastric cancer (with videos). *Gastrointest Endosc* 2009; **69**: 10-15 [PMID: 18599053 DOI: 10.1016/j.gie.2008.03.1127]

16 **Oyama T**. Counter traction makes endoscopic submucosal dissection easier. *Clin Endosc* 2012; **45**: 375-378 [PMID: 23251884 DOI: 10.5946/ce.2012.45.4.375]

17 **Sakamoto N**, Osada T, Shibuya T, Beppu K, Matsumoto K, Mori H, Kawabe M, Nagahara A, Otaka M, Ogihara T, Watanabe S. Endoscopic submucosal dissection of large colorectal tumors by using a novel spring-action S-O clip for traction (with video). *Gastrointest Endosc* 2009; **69**: 1370-1374 [PMID: 19403131 DOI: 10.1016/j.gie.2008.12.245]

18 **Parra-Blanco A**, Nicolas D, Arnau MR, Gimeno-Garcia AZ, Rodrigo L, Quintero E. Gastric endoscopic submucosal dissection assisted by a new traction method: the clip-band technique. A feasibility study in a porcine model (with video). *Gastrointest Endosc* 2011; **74**: 1137-1141 [PMID: 22032320 DOI: 10.1016/j.gie.2011.07.037]

19 **Neuhaus H**, Costamagna G, Devière J, Fockens P, Ponchon T, Rösch T; ARCADE Group. Endoscopic submucosal dissection (ESD) of early neoplastic gastric lesions using a new double-channel endoscope (the "R-scope"). *Endoscopy* 2006; **38**: 1016-1023 [PMID: 17058167 DOI: 10.1055/s-2006-944830]

20 **Yamamoto K**, Hayashi S, Nakabori T, Shibuya M, Ichiba M, Inada M. Endoscopic submucosal dissection using endoclips to assist in mucosal flap formation (novel technique: "clip flap method"). *Endoscopy* 2012; **44** Suppl 2 UCTN: E334-E335 [PMID: 23012008 DOI: 10.1055/s-0032-1309860]

21 **Yamamoto K**, Hayashi S, Nishida T, Saiki H, Naito M, Michida T, Ito T. Effective use of the "clip-flap" method for the endoscopic submucosal dissection of a difficult-to-approach superficial gastric tumor. *Endoscopy* 2015; **47** Suppl 1 UCTN: E318-E319 [PMID: 26115394 DOI: 10.1055/s-0034-1392319]

22 **Yamamoto K**, Hayashi S, Saiki H, Indo N, Nakabori T, Yamamoto M, Shibuya M, Nishida T, Ichiba M, Inada M. Endoscopic submucosal dissection for large superficial colorectal tumors using the "clip-flap method". *Endoscopy* 2015; **47**: 262-265 [PMID: 25412089 DOI: 10.1055/s-0034-1390739]

23 **Kamada T**, Haruma K, Inoue K, Shiotani A. [Helicobacter pylori infection and endoscopic gastritis -Kyoto classification of gastritis]. *Nihon Shokakibyo Gakkai Zasshi* 2015; **112**: 982-993 [PMID: 26050720 DOI: 10.11405/nisshoshi.112.982]

24 **Sugimoto M**, Ban H, Ichikawa H, Sahara S, Otsuka T, Inatomi O, Bamba S, Furuta T, Andoh A. Efficacy of the Kyoto Classification of Gastritis in Identifying Patients at High Risk for Gastric Cancer. *Intern Med* 2017; **56**: 579-586 [PMID: 28321054 DOI: 10.2169/internalmedicine.56.7775]

P- Reviewer: Matowicka-Karna J, Sitarz R, Zhang CW
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 21; 24(35): 4086-4092

DOI: 10.3748/wjg.v24.i35.4086

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

CASE REPORT

Infant cholestasis patient with a novel missense mutation in the *AKR1D1* gene successfully treated by early adequate supplementation with chenodeoxycholic acid: A case report and review of the literature

Hui-Hui Wang, Fei-Qiu Wen, Dong-Ling Dai, Jian-She Wang, Jing Zhao, Kenneth DR Setchell, Li-Na Shi, Shao-Ming Zhou, Si-Xi Liu, Qing-Hua Yang

Hui-Hui Wang, Fei-Qiu Wen, Dong-Ling Dai, Shao-Ming Zhou, Si-Xi Liu, Qing-Hua Yang, Gastroenterology Department, Shenzhen Children's Hospital, Shenzhen 518036, Guangdong Province, China

Jian-She Wang, Jing Zhao, Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, Shanghai 201102, China

Kenneth DR Setchell, Department of Pathology and Laboratory Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, United States

Li-Na Shi, MyGenostics Incorporation, Konggang Industrial Park, Beijing 101318, China

ORCID number: Hui-Hui Wang (0000-0002-0469-1641); Fei-Qiu Wen (0000-0002-3551-738X); Dong-Ling Dai (0000-0002-3002-4631); Jian-She Wang (0000-0003-0823-586X); Jing Zhao (0000-0002-4982-9843); Kenneth DR Setchell (0000-0002-2472-2476); Li-Na Shi (0000-0002-7629-7301); Shao-Ming Zhou (0000-0001-7269-4214); Si-Xi Liu (0000-0003-1674-2685); Qing-Hua Yang (0000-0002-7325-2968).

Author contributions: Wang HH drafted the manuscript and analyzed the data; Wen FQ, Wang JS and Zhao J contributed to sample analysis and patients' treatment regimens; Setchell KD contributed to the analysis, interpretation of urinary bile acids and finalized the manuscript; Wang HH, Dai DL, Zhou SM, Liu SX and Yang QH were involved in patient management and follow-up; Shi LN performed gene sequencing and analysis; Dai DL conceived and supervised the study; Wang HH and Wen FQ contributed equally to this work. All of the authors approved submission.

Supported by the Guangdong Medical Research Foundation, No. A2018550.

Informed consent statement: Consent was obtained from the parents of the patient for publication of the case report and any accompanying images.

Conflict-of-interest statement: The authors who took part in this study declare that they do not have anything to disclose regarding funding or a conflict of interest with respect to this manuscript. Setchell KD is a consultant to Retrophin and has minor equity in Asklepion Pharmaceuticals.

CARE Checklist (2013) statement: The authors have read the CARE Checklist (2013). The manuscript was prepared and revised according to the CARE Checklist (2013).

Manuscript source: Unsolicited manuscript

Correspondence to: Dong-Ling Dai, MD, PhD, Chief Doctor, Gastroenterology Department, Shenzhen Children's Hospital, 7019 Yitian Road, Futian District, Shenzhen 518036, Guangdong Province, China. daidong3529@sina.com
Telephone: +86-755-83008333
Fax: +86-755-83008333

Received: June 7, 2018
Peer-review started: June 7, 2018
First decision: June 20, 2018
Revised: July 17, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 21, 2018

Abstract

Steroid 5β-reductase [aldo-keto reductase family 1 mem-

ber D1 (*AKR1D1*)] is essential for bile acid biosynthesis. Bile acid deficiency caused by genetic defects in *AKR1D1* leads to life-threatening neonatal hepatitis and cholestasis. There is still limited experience regarding the treatment of this disease. We describe an infant who presented with hyperbilirubinemia and coagulopathy but normal bile acid and γ-glutamyltransferase. Gene analysis was performed using genomic DNA from peripheral lymphocytes from the patient, his parents, and his elder brother. The patient was compound heterozygous for c.919C>T in exon 8 and exhibited a loss of heterozygosity of the *AKR1D1* gene, which led to an amino acid substitution of arginine by cysteine at amino acid position 307 (p.R307C). Based on these mutations, the patient was confirmed to have primary 5β-reductase deficiency. Ursodeoxycholic acid (UDCA) treatment did not have any effect on the patient. However, when we changed to chenodeoxycholic acid (CDCA) treatment, his symptoms and laboratory tests gradually improved. It is therefore crucial to supplement with an adequate dose of CDCA early to improve clinical symptoms and to normalize laboratory tests.

Key words: Aldo-keto reductase family 1 member D1; Cholestasis; Congenital bile acid synthesis defect; Gene mutation

Core tip: We report a case of an infant with primary 3-oxo-Δ^4-steroid 5β-reductase deficiency with a novel missense mutation in the aldo-keto reductase family 1 member D1 (*AKR1D1*) gene. The patient was successfully treated by early adequate supplementation with chenodeoxycholic acid (CDCA). This case suggests that a novel compound heterozygous R307C mutation and loss of heterozygosity in the *AKR1D1* gene play a pathogenic role in congenital bile acid synthesis defect type 2. Accurate diagnosis of the disease and early adequate supplementation with CDCA are vital for the amelioration of symptoms in clinical practice.

Wang HH, Wen FQ, Dai DL, Wang JS, Zhao J, Setchell KD, Shi LN, Zhou SM, Liu SX, Yang QH. Infant cholestasis patient with a novel missense mutation in the *AKR1D1* gene successfully treated by early adequate supplementation with chenodeoxycholic acid: A case report and review of the literature. *World J Gastroenterol* 2018; 24(35): 4086-4092 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i35/4086.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i35.4086

INTRODUCTION

Congenital bile acid synthesis defect type 2 (CBS2) is a rare and autosomal recessive inherited disease presenting with infant intrahepatic cholestasis, normal or slightly elevated total bile acids and γ-glutamyltransferase in serum[1,2]. This inborn error of bile acid synthesis is caused by a defect in the aldo-ketoreductase family 1 member D1 (*AKR1D1*) gene, which encodes Δ^4-3-oxosteroid 5β-reductase, the key enzyme involved in bile acid biosynthesis[3]. This enzyme catalyzes the reduction of the Δ^4-3-ketosteroid to form the AB *cis* ring structure; its deficiency results in a lack of primary bile acids and an increase in the synthesis of 3-oxo-Δ^4 bile and allo-bile acids[4].

In 1988, Clayton *et al*[5] reported that severe liver disease in pediatric patients was detected with predominant unusual 3-oxo-Δ^4 bile acids secondary to 5β-reductase deficiency. Primary 5β-reductase deficiency was first characterized by Setchell *et al*[6] the same year. It is difficult to distinguish primary 5β-reductase deficiency from another cholestasis secondary to a variety of severe liver diseases based on clinical symptoms and regular laboratory tests[1,7,8]. Thus, genetic analysis of the *AKR1D1* gene is essential for the accurate diagnosis of primary 5β-reductase deficiency. Thus far, more than 20 cases of this inborn error have been reported, and over ten variant mutations of the *AKR1D1* gene are attributed to a defect in 5β-reductase[1,7-13]. Most of these mutations are missense mutations, causing an amino acid alteration in the protein. Drury *et al*[3] further investigated five reported point mutations (L106F, P133R, P198L, G223E, and R261C) in the *AKR1D1* gene to evaluate their effects on the enzymatic properties of 5β-reductase. They found that these mutations result in significantly decreased 5β-reductase activity and subsequently contribute to the progression of bile acid deficiency.

Primary bile acid supplementation can ameliorate the symptoms of CBSA2 and normalize liver function by offering feedback repression of the cholesterol 7α-hydroxylase gene and improving the absorption of fat and fat-soluble vitamins[14]. Treatment with primary bile acids includes monotherapy or the combination of cholic acid (CA), ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA). Early treatment of these bile acids, especially CA and CDCA, is essential to reserve liver function and avoid liver transplantation. A delayed diagnosis would lead to a poor response to primary bile acid treatment and an unfavorable prognosis. There is still limited experience with the treatment of this disease. Here, we describe a case of CBS2 diagnosed by genetic analysis with a novel compound heterozygous mutation in the *AKR1D1* gene, and review both the treatments and prognoses of genetically diagnosed CBS2 cases.

CASE REPORT

A male patient was delivered *via* Caesarean section at term after an uneventful pregnancy with a birth weight of 3400 g. He was the second child of his family and the third pregnancy of his mother. One of his mother's pregnancies was terminated by abortion for social reasons. His parents were non-consanguineous and healthy, his elder brother was healthy, and none of them presented with any liver disease. The patient soon developed progressive jaundice after birth, with dark

Table 1 Summary of the mutations reported in the *AKR1D1* gene and patient prognoses

Variant	Zygotic type	Age	Sex	Treatment	Outcome	INR	Ref.
c.662C > T (p. P198L)	Homozygote	8 mo	F	CDCA 8 mg/kg/d CA 8 mg/kg/d	Alive and well	1.00	[7]
c.511delT (frameshift)	Homozygote	8 wk	M	CDCA 8 mg/kg/d CA 8 mg/kg/d	Liver transplantation; alive and well	1.40	
c.385C > T (p. L106F)	Homozygote	6 wk	F	UDCA 60 mg/d CDCA 30 mg/d	Liver transplantation and died	2.00	
c.467C > G (p. P133R) c.850C > T (p. R261C)	Heterozygote	8 mo	F	CA 10 mg/kg/d	Alive and well	/	[10]
c.737G > A (p. G223Q)	Heterozygote	3 mo	F	UDCA 5-10 mg/kg/d	Alive and well	/	[8]
c.217C > T (Arg50 stop)	Heterozygote	2 mo	F	CDCA 12 mg/kg/d	Liver transplantation	/	
c.850C > T (p.R261C)	Homozygote	6 mo	/	CA 8 mg/kg/d	Died	2.50	[1]
c.797G > A (p. R266Q)	Heterozygote	11 mo	M	UDCA 40 mg/kg/d	Alive and well	/	[13]
c.396C > A (nonsense mutation) c.722A >T (p. D241V)	Heterozygote	11wk	M	UDCA 40 mg/kg/d for 4 mo; CDCA 25 mg/kg/d	Alive and cerebral dysplasia	/	
c.866G > A (p. R266Q)	Heterozygote	6 mo	M	UDCA 7.5 mg/kg/d; CDCA 5 mg/kg/d	Alive and well	/	[12]
c.737G >A (p. G223E) c.850C >T (p. R261C)	Heterozygote	8 mo	F	UDCA 7.5 mg/kg/d; CDCA 10 mg/kg/d	Alive and well	/	
c.587delG (frameshift)	Homozygote	9 wk	F	UCDA	Died	/	[11]
c.587delG (frameshift)	Homozygote	6 mo	F	UCDA	Died	/	
c.587delG (frameshift)	Homozygote	5 wk	F	CA 15 mg/kg/d	Alive and well	/	
c.579 + 2delT, c.853C > T (p. Q285X)	Heterozygote	8 mo	M	CDCA	Alive and well	/	[9]

/: No data; CA: Cholic acid; CDCA: Chenodeoxycholic acid; INR: International normalized ratio; UDCA: Ursodeoxycholic acid; F: Female; M: Male.

urine and pale stool. He was referred to our hospital at the age of two months. Laboratory tests indicated total bilirubin levels of 204.8 μmol/L, direct bilirubin levels of 112.4 μmol/L, alanine aminotransferase levels of 339 IU/L, aspartate aminotransferase levels of 619 IU/L, γ-glutamyltransferase levels of 50 IU/L, total bile acid levels of 1.8 μmol/L, an activated partial thromboplastin time of 62.6 s, a prothrombin time (PT) of 23.6 s, and an international normalized ratio of 2.1. Chronic hepatitis virus tests, including hepatitis B, hepatitis C and cytomegalovirus, were negative, and autoimmune hepatitis was ruled out by an appropriate laboratory test. Abdominal ultrasound showed a visible gallbladder and hepatomegaly; no other bile duct dysplasia was observed. Analysis of the amino acid and acylcarnitine spectrum of genetic metabolic diseases showed elevated tyrosine, which was speculated to be secondary to impaired liver function. Comprehensive analysis of urinary organic acids was normal.

Since we were unavailable to perform bile acid analysis in our hospital, we performed genetic analysis with a cholestasis panel (Supplementary Table 1), which included prevalent pathogenic genes associated with infant cholestasis, to confirm the patient's diagnosis. With informed consent, gene analysis was performed using genomic DNA from peripheral lymphocytes from the patient (Figure 1A), his parents (Figure 1B and C), and his elder brother (Figure 1D). The patient was compound heterozygous for c.919C>T in exon 8 (Figure 1A) and exhibited loss of heterozygosity of the *AKR1D1* gene (Figure 1E), leading to an amino acid substitution of arginine by cysteine at amino acid position 307 (p. R307C) (Figure 2).

The patient was initially given UDCA treatment; however, there was no improvement in his clinical symptoms or liver function. UDCA was then changed to CDCA (80 mg/d) after one week of UCDA treatment. The jaundice began to alleviate after five days of CDCA treatment and his liver function gradually improved (Figure 3). To evaluate the response of bile acid metabolism subsequent to CDCA treatment, we sent the patient's urine sample to Cincinnati Children's Hospital Medical Center *via* the Children's Hospital of Fudan University. Urine bile acid analysis was performed using fast atom bombardment ionization mass spectrometry after two months of CDCA treatment (80 mg/d). The profile revealed significant elevations in taurine and glycine conjugates of unsaturated oxo-dihydroxy and oxo-trihydroxy bile acids. Ions at m/z 444, 460, 494 and 510 reflected the presence of Δ^4-3-oxo bile acids that are characteristic of the bile acid synthetic disorder involving a deficiency in the activity of the Δ^4-3-oxo-steroid 5β-reductase enzyme. Although these are not exceptionally high in concentration, it is difficult to know how responsive the patient was to CDCA therapy because we had no record of having analyzed a urine sample before treatment began. There is clear evidence of compliance to therapy from the presence of ions that reflect metabolites of CDCA. However, based on this mass spectrum, it appeared that the current dose of CDCA was not sufficient to complete the suppression of atypical bile acids. Thus, we increased the dose of CDCA to 100 mg/d and sent a second urine sample for bile acid analyses one month later. The profile showed a good response in terms of the down-regulation of hepatic bile acid synthesis. Thus, the increased dose of CDCA

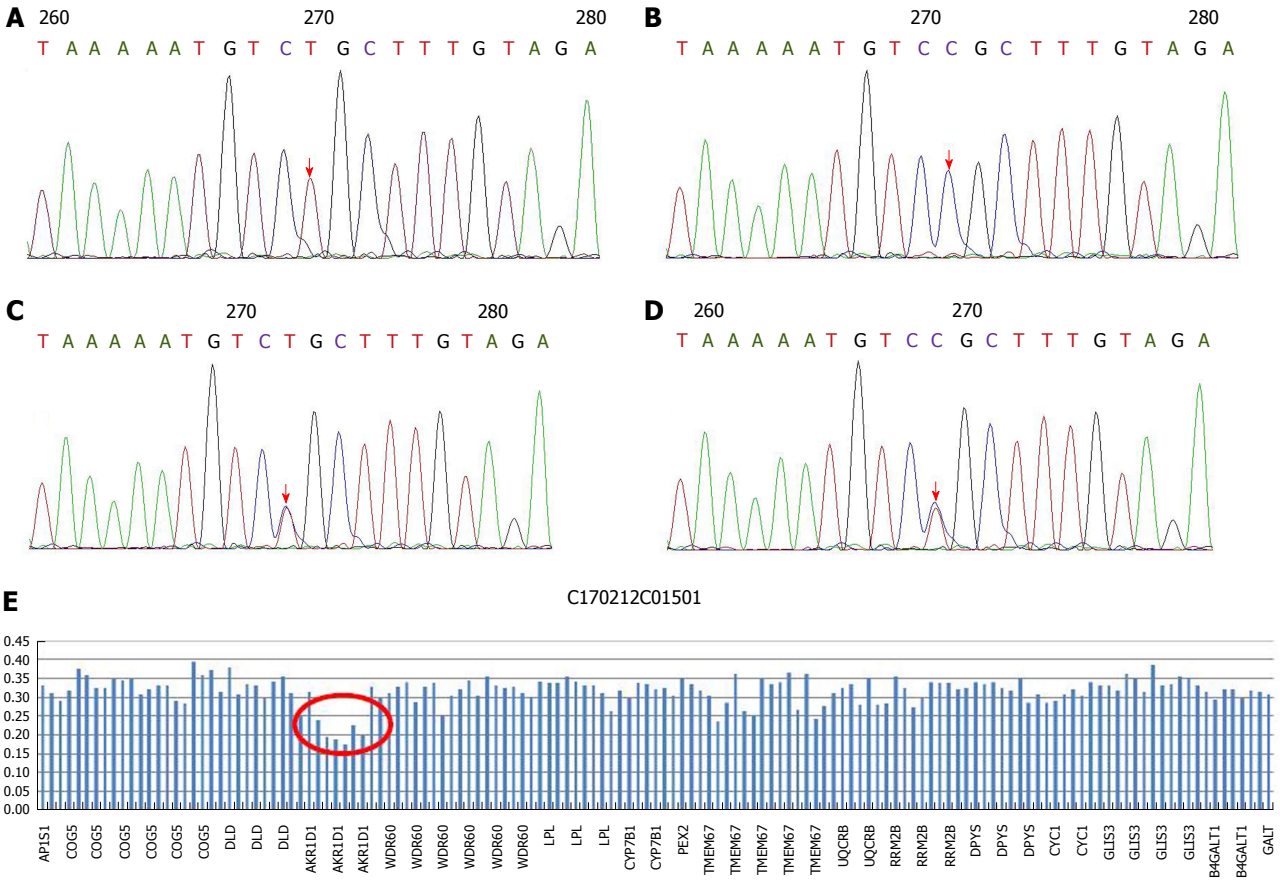


Figure 1 Genomic DNA sequences in exon 8 of the *AKR1D1* gene in the patient and his family. A: Compound heterozygote in the patient (c.919C > T, R307C); B: No variant in his father; C: Heterozygote in his mother; D: Heterozygote in his brother; E: Loss of heterozygosity in exons 1-9 of *AKR1D1* in the patient. *AKR1D1*: Aldo-ketoreductase family 1 member D1.

appeared adequate.

DISCUSSION

We summarized published CBS2 cases with a confirmed *AKR1D1* mutation reported in the NCBI database through the end of December 2017 (Table 1). As demonstrated, missense mutations were present in 11 of 15 cases; the other four cases had a frameshift mutation. These cases consisted of seven homozygous and eight heterozygous mutations. All four cases in which the patient was deceased were homozygous and had a remarkably prolonged international normalized ratio (INR) (1.8 or above), which comprised three frameshift mutations and one missense mutation. In the other three homozygous cases, two showed a good response to primary bile acid treatment and had good prognoses; one patient was referred for liver transplantation and remains alive. All heterozygous cases remain alive and were effectively treated with primary bile acid treatment; only one patient required liver transplantation.

The patient we describe herein developed progressive jaundice in early infancy, with elevated direct bilirubin and alanine aminotransferase but normal total bile acids and γ-glutamyltransferase. After exclusion of bile duct dysplasia, metabolic disorder, viral hepatitis and autoimmune hepatitis, we highly suspected hereditary cholestasis. We were unable to perform bile acid profile analyses in our hospital at that time. To identify the cause of cholestasis, we screened gene disorders using a hereditary cholestasis panel. Genetic analyses revealed that the patient had one heterozygous mutation (R307C) in the *AKR1D1* gene from his mother and loss of heterozygosity in the *AKR1D1* gene from his father, making him compound heterozygous. Family genetic analyses indicated that the R307C mutation in the *AKR1D1* gene was heterozygous both in the patient's mother and brother but absent in his father. On the other hand, the loss of heterozygosity in the *AKR1D1* gene was found in the patient and his father but was absent in his mother and brother. As predicted by SWISS-MODEL Homology Modeling, the R307C mutation could cause an alteration in the amino acid side chain, which may subsequently lead to 5β-reductase deficiency.

However, the patient's brother did not develop cholestasis even though he also had the heterozygous R307C mutation, but without loss of heterozygosity in the *AKR1D1* gene. Accordingly, we speculate that the combination of the R307C mutation and loss of heterozygosity cause the loss of 5β-reductase function.

The patient described herein showed an effective response to CDCA monotherapy (80 mg/d), consistent

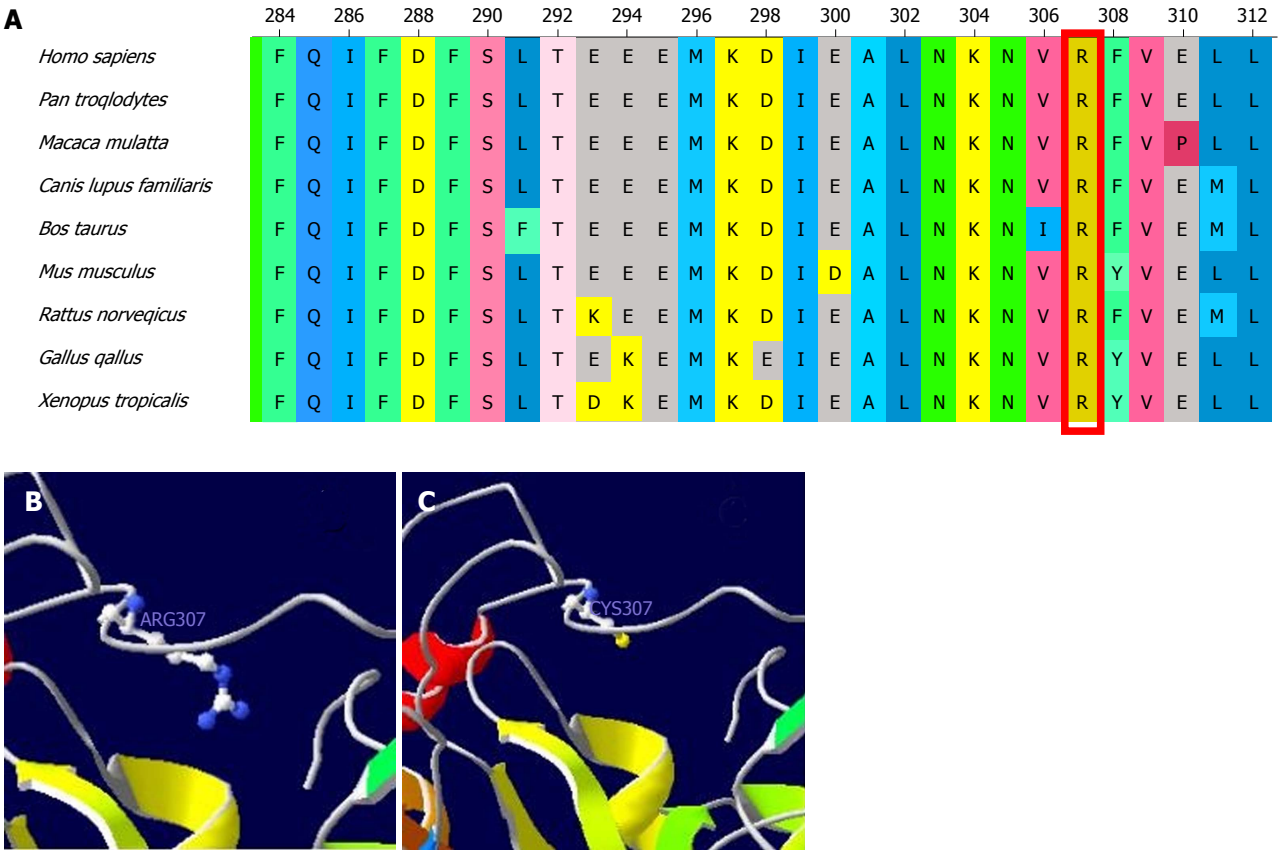


Figure 2 Multiple sequence alignments from different species and structural model of the aldo-ketoreductase family 1 member D1 protein. A: Multiple sequence alignments; the red outline in the alignments shows the amino acid affected by the mutation; B: Wild-type model; C: The mutant model shows the alteration of the amino acid side chain caused by the R307C mutation.

with a previous report[12]. After two months of oral CDCA treatment, the laboratory tests and clinical presence of the patient improved. However, urine bile acid analyses indicated that the CDCA dose of 80 mg/d was insufficient to complete the suppression of atypical bile acids. Thus, we increased the dose of CDCA to 100 mg/d, which proved adequate to down-regulate hepatic bile acid synthesis according to the second urine bile acid analyses. All laboratory tests had normalized when the patient was eight months old, and 100 mg/d CDCA was used to maintain treatment. Seki *et al*[12] reported that 5 mg/kg/d CDCA may not be able to induce negative feedback, and Gonzales *et al*[15] suggested a CDCA dose of 10 mg/kg/d may provide effective negative feedback against cholesterol 7α-hydroxylase. Our case required an even higher dose of CDCA to maintain effective feedback repression of 7α-hydroxylase. CA is considered more effective than CDCA in activating negative feedback of 7α-hydroxylase and is less hepatotoxic[15]. Clayton *et al*[16] reported that 5β-reductase deficiency was responsive to the combination of CDCA and CA treatment, but irresponsive to UCDA. As illustrated in Table 1, Lemonde *et al*[7] was also successful when combining CDCA (8 mg/kg/d) and CA (8 mg/kg/d) to treat a homozygous patient with normal PT. Nevertheless, the same treatment failed in two other homozygous patients with prolonged PT. The combination of CDCA and CA requires a smaller dose of CDCA, which may reduce the accumulation of potential hepatotoxic CDCA metabolites. According to our experience, an adequate dose of CDCA monotherapy was effective in alleviating clinical symptoms and normalizing laboratory tests of *AKR1D1* deficiency, and the adjustment of bile acid dose should be based on urine bile acid analyses. Long-term follow-up, including liver function monitoring and urine bile acid analyses, are required to evaluate the hepatotoxicity of CDCA monotherapy and dose regulation. Although it is well-accepted that UCDA is not an optimal choice for the treatment of 5β-reductase deficiency[16,17], some reported cases, all of which were heterozygous, still benefited from UCDA treatment[8,13]. The natural immaturity of 5β-reductase during early infancy may promote the advancement of cholestasis caused by a defect in *AKR1D1*[18,19]. Thus, the presence of cholestasis and liver dysfunction in cases with a heterozygous mutation in the *AKR1D1* gene may not require bile acid supplementation due to the natural physiological maturation of 5β-reductase.

Clayton *et al*[1] reported that patients with an INR of 1.4 or above at diagnosis were not responsive to bile acid treatment and had unfavorable outcomes. As more cumulative cases have been reported, it has been revealed that patients with significantly prolonged INR are predisposed to bad prognoses. Moreover, all

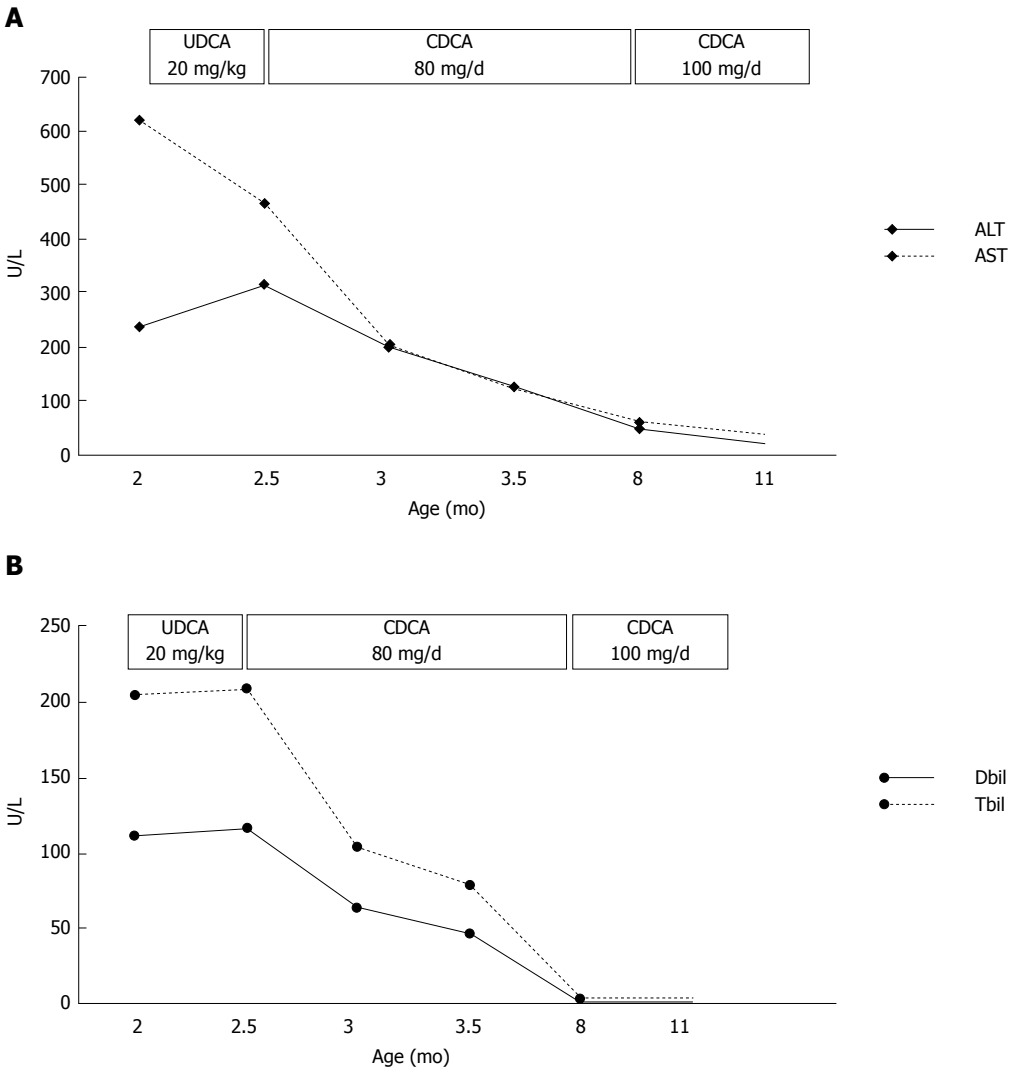


Figure 3 Responses of liver function after treatment with ursodeoxycholic acid and chenodeoxycholic acid. A: Transaminase 1; B: Bilirubin. UDCA: Ursodeoxycholic acid; CDCA: Chenodeoxycholic acid.

reported cases in which the patients are deceased were homozygous and had an INR of 1.8 or above. However, although the case we encountered had an INR of 2.1, the patient had a good response to primary bile acid treatment. Due to the suspicion of an inborn error of bile acid synthesis, we soon substituted UDCA with CDCA after one week of invalid UDCA treatment. We believe that early supplementation with CDCA in our case may have prevented the deterioration of the patient's liver function despite impaired coagulation function.

In conclusion, the case described herein was confirmed to involve a novel compound heterozygous R307C mutation and loss of heterozygosity in the *AKR1D1* gene. Both early supplementation with and an adequate dose of CDCA monotherapy showed a favorable response, resulting in both improved clinical symptoms and the normalization of laboratory tests.

ARTICLE HIGHLIGHTS

Case characteristics

A 2 mo old male infant presented with hyperbilirubinemia and coagulopathy, but normal bile acid and γ-glutamyltransferase.

Clinical diagnosis

Infant cholestatic liver disease, diagnosed by elevated direct bilirubin and alanine aminotransferase.

Differential diagnosis

Virus hepatitis, congenital bile duct dysplasia, genetic metabolic diseases, and autoimmune hepatitis.

Laboratory diagnosis

Hyperbilirubinemia, coagulopathy, and impaired liver function.

Treatment

The patient was initially given ursodeoxycholic acid (UDCA) treatment. We changed UDCA to chenodeoxycholic acid (CDCA) (80 mg/d) after one week of ineffective UCDA treatment. After two months of oral CDCA treatment, urine bile acid analyses indicated that the CDCA dose of 80 mg/d was insufficient to complete the suppression of atypical bile acids. We thus increased the dose of CDCA to 100 mg/d, which proved adequate to down-regulate hepatic bile acid synthesis based on the second urine bile acid analyses.

Related reports

More than 20 cases of primary 5β-reductase deficiency have been reported,

and over ten variant mutations in the aldo-ketoreductase family 1 member D1 (*AKR1D1*) gene are attributed to a defect in 5β-reductase.

Term explanation

Aldo-ketoreductase family 1 member D1 (*AKR1D1*) encodes Δ^4-3-oxosteroid 5β-reductase; its deficiency results in a lack of primary bile acids and increased synthesis of 3-oxo-Δ^4 bile and allo-bile acids.

Experiences and lessons

Gene analysis is essential for the accurate diagnosis of primary 3-oxo-Δ^4-steroid 5β-reductase deficiency. Early diagnosis and adequate supplementation with CDCA are vital for the amelioration of clinical symptoms.

ACKNOWLEDGMENTS

We thank the patient's family for providing background information and allowing us to publish this manuscript.

REFERENCES

1 **Clayton PT**. Disorders of bile acid synthesis. *J Inherit Metab Dis* 2011; **34**: 593-604 [PMID: 21229319 DOI: 10.1007/s10545-010-9259-3]

2 **Heubi JE**, Setchell KD, Bove KE. Inborn errors of bile acid metabolism. *Semin Liver Dis* 2007; **27**: 282-294 [PMID: 17682975 DOI: 10.1055/s-2007-985073]

3 **Drury JE**, Mindnich R, Penning TM. Characterization of disease-related 5beta-reductase (AKR1D1) mutations reveals their potential to cause bile acid deficiency. *J Biol Chem* 2010; **285**: 24529-24537 [PMID: 20522910 DOI: 10.1074/jbc.M110.127779]

4 **Kondo KH**, Kai MH, Setoguchi Y, Eggertsen G, Sjöblom P, Setoguchi T, Okuda KI, Björkhem I. Cloning and expression of cDNA of human delta 4-3-oxosteroid 5 beta-reductase and substrate specificity of the expressed enzyme. *Eur J Biochem* 1994; **219**: 357-363 [PMID: 7508385 DOI: 10.1111/j.1432-1033.1994.tb19947.x]

5 **Clayton PT**, Lake BD, Hjelm M, Stephenson JB, Besley GT, Wanders RJ, Schram AW, Tager JM, Schutgens RB, Lawson AM. Bile acid analyses in "pseudo-Zellweger" syndrome; clues to the defect in peroxisomal beta-oxidation. *J Inherit Metab Dis* 1988; **11** Suppl 2: 165-168 [PMID: 3141700 DOI: 10.1007/BF01804226]

6 **Setchell KD**, Suchy FJ, Welsh MB, Zimmer-Nechemias L, Heubi J, Balistreri WF. Delta 4-3-oxosteroid 5 beta-reductase deficiency described in identical twins with neonatal hepatitis. A new inborn error in bile acid synthesis. *J Clin Invest* 1988; **82**: 2148-2157 [PMID: 3198770 DOI: 10.1172/JCI113837]

7 **Lemonde HA**, Custard EJ, Bouquet J, Duran M, Overmars H, Scambler PJ, Clayton PT. Mutations in SRD5B1 (AKR1D1), the gene encoding delta(4)-3-oxosteroid 5beta-reductase, in hepatitis and liver failure in infancy. *Gut* 2003; **52**: 1494-1499 [PMID: 12970144 DOI: 10.1136/gut.52.10.1494]

8 **Ueki I**, Kimura A, Chen HL, Yorifuji T, Mori J, Itoh S, Maruyama K, Ishige T, Takei H, Nittono H, Kurosawa T, Kage M, Matsuishi T. SRD5B1 gene analysis needed for the accurate diagnosis of primary 3-oxo-Delta4-steroid 5beta-reductase deficiency. *J Gastroenterol Hepatol* 2009; **24**: 776-785 [PMID: 19175828 DOI: 10.1111/j.1440-1746.2008.05669.x]

9 **Cheng Y**, Guo L, Deng M, Song YZ. [Clinical feature and genetic analysis of a family affected by congenital bile acid synthesis defect type 2: identification of 2 novel mutations in AKR1D1 gene]. *Zhongguo Dang Dai Er Ke Za Zhi* 2017; **19**: 734-740 [PMID: 28697823]

10 **Gonzales E**, Cresteil D, Baussan C, Dabadie A, Gerhardt MF, Jacquemin E. SRD5B1 (AKR1D1) gene analysis in delta(4)-3-oxosteroid 5beta-reductase deficiency: evidence for primary genetic defect. *J Hepatol* 2004; **40**: 716-718 [PMID: 15030995 DOI: 10.1016/j.jhep.2003.12.024]

11 **Morgan NV**, Hartley JL, Setchell KD, Simpson MA, Brown R, Tee L, Kirkham S, Pasha S, Trembath RC, Maher ER, Gissen P, Kelly DA. A combination of mutations in AKR1D1 and SKIV2L in a family with severe infantile liver disease. *Orphanet J Rare Dis* 2013; **8**: 74 [PMID: 23679950 DOI: 10.1186/1750-1172-8-74]

12 **Seki Y**, Mizuochi T, Kimura A, Takahashi T, Ohtake A, Hayashi S, Morimura T, Ohno Y, Hoshina T, Ihara K, Takei H, Nittono H, Kurosawa T, Homma K, Hasegawa T, Matsuishi T. Two neonatal cholestasis patients with mutations in the SRD5B1 (AKR1D1) gene: diagnosis and bile acid profiles during chenodeoxycholic acid treatment. *J Inherit Metab Dis* 2013; **36**: 565-573 [PMID: 23160874 DOI: 10.1007/s10545-012-9526-6]

13 **Zhao J**, Fang LJ, Setchell KD, Chen R, Li LT, Wang JS. Primary Δ4-3-oxosteroid 5β-reductase deficiency: two cases in China. *World J Gastroenterol* 2012; **18**: 7113-7117 [PMID: 23323017 DOI: 10.3748/wjg.v18.i47.7113]

14 **Russell DW**, Setchell KD. Bile acid biosynthesis. *Biochemistry* 1992; **31**: 4737-4749 [PMID: 1591235]

15 **Gonzales E**, Gerhardt MF, Fabre M, Setchell KD, Davit-Spraul A, Vincent I, Heubi JE, Bernard O, Jacquemin E. Oral cholic acid for hereditary defects of primary bile acid synthesis: a safe and effective long-term therapy. *Gastroenterology* 2009; **137**: 1310-1320.e1-e3 [PMID: 19622360 DOI: 10.1053/j.gastro.2009.07.043]

16 **Clayton PT**, Mills KA, Johnson AW, Barabino A, Marazzi MG. Delta 4-3-oxosteroid 5 beta-reductase deficiency: failure of ursodeoxycholic acid treatment and response to chenodeoxycholic acid plus cholic acid. *Gut*1996; **38**: 623-628 [PMID: 8707100 DOI: 10.1136/gut.38.4.623]

17 **Setchell KD**, Heubi JE. Defects in bile acid biosynthesis--diagnosis and treatment. *J Pediatr Gastroenterol Nutr* 2006; **43** Suppl 1: S17-S22 [PMID: 16819396 DOI: 10.1097/01.mpg.0000226386.79483.7b]

18 **Inoue T**, Kimura A, Aoki K, Tohma M, Kato H. Developmental pattern of 3-oxo-delta 4 bile acids in neonatal bile acid metabolism. *Arch Dis Child Fetal Neonatal Ed* 1997; **77**: F52-F56 [PMID: 9279184 DOI: 10.1136/fn.77.1.F52]

19 **Kimura A**, Mahara R, Inoue T, Nomura Y, Murai T, Kurosawa T, Tohma M, Noguchi K, Hoshiyama A, Fujisawa T, Kato H. Profile of urinary bile acids in infants and children: developmental pattern of excretion of unsaturated ketonic bile acids and 7beta-hydroxylated bile acids. *Pediatr Res* 1999; **45**: 603-609 [PMID: 10203155 DOI: 10.1203/00006450-199904010-00022]

P- Reviewer: Deneau M, Schwarz SM **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Yin SY

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 September 28; 24(36): 4093-4216

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 36 September 28, 2018

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
September 28, 2018

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4093-4103

DOI: 10.3748/wjg.v24.i36.4093

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

EDITORIAL

Interleukin 12/interleukin 23 pathway: Biological basis and therapeutic effect in patients with Crohn's disease

Ioanna Aggeletopoulou, Stelios F Assimakopoulos, Christos Konstantakis, Christos Triantos

Ioanna Aggeletopoulou, Christos Konstantakis, Christos Triantos, Division of Gastroenterology, Department of Internal Medicine, Medical School, University of Patras, Patras 26504, Greece

Stelios F Assimakopoulos, Department of Internal Medicine, University Hospital of Patras, Patras 26504, Greece

ORCID number: Ioanna Aggeletopoulou (0000-0003-4489-1485); Stelios F Assimakopoulos (0000-0002-6901-3681); Christos Konstantakis (0000-0001-5834-9182); Christos Triantos (0000-0003-3094-8209).

Author contributions: Aggeletopoulou I and Konstantakis C were responsible for the literature review and analysis; Aggeletopoulou I, Assimakopoulos SF and Triantos C were responsible for the drafting of the manuscript and the data interpretation; Assimakopoulos SF and Triantos C were responsible for the revision of the manuscript for important intellectual content; all authors issued final approval for the version to be submitted.

Conflict-of-interest statement: Not related to this article.

Manuscript source: Invited manuscript

Correspondence to: Christos Triantos, PhD, Assistant Professor in Internal Medicine and Gastroenterology, Department of Gastroenterology, University Hospital of Patras, D. Stamatopoulou 4, Patras 26504, Greece. chtriantos@upatras.gr
Telephone: +30-69-72894651
Fax: +30-26-10625382

Received: July 16, 2018
Peer-review started: July 16, 2018
First decision: July 31, 2018
Revised: August 2, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 28, 2018

Abstract

Considering that both innate and adaptive immune responses are involved in the pathogenesis of Crohn's disease (CD), novel therapeutic options have significantly been developed. Biological agents represent an important addition to the conventional treatments for immuno-inflammatory conditions, acting as antagonists of adhesion molecules or various inflammatory cytokines. The interleukin 12 (IL-12)/IL-23 common pathway has been found to play a determinant role in the induction of inflammation in adaptive immune responses. In particular, IL-23 promotes the differentiation of naïve T helper cells into Th17 phenotype with the concomitant secretion of several inflammatory cytokines such as IL-17 and IL-22, whereas IL-12 induces the Th1 polarization and production of critical cytokines such as interferon-γ and tumor necrosis factor. Nowadays, there is increased interest regarding the role of IL-23 as a therapeutic target of CD through the blockage of IL-23 mediated pathways. In this editorial, we focus on the role of IL-12/IL-23 pathway in the regulation of mucosal immunity and in the induction and maintenance of chronic inflammation. In parallel, we critically discuss the available data regarding the therapeutic effect of the IL-12/IL-23 inhibitors and especially of ustekinumab, a human monoclonal antibody which has been recently approved by the United States Food and Drug Administration for the management of moderate-to-severe CD and its potential to be used as first-line therapy in everyday clinical practice.

Key words: Crohn's disease; Interleukin 12; Interleukin

23; Monoclonal antibodies; Ustekinumab; Biological agents; Interleukin 12/interleukin 23 blockade

Core tip: The therapeutic management of Crohn's disease patients not responding to treatment with anti-tumor necrosis factor agents remains a clinical challenge. Recently, there has been increased evidence regarding the development of new drugs with alternative mechanisms of action. Interleukin (IL)-12 and IL-23 are important cytokines which are involved in the adaptive immune responses and their common pathway has been found to play a determinant role in the induction of inflammation. Clinical trials have assessed the therapeutic effect of an IL-12/IL-23 inhibitor (ustekinumab), demonstrating rapid clinical effect with a safety profile. Further studies are needed to elucidate its potential role as first-line therapy in Crohn's disease.

Aggeletopoulou I, Assimakopoulos SF, Konstantakis C, Triantos C. Interleukin 12/interleukin 23 pathway: Biological basis and therapeutic effect in patients with Crohn's disease. *World J Gastroenterol* 2018; 24(36): 4093-4103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4093.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4093

INTRODUCTION

Crohn's disease (CD) is an immune-mediated inflammatory disorder characterized by chronic relapsing inflammation in different segments of the gastrointestinal tract. Although the typical preferential regions of involvement are the distal ileum, the ileocaecal region, the colon and the perianal region, extra-intestinal manifestations are not uncommon[1]. The etiology of this disease in not yet fully understood. However, it is currently considered that genetic and environmental factors, impaired immune regulation, gut barrier dysfunction and changes in the intestinal microbiome are involved in the pathogenesis and development of this condition[2-4]. CD treatment is generally individualized and is associated with several factors including disease phenotype, disease severity, affected region, and related luminal or extraluminal complications. The treatment strategy is mainly classified into two stages: (1) Appropriate treatment of the acute flare aiming to induce remission (2) maintenance of remission[5]. Until recently, the initial choice of treatment has focused on long-term use of corticosteroids and immunosuppressants like thiopurines and methotrexate for the induction and the maintenance of remission, respectively[6-8]. During the last years, therapeutic options have significantly benefited from the introduction of biological agents, which became the mainstay of moderate to severe CD treatment, using monoclonal antibodies targeting tumor necrosis factor (TNF)[9-11] or adhesion molecules (integrins)[12,13]. However, a significant proportion of patients (about 30%) will not respond adequately to induction therapy with TNF inhibitors. Furthermore, another subgroup of patients that achieve initial (short-term) response, run a risk of secondary loss which occurs in approximately 40% of patients[14,15]. The main causes of secondary failure are non-compliance to anti-TNF treatment, drug immunogenicity and non-immune clearance of anti-TNF or the persistence of inflammatory activity in spite of sufficient anti-TNF levels[16]. This latter clinical scenario is usually performed by switching to another class of biological agents[16].Moreover, the humanized anti-$\alpha 4\beta 7$ integrin antibody that has been recently introduced in clinical practice, has displayed efficacy on the induction and maintenance of remission in moderate-to-severe refractory CD patients; however, safety concerns have been raised due to rare but possible adverse events[17].

Current data suggest that the initiation and perpetuation of inflammation in CD are associated with a disruption in the balance among the intestinal epithelium, the commensal microbiota and the innate immune response[18]. This condition is maintained by the presence of defects in the intestinal wall, environmental factors, genetic predisposition and dysfunction in regulatory mechanisms, which in turn lead to the release of an array of cytokines that promote the inflammatory immune response[18].

Taking into consideration the adverse events resulted from previous treatments regiments, target tailored treatment options that aim at specific pathways of inflammation have emerged. CD is characterized by dysfunction in both innate and adaptive immune responses. Disturbances in adaptive immune response are closely related to tissue damage, mainly driven by interleukin (IL)-12 and IL-23[4]. Therefore, inhibitors of IL-12/IL-23 and specific inhibitors of IL-23 have been developed for the management of CD. In this editorial, we focus on the role of IL-12/IL-23 pathway in the modulation of mucosal immunity and in the induction and maintenance of remission of the associated chronic inflammation of the intestinal epithelium. Moreover, we critically discuss the therapeutic effects of the IL-12/IL-23 blocker in patients with CD and its potential position as first-line therapy in everyday clinical practice.

IL-12 AND IL-23 ROLE IN CD/ MECHANISM OF ACTION

Both innate and adaptive immune responses are involved in the pathogenesis of CD. Adaptive immunity is provided by resident and recruited cells, including mucosal B cells which produce the secretary immunoglobulins A and G, T cells subpopulations and

especially T helper 1 (Th1), Th17, or Th2 cells, and T and B regulatory cells[4]. Th1 phenotype is induced by microbes which in turn activate the excretion of interferon (IFN)-γ and IL-12p40 through the signal transducer and activator of transcription 1 (STAT1), T-box factor 21 (TBX21) and STAT4 signaling pathways[19]. CD seems to be driven by a Th1-mediated pathology, with an increased synthesis of IFN-γ[20]. In parallel, inflamed intestinal mucosa is infiltrated by Th17 cells with a concomitant production of IL-17 cytokine[21]. Th17 lineage commitment is directed by transforming growth factor beta (TGF-β) in the presence of a proinflammatory environment, and IL-23 is related to the expansion and maintenance of Th17 cells[22]. Moreover, CD is characterized by an increased production of IL-12, the major Th1-stimulating factor[23,24].

IL-12 family includes IL-12, IL-2, IL-35 and IL-27, key mediators of inflammatory response[25]. IL-12 is a heterodimeric cytokine comprising of two covalently linked subunits (p40 and p35) and is mainly produced by activated phagocytic cells [monocytes/macrophages, neutrophils, dendritic cells (DCs)] in response to bacterial stimulation, intracellular pathogens and intestinal inflammation[26,27]. IL-12 exerts its biological function through the binding to its heterodimeric receptor formed by IL-12R-β1 and IL-12R-β2. β2 receptor subunit plays a major role in IL-12 function, as it controls the Th1 cell lineage commitment. Moreover, IL-12R-β2 is overexpressed by T cells in inflamed mucosa[26] as well as in CD T-lamina propria lymphocytes (T-LPL)[28]. Data has shown that the inhibition of IL-12 resulted in reduced production of IFN-γ and IL-21 in CD mucosal T cells cultures[23], and T cell stimulation in T cell cultures from fetal gut explants, promoting Th1 immune response and causing mucosal degradation[29]. Beyond the role of IL-12 in T cells, a recent study has demonstrated its role in a distinct population, the innate lymphoid cells (ILCs), which are considerably encountered in inflamed tissue of intestinal wall of CD patients. IL-12 stimulates these cells to produce IFN-γ indicating the role of ILCs in the pathogenesis of gut mucosal inflammation[30].

Recent studies have demonstrated the crucial role of IL-23 in the regulation of Th1 cell responses and its potential role in the CD pathogenesis. IL-23 belongs to the IL-12 family, it is composed of one subunit of p40, that is shared with IL12, and one subunit of p19, which is unique[31]. IL-23 is produced by myeloid DCs or conventional DCs and macrophages in response to bacterial stimulation, endogenous signals or CD40L activation[32,33]. Depending on the various environmental signals, macrophages can obtain distinct functional phenotypes through undergoing different polarization[34]. Macrophage M1 phenotype is induced by microbial products or proinflammatory cytokines and is characterized by high production of IL-12 and IL-23 cytokines, in contrast to M2 phenotype which is mainly associated with Th2 immune responses and promotes tissue repair[35]. The binding of IL-23 on its receptor, which is composed of IL-12R β1 and IL-23R results in the specific induction of naïve $CD4^+$ T cells into Th17 cells, with a concomitant activation of numerous proinflammatory cytokines such as IL-17, IL-17F, TNF-α and IL-6[36]. Beyond $CD4^+$ T induction, IL-23 participates in the ILCs[37], $CD8^+$ T[38], natural killer (NK), NKT[39] and γδ T cells[40] activation. The presence of inflammation in the intestinal wall stems from the pathological Th1 immune response against the bacterial microbiota which are closely related to the IL-12 and IL-23 expression[41]. IL-23 expression is highly increased in ILCs in the inflamed intestine in CD patients, indicating the presence of IL-23-responsive ILCs in the human gut and promoting IL-17A and IFN-γ production[37,42]. Moreover, studies have demonstrated the existence of single nucleotide polymorphisms (SNPs) in the IL-23R gene, which are highly protective in CD patients, suggesting that the blockade of IL-23 signaling could decrease the risk of CD development[43-45].

The above data highlighted the determinant role of IL-12 and IL-23 in intestinal inflammation, since they are able to trigger signals in different cell populations and lead to the introduction of monoclonal antibodies as therapeutic agents for CD, targeting the common p40 subunit of IL-12 and IL-23.

IL-12/IL-23 BLOCKADE/EMERGING BIOLOGICAL AGENTS

Anti-IL-12/IL-23p40 antibodies

Ustekinumab is an IgG1 humanized monoclonal antibody directed against the common p40 subunit of the IL-12 and IL-23. It binds to the p40 subunit and impedes the interaction with the IL-12Rβ1 on the cell surface of NK, T cells, or antigen-presenting cells[46]. This process results in the blockade of IL-12 and IL-23 mediated downstream cell signaling, gene activation and cytokine production[46]. Ustekinumab binding to the IL-12 and IL-23, equally neutralizes IL-12 mediated responses, including the intracellular phosphorylation of STAT4, the expression of cell surface markers and the production of IFN-γ and IL-23 mediated responses including the intracellular phosphorylation of STAT3 and IL-17A, IL-17F, and the production of IL-22[46] (Figure 1).

Briakinumab is an IgG1 monoclonal antibody with similar mechanism of action as ustekinumab, which was tested for the induction and maintenance of remission in patients with moderately to severely active CD. The results of a placebo-controlled phase 2b trial showed that although briakinumab presented a similar safety and tolerability profile to placebo in the induction and maintenance phases, it did not achieve the primary end point of clinical remission at week 6[47].

Anti-IL-23p19 antibodies

MEDI2070 (or AMG 139) is a humanized monoclonal

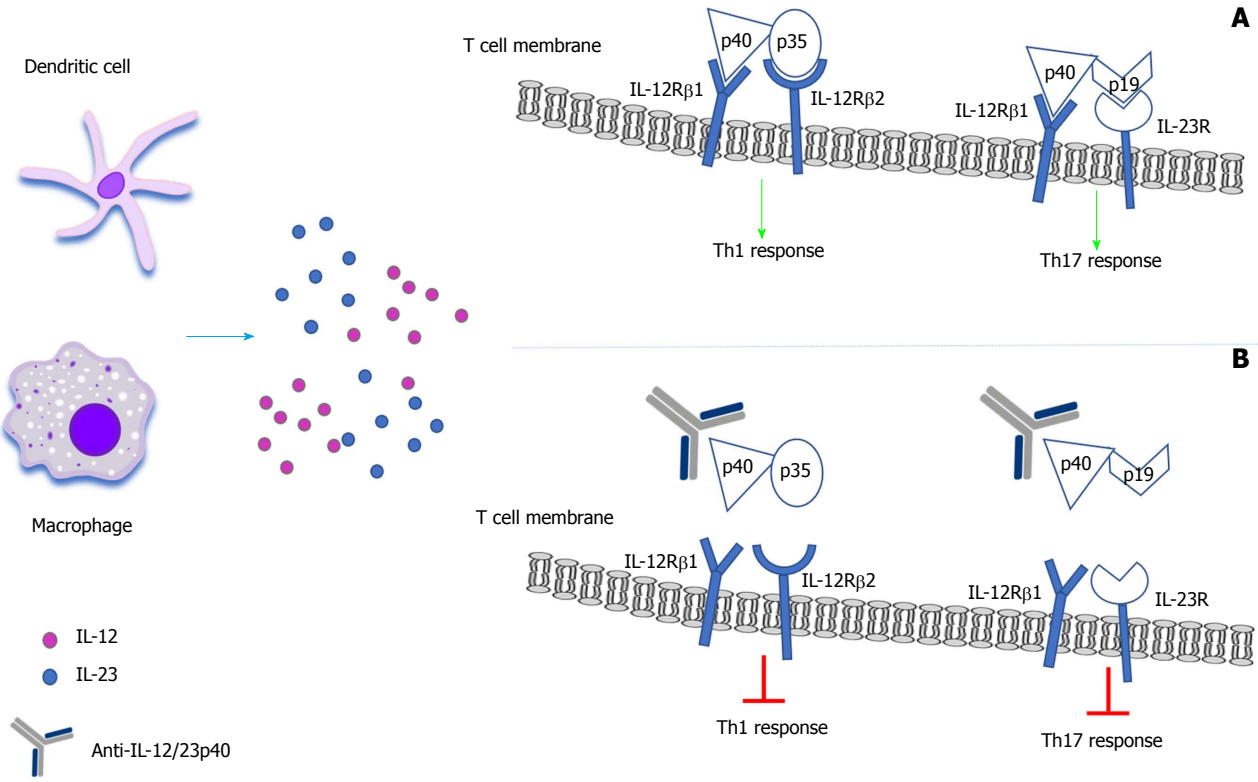


Figure 1 Neutralization of the interleukin-12/interleukin-23 pathways associated with T-cell activation and differentiation. A: In inflamed tissue, bacterial stimulation can lead to the activation of dendritic cells and macrophages. This process results in the activation of T cells and the secretion of inflammatory cytokines such as IL-12 and IL-23. The binding of IL-12 in its receptor, which is composed of IL-12R-β1 and IL-12R-β2 results in the preferential T cell differentiation into Th1 cells, promoting the Th1 cell response and secreting cytokines such as IFN-γ and TNF. The binding of IL-23 in its receptor, which is composed of IL-12R-β1 and IL-23R results in the preferential induction of T cells into Th17 cells, inducing Th17 cell response and secreting cytokines such as IL-17 and IL-22; B: The use of a monoclonal antibody against the common subunit of IL-12 and IL-23 (IL-12/23p40) that selectively targets both IL-23 and IL-12 cytokines, disrupts their mediated signaling pathway and cytokine cascade, through the prevention of these cytokines' interaction with their shared cell-surface receptor, IL-12R-β1. This process results in the inhibition of IL-12 and IL-23 signaling and further activation of Th1 and Th17 phenotypes. IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor.

IgG2 antibody that selectively binds the p19 subunit, specifically blocking the binding of IL-23 to its receptor. In a phase 2a trial of patients with moderate to severe CD who had failed to anti-TNF treatment, the use of MEDI2070 showed induction of clinical response in CD patients compared to placebo[48].

Risankizumab, a humanized monoclonal antibody targeting the p19 subunit of IL-23, resembles the mechanism of action of MEDI2070. The efficacy and safety of this antibody were assessed in a randomized, double-blind, placebo-controlled phase 2 study and the results showed that it was significantly better than placebo in inducing clinical remission[49].

USTEKINUMAB

Ustekinumab was approved in 2017 by the United States Food and Drug Administration (FDA) for the treatment of moderate to severe active CD in patients who have failed or were intolerant to therapy with corticosteroids or other immunomodulators but have never failed anti-TNF treatment, or in patients who have failed or were intolerant to therapy with one or more anti-TNF agents[50]. In parallel, in the same year, the use of ustekinumab was approved by the European Medicines Agency (EMA) for adults with moderate to severe active CD with inadequate response, or loss of response, or intolerance to either conventional treatment, or anti-TNF agents, or with medical contraindications to such therapies[51].

CLINICAL EFFICACY OF USTEKINUMAB IN CD/ RANDOMIZED CLINICAL TRIALS

The clinical efficacy of ustekinumab in humans was first evaluated in immune-mediated diseases, such as psoriasis[52,53], psoriatric arthritis[54-56] and multiple sclerosis[57]. The current use of ustekinumab in patients with CD has been assessed by multiple clinical trials (Tables 1 and 2).

Phase II studies

The use of ustekinumab in the treatment of moderate to severe CD was first investigated in 2008 in a randomized, placebo-controlled, phase 2a induction trial[58]. The study comprised of two patient groups. Population 1 (the double-blind, cross-over phase IIa arm of the study) included 104 patients who had previously

Table 1 Summary of randomized, placebo-controlled trials on ustekinumab in Crohn's disease

Study (reference)	Publication year	Type of publication	Study design	Study phase
Sandborn *et al*[58]	2008	Full paper	Multicenter, double-blind, placebo-controlled, parallel cross-over	IIa
Sandborn *et al*[59] (CERTIFI)	2012	Full paper	Randomized, multicenter, double-blind, placebo-controlled	IIb induction IIb maintenance
Feagan *et al*[60] (UNITI-1)	2016	Full paper	Randomized, multicenter, double-blind, placebo-controlled	III
Feagan *et al*[60] (UNITI-2)	2016	Full paper	Randomized, multicenter, double-blind, placebo-controlled	III
Feagan *et al*[60] (IM-UNITI)	2016	Full paper	Randomized, multicenter, double-blind, placebo-controlled	Maintenance phase of UNITI 1 and 2 responders
Sandborn *et al*[63] (IM-UNITI long term extension)	2017	Abstract	Randomized, multicenter, double-blind, placebo-controlled	Maintenance phase of UNITI 1 and 2 responders
Sands *et al*[62] (UNITI-IM)	2018	Abstract	Randomized, multicenter, double-blind, placebo-controlled	Maintenance phase of UNITI 1 and 2 responders
Rutgeerts *et al*[61]	2018	Full paper	Randomized, multicenter, double-blind, placebo-controlled	Induction and maintenance of endoscopic healing

received conventional therapy or anti-TNF regimens. The second group, population 2 - open-label arm, consisted of 27 non-responders (primary or secondary) to infliximab. The results showed that ustekinumab could induce clinical response in patients with moderate-to-severe active CD, especially in those who were previously treated with infliximab[58]. Regarding the development of serious adverse events, there was no difference in patients receiving ustekinumab compared to placebo[58]. The above results led to the conduct of a 36-wk, randomized, double-blind, placebo-controlled phase IIb trial (CERTIFI) on the role of ustekinumab in the induction and maintenance of remission in patients with moderate-to-severe CD who were resistant to anti-TNF treatment[59]. The study enlisted 526 patients in the induction arm and 145 responders in the maintenance phase. The results demonstrated that patients who were resistant to anti-TNF therapy showed an increased response rate to induction with ustekinumab compared to placebo, although remission rates were comparable[59]. However, ustekinumab induction responders showed significantly increased rates of response and remission during the maintenance phase[59]. No difference was reported in the incidence of adverse events between examined groups during the maintenance phase[59]. Basal-cell carcinoma developed in 1 patient receiving ustekinumab.

Phase III studies

Phase III, multicentre, double-blind, placebo-controlled, trials for the evaluation of ustekinumab in patients with moderate to severe CD have been recently completed. The first trial (UNITI-1) included 741 patients who were primary or secondary non-responders to anti-TNF treatment or had severe side effects[60]. In the second trial (UNITI-2) 628 patients who had failed the conventional therapy or had experienced severe side effects were enrolled[60]. The results showed that intravenous ustekinumab induced clinical response and remission in patients from both trials (UNITI 1-2)[60]. No difference in adverse and serious adverse events was reported between the groups. Moreover, there was no report of death, malignancy, opportunistic infections or tuberculosis in ustekinumab treated patients[60]. The 397 patients who completed the induction trials (UNITI 1 and 2) and were responders to ustekinumab, were enrolled in the IM-UNITI trial[60]. Primary endpoint for this trial was the maintenance of remission at week 44 and the results showed that treatment with ustekinumab was more effective than placebo for maintaining remission[60]. Between the placebo and the ustekinumab groups, the rates of adverse events development and severity were similar[60].

Effect of ustekinumab in endoscopic activity

A sub-study of the UNITI trial enrolled 334 patients with moderate to severe CD and assessed the clinical effect of ustekinumab in the simplified endoscopic activity score for CD (SES-CD) and the efficacy of maintenance therapy[61]. Patients treated with ustekinumab had higher reduction in SES-CD compared to placebo during the induction phase[61]. The results were similar in patients from different induction trials (UNITI 1 or 2) and in those receiving different ustekinumab doses. Greater reduction in the SES-CD at week 44 was also observed in the ustekinumab group compared to placebo[61].

Dose adjustment effect of ustekinumab in patients with loss of response or in delayed responders

Another sub-study of the UNITI-IM maintenance programme addressed important points of clinical application of ustekinumab. This trial evaluated the clinical effect of dose adjustment of ustekinumab in patients who (1) entered the maintenance trial in response and subsequently lost response (LOR) (2) were non-responders to intravenous ustekinumab during induction phase[62]. The results showed that in patients

Table 2 Characteristics of randomized, placebo-controlled trials evaluating the efficacy of ustekinumab in Crohn's disease

Study (reference)	Patients	Endpoints	Intervention parameters	Outcomes
Sandbornetal[58]	25	Clinical response at week 4/week 8	90 mg SC week 0-3→Placebo SC week 8-11	Ustekinumab: 53%/49%
	26		Placebo SC week 0-3→90mg SC week 8-11	
	26		4.5 mg/Kg IV week 0→Placebo IV week 8	Placebo: 30%/40%
	27		Placebo IV week 0→4.5 mg/Kg IV week 8	
	27 (primary or secondary non-responders to infliximab)	Clinical response at week 8	90 mg SC	43%
			4.5 mg/kg IV	54%
Sandborn *et al*[59] (CERTIFI) Induction	131	Clinical response at week 6	1 mg/kg IV	36.60%
	132		3 mg/kg IV	34.10%
	131		6 mg/kg IV	39.70%
	132		Placebo IV	23.50%
Sandborn *et al*[59] (CERTIFI) Maintenance	145 Responders 72 Ustekinumab	Clinical response at week 22	90 mg SC	69.40%
			Placebo SC	42.50%
	73 Placebo	Clinical remission at week 22	90 mg SC	41.70%
			Placebo SC	27.40%
Feagan *et al*[60] UNITI-1 induction	245	Clinical response at week 6	130 mg IV	34.30%
	249		6 mg/kg IV	33.70%
	247		Placebo IV	21.50%
Feagan *et al*[60] UNITI-2 induction	209	Clinical response at week 6	130 mg IV	51.70%
	209		6 mg/kg IV	55.50%
	210		Placebo IV	28.70%
Feagan *et al*[60] IM- UNITI maintenance	132	Clinical remission at week 44	90 mg SC every 8 wk	53.10%
	132		90 mg SC every 12 wk	48.80%
	133		Placebo SC	35.90%
Sandborn *et al*[63] (IM- UNITI long term extension)	1281	Clinical remission at week 92	90 mg SC every 8 wk	74.40%
			90 mg SC every 12 wk	72.60%
			Subjects with prior dose adjustment	53.50%
			All ustekinumab treated	67.50%
Sands *et al*[62] (IM-UNITI patients with dose adjustment following loss of response)	51	Clinical response [CR-100][1]	Placebo to 90 mg SC ustekinumab every 8 wk	71%
	29		Ustekinumab 90 mg SC every 12 wk to ustekinumab 90 mg SC every 8 wk	55%
	28		No dose adjustment	46%
Sands *et al*[62] (IM-UNITI non-responders during induction phase having an additional SC dose)	467	Clinical response 8 wk after one additional dose	Additional dose of 90 mg SC	50.50%
		Clinical remission 8 wk after one additional dose		28.90%
Rutgeerts *et al*[61] Induction week 8	155	SES-CD Change from baseline, mean (SD)	130 mg IV/6 mg/kg	-2.8 (8.10)[a]
	97		Placebo IV	-0.7 (4.97)
Rutgeerts *et al*[61] Maintenance week 44	47	SES-CD Change from baseline, mean (SD)	90mg SC every 12 wk	-1.5 (4.22)
	74		90mg SC every 8 wk	-3.8 (6.02)
	51		Placebo SC	-2.0 (5.35)

[1]CR-100, ≥ 100-point decrease in Crohn's Disease Activity Index; [a]$P < 0.05$. SC: Subcutaneous; IV: Intravenous; SES-CD: Simplified endoscopic activity score for Crohn's disease; SD: Standard deviation.

with LOR, the dose adjustment of ustekinumab (12-wk interval to 8-wk interval) provided clinical benefits compared to patients who remained to the 8-wk interval. Moreover, patients who were initial non-responders to induction treatment benefited from continued treatment (at least 1 additional subcutaneous dose) following the initial intravenous dose (rescue therapy - late responders)[62].

Long-term efficacy and safety of ustekinumab

The long-term efficacy and safety of ustekinumab were evaluated in an ongoing IM-UNITI study with a duration of approximately 5 years[63]. The preliminary results through week 96 showed that the clinical response and remission were maintained in patients who were under treatment with subcutaneous ustekinumab. There was no difference in adverse events and infection rates between patients treated with ustekinumab or placebo from week 44 through week 96[63].

DISCUSSION

The introduction of monoclonal antibodies in the last decades has changed the therapeutic strategy of CD. The biological factors that have been approved to date for the management of CD include anti-TNF agents (adalimumab, certolizumab and infliximab), anti-integrins (natalizumab and vedolizumab) and the anti-IL-12/IL-23p40 agent (ustekinumab). The clinical benefits of monoclonal antibodies are the efficacy and safety during the induction and maintenance of clinical response as well as a decreased risk of hospitalization and surgery. Anti-TNF agents are currently positioned as first line biologic treatment for the management of

moderate to severe CD and have been proven to be effective for both induction and maintenance of CD patients[64]. However, these agents do not fully cover the needs of all patients as there is significant percentage who was not respond to treatment, or even if they achieved an initial short-term response, they could undergo secondary failure to anti-TNF agents or develop unacceptable adverse events, which lead to treatment discontinuation. Currently, for patients with primary failure to anti-TNF treatment, the use of a second TNF agonist is not indicated and a switch to another agent with a different mechanism of action is suggested[65,66]. The anti-$\alpha 4\beta 7$ integrin antibody was approved for induction and maintenance of CD patients, but due to its fairly slow-action, it is considered better for the maintenance phase.

Clinical evidence suggests that ustekinumab may be preferred over the anti-integrin treatment given its rapid onset of action[60]. In particular, the clinical benefits of ustekinumab over vedolizumab in inducing clinical response and remission have been shown in patients who were non-responders or were intolerant to anti-TNF treatment, since ustekinumab treated patients responded as early as week 3[60] compared to patients treated with vedolizumab who responded at week 10[67]. The results of the study IM-UNITI LTE have shown that the rapid onset of ustekinumab action is accompanied by a long duration of action, as 75% of patients in remission at year 1, were still in remission at year 2, indicating one more advantage over the anti-TNF or anti-integrins agents[63].

The route of ustekinumab administration could be considered as an important benefit over the other treatment options. During induction phase, only one intravenous dose of ustekinumab is required for the development of clinical response and during maintenance phase, a single subcutaneous dose is able to induce clinical response up to 44 wk in one-third of the patients[60]. These results highlight the usefulness of ustekinumab as a more convenient option with its potential for a home-based therapy. Moreover, a sub-study of the IM-UNITI programme has shown that ustekinumab dose adjustment can provide additional clinical advantage in patients with loss of response. The results demonstrated that initial non-responders to the induction therapy could benefit more from continued treatment with at least one additional dose[62].

The study by Fegan *et al*[60], has shown that the rapid onset of clinical efficacy was accompanied by a reduction in CRP and fecal calprotectin levels which persisted during the maintenance phase up to week 44. The improvement in CRP and fecal calprotectin levels following ustekinumab treatment suggests that decrease of inflammation occurred along with the clinical improvement[60]. In parallel, recent data has demonstrated the ability of ustekinumab to induce endoscopic healing during the induction phase (at week 8) in patients with moderate to severe CD[61].

Beyond the obvious benefits of ustekinumab in the management of moderate to severe CD, patient safety is an important factor of determining the risk/benefit ratio of each treatment option. Although the data from studies evaluating the long-term safety of ustekinumab in patients with moderate to severe psoriasis and multiple sclerosis suggests an increased risk of serious infections, in CD patients the drug seems to have a rather favorable safety profile[58-60,63], which is very important considering the role of IL-12/IL-23 in maintaining immune homeostasis[68-71].

Taking into consideration the above advantages, we can speculate the use of ustekinumab as a first-line treatment or its use in conjunction with or before other biological agents, following corticosteroid failure. Ustekinumab may be the ideal option for frail patients, considering its safety profile and the mode of administration. In addition, CD patients with other immune mediated diseases such as previous history of multiple sclerosis and psoriasis or patients with TNF agonist induced psoriasis represent promising candidates for ustekinumab treatment considering its systemic anti-inflammatory action[52-56,72,73]. On the other hand, the use of ustekinumab may not be favored in patients with perianal fistulizing CD in whom the use of infliximab is proposed[74]. Furthermore, there is limited evidence concerning the use of ustekinumab in pregnancy and breast feeding. Studies in animals have shown no developmental toxicity due to ustekinumab exposure[75]. In humans, there are two case reports of abortions in ustekinumab-exposed pregnancies[76,77] and two successful pregnancies after prolonged ustekinumab treatment[78,79].

CONCLUSION

Ustekinumab has exhibited considerable potential in the management of intestinal inflammation by downregulating the immune system through its binding in the common subunit of IL-12 and IL-23 and by blocking their action. In light of current evidence, ustekinumab is considered to be an effective drug with a favorable safety profile for the management of patients with moderate-to-severe active CD. Its use appears to be very promising in patients with an inadequate response, loss of response, intolerance or contraindications to treatment with traditional anti-TNF agents. Moreover, ustekinumab could be considered as first line biological treatment in patients who failed conventional therapy, although the high treatment cost poses severe limitations to this alternative. Large scale-multicentre trials with long term follow up and high-quality evidence are required to further explore the spot that this novel agent should hold in the CD treatment algorithms, and its role in specific disease phenotypes (such as fistulizing disease, early-onset

CD or postoperative setting). Lastly, conduct of head-to-head comparison studies of ustekinumab with other biological agents, evaluation of drug to drug interactions and pharmacoeconomic (cost effective) analysis of ustekinumab are the next steps towards thoroughly delineating the place of ustekinumab in clinical practice.

REFERENCES

1 **Freeman HJ**. Natural history and long-term clinical course of Crohn's disease. *World J Gastroenterol* 2014; **20**: 31-36 [PMID: 24415855 DOI: 10.3748/wjg.v20.i1.31]

2 **Cosnes J**, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785-1794 [PMID: 21530745 DOI: 10.1053/j.gastro.2011.01.055]

3 **Liu TC**, Stappenbeck TS. Genetics and Pathogenesis of Inflammatory Bowel Disease. *Annu Rev Pathol* 2016; **11**: 127-148 [PMID: 26907531 DOI: 10.1146/annurev-pathol-012615-044152]

4 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434 [PMID: 17653185 DOI: 10.1038/nature06005]

5 **Raad MA**, Chams NH, Sharara AI. New and Evolving Immunotherapy in Inflammatory Bowel Disease. *Inflamm Intest Dis* 2016; **1**: 85-95 [PMID: 29922662 DOI: 10.1159/000445986]

6 **Baumgart DC**, Sandborn WJ. Crohn's disease. *Lancet* 2012; **380**: 1590-1605 [PMID: 22914295 DOI: 10.1016/S0140-6736(12)60026-9]

7 **Feagan BG**, Rochon J, Fedorak RN, Irvine EJ, Wild G, Sutherland L, Steinhart AH, Greenberg GR, Gillies R, Hopkins M. Methotrexate for the treatment of Crohn's disease. The North American Crohn's Study Group Investigators. *N Engl J Med* 1995; **332**: 292-297 [PMID: 7816064 DOI: 10.1056/nejm199502023320503]

8 **Present DH**, Korelitz BI, Wisch N, Glass JL, Sachar DB, Pasternack BS. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. *N Engl J Med* 1980; **302**: 981-987 [PMID: 6102739 DOI: 10.1056/nejm198005013021801]

9 **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65 [PMID: 17241859 DOI: 10.1053/j.gastro.2006.11.041]

10 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P; ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549 [PMID: 12047962 DOI: 10.1016/s0140-6736(02)08512-4]

11 **Sandborn WJ**, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S; PRECISE 1 Study Investigators. Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 2007; **357**: 228-238 [PMID: 17634458 DOI: 10.1056/NEJMoa067594]

12 **Sandborn WJ**, Feagan BG, Rutgeerts P, Hanauer S, Colombel JF, Sands BE, Lukas M, Fedorak RN, Lee S, Bressler B, Fox I, Rosario M, Sankoh S, Xu J, Stephens K, Milch C, Parikh A; GEMINI 2 Study Group. Vedolizumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2013; **369**: 711-721 [PMID: 23964933 DOI: 10.1056/NEJMoa1215739]

13 **Vermeire S**, Loftus EV Jr, Colombel JF, Feagan BG, Sandborn WJ, Sands BE, Danese S, D'Haens GR, Kaser A, Panaccione R, Rubin DT, Shafran I, McAuliffe M, Kaviya A, Sankoh S, Mody R, Abhyankar B, Smyth M. Long-term Efficacy of Vedolizumab for Crohn's Disease. *J Crohns Colitis* 2017; **11**: 412-424 [PMID: 27683798 DOI: 10.1093/ecco-jcc/jjw176]

14 **Hazlewood GS**, Rezaie A, Borman M, Panaccione R, Ghosh S, Seow CH, Kuenzig E, Tomlinson G, Siegel CA, Melmed GY, Kaplan GG. Comparative effectiveness of immunosuppressants and biologics for inducing and maintaining remission in Crohn's disease: a network meta-analysis. *Gastroenterology* 2015; **148**: 344-354.e5; quiz e14-e15 [PMID: 25448924 DOI: 10.1053/j.gastro.2014.10.011]

15 **Danese S**, Fiorino G, Reinisch W. Review article: Causative factors and the clinical management of patients with Crohn's disease who lose response to anti-TNF-α therapy. *Aliment Pharmacol Ther* 2011; **34**: 1-10 [PMID: 21539588 DOI: 10.1111/j.1365-2036.2011.04679.x]

16 **Ding NS**, Hart A, De Cruz P. Systematic review: predicting and optimising response to anti-TNF therapy in Crohn's disease - algorithm for practical management. *Aliment Pharmacol Ther* 2016; **43**: 30-51 [PMID: 26515897 DOI: 10.1111/apt.13445]

17 **Pagnini C**, Arseneau KO, Cominelli F. Natalizumab in the treatment of Crohn's disease patients. *Expert Opin Biol Ther* 2017; **17**: 1433-1438 [PMID: 28832222 DOI: 10.1080/14712598.2017.1366444]

18 **Kaser A**, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol* 2010; **28**: 573-621 [PMID: 20192811 DOI: 10.1146/annurev-immunol-030409-101225]

19 **Weaver CT**, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007; **25**: 821-852 [PMID: 17201677 DOI: 10.1146/annurev.immunol.25.022106.141557]

20 **Neurath MF**, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A, Mizoguchi E, Mudter J, Galle PR, Bhan A, Autschbach F, Sullivan BM, Szabo SJ, Glimcher LH, Blumberg RS. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 2002; **195**: 1129-1143 [PMID: 11994418 DOI: 10.1084/jem.20011956]

21 **Fujino S**, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 2003; **52**: 65-70 [PMID: 12477762]

22 **Bettelli E**, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 2007; **8**: 345-350 [PMID: 17375096 DOI: 10.1038/ni0407-345]

23 **Monteleone G**, Biancone L, Marasco R, Morrone G, Marasco O, Luzza F, Pallone F. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 1997; **112**: 1169-1178 [PMID: 9098000 DOI: 10.1016/S0016-5085(97)70128-8]

24 **Parronchi P**, Romagnani P, Annunziato F, Sampognaro S, Becchio A, Giannarini L, Maggi E, Pupilli C, Tonelli F, Romagnani S. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol* 1997; **150**: 823-832 [PMID: 9060820]

25 **Gee K**, Guzzo C, Che Mat NF, Ma W, Kumar A. The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflamm Allergy Drug Targets* 2009; **8**: 40-52 [PMID: 19275692 DOI: 10.2174/187152809787582507]

26 **Trinchieri G**. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 1994; **84**: 4008-4027 [PMID: 7994020 DOI: 10.1017/CBO9781107415324.004]

27 **Trinchieri G**. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; **3**: 133-146 [PMID: 12563297 DOI: 10.1038/nri1001]

28 **Parrello T**, Monteleone G, Cucchiara S, Monteleone I, Sebkova L, Doldo P, Luzza F, Pallone F. Up-regulation of the IL-12 receptor beta 2 chain in Crohn's disease. *J Immunol* 2000; **165**: 7234-7239 [PMID: 11120856 DOI: 10.4049/jimmunol.165.12.7234]

29 **Monteleone G**, MacDonald TT, Wathen NC, Pallone F, Pender SL. Enhancing Lamina propria Th1 cell responses with interleukin 12

produces severe tissue injury. *Gastroenterology* 1999; **117**: 1069-1077 [PMID: 10535869 DOI: 10.1016/S0016-5085(99)70391-4]
30 **Bernink JH**, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, Hreggvidsdottir HS, Heinsbroek SE, Legrand N, Buskens CJ, Bemelman WA, Mjösberg JM, Spits H. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol* 2013; **14**: 221-229 [PMID: 23334791 DOI: 10.1038/ni.2534]
31 **Hunter CA**. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 2005; **5**: 521-531 [PMID: 15999093 DOI: 10.1038/nri1648]
32 **Vignali DA**, Kuchroo VK. IL-12 family cytokines: immunological playmakers. *Nat Immunol* 2012; **13**: 722-728 [PMID: 22814351 DOI: 10.1038/ni.2366]
33 **Lyakh L**, Trinchieri G, Provezza L, Carra G, Gerosa F. Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. *Immunol Rev* 2008; **226**: 112-131 [PMID: 19161420 DOI: 10.1111/j.1600-065X.2008.00700.x]
34 **O'Shea JJ**, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* 2010; **327**: 1098-1102 [PMID: 20185720 DOI: 10.1126/science.1178334]
35 **Martinez FO**, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 2009; **27**: 451-483 [PMID: 19105661 DOI: 10.1146/annurev.immunol.021908.132532]
36 **Iwakura Y**, Ishigame H. The IL-23/IL-17 axis in inflammation. *J Clin Invest* 2006; **116**: 1218-1222 [PMID: 16670765 DOI: 10.1172/jci28508]
37 **Geremia A**, Arancibia-Cárcamo CV, Fleming MP, Rust N, Singh B, Mortensen NJ, Travis SP, Powrie F. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med* 2011; **208**: 1127-1133 [PMID: 21576383 DOI: 10.1084/jem.20101712]
38 **Vanden Eijnden S**, Goriely S, De Wit D, Willems F, Goldman M. IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human. *Eur J Immunol* 2005; **35**: 469-475 [PMID: 15682457 DOI: 10.1002/eji.200425677]
39 **van de Wetering D**, de Paus RA, van Dissel JT, van de Vosse E. IL-23 modulates CD56+/CD3- NK cell and CD56+/CD3+ NK-like T cell function differentially from IL-12. *Int Immunol* 2009; **21**: 145-153 [PMID: 19088061 DOI: 10.1093/intimm/dxn132]
40 **Ness-Schwickerath KJ**, Jin C, Morita CT. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *J Immunol* 2010; **184**: 7268-7280 [PMID: 20483730 DOI: 10.4049/jimmunol.1000600]
41 **Hue S**, Ahern P, Buonocore S, Kullberg MC, Cua DJ, McKenzie BS, Powrie F, Maloy KJ. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 2006; **203**: 2473-2483 [PMID: 17030949 DOI: 10.1084/jem.20061099]
42 **Buonocore S**, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, Powrie F. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* 2010; **464**: 1371-1375 [PMID: 20393462 DOI: 10.1038/nature08949]
43 **Duerr RH**, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A, Dassopoulos T, Bitton A, Yang H, Targan S, Datta LW, Kistner EO, Schumm LP, Lee AT, Gregersen PK, Barmada MM, Rotter JI, Nicolae DL, Cho JH. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 2006; **314**: 1461-1463 [PMID: 17068223 DOI: 10.1126/science.1135245]
44 **Pidasheva S**, Trifari S, Phillips A, Hackney JA, Ma Y, Smith A, Sohn SJ, Spits H, Little RD, Behrens TW, Honigberg L, Ghilardi N, Clark HF. Functional studies on the IBD susceptibility gene IL23R implicate reduced receptor function in the protective genetic variant R381Q. *PLoS One* 2011; **6**: e25038 [PMID: 22022372 DOI: 10.1371/journal.pone.0025038]
45 **Van Limbergen J**, Wilson DC, Satsangi J. The genetics of Crohn's disease. *Annu Rev Genomics Hum Genet* 2009; **10**: 89-116 [PMID: 19453248 DOI: 10.1146/annurev-genom-082908-150013]
46 **Benson JM**, Peritt D, Scallon BJ, Heavner GA, Shealy DJ, Giles-Komar JM, Mascelli MA. Discovery and mechanism of ustekinumab: a human monoclonal antibody targeting interleukin-12 and interleukin-23 for treatment of immune-mediated disorders. *MAbs* 2011; **3**: 535-545 [PMID: 22123062 DOI: 10.4161/mabs.3.6.17815]
47 **Panaccione R**, Sandborn WJ, Gordon GL, Lee SD, Safdi A, Sedghi S, Feagan BG, Hanauer S, Reinisch W, Valentine JF, Huang B, Carcereri R. Briakinumab for treatment of Crohn's disease: results of a randomized trial. *Inflamm Bowel Dis* 2015; **21**: 1329-1340 [PMID: 25989338 DOI: 10.1097/MIB.0000000000000366]
48 **Sands BE**, Chen J, Feagan BG, Penney M, Rees WA, Danese S, Higgins PDR, Newbold P, Faggioni R, Patra K, Li J, Klekotka P, Morehouse C, Pulkstenis E, Drappa J, van der Merwe R, Gasser RA Jr. Efficacy and Safety of MEDI2070, an Antibody Against Interleukin 23, in Patients With Moderate to Severe Crohn's Disease: A Phase 2a Study. *Gastroenterology* 2017; **153**: 77-86.e6 [PMID: 28390867 DOI: 10.1053/j.gastro.2017.03.049]
49 **Feagan BG**, Sandborn WJ, D'Haens G, Panés J, Kaser A, Ferrante M, Louis E, Franchimont D, Dewit O, Seidler U, Kim KJ, Neurath MF, Schreiber S, Scholl P, Pamulapati C, Lalovic B, Visvanathan S, Padula SJ, Herichova I, Soaita A, Hall DB, Böcher WO. Induction therapy with the selective interleukin-23 inhibitor risankizumab in patients with moderate-to-severe Crohn's disease: a randomised, double-blind, placebo-controlled phase 2 study. *Lancet* 2017; **389**: 1699-1709 [PMID: 28411872 DOI: 10.1016/S0140-6736(17)30570-6]
50 FDA approves STELARA® (Ustekinumab) for treatment of moderate to severe Crohn's disease. New York, CCFA, 2016. Available from: URL: http://www.ccfa.org/news/Stelara.html
51 European Commission approves Stelara® (Ustekinumab) for treatment of adults with moderately to severely active Crohn's disease. Beerse, Belgium: Johnson Johnson Media Center, 2016. Available from: URL: https://www.jnj.com/media-center/press-releases/european-commission-approves-stelara-ustekinumab-for-treatment-of-adults-with-moderately-to-severely-active-crohns-disease
52 **Leonardi CL**, Kimball AB, Papp KA, Yeilding N, Guzzo C, Wang Y, Li S, Dooley LT, Gordon KB; PHOENIX 1 study investigators. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). *Lancet* 2008; **371**: 1665-1674 [PMID: 18486739 DOI: 10.1016/S0140-6736(08)60725-4]
53 **Papp KA**, Langley RG, Lebwohl M, Krueger GG, Szapary P, Yeilding N, Guzzo C, Hsu MC, Wang Y, Li S, Dooley LT, Reich K; PHOENIX 2 study investigators. Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). *Lancet* 2008; **371**: 1675-1684 [PMID: 18486740 DOI: 10.1016/S0140-6736(08)60726-6]
54 **Gottlieb A**, Menter A, Mendelsohn A, Shen YK, Li S, Guzzo C, Fretzin S, Kunynetz R, Kavanaugh A. Ustekinumab, a human interleukin 12/23 monoclonal antibody, for psoriatic arthritis: randomised, double-blind, placebo-controlled, crossover trial. *Lancet* 2009; **373**: 633-640 [PMID: 19217154 DOI: 10.1016/S0140-6736(09)60140-9]
55 **McInnes IB**, Kavanaugh A, Gottlieb AB, Puig L, Rahman P, Ritchlin C, Brodmerkel C, Li S, Wang Y, Mendelsohn AM, Doyle MK; PSUMMIT 1 Study Group. Efficacy and safety of ustekinumab in patients with active psoriatic arthritis: 1 year results of the phase 3, multicentre, double-blind, placebo-controlled PSUMMIT 1 trial. *Lancet* 2013; **382**: 780-789 [PMID: 23769296 DOI: 10.1016/S0140-6736(13)60594-2]
56 **Ritchlin C**, Rahman P, Kavanaugh A, McInnes IB, Puig L, Li S, Wang Y, Shen YK, Doyle MK, Mendelsohn AM, Gottlieb AB; PSUMMIT 2 Study Group. Efficacy and safety of the anti-IL-12/23 p40 monoclonal antibody, ustekinumab, in

patients with active psoriatic arthritis despite conventional non-biological and biological anti-tumour necrosis factor therapy: 6-mo and 1-year results of the phase 3, multicentre, double-blind, placebo-controlled, randomised PSUMMIT 2 trial. *Ann Rheum Dis* 2014; **73**: 990-999 [PMID: 24482301 DOI: 10.1136/annrheumdis-2013-204655]

57 **Segal BM**, Constantinescu CS, Raychaudhuri A, Kim L, Fidelus-Gort R, Kasper LH; Ustekinumab MS Investigators. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol* 2008; **7**: 796-804 [PMID: 18703004 DOI: 10.1016/S1474-4422(08)70173-X]

58 **Sandborn WJ**, Feagan BG, Fedorak RN, Scherl E, Fleisher MR, Katz S, Johanns J, Blank M, Rutgeerts P; Ustekinumab Crohn's Disease Study Group. A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology* 2008; **135**: 1130-1141 [PMID: 18706417 DOI: 10.1053/j.gastro.2008.07.014]

59 **Sandborn WJ**, Gasink C, Gao LL, Blank MA, Johanns J, Guzzo C, Sands BE, Hanauer SB, Targan S, Rutgeerts P, Ghosh S, de Villiers WJ, Panaccione R, Greenberg G, Schreiber S, Lichtiger S, Feagan BG; CERTIFI Study Group. Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med* 2012; **367**: 1519-1528 [PMID: 23075178 DOI: 10.1056/NEJMoa1203572]

60 **Feagan BG**, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, Blank MA, Johanns J, Gao LL, Miao Y, Adedokun OJ, Sands BE, Hanauer SB, Vermeire S, Targan S, Ghosh S, de Villiers WJ, Colombel JF, Tulassay Z, Seidler U, Salzberg BA, Desreumaux P, Lee SD, Loftus EV Jr, Dieleman LA, Katz S, Rutgeerts P; UNITI–IM-UNITI Study Group. Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. *N Engl J Med* 2016; **375**: 1946-1960 [PMID: 27959607 DOI: 10.1056/NEJMoa1602773]

61 **Rutgeerts P**, Gasink C, Chan D, Lang Y, Pollack P, Colombel JF, Wolf DC, Jacobstein D, Johanns J, Szapary P, Adedokun OJ, Feagan BG, Sandborn WJ. Efficacy of Ustekinumab in Inducing Endoscopic Healing in Patients with Crohn's Disease. *Gastroenterology* 2018 [PMID: 29909019 DOI: 10.1053/j.gastro.2018.06.035]

62 **Sands B**, Gasink C, Jacobstein D, Gao L, Johanns J, Szapary P, Colombel J, Targan S, Ghosh S, Sandborn W. A85 Efficacy and safety of dose adjustment and delayed response to ustekinumab in moderate-severe Crohn's disease patients: results from the IM-UNIT maintenance study. *Can J Gastroenterol Hepato*l 2018; **1** Suppl 1: 147-148

63 **Sandborn W**, Rutgeerts P, Gasink C, Jacobstein D, Gao L-L, Johanns J, Sands B, Hanauer S, Targan S, Ghosh S. OP010 Long term efficacy and safety of Ustekinumab for Crohn's disease: results from IM-UNITI long-term extension through 2 years. *J Crohns Colitis* 2017; **11** Suppl 1: S6-S6

64 **Terdiman JP**, Gruss CB, Heidelbaugh JJ, Sultan S, Falck-Ytter YT; AGA Institute Clinical Practice and Quality Management Committee. American Gastroenterological Association Institute guideline on the use of thiopurines, methotrexate, and anti-TNF-α biologic drugs for the induction and maintenance of remission in inflammatory Crohn's disease. *Gastroenterology* 2013; **145**: 1459-1463 [PMID: 24267474 DOI: 10.1053/j.gastro.2013.10.047]

65 **Gisbert JP**, Marín AC, McNicholl AG, Chaparro M. Systematic review with meta-analysis: the efficacy of a second anti-TNF in patients with inflammatory bowel disease whose previous anti-TNF treatment has failed. *Aliment Pharmacol Ther* 2015; **41**: 613-623 [PMID: 25652884 DOI: 10.1111/apt.13083]

66 **Armuzzi A**, Gionchetti P, Daperno M, Danese S, Orlando A, Lia Scribano M, Vecchi M, Rizzello F; GIVI (Gruppo Italiano su Vedolizumab nelle IBD) Group. Expert consensus paper on the use of Vedolizumab for the management of patients with moderate-to-severe Inflammatory Bowel Disease. *Dig Liver Dis* 2016; **48**: 360-370 [PMID: 26821929 DOI: 10.1016/j.dld.2015.12.016]

67 **Sands BE**, Feagan BG, Rutgeerts P, Colombel JF, Sandborn WJ, Sy R, D'Haens G, Ben-Horin S, Xu J, Rosario M, Fox I, Parikh A, Milch C, Hanauer S. Effects of vedolizumab induction therapy for patients with Crohn's disease in whom tumor necrosis factor antagonist treatment failed. *Gastroenterology* 2014; **147**: 618-627.e3 [PMID: 24859203 DOI: 10.1053/j.gastro.2014.05.008]

68 **Dávila-Seijo P**, Dauden E, Descalzo MA, Carretero G, Carrascosa JM, Vanaclocha F, Gómez-García FJ, De la Cueva-Dobao P, Herrera-Ceballos E, Belinchón I, López-Estebaranz JL, Alsina M, Sánchez-Carazo JL, Ferrán M, Torrado R, Ferrandiz C, Rivera R, Llamas M, Jiménez-Puya R, García-Doval I; BIOBADADERM Study Group. Infections in Moderate to Severe Psoriasis Patients Treated with Biological Drugs Compared to Classic Systemic Drugs: Findings from the BIOBADADERM Registry. *J Invest Dermatol* 2017; **137**: 313-321 [PMID: 27677836 DOI: 10.1016/j.jid.2016.08.034]

69 **Fiorentino D**, Ho V, Lebwohl MG, Leite L, Hopkins L, Galindo C, Goyal K, Langholff W, Fakharzadeh S, Srivastava B, Langley RG. Risk of malignancy with systemic psoriasis treatment in the Psoriasis Longitudinal Assessment Registry. *J Am Acad Dermatol* 2017; **77**: 845-854.e5 [PMID: 28893407 DOI: 10.1016/j.jaad.2017.07.013]

70 **Papp K**, Gottlieb AB, Naldi L, Pariser D, Ho V, Goyal K, Fakharzadeh S, Chevrier M, Calabro S, Langholff W, Krueger G. Safety Surveillance for Ustekinumab and Other Psoriasis Treatments From the Psoriasis Longitudinal Assessment and Registry (PSOLAR). *J Drugs Dermatol* 2015; **14**: 706-714 [PMID: 26151787]

71 **Singh S**, Fumery M, Sandborn WJ, Murad MH. Systematic review and network meta-analysis: first- and second-line biologic therapies for moderate-severe Crohn's disease. *Aliment Pharmacol Ther* 2018 [PMID: 29920733 DOI: 10.1111/apt.14852]

72 **Tillack C**, Ehmann LM, Friedrich M, Laubender RP, Papay P, Vogelsang H, Stallhofer J, Beigel F, Bedynek A, Wetzke M, Maier H, Koburger M, Wagner J, Glas J, Diegelmann J, Koglin S, Dombrowski Y, Schauber J, Wollenberg A, Brand S. Anti-TNF antibody-induced psoriasiform skin lesions in patients with inflammatory bowel disease are characterised by interferon-γ-expressing Th1 cells and IL-17A/IL-22-expressing Th17 cells and respond to anti-IL-12/IL-23 antibody treatment. *Gut* 2014; **63**: 567-577 [PMID: 23468464 DOI: 10.1136/gutjnl-2012-302853]

73 **Wils P**, Bouhnik Y, Michetti P, Flourie B, Brixi H, Bourrier A, Allez M, Duclos B, Grimaud JC, Buisson A, Amiot A, Fumery M, Roblin X, Peyrin-Biroulet L, Filippi J, Bouguen G, Abitbol V, Coffin B, Simon M, Laharie D, Pariente B; Groupe d'Etude Thérapeutique des Affections Inflammatoires du Tube Digestif. Subcutaneous Ustekinumab Provides Clinical Benefit for Two-Thirds of Patients With Crohn's Disease Refractory to Anti-Tumor Necrosis Factor Agents. *Clin Gastroenterol Hepatol* 2016; **14**: 242-50.e1-2 [PMID: 26432476 DOI: 10.1016/j.cgh.2015.09.018]

74 **Lee MJ**, Parker CE, Taylor SR, Guizzetti L, Feagan BG, Lobo AJ, Jairath V. Efficacy of Medical Therapies for Fistulizing Crohn's Disease: Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2018 [PMID: 29374617 DOI: 10.1016/j.cgh.2018.01.030]

75 **Martin PL**, Sachs C, Imai N, Tsusaki H, Oneda S, Jiao Q, Treacy G. Development in the cynomolgus macaque following administration of ustekinumab, a human anti-IL-12/23p40 monoclonal antibody, during pregnancy and lactation. *Birth Defects Res B Dev Reprod Toxicol* 2010; **89**: 351-363 [PMID: 20540088 DOI: 10.1002/bdrb.20250]

76 **Fotiadou C**, Lazaridou E, Sotiriou E, Ioannides D. Spontaneous abortion during ustekinumab therapy. *J Dermatol Case Rep* 2012; **6**: 105-107 [PMID: 23329988 DOI: 10.3315/jdcr.2012.1116]

77 **Venturin C**, Nancey S, Danion P, Uzzan M, Chauvenet M, Bergoin C, Roblin X, Flourié B, Boschetti G. Fetal death in utero and miscarriage in a patient with Crohn's disease under therapy with ustekinumab: case-report and review of the literature. *BMC Gastroenterol* 2017; **17**: 80 [PMID: 28629323 DOI: 10.1186/s12876-017-0633-6]

78 **Cortes X**, Borrás-Blasco J, Antequera B, Fernandez-Martinez S, Casterá E, Martin S, Molés JR. Ustekinumab therapy for Crohn's disease during pregnancy: a case report and review of the literature. *J Clin Pharm Ther* 2017; **42**: 234-236 [PMID: 28004853 DOI: 10.1111/jcpt.12492]

79 **Galli-Novak E**, Mook SC, Büning J, Schmidt E, Zillikens D, Thaci D, Ludwig RJ. Successful pregnancy outcome under prolonged ustekinumab treatment in a patient with Crohn's disease and paradoxical psoriasis. *J Eur Acad Dermatol Venereol* 2016; **30**: e191-e192 [PMID: 26559393 DOI: 10.1111/jdv.13499]

P- Reviewer: Gassler N, Naito Y **S- Editor**: Wang XJ **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4104-4118

DOI: 10.3748/wjg.v24.i36.4104

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

REVIEW

Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease

Jorge-Luis Torres, Ignacio Novo-Veleiro, Laura Manzanedo, Lucía Alvela-Suárez, Ronald Macías, Francisco-Javier Laso, Miguel Marcos

Jorge-Luis Torres, Laura Manzanedo, Ronald Macías, Francisco-Javier Laso, Miguel Marcos, Department of Internal Medicine, University Hospital of Salamanca, Institute of Biomedical Research of Salamanca-IBSAL, Salamanca 37007, Spain

Francisco-Javier Laso, Miguel Marcos, Department of Medicine, Faculty of Medicine, University of Salamanca, Salamanca 37007, Spain

Ignacio Novo-Veleiro, Department of Internal Medicine, University Hospital of Santiago de Compostela, A Coruña 15706, Spain

Lucía Alvela-Suárez, Department of Internal Medicine, HM Rosaleda Hospital, Santiago de Compostela, A Coruña 15701, Spain

Jorge-Luis Torres, Ignacio Novo-Veleiro, Francisco-Javier Laso, Miguel Marcos, Spanish Working Group on Alcohol and Alcoholism, Spanish Society of Internal Medicine, Madrid 28016, Spain

ORCID number: Jorge-Luis Torres (0000-0001-6853-9115); Ignacio Novo-Veleiro (0000-0003-0948-2440); Laura Manzanedo (0000-0001-8394-5218); Lucía Alvela-Suárez (0000-0001-6106-2174); Ronald Macías (0000-0001-8734-7872); Francisco-Javier Laso (0000-0003-0945-5186); Miguel Marcos (0000-0003-1269-4487).

Author contributions: Torres JL and Novo-Veleiro I contributed equally as first authors of this manuscript; Torres JL, Novo-Veleiro I, Manzanedo L, Alvela-Suárez L, Macías R and Marcos M carried out the literature research and drafted the manuscript; Torres JL, Novo-Veleiro I, Laso FJ and Marcos M contributed to manuscript conception and design; all authors approved the final version of the manuscript.

Supported by the Spanish Ministry of Science and Innovation, Instituto de Salud Carlos III and the European Union FEDER funds, Una manera de hacer Europa, (No. PI16/01548 to Miguel Marcos and No. RD16/0017/0023 to Francisco-Javier Laso) and Junta de Castilla y León GRS 1362/A/16 and INT/M/17/17 to Miguel Marcos and GRS 1587/A/17 to Jorge-Luis Torres).

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Miguel Marcos, MD, PhD, Associate Professor, Staff Physician, Department of Internal Medicine, University Hospital of Salamanca, Paseo de San Vicente 182, Salamanca 37007, Spain. mmarcos@usal.es
Telephone: +34-923-291100-55437
Fax: +34-923-294739

Received: April 14, 2018
Peer-review started: April 14, 2018
First decision: May 21, 2018
Revised: June 25, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: September 28, 2018

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate multiple physiological and pathological functions through the modulation of gene expression at the post-transcriptional level. Accumulating evidence has established a role for miRNAs in the development and pathogenesis of liver disease. Specifically, a large number of studies have assessed the role of miRNAs

in alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), two diseases that share common underlying mechanisms and pathological characteristics. The purpose of the current review is to summarize and update the body of literature investigating the role of miRNAs in liver disease. In addition, the potential use of miRNAs as biomarkers and/or therapeutic targets is discussed. Among all miRNAs analyzed, miR-34a, miR-122 and miR-155 are most involved in the pathogenesis of NAFLD. Of note, these three miRNAs have also been implicated in ALD, reinforcing a common disease mechanism between these two entities and the pleiotropic effects of specific miRNAs. Currently, no single miRNA or panel of miRNAs has been identified for the detection of, or staging of ALD or NAFLD. While promising results have been shown in murine models, no therapeutic based-miRNA agents have been developed for use in humans with liver disease.

Key words: Alcohol use disorder; Alcoholic liver disease; Non-alcoholic fatty liver disease; Steatosis; Obesity; miRNA; Biomarkers

Core tip: MicroRNAs (miRNAs) are small RNAs that regulate gene expression at a post-transcriptional level. Altered miRNA expression has been found in a variety of liver diseases, including non-alcoholic fatty liver disease and alcoholic liver disease. A group of miRNAs (miR-155, miR-122 and miR-34a) contributes to the pathogenesis of these two diseases and these miRNAs have potential use as biomarkers or therapeutic targets. Several technical limitations and a lack of clinical studies, however, preclude their clinical use.

Torres JL, Novo-Veleiro I, Manzanedo L, Alvela-Suárez L, Macías R, Laso FJ, Marcos M. Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease. *World J Gastroenterol* 2018; 24(36): 4104-4118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4104.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4104

INTRODUCTION

MicroRNAs (miRNAs), small non-coding RNAs, can modulate gene expression at the post-transcriptional level by targeting messenger RNAs and inhibiting their translation or promoting their degradation[1,2]. Since the discovery of the first miRNA in 1993, lin-4[3], more than 2000 miRNAs have been described in humans and they are believed to regulate up to 60% of protein-coding genes in the human genome[4].

Human miRNAs are involved in virtually all physiological and pathological processes, including cell differentiation and proliferation, signal transduction, inflammation and immune response, metabolism, viral-host interaction, and oncogenesis[1,2]. The expression of a wide variety of miRNAs is potentially regulated by many factors, such as alcohol, but also diet, cigarette smoking and other drugs[5]. Therefore, it is not surprising that miRNAs have been increasingly recognized as key actors in the pathogenesis of a variety of diseases and as potential biomarkers for diagnosis or therapeutic targets[2]. The role of miRNAs in liver inflammation, fibrosis and cirrhosis has been widely described in the last twenty years[6-8]. The current paper reviews the existing literature pertaining to miRNA alteration, function, and the potential clinical application of miRNAs in alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). While ALD and NAFLD differ in some aspects, they also share common features, including underlying mechanisms and clinical and histopathological characteristics[9]. Given the rapid expansion of research in miRNAs in recent years, an updated review on the topic will first be presented, followed by a summary of miRNA alterations that are common to both ALD and NAFLD.

ROLE OF MIRNAS IN ALD

Pathogenic role of miRNAs in ALD

The development of the different forms of ALD (steatosis, alcoholic hepatitis and cirrhosis) requires prolonged and heavy alcohol consumption along with susceptibility to the disease. Pathophysiological mechanisms of ALD are based both on the direct toxic effect of alcohol and also on ethanol-induced alterations in the inflammatory response[10]. A variety of enzymes, such as alcohol dehydrogenase (ADH) and the cytochrome P450 2E1 (CYP2E1), contribute to alcohol metabolism[11], leading to oxygen free radicals, nitric oxide and acetaldehyde, which ultimately can cause cellular damage and liver inflammation[12]. In addition, the toxic effect of acetaldehyde increases intestinal permeability to bacterial lipopolysaccharide (LPS)[13], which binds to toll-like receptors 4 (TLR4) and activates Kupffer and stellate cells through pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, production[14]. This inflammatory signal is transmitted *via* the nuclear factor-κB (NF-κB) pathway, ultimately leading to liver damage[14].

While most immune mechanisms involved in ALD development are related to the TLR4-NF-κB pathway, the activation of TLR4 also triggers the transmission of pro-inflammatory stimuli through other signaling pathways, such us mitogen-activated protein kinases (MAPK) or TIR-domain-containing adapter-inducing interferon-β (TRIF)[14]. miRNAs can regulate this complex interplay between inflammatory signals *via* the regulation of cytokines and other components of the pathways[15]. Oxidative stress and free oxygen radicals generation involved in ALD development are also regulated by miRNAs through different pathways like Kelch-like ECH-associated protein 1 Kelch-like ECH-associated protein 1 (Keap1) / Nuclear factor-erythroid-2-related factor 2 (Nrf2) pathway[16-20]. In addition to this, miRNAs have also

Table 1 MicroRNA targets involved in alcoholic liver disease pathogenesis

miRNA	Source of sample	miRNA target
let-7[27]	Animal models	Lin28, HMGA2
	Human HSCs	
miR-19b[28]	Animal models	TGFβRII, Col1α2, MeCP2
	Human HSCs	
miR-21[36,37]	Animal models	FASLG, DR5, Crebl2
miR-26a[35]	Animal models	DUSP4, DUSP5
miR-27a[44,52]	Animal models	Sprouty2, CD206
	HMC	
	Humans (plasma)	
miR-34a[29,43]	Animal models	SIRT1, CASP2
	Human HSCs	
	NHH	
	HiBECs	
	Humans (liver biopsy)	
miR-103 and miR-107[53]	Humans (liver biopsy)	Caveolin-1
miR-122[32,124,125]	Animal models	P4HA1, HO-1, Cyclin G1, Bcl-w, HIF-1α
miR-155[38,39,97,126,127]	Animal models	TNFα, SHIP1, SOCS1, IRAKM, C/EBPβ
miR-181b-3p[40]	Animal models	Importin α1
miR-182[30]	Animal models	SLC1A1, Cofilin 1, CCL20, CXCL1, IL-8, Cyclin D1, IL-6
	Humans (serum samples and liver biopsy)	
miR-199[128]	Animal models	ET-1, ET-BR
miR-200a[31]	Animal models	ZEB-2
miR-212[46]	Caco-2 cells	ZO-1
	Humans (colon biopsy)	
miR-214[24,34]	Animal models	POR, GSR, CYP2E1
	HHCs	
miR-217[41]	Animal models	SIRT-1
miR-223[45]	Animal models	$p47^{phox}$, IL-6
	Humans (serum)	
miR-291b[42]	Animal models	Tollip
	HPBMs	
miR-378[59]	Animal models	Gli-3
miR-497[25]	Animal models	Btg2, Yy1

HSCs: Hepatic stellate cells; HMGA2: High mobility group AT-hook 2; TGFβRII: Transforming growth factor β receptor II; Col1α2: Collagen type I α 2 chain; MeCP2: Methyl-CpG binding protein 2; FASLG: Fas ligand; DR5: Death receptor 5; Crebl2: cAMP responsive element binding protein like 2; DUSP: Dual specificity phosphatase; HMC: Human Monocyte Cells; NHH: Normal Human Hepatocytes; HiBECs: Human intrahepatic Biliary Epithelial Cells; SIRT1: sirtuin 1; CASP2: caspase 2; P4HA1: prolyl 4-hydroxylase subunit α 1; HO-1: heme oxygenase-1; BCL-W: Bcl-2-like protein 2; HIF-1α: Hypoxia inducible factor 1 α; TNFα: Tumor necrosis factor α; SHIP1: Src homology 2 domain-containing inositol phosphatase 1; SOCS1: Suppressor of cytokine signaling 1; IRAKM: Interleukin 1 receptor associated kinase 3; C/EBPβ: CCAAT/enhancer binding protein β; SLC1A1: Solute carrier family 1 member 1; CCL20: C-C motif chemokine ligand 20; CXCL1: C-X-C motif chemokine ligand 1; IL: Interleukin; ET-1: Endothelin-1; ET-BR: Endothelin-B receptor; ZEB-2: Zinc finger E-box binding homeobox 2; ZO-1: Zonula occludens 1; HHCs: Human Hepatoma Cells; POR: Cytochrome P450 oxidoreductase; GSR: Glutathione reductase; CYP2E1: Cytochrome P450 2E1; p47phox: Neutrophil cytosolic factor 1-like; HPBMs: Human Peripheral Blood Monocytes; Tollip: Toll interacting protein; Gli3: GLI Family Zinc Finger 3; Btg2: B-cell translocation gene 2; YY1: Yin yang 1; miRNA: MicroRNA.

been shown to exert an important modulatory function on macrophage activation and differentiation[21,22]. Moreover, recent studies have shown even broader effects of miRNAs in ALD development, including a role in intercellular communication, in secretion in exosomes[23], in the expression of enzymes directly linked to alcohol metabolism (*e.g.*, regulation of CYP2E1 by miR-214[24]) and in the modulation of pro-inflammatory pathways such as the B-cell translocation gene 2/Yin-yang 1 (BTG2/YY1) signaling pathway by miR-497[25]. Finally, alcohol consumption, with or without concurrent ALD, has also been linked to altered expression of several miRNAs[5,26].

Numerous studies, therefore, have addressed the relationship between ALD development and miRNAs. While animal models have been used in the majority of these studies, there is an increasing number of studies in human cells, tissues and serum, confirming the key role of miRNAs in ALD[27-30]. A summary of all available studies is shown in Table 1. In addition, a summary of the regulatory actions of miRNAs in the inflammatory response according to the different cell types involved, is displayed in Figure 1.

Hepatocytes: Some miRNAs (*e.g.*, miR-34a and miR-200a) are responsible for the induction of hepatocytic apoptosis during ALD development[29,31]. In addition, secretion of miRNAs in exosomes (*e.g.*, miR-122) can cause an increase in inflammatory response by targeting monocyte/macrophage cells[32], ultimately leading to hepatocytic injury. MiRNAs action and pleiotropic effects could be different depending on the cell in which they act; thus, miR-122 could have a protective role inside the hepatocyte during alcohol-induced liver damage[33]. Increase in oxidative stress

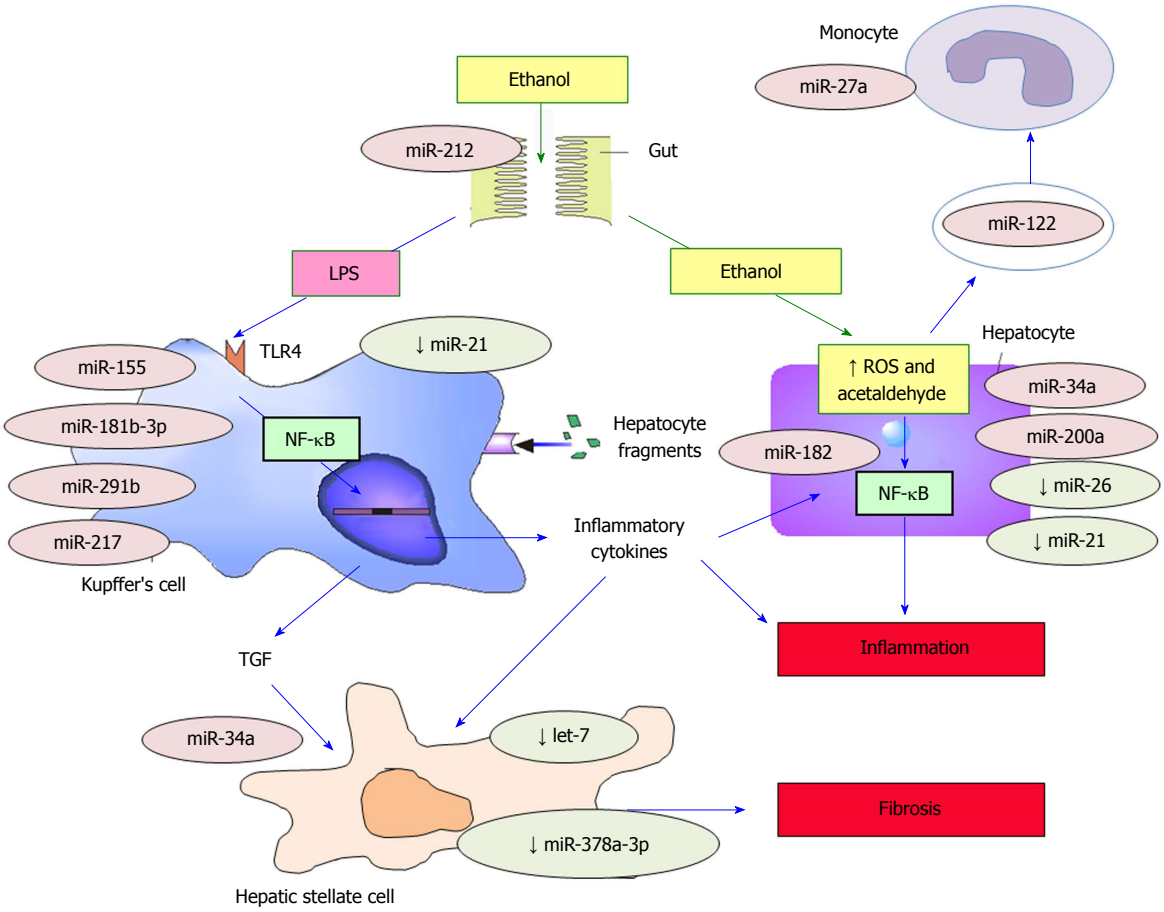


Figure 1 MicroRNAs involved in the pathogenesis of alcoholic liver disease. miRNAs preceded by a ↓ symbol are decreased in ALD or inhibit the development of ALD. The remainder of miRNAs promotes the development of ALD. TLR4: Toll-like receptor 4; TFG: Transforming growth factor; ALD: Alcoholic liver disease; ROS: Reactive oxygen species; NF-κB: Nuclear factor-κB. Figure adapted from Laso *et al*[10].

and alterations of enzymatic function in hepatocytes are also regulated by miRNAs[24,34]. Conversely, miRNAs may also have a protective role in ALD. For example miR-26a can increase autophagy[35] and miR-21 can inhibit alcohol-induced apoptosis[36,37].

Kupffer cells (KCs): miR-155, which is increased by chronic alcohol consumption through NF-κB induction, has been shown to be the main regulator of KC activation and function[38]. miR-155 inhibits the expression of multiple TLR4/NF-κB inhibitory regulators such as Src homology 2 domain-containing inositol phosphatase 1 (SHIP1) and Suppressor of cytokine signaling 1 (SOCS1)[38,39] leading to an increase in KC response to LPS and ultimately the development of liver fibrosis[39]. The Keap1/Nrf2 pathway could also be involved in miR-155 role in ALD development and KCs regulation[17]. Other miRNAs, such as miR-181b-3p, are also linked to increased LPS-sensitivity through the TLR4-NF-κB pathway[40]. In addition, miRNAs have been shown to regulate Sirtuin-1-Lipin-1, an inflammatory response mediator, leading to the down-regulation of the NF-κB pathway *via* de-acetylation. Alcohol consumption increases miR-217 expression, which in turn down-regulates sirtuin-1-Lipin-1[41], consequently leading to more hepatic inflammation[41]. Toll Interacting Protein (Tollip), another down-regulator of the TLR4-NF-κB pathway, is inhibited by miR-291b[42].

Hepatic stellate cells (HSCs): HSCs, responsible for the development of liver fibrosis, are regulated by several miRNAs, including let-7. The downregulation of let-7 by LPS and alcohol use causes an increase in HSCs activation[27]. In addition, chronic alcohol consumption has been linked to an overexpression of miR-34a, which increases the expression of proteins such as transforming growth factor-β1 (TFG-β1), leading to a higher survival of HSCs through apoptosis inhibition[43].

Other cell types: In addition to the cell types described above, other cells involved in ALD development, such as circulating monocytes (by miR-27a[44]), and circulating neutrophils, (by miR-223[45]) are regulated by miRNAs. In addition, miR-212 has been shown to increase permeability to LPS by altering cells of the intestinal mucosa[46].

Due to the role of miRNAs in ALD and the modulatory effects of alcohol consumption on miRNA expression, it is plausible to hypothesize that genetic variations in certain miRNAs may lead to altered miRNA function and

an increased risk of liver damage. Consequently, we and others have analyzed the relationship of alcohol-related diseases and polymorphisms within miRNA genes or miRNA targets[47,48]. Interestingly, the miR-146a C>G rs2910164 variant is linked to a susceptibility to alcohol use disorder[47] and the pre-miR-27a A>G rs895819 polymorphism is linked to a higher alcohol intake[49], suggesting a potential relationship between these genetic variants and alcohol-related diseases. The lack of replication studies precludes any conclusions regarding these SNPs, and to date, only rs738409 polymorphism within the *PNPLA3* gene is clearly linked to a higher susceptibility to ALD[50].

miRNAs as a target for diagnosis and treatment of ALD

The clinical use of miRNAs as a diagnostic tool or therapeutic agent in ALD has not been well studied[51]. However, over the last years, an increasing number of miRNAs have been proposed as potential biomarkers of ALD. The following is a review of the most promising results.

miR-192 and miR-30a: It has been shown that serum levels of miR-192 and miR-30a are significantly correlated with the diagnosis of alcoholic hepatitis. Therefore, these miRNAs may be useful in the diagnosis, staging, and monitoring of patients with this specific form of ALD[23].

miR-27a: miR-27a has been linked to monocyte differentiation and is increased in extracellular plasmatic vesicles of patients with alcoholic hepatitis, making it a potentially useful diagnostic tool[52].

miR-182: An elevated level of miR-182 has been linked to greater disease severity and liver injury in alcoholic hepatitis. The correlation between miR-182 and disease severity, however, has only been shown in liver biopsies, limiting its application as a diagnostic tool[30].

miR-103 and miR-107: A prior study found that miR-103 and miR-107 were increased in liver from patients with ALD and with NAFLD, but not in healthy livers or in subjects with viral hepatitis[53].

miR-155 and miR-122: Increased blood levels of miR-155[32,54] and miR-122[55] have been found in healthy individuals after binge drinking and in a murine model of liver damage. While these miRNAs could be potential biomarkers of alcohol intake or alcohol liver damage, they are increased in several types of liver disease and therefore are unlikely to be specific to ALD[54].

Therapeutic application of miRNAs in ALD

There are no studies to date supporting a therapeutic role for miRNAs in ALD. Available data, however, suggest a potential role for the inhibition or activation of some miRNAs in the treatment of liver disease. A recent study found that treatment with hyaluronic acid normalized miR-181b-3p and Importin α5 levels in ethanol-fed mice, protecting them from ethanol-induced liver and intestinal damage[40]. In addition, hyaluronic acid normalized the miR-291b/Tollip pathway, leading to a lower sensitization of monocytes/macrophages to ethanol-induced activation *via* TLR4[42]. While both studies were performed in animal models, taken together they suggest a potential role for hyaluronic acid as a therapeutic regulator of the KC response to ethanol *via* miRNA modulation.

The role of miR-155 in KC and miR-122 in hepatocytes suggest that these miRNAs may serve as potential targets for treatment of ALD. Miravirsen, an miR-122 inhibitor, has shown promising results in chronic hepatitis C treatment[56,57], suggesting its potential usefulness in ALD. A recent study showed that the restoration of miR-122 in hepatocytes could have a protective role against ALD development[33]. These apparently contradictory results could reflect the ability of miRNAs to develop different actions in different cells and also its relevance in inter-cellular communications[32]. In this sense, the therapeutic action of Miravirsen over viral replication could be explained by the interruption of these communications[57]. In addition, other potential therapeutic miRNAs currently under development for other diseases, such as cardiac fibrosis and remodeling or vascular disease[58], could serve as potential targets for ALD. There is indirect data that inhibition of miR-155, may lead to decreased sensitivity of KC to LPS-mediated activation[39].

In addition to the inhibition of detrimental miRNAs, stimulation of protective miRNAs could also serve as a potential therapeutic target. For example, miR-21, which aids hepatocyte regeneration[36]; miR26a, which protects hepatocytes from fibrosis development[35]; miR-223, which inhibits neutrophil activation and liver infiltration[45]; and miR-378, which exerts a stop-signaling action in HSC[59], are all potential targets for treatment. There are no clinical trials to date involving these miRNAs as therapeutic targets in ALD and further studies will be necessary before clinical application.

ROLE OF MIRNAS IN NAFLD

NAFLD is defined as the accumulation of fat in the liver in the absence of alcohol intake, viral infection or other specific causes of liver disease. NAFLD represents a spectrum of disorders ranging from the simple accumulation of triglycerides in hepatocytes (hepatic steatosis) to steatosis with inflammation [non-alcoholic steatohepatitis (NASH)], fibrosis and cirrhosis[60]. NAFLD and NASH have rapidly become the most common cause of chronic liver disease worldwide in recent decades. The prevalence of these diseases has been estimated between 25% to 45% of the general population[61] with a greater prevalence in patients with obesity, diabetes mellitus or metabolic syndrome, in which case, the

prevalence of NAFLD can reach 70% to 90%[62-64]. It is estimated that by 2020 cirrhosis related to NAFLD will be the first indication for liver transplantation[65].

Pathogenic role of miRNAs in NAFLD

The pathogenesis of NAFLD, along with the underlying mechanisms of progression from steatosis to steatohepatitis, has not been fully elucidated. Traditionally, the "two hit" theory[66] has been upheld. The "first hit", which includes insulin resistance leading to the accumulation of fat in the liver, is followed by a "second hit", consisting of the interaction of inflammatory cytokines, mitochondrial dysfunction and oxidative stress, leading to hepatocellular injury, inflammation and fibrosis[67]. However, more recently, multiple factors have been implicated in the pathogenesis of NAFLD, such that the "two hit" theory has been replaced by a "multiple-hit" hypothesis[68]. The "multiple-hit" theory includes the involvement of insulin resistance, adipose tissue dysfunction, mitochondrial dysfunction, endoplasmic reticulum stress, dietary factors, fatty acids, iron overload, inflammatory activation, LPS produced by gut microbiota, a chronic inflammatory state, and genetic and epigenetic factors in the pathogenesis and progression of NAFLD[68-70]. Accordingly, the following is a summary of the research implicating several miRNAs in the regulation of key targets in the development of NAFLD[8]. It is of special interest that recent studies have reported differences in miRNA expression between liver samples from patients with NAFLD and controls. Specifically, livers from patients with NAFLD express an upregulation of miR-31, miR-33a, miR-34a, miR-144, miR-146b, miR-150, miR-182, miR-183, miR-200a, miR-224, and miR-301a and a down regulation of miR-17, miR-122, miR-296, miR-373, miR-375 and miR-378c[71-76]. Among these miRNAs, miR-34a, miR-122, and miR-155 have been most often associated with the pathogenesis of NAFLD and as such, the following is a review of these miRNAs in detail. Table 2 displays a list of all miRNAs that have been associated with NAFLD through February 2018.

miR-122: miR-122 is the most abundant miRNA in the liver and plays a fundamental role in liver physiology[77-79] and lipid metabolism[80]. miR-122 interacts with multiple important lipogenic factors in human NAFLD, such as acetyl coA carboxylase-2 (ACC2) and the sterol regulatory element binding protein (SREBP)[71,81,82]. miR-122 is decreased in liver samples[83-85] but increased in serum[84,86,87] from patients with NAFLD compared to healthy controls. Despite this somewhat paradoxical finding, the association of miR-122 with NAFLD pathogenesis is well established. Inhibition of miR-122 in high-fat fed mice is associated with a significant reduction in hepatic steatosis and plasma cholesterol levels, which was associated with a reduction in hepatic sterol and fatty acid synthesis rates and stimulation of hepatic fatty-acid oxidation mediated by activation of adenosine 5'-monophosphate-activated protein kinase (AMPK)[80]. Moreover, the relationship of miR-122 with the development and progression of hepatic fibrosis has been demonstrated *in vitro*, through the regulation of HSC proliferation and production of collagen by targeting prolyl 4-hydroxylase subunit α-1 (P4HA1)[88].

miR-34a: miR-34a is overexpressed in both murine models of NAFLD (*e.g.*, mice fed a high-fat diet) and liver and serum from patients with NAFLD[81,87,89,90]. The main target of miR-34a is Sirtuin 1 (SIRT1), which regulates energy homeostasis by activating transcription factors such as peroxisome proliferator activated receptors (PPAR) α and liver X receptor (LXR). In addition, SIRT1 inhibits the co-activator 1α of the PPAR-γ (PGC1-α), the SREBP-1c and the farnesoid X receptor (FXR). SIRT1 is downregulated in the liver of NAFLD patients[91] and the inhibition of miR-34a restores the expression of SIRT1 and PPAR-α, leading to the activation of AMP-activated protein kinase (AMPK) and several target genes of PPAR-α. These findings suggest a fundamental role for miR-34a in the dysregulation of lipid metabolism associated with NAFLD[92].

miR-155: miR-155 is an important regulator of immune cells in both humans and mice and is involved in several inflammatory processes, such as rheumatic diseases[93], lipid metabolism[94] and in ALD (as described above). In patients with NAFLD, miR-155 is dysregulated by adipogenic transcription factors CCAAT/enhancer binding protein (C/EBP)-α, C/EBP-β, PPAR-γ and LXRα[95,96], fibrosis targets platelet derived growth factor (PDGF), Smad3 and C/EBP-β[97], and a tumor suppressor in the liver, SOCS-1[90,98]. However, animal models of NAFLD show contradictory results. For example, miR-155 deficient mice fed a high-fat diet showed a significant increase in hepatic steatosis[98], while miR-155 KO mice fed a methionine-choline-deficient diet showed a decrease in steatosis and expression of genes involved in fatty acid metabolism and fibrosis, with no concomitant liver injury or inflammation[97]. In addition, miR-155 may also be involved in hepatocarcinoma development[99]. These findings suggest that miR-155 may have different roles in fat storage and lipid accumulation in liver diseases and healthy subjects. However, additional research is warranted[97].

miRNAs as biomarkers in the diagnosis of NAFLD

As shown in Table 2, many miRNAs are differentially expressed in patients with NAFLD compared to healthy controls. These miRNAs may serve as potential biomarkers in the diagnosis and staging of NAFLD.

miR-122: Several studies have found that miR-122 is elevated in serum in NAFLD patients[81,86,100-102], even long before an alteration in transaminase levels occurs[103]. The diagnostic potential of miR-122 may extend to an indicator of disease severity and as a

Table 2 Summary of microRNAs associated with non-alcoholic fatty liver disease

miRNA	Source of samples	Change	Main targets
miR-9[129]	Human serum; Human hepatocyte cell line	Upregulated	Onecut2; SIRT1
miR-10b[130]	Human hepatocyte cell line	Downregulated	PPARα
miR-15b[131,132]	Animal models Human serum	Upregulated	
miR-16[104]	Human serum	Upregulated	
miR-17[74]	Human liver	Downregulated	
miR-19[84]	Human serum	Upregulated	
miR-21[86,87,99,133-136]	Animal models Human hepatocyte cell line Human liver and serum	Upregulated	PPARα; TGF-β PTEN
miR-21[85,89,137,138]	Animal models Human liver Human hepatocyte cell line	Downregulated	HMGCR; FABP7
miR-24[139]	Animal models Human hepatocyte cell line	Upregulated	Insig1; SREBP
miR-26[140]	Animal models	Downregulated	IL-6, IL-7
miR-27a[141]	Animal models	Downregulated	
miR-27b[102]	Human serum	Upregulated	
miR-29a[142,143]	Animal models	Downregulated	HMGCR; LPL
miR-29c[85,89,90]	Animal models	Downregulated	DNMT3A; DNMT3B
miR-30b[83]	Human liver Human hepatocyte cell line	Downregulated	ITGAX; FABP4
miR-30c[144]	Human serum	Upregulated	
miR-31[74,89]	Human liver Animal models	Upregulated	
miR-33a[73,76]	Human liver	Upregulated	ABCA1; ABCA2
miR-33a[85]	Human liver	Downregulated	
MiR-34a[71,81,82,85,87,89,90,92,104,105,145-148]	Animal models Human hepatocyte cell line Human liver and serum	Upregulated	SIRT1; HNF4α; PPARα
miR-99a[149]	Human serum	Downregulated	
miR-101[150]	Human hepatocyte cell line Human monocyte cell line	Upregulated	ABCA1
miR-103[53,89,151]	Animal models Human liver and serum	Upregulated	Cav1
miR-103a[152]	Human liver Human hepatocyte cell line	Upregulated	
miR-106b[152]	Human liver	Upregulated	
miR-107[53,89]	Animal models Human liver	Upregulated	Cav1
miR-122[81,84,86,87,101-104,106,153]	Animal models Human Serum	Upregulated	
miR-122[71,82-85,89,90,99,106,141,154,155]	Animal models Human liver	Downregulated	ACC-2; HAMP; FAS; HMGCR; SREBF-1c SREPBF-2; HIF-1α; Vimentin; MAP3K3
miR-125b[84]	Human serum	Upregulated	
miR-125b[156]	Animal models	Downregulated	FAS
miR-139-5p[83]	Human liver	Downregulated	TNFα
miR-144[76]	Human Liver	Upregulated	ABCA1
miR-144[157]	Animal models	Downregulated	TLR-2
miR-146a[158]	Animal models Human hepatocyte cell line	Upregulated	
miR-146a[132]	Animal models Human hepatocyte cell line	Downregulated	Wnt1; Wnt5
Mir-146b[149,159]	Animal models Human serum Human hepatocyte cell line	Downregulated	IRAK1 TRAF6
miR-146b[71,83,158]	Animal models Human liver Human hepatocyte cell line	Upregulated	
miR-149[160]	Animal models Human hepatocyte cell line	Upregulated	FGF-21
miR-150[74]	Human liver	Upregulated	
miR-152[158]	Animal models Human hepatocyte cell line	Upregulated	

miR-155[90,97-99,161,162]	Animal models	Upregulated	SOCS1; C/EBP-B; CES3;
	Human hepatocyte cell line		PDGF; SMAD3
miR-155[96]	Animal models	Downregulated	LXRα
	Human liver and serum		
miR-181a[82]	Animal models	Upregulated	
miR-181d[149]	Human serum	Downregulated	
miR-182[74]	Human liver	Upregulated	FOXO3
miR-183[74]	Human liver	Upregulated	
miR-192[84,90]	Animal models	Downregulated	
	Human liver		
miR-192-5p[82,84,86,102,106]	Animal models	Upregulated	
	Human liver and serum		
miR-194[89]	Animal models	Upregulated	
miR-197[149]	Human serum	Downregulated	
miR-199[163]	Animal models	Upregulated	Cav1; PPARα
	Human hepatocyte cell line		
	Human liver		
miR-200a/b/c[74,82,89,90,141,158,162,164]	Animal models	Upregulated	ZEB1; CDH1; EZH2; IRP1
	Human hepatocyte cell line		
miR-203[90,132]	Animal models	Downregulated	
miR-212[165]	Animal models	Upregulated	FGF-21
	Human hepatocyte cell line		
miR-214[71,166]	Human liver	Upregulated	
	Animal models		
miR-216[167]	Animal models	Downregulated	
miR-219a[74]	Human liver	Downregulated	
miR-221[73]	Human liver	Downregulated	
miR-221[89,90,99]	Animal models	Upregulated	
miR-222[99]	Animal models	Upregulated	
miR-223[86,164]	Animals models	Upregulated	IRP1
	Human serum		
miR-224[73,74]	Human liver	Upregulated	
miR-291b[168]	Animal models	Upregulated	AMPKα1
miR-302a[167]	Animals model	Downregulated	ELOVL6
miR-331[144]	Human serum	Upregulated	
miR-335[89]	Animal models	Upregulated	
miR-375[84]	Human serum	Upregulated	
miR-378i[74]	Human liver	Downregulated	
miR-421[169]	Animal models	Upregulated	SIRT-3
miR-422a[83]	Human liver	Downregulated	
miR-429[141]	Animal models	Upregulated	
miR-451[87]	Human Serum	Upregulated	
miR-451[89,141,170]	Animal models	Downregulated	AMPK/AKT
	Human liver		
miR-467b[171]	Animal models	Downregulated	LPL
miR-576[152]	Human liver	Downregulated	RAC1
	Human hepatocyte cell line		
miR-590[74]	Human liver	Downregulated	
miR-892a[152]	Human liver	Upregulated	
	Human hepatocyte cell line		
miR-1290[102]	Human serum	Upregulated	

Onecut2: One cut homeobox 2; SIRT: Sirtuin; PPARα: Peroxisome proliferator activated receptor α; TGF-β: Transforming growth factor β; PTEN: Phosphatase and tensin homolog; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; FABP: Fatty acid binding protein; Insig1: Insulin induced gene 1; SREBP: Sterol regulatory element binding protein; IL: Interleukin; LPL: Lipoprotein lipase; DNMT: DNA methyltransferase; ITGAX: Integrin subunit α X; ABCA: ATP binding cassette subfamily A; HNF4α: Hepatocyte nuclear factor 4 α; Cav1: Caveolin 1; ACC-2: Acetyl-CoA carboxylase 2; SREBF: Sterol regulatory element binding transcription factor; HIF-1α: Hypoxia inducible factor 1 α; MAP3K3: Mitogen-activated protein kinase kinase kinase 3; FAS: Fatty acid synthase; TNFα: Tumour necrosis factor α; TLR-2: Toll-like receptor 2; Wnt: Wnt family member; IRAK1: Interleukin 1 receptor associated kinase 1; TRAF6: TNF receptor associated factor 6; FGF-21: Fibroblast growth factor 21; SOCS1: Suppressor of cytokine signaling 1; C/EBPβ: CCAAT/enhancer binding protein β; CES3: Carboxylesterase 3; PDGF: Platelet derived growth factor; SMAD3: SMAD family member 3; LXRα: Liver X receptor; FOXO3: Forkhead box O3; ZEB-1: Zinc finger E-box binding homeobox 1; CDH1: Cadherin 1; EZH2: Enhancer of zeste 2 polycomb repressive complex 2; IRP1: Iron regulatory protein 1; AMPKα1: AMPK: Adenosine monophosphate activated protein kinase α 1; ELOVL6: ELOVL fatty acid elongase 6; AMPK: Adenosine monophosphate activated protein kinase; AKT: AKT serine/threonine kinase 1; RAC1: Ras-related C3 botulinum toxin substrate 1.

predictor of hepatic fibrosis[82,84,87,104].

miR-34a: Similar to miR-122, miR-34a has also been shown to have potential as a biomarker of diagnosis and severity of NAFLD. Several studies have shown that miR-34a is upregulated in the liver and serum of patients with NAFLD[71,81,82,104]. Additionally, elevated serum levels of miR-34a correlate with disease severity from simple

steatosis to steatohepatitis, with liver enzyme levels, with fibrosis stage and with inflammation activity[82,104,105].

miR-192: Serum miR-192 levels are positively correlated with the severity of NAFLD-specific liver pathomorphological changes in mice fed a choline and folate deficient diet[82] and miR-192 upregulation in human serum has been demonstrated[82,84,86,102,106]. Interestingly, serum levels of miR-122 and mir-192 have been shown to be strongly correlated[84,86].

Panels: In addition to individual miRNAs, a serum panel comprised of hsa-miR-122-5p, hsa-miR-1290, hsa-miR-27b-3p, and hsa-miR-192-5p has shown high NAFLD diagnostic accuracy, regardless of NAFLD activity score (NAS) status[102]. Another research group found that NAFLD was associated with an miRNA signature based on up-regulation of miR-122, miR-192, miR-19a, miR-19b, miR-125b, and miR-375[84].

It is important to mention that most studies have compared patients with NAFLD to healthy controls or patients with chronic viral hepatitis B[105] or C[104]. However, no comparisons have been performed, to our knowledge, between patients with NAFLD and patients with ALD.

Therapeutic application of miRNAs in NAFLD

As previously mentioned, miRNAs are involved in several stages of NAFLD development (from lipid metabolism or diabetes to liver inflammation), and are therefore potential therapeutic targets[7,107]. The expression of miR-103 and miR-107 is upregulated in obese mice[53,89]. Inactivation of miR-103/107 in murine adipocytes upregulates caveolin-1 (a critical mediator of the insulin receptor) leading to enhanced insulin signaling, decreased adipocyte size and enhanced insulin-stimulated glucose uptake[53,108]. An N-acetylgalactosamine (GalNAc)-conjugated anti-miR-103/107 (RG-125/AZD4076, Regulus Therapeutics) has been developed for the treatment of NAFLD and type 2 diabetes or pre-diabetes[108-110]. Currently, two clinical trials are registered using this drug in patients with NAFLD (ClinicalTrials.gov Identifier: NCT02826525 and NCT02612662), although Regulus has acknowledged that AstraZeneca intends to terminate the clinical development of RG-125/AZD4076[108,111].

miR-122 has also shown promising results as a treatment for NAFLD. There is a high concentration of miR-122 in liver tissue[112] and this miRNA plays an important role in liver development, differentiation, homeostasis and functioning[113]. Over-expression of miR-122 may affect the Ying Yan 1 and Farnesoid X Receptor (YY1-FXR-SHP) regulatory axis leading to a reduction in hepatic triglyceride levels, potentially serving as a target for NAFLD treatment[114]. miR-122 is also an essential host factor for hepatitis C virus (HCV) replication and anti-miR-122 efficiently reduces viral load in chronically infected HCV patients without detectable resistance[108]. The fact that miR-122 has protective effects on NAFLD, while imposing a deleterious impact on HCV infection, emphasizes the importance of cautious targeting of miRNAs therapy since the role of miRNAs can be highly context dependent[115].

circRNA_0046366 antagonizes miR-34a and normalizes PPARα signaling, leading to the amelioration of liver steatosis in a murine model[116]. However, a phase I study on the effects of a miR-34 mimic (MRX34) on primary liver cancer and advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971) was prematurely terminated due to serious immune-related adverse events[108], highlighting the potential risks of miRNA based-therapies.

CONCLUSION

All except four (miR-199, miR-212, miR-214 and miR-497) of the 21 miRNAs associated with ALD, listed in Table 1, are also related to NAFLD or lipid metabolism (although the four have been associated with other diseases, such as cancer[117]). Conversely, miRNAs that are related to the pathogenesis of NAFLD (miR-122, miR-34a and miR-155) are also clearly linked to ALD. These results reflect the common mechanisms between NAFLD and ALD and also the pleiotropic effects of any particular miRNA.

Due to the lack of specificity of miRNAs, the development of a biomarker or treatment specific to ALD or NAFLD is difficult. It is more feasible that individual miRNAs or a panel of miRNAs would be useful in the staging of liver disease (*e.g.*, distinguishing simple steatosis in ALD or NAFLD from steatohepatitis)[118]. miR-122 is the most promising candidate as a biomarker due to its liver specificity. It is clear however, that miR-122 is also a marker of liver damage regardless of etiology[119]. Technical limitations, such as standardization of techniques and potential costs, add to the difficulties inherent to the development of a validated diagnostic biomarker. Circulating miRNAs are promising as biomarkers due to their stability and potential ability to detect advanced liver disease without a biopsy. However, rigorously validated studied are needed before they can be brought to the clinic[119].

The development of miRNA-targeted interventions for ALD and NAFLD is an intriguing area of research. However, despite the success in animal models and the potential targets described in this review, to the best of our knowledge there are no current clinical trials for miRNA interventions in ALD or NAFLD. The few studies that are being conducted on miRNA treatment in other diseases are phase 1 studies in the field of cancer research (*e.g.*, assessing the activity of miRNA-loaded minicells or TargomiRs in malignant pleural mesothelioma[120]). Theoretical miRNA-based therapies are pharmacologically complex and include miRNA inhibition (*e.g.*, synthetic

anti-miRNAs) or miRNA replacement therapy (*e.g.*, lipid vesicles or gold nanoparticles)[121]. One major challenge to the development of miRNA-based therapies is the improvement of drug delivery systems. Due to the biochemical instability of unmodified miRNAs and potential immunogenicity, specific delivery to target organs should be achieved. The high degree of redundancy among miRNAs and the multiple binding sites for any given miRNA must also be taken into account when designing efficacious and safe miRNA-based therapies[122].

To sum up, there is a large body of literature regarding miRNAs in NAFLD and ALD at various stages of the disease. These studies include expression data from microarrays and next generation sequencing from animal models and human studies, and cell-specific data from in situ hybridization and sensor constructs. The role of miRNAs in pathogenesis is well-documented and as such, their potential value as biomarkers or therapeutic targets is warranted. However, most miRNA modifications have a modest phenotypic effect, since miRNAs are unlikely to be the single key factor in chronic and multifactorial diseases such as liver steatosis[123]. Instead, most miRNAs act as fine-tuners in disease pathways and this characteristic, along with their lack of specificity must be considered before use in the clinic. To this end, we must improve our understanding of the interaction of different miRNAs in the development of advanced liver disease.

REFERENCES

1 **Ambros V**. The functions of animal microRNAs. *Nature* 2004; **431**: 350-355 [PMID: 15372042 DOI: 10.1038/nature02871]
2 **Hammond SM**. An overview of microRNAs. *Adv Drug Deliv Rev* 2015; **87**: 3-14 [PMID: 25979468 DOI: 10.1016/j.addr.2015.05.001]
3 **Lee RC**, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; **75**: 843-854 [PMID: 8252621]
4 **Friedman RC**, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; **19**: 92-105 [PMID: 18955434 DOI: 10.1101/gr.082701.108]
5 **Dolganiuc A**, Petrasek J, Kodys K, Catalano D, Mandrekar P, Velayudham A, Szabo G. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcohol Clin Exp Res* 2009; **33**: 1704-1710 [PMID: 19572984 DOI: 10.1111/j.1530-0277.2009.01007.x]
6 **Szabo G**, Bala S. MicroRNAs in liver disease. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 542-552 [PMID: 23689081 DOI: 10.1038/nrgastro.2013.87]
7 **Bala S**, Marcos M, Szabo G. Emerging role of microRNAs in liver diseases. *World J Gastroenterol* 2009; **15**: 5633-5640 [PMID: 19960558]
8 **Szabo G**, Csak T. Role of MicroRNAs in NAFLD/NASH. *Dig Dis Sci* 2016; **61**: 1314-1324 [PMID: 26769057 DOI: 10.1007/s10620-015-4002-4]
9 **Sanyal AJ**, Mathurin P, Nagy LA. Commonalities and Distinctions Between Alcoholic and Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2016; **150**: 1695-1697 [PMID: 27155520 DOI: 10.1053/j.gastro.2016.04.038]
10 **Laso FJ**, Pastor I, Orfao A. Immune system and alcoholic liver disease. *Medic Clin* 2005; **125**: 263-269
11 **Lieber CS**. Hepatic and metabolic effects of ethanol: pathogenesis and prevention. *Ann Med* 1994; **26**: 325-330 [PMID: 7826592]
12 **Mello T**, Ceni E, Surrenti C, Galli A. Alcohol induced hepatic fibrosis: role of acetaldehyde. *Mol Aspects Med* 2008; **29**: 17-21 [PMID: 18164754 DOI: 10.1016/j.mam.2007.10.001]
13 **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]
14 **Mandrekar P**, Szabo G. Signalling pathways in alcohol-induced liver inflammation. *J Hepatol* 2009; **50**: 1258-1266 [PMID: 19398236 DOI: 10.1016/j.jhep.2009.03.007]
15 **O'Neill LA**, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat Rev Immunol* 2011; **11**: 163-175 [PMID: 21331081 DOI: 10.1038/nri2957]
16 **Boccuto L**, Abenavoli L. Genetic and Epigenetic Profile of Patients With Alcoholic Liver Disease. *Ann Hepatol* 2017; **16**: 490-500 [PMID: 28612750 DOI: 10.5604/01.3001.0010.0274]
17 **Wan C**, Han R, Liu L, Zhang F, Li F, Xiang M, Ding W. Role of miR-155 in fluorooctane sulfonate-induced oxidative hepatic damage via the Nrf2-dependent pathway. *Toxicol Appl Pharmacol* 2016; **295**: 85-93 [PMID: 26844784 DOI: 10.1016/j.taap.2016.01.023]
18 **Kurinna S**, Werner S. NRF2 and microRNAs: new but awaited relations. *Biochem Soc Trans* 2015; **43**: 595-601 [PMID: 26551699 DOI: 10.1042/BST20140317]
19 **Yang JJ**, Tao H, Hu W, Liu LP, Shi KH, Deng ZY, Li J. MicroRNA-200a controls Nrf2 activation by target Keap1 in hepatic stellate cell proliferation and fibrosis. *Cell Signal* 2014; **26**: 2381-2389 [PMID: 25049078 DOI: 10.1016/j.cellsig.2014.07.016]
20 **Shi L**, Wu L, Chen Z, Yang J, Chen X, Yu F, Zheng F, Lin X. MiR-141 Activates Nrf2-Dependent Antioxidant Pathway via Down-Regulating the Expression of Keap1 Conferring the Resistance of Hepatocellular Carcinoma Cells to 5-Fluorouracil. *Cell Physiol Biochem* 2015; **35**: 2333-2348 [PMID: 25896253 DOI: 10.1159/000374036]
21 **O'Connell RM**, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. *Annu Rev Immunol* 2012; **30**: 295-312 [PMID: 22224773 DOI: 10.1146/annurev-immunol-020711-075013]
22 **Wu XQ**, Dai Y, Yang Y, Huang C, Meng XM, Wu BM, Li J. Emerging role of microRNAs in regulating macrophage activation and polarization in immune response and inflammation. *Immunology* 2016; **148**: 237-248 [PMID: 27005899 DOI: 10.1111/imm.12608]
23 **Momen-Heravi F**, Saha B, Kodys K, Catalano D, Satishchandran A, Szabo G. Increased number of circulating exosomes and their microRNA cargos are potential novel biomarkers in alcoholic hepatitis. *J Transl Med* 2015; **13**: 261 [PMID: 26264599 DOI: 10.1186/s12967-015-0623-9]
24 **Wang Y**, Yu D, Tolleson WH, Yu LR, Green B, Zeng L, Chen Y, Chen S, Ren Z, Guo L, Tong W, Guan H, Ning B. A systematic evaluation of microRNAs in regulating human hepatic CYP2E1. *Biochem Pharmacol* 2017; **138**: 174-184 [PMID: 28438567 DOI: 10.1016/j.bcp.2017.04.020]
25 **Kim YD**, Hwang SL, Lee EJ, Kim HM, Chung MJ, Elfadl AK, Lee SE, Nedumaran B, Harris RA, Jeong KS. Melatonin ameliorates alcohol-induced bile acid synthesis by enhancing miR-497 expression. *J Pineal Res* 2017; **62** [PMID: 28095641 DOI: 10.1111/jpi.12386]
26 **Dippold RP**, Vadigepalli R, Gonye GE, Patra B, Hoek JB. Chronic ethanol feeding alters miRNA expression dynamics during liver regeneration. *Alcohol Clin Exp Res* 2013; **37** Suppl 1: E59-E69 [PMID: 22823254 DOI: 10.1111/j.1530-0277.2012.01852.x]
27 **McDaniel K**, Huang L, Sato K, Wu N, Annable T, Zhou T, Ramos-Lorenzo S, Wan Y, Huang Q, Francis H, Glaser S, Tsukamoto H, Alpini G, Meng F. The let-7/Lin28 axis regulates activation of hepatic stellate cells in alcoholic liver injury. *J Biol Chem* 2017; **292**: 11336-11347 [PMID: 28536261 DOI: 10.1074/jbc.M116.773291]
28 **Brandon-Warner E**, Feilen NA, Culberson CR, Field CO, deLemos AS, Russo MW, Schrum LW. Processing of miR17-92

Cluster in Hepatic Stellate Cells Promotes Hepatic Fibrogenesis During Alcohol-Induced Injury. *Alcohol Clin Exp Res* 2016; **40**: 1430-1442 [PMID: 27291156 DOI: 10.1111/acer.13116]

29 **Meng F**, Glaser SS, Francis H, Yang F, Han Y, Stokes A, Staloch D, McCarra J, Liu J, Venter J, Zhao H, Liu X, Francis T, Swendsen S, Liu CG, Tsukamoto H, Alpini G. Epigenetic regulation of miR-34a expression in alcoholic liver injury. *Am J Pathol* 2012; **181**: 804-817 [PMID: 22841474 DOI: 10.1016/j.ajpath.2012.06.010]

30 **Blaya D**, Coll M, Rodrigo-Torres D, Vila-Casadesús M, Altamirano J, Llopis M, Graupera I, Perea L, Aguilar-Bravo B, Díaz A, Banales JM, Clària J, Lozano JJ, Bataller R, Caballería J, Ginès P, Sancho-Bru P. Integrative microRNA profiling in alcoholic hepatitis reveals a role for microRNA-182 in liver injury and inflammation. *Gut* 2016; **65**: 1535-1545 [PMID: 27196584 DOI: 10.1136/gutjnl-2015-311314]

31 **Zhao YX**, Sun YY, Huang AL, Li XF, Huang C, Ma TT, Li J. MicroRNA-200a induces apoptosis by targeting ZEB2 in alcoholic liver disease. *Cell Cycle* 2018; **17**: 250-262 [PMID: 29251244 DOI: 10.1080/15384101.2017.1417708]

32 **Momen-Heravi F**, Bala S, Kodys K, Szabo G. Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. *Sci Rep* 2015; **5**: 9991 [PMID: 25973575 DOI: 10.1038/srep09991]

33 **Satishchandran A**, Ambade A, Rao S, Hsueh YC, Iracheta-Vellve A, Tornai D, Lowe P, Gyongyosi B, Li J, Catalano D, Zhong L, Kodys K, Xie J, Bala S, Gao G, Szabo G. MicroRNA 122, Regulated by GRLH2, Protects Livers of Mice and Patients From Ethanol-Induced Liver Disease. *Gastroenterology* 2018; **154**: 238-252.e7 [PMID: 28987423 DOI: 10.1053/j.gastro.2017.09.022]

34 **Dong X**, Liu H, Chen F, Li D, Zhao Y. MiR-214 promotes the alcohol-induced oxidative stress via down-regulation of glutathione reductase and cytochrome P450 oxidoreductase in liver cells. *Alcohol Clin Exp Res* 2014; **38**: 68-77 [PMID: 23905773 DOI: 10.1111/acer.12209]

35 **Han W**, Fu X, Xie J, Meng Z, Gu Y, Wang X, Li L, Pan H, Huang W. MiR-26a enhances autophagy to protect against ethanol-induced acute liver injury. *J Mol Med* (Berl) 2015; **93**: 1045-1055 [PMID: 25877859 DOI: 10.1007/s00109-015-1282-2]

36 **Dippold RP**, Vadigepalli R, Gonye GE, Hoek JB. Chronic ethanol feeding enhances miR-21 induction during liver regeneration while inhibiting proliferation in rats. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G733-G743 [PMID: 22790595 DOI: 10.1152/ajpgi.00019.2012]

37 **Francis H**, McDaniel K, Han Y, Liu X, Kennedy L, Yang F, McCarra J, Zhou T, Glaser S, Venter J, Huang L, Levine P, Lai JM, Liu CG, Alpini G, Meng F. Regulation of the extrinsic apoptotic pathway by microRNA-21 in alcoholic liver injury. *J Biol Chem* 2014; **289**: 27526-27539 [PMID: 25118289 DOI: 10.1074/jbc.M114.602383]

38 **Bala S**, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P, Szabo G. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor {alpha} (TNF{alpha}) production via increased mRNA half-life in alcoholic liver disease. *J Biol Chem* 2011; **286**: 1436-1444 [PMID: 21062749 DOI: 10.1074/jbc.M110.145870]

39 **Bala S**, Csak T, Kodys K, Catalano D, Ambade A, Furi I, Lowe P, Cho Y, Iracheta-Vellve A, Szabo G. Alcohol-induced miR-155 and HDAC11 inhibit negative regulators of the TLR4 pathway and lead to increased LPS responsiveness of Kupffer cells in alcoholic liver disease. *J Leukoc Biol* 2017; **102**: 487-498

40 **Saikia P**, Bellos D, McMullen MR, Pollard KA, de la Motte C, Nagy LE. MicroRNA 181b-3p and its target importin α5 regulate toll-like receptor 4 signaling in Kupffer cells and liver injury in mice in response to ethanol. *Hepatology* 2017; **66**: 602-615 [PMID: 28257601 DOI: 10.1002/hep.29144]

41 **Yin H**, Liang X, Jogasuria A, Davidson NO, You M. miR-217 regulates ethanol-induced hepatic inflammation by disrupting sirtuin 1-lipin-1 signaling. *Am J Pathol* 2015; **185**: 1286-1296 [PMID: 25797648 DOI: 10.1016/j.ajpath.2015.01.030]

42 **Saikia P**, Roychowdhury S, Bellos D, Pollard KA, McMullen MR, McCullough RL, McCullough AJ, Gholam P, de la Motte C, Nagy LE. Hyaluronic acid 35 normalizes TLR4 signaling in Kupffer cells from ethanol-fed rats via regulation of microRNA291b and its target Tollip. *Sci Rep* 2017; **7**: 15671 [PMID: 29142263 DOI: 10.1038/s41598-017-15760-4]

43 **Wan Y**, McDaniel K, Wu N, Ramos-Lorenzo S, Glaser T, Venter J, Francis H, Kennedy L, Sato K, Zhou T, Kyritsi K, Huang Q, Annable T, Wu C, Glaser S, Alpini G, Meng F. Regulation of Cellular Senescence by miR-34a in Alcoholic Liver Injury. *Am J Pathol* 2017; **187**: 2788-2798 [PMID: 29128099 DOI: 10.1016/j.ajpath.2017.08.027]

44 **Saha B**, Bruneau JC, Kodys K, Szabo G. Alcohol-induced miR-27a regulates differentiation and M2 macrophage polarization of normal human monocytes. *J Immunol* 2015; **194**: 3079-3087 [PMID: 25716995 DOI: 10.4049/jimmunol.1402190]

45 **Li M**, He Y, Zhou Z, Ramirez T, Gao Y, Gao Y, Ross RA, Cao H, Cai Y, Xu M, Feng D, Zhang P, Liangpunsakul S, Gao B. MicroRNA-223 ameliorates alcoholic liver injury by inhibiting the IL-6-p47 phox -oxidative stress pathway in neutrophils. *Gut* 2017; **66**: 705-715 [PMID: 27679493 DOI: 10.1136/gutjnl-2016-311861]

46 **Tang Y**, Banan A, Forsyth CB, Fields JZ, Lau CK, Zhang LJ, Keshavarzian A. Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. *Alcohol Clin Exp Res* 2008; **32**: 355-364 [PMID: 18162065 DOI: 10.1111/j.1530-0277.2007.00584.x]

47 **Novo-Veleiro I**, González-Sarmiento R, Cieza-Borrella C, Pastor I, Laso FJ, Marcos M. A genetic variant in the microRNA-146a gene is associated with susceptibility to alcohol use disorders. *Eur Psychiatry* 2014; **29**: 288-292 [PMID: 24630744 DOI: 10.1016/j.eurpsy.2014.02.002]

48 **Novo-Veleiro I**, Cieza-Borrella C, Pastor I, González-Sarmiento R, Laso FJ, Marcos M. Analysis of the relationship between interleukin polymorphisms within miRNA-binding regions and alcoholic liver disease. *Rev Clin Esp* 2018; **218**: 170-176 [PMID: 29566963 DOI: 10.1016/j.rce.2018.02.005]

49 **Barragán R**, Coltell O, Asensio EM, Francés F, Sorlí JV, Estruch R, Salas-Huetos A, Ordovas JM, Corella D. MicroRNAs and Drinking: Association between the Pre-miR-27a rs895819 Polymorphism and Alcohol Consumption in a Mediterranean Population. *Int J Mol Sci* 2016; **1** [PMID: 27537871 DOI: 10.3390/ijms17081338]

50 **Chamorro AJ**, Torres JL, Mirón-Canelo JA, González-Sarmiento R, Laso FJ, Marcos M. Systematic review with meta-analysis: the I148M variant of patatin-like phospholipase domain-containing 3 gene (PNPLA3) is significantly associated with alcoholic liver cirrhosis. *Aliment Pharmacol Ther* 2014; **40**: 571-581 [PMID: 25060292 DOI: 10.1111/apt.12890]

51 **Afonso MB**, Rodrigues PM, Simão AL, Castro RE. Circulating microRNAs as Potential Biomarkers in Non-Alcoholic Fatty Liver Disease and Hepatocellular Carcinoma. *J Clin Med* 2016; **5** [PMID: 26950158 DOI: 10.3390/jcm5030030]

52 **Saha B**, Momen-Heravi F, Kodys K, Szabo G. MicroRNA Cargo of Extracellular Vesicles from Alcohol-exposed Monocytes Signals Naive Monocytes to Differentiate into M2 Macrophages. *J Biol Chem* 2016; **291**: 149-159 [PMID: 26527689 DOI: 10.1074/jbc.M115.694133]

53 **Trajkovski M**, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH, Stoffel M. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* 2011; **474**: 649-653 [PMID: 21654750 DOI: 10.1038/nature10112]

54 **Bala S**, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, Alao H, Kodys K, Szabo G. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology* 2012; **56**: 1946-1957 [PMID: 22684891 DOI: 10.1002/hep.25873]

55 **McCrae JC**, Sharkey N, Webb DJ, Vliegenthart AD, Dear JW. Ethanol consumption produces a small increase in circulating miR-122 in healthy individuals. *Clin Toxicol* (Phila) 2016; **54**: 53-55 [PMID: 26574140 DOI: 10.3109/15563650.2015.1112015]

56 **Janssen HL**, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson

R, King BD, Kauppinen S, Levin AA, Hodges MR. Treatment of HCV infection by targeting microRNA. *N Engl J Med* 2013; **368**: 1685-1694 [PMID: 23534542 DOI: 10.1056/NEJMoa1209026]

57 **Gebert LF**, Rebhan MA, Crivelli SE, Denzler R, Stoffel M, Hall J. Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. *Nucleic Acids Res* 2014; **42**: 609-621 [PMID: 24068553 DOI: 10.1093/nar/gkt852]

58 **Chakraborty C**, Sharma AR, Sharma G, Doss CGP, Lee SS. Therapeutic miRNA and siRNA: Moving from Bench to Clinic as Next Generation Medicine. *Mol Ther Nucleic Acids* 2017; **8**: 132-143 [PMID: 28918016 DOI: 10.1016/j.omtn.2017.06.005]

59 **Hyun J**, Wang S, Kim J, Rao KM, Park SY, Chung I, Ha CS, Kim SW, Yun YH, Jung Y. MicroRNA-378 limits activation of hepatic stellate cells and liver fibrosis by suppressing Gli3 expression. *Nat Commun* 2016; **7**: 10993 [PMID: 27001906 DOI: 10.1038/ncomms10993]

60 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005-2023 [PMID: 22488764 DOI: 10.1002/hep.25762]

61 **Loomba R**, Sanyal AJ. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 686-690 [PMID: 24042449 DOI: 10.1038/nrgastro.2013.171]

62 **Gholam PM**, Kotler DP, Flancbaum LJ. Liver pathology in morbidly obese patients undergoing Roux-en-Y gastric bypass surgery. *Obes Surg* 2002; **12**: 49-51 [PMID: 11868297 DOI: 10.1381/096089202321144577]

63 **Praveenraj P**, Gomes RM, Kumar S, Karthikeyan P, Shankar A, Parthasarathi R, Senthilnathan P, Rajapandian S, Palanivelu C. Prevalence and Predictors of Non-Alcoholic Fatty Liver Disease in Morbidly Obese South Indian Patients Undergoing Bariatric Surgery. *Obes Surg* 2015; **25**: 2078-2087 [PMID: 25835982 DOI: 10.1007/s11695-015-1655-1]

64 **Williamson RM**, Price JF, Glancy S, Perry E, Nee LD, Hayes PC, Frier BM, Van Look LA, Johnston GI, Reynolds RM, Strachan MW; Edinburgh Type 2 Diabetes Study Investigators. Prevalence of and risk factors for hepatic steatosis and nonalcoholic Fatty liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care* 2011; **34**: 1139-1144 [PMID: 21478462 DOI: 10.2337/dc10-2229]

65 **Charlton MR**, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology* 2011; **141**: 1249-1253 [PMID: 21726509 DOI: 10.1053/j.gastro.2011.06.061]

66 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102]

67 **Videla LA**, Rodrigo R, Araya J, Poniachik J. Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease. *Trends Mol Med* 2006; **12**: 555-558 [PMID: 17049925 DOI: 10.1016/j.molmed.2006.10.001]

68 **Tilg H**, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836-1846 [PMID: 21038418 DOI: 10.1002/hep.24001]

69 **Buzzetti E**, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 2016; **65**: 1038-1048 [PMID: 26823198 DOI: 10.1016/j.metabol.2015.12.012]

70 **Peverill W**, Powell LW, Skoien R. Evolving concepts in the pathogenesis of NASH: beyond steatosis and inflammation. *Int J Mol Sci* 2014; **15**: 8591-8638 [PMID: 24830559 DOI: 10.3390/ijms15058591]

71 **Cheung O**, Puri P, Eicken C, Contos MJ, Mirshahi F, Maher JW, Kellum JM, Min H, Luketic VA, Sanyal AJ. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 2008; **48**: 1810-1820 [PMID: 19030170 DOI: 10.1002/hep.22569]

72 **Cazanave SC**, Mott JL, Elmi NA, Bronk SF, Masuoka HC, Charlton MR, Gores GJ. A role for miR-296 in the regulation of lipoapoptosis by targeting PUMA. *J Lipid Res* 2011; **52**: 1517-1525 [PMID: 21633093 DOI: 10.1194/jlr.M014654]

73 **Lendvai G**, Jármay K, Karácsony G, Halász T, Kovalszky I, Baghy K, Wittmann T, Schaff Z, Kiss A. Elevated miR-33a and miR-224 in steatotic chronic hepatitis C liver biopsies. *World J Gastroenterol* 2014; **20**: 15343-15350 [PMID: 25386083 DOI: 10.3748/wjg.v20.i41.15343]

74 **Leti F**, Malenica I, Doshi M, Courtright A, Van Keuren-Jensen K, Legendre C, Still CD, Gerhard GS, DiStefano JK. High-throughput sequencing reveals altered expression of hepatic microRNAs in nonalcoholic fatty liver disease-related fibrosis. *Transl Res* 2015; **166**: 304-314 [PMID: 26001595 DOI: 10.1016/j.trsl.2015.04.014]

75 **Guo Y**, Xiong Y, Sheng Q, Zhao S, Wattacheril J, Flynn CR. A micro-RNA expression signature for human NAFLD progression. *J Gastroenterol* 2016; **51**: 1022-1030 [PMID: 26874844 DOI: 10.1007/s00535-016-1178-0]

76 **Vega-Badillo J**, Gutiérrez-Vidal R, Hernández-Pérez HA, Villamil-Ramírez H, León-Mimila P, Sánchez-Muñoz F, Morán-Ramos S, Larrieta-Carrasco E, Fernández-Silva I, Méndez-Sánchez N, Tovar AR, Campos-Pérez F, Villarreal-Molina T, Hernández-Pando R, Aguilar-Salinas CA, Canizales-Quinteros S. Hepatic miR-33a/miR-144 and their target gene ABCA1 are associated with steatohepatitis in morbidly obese subjects. *Liver Int* 2016; **36**: 1383-1391 [PMID: 26945479 DOI: 10.1111/liv.13109]

77 **Xu H**, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, Qu LH. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology* 2010; **52**: 1431-1442 [PMID: 20842632 DOI: 10.1002/hep.23818]

78 **Laudadio I**, Manfroid I, Achouri Y, Schmidt D, Wilson MD, Cordi S, Thorrez L, Knoops L, Jacquemin P, Schuit F, Pierreux CE, Odom DT, Peers B, Lemaigre FP. A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation. *Gastroenterology* 2012; **142**: 119-129 [PMID: 21920465 DOI: 10.1053/j.gastro.2011.09.001]

79 **Deng XG**, Qiu RL, Wu YH, Li ZX, Xie P, Zhang J, Zhou JJ, Zeng LX, Tang J, Maharjan A, Deng JM. Overexpression of miR-122 promotes the hepatic differentiation and maturation of mouse ESCs through a miR-122/FoxA1/HNF4a-positive feedback loop. *Liver Int* 2014; **34**: 281-295 [PMID: 23834235 DOI: 10.1111/liv.12239]

80 **Esau C**, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Monia BP. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 2006; **3**: 87-98 [PMID: 16459310 DOI: 10.1016/j.cmet.2006.01.005]

81 **Salvoza NC**, Klinzing DC, Gopez-Cervantes J, Baclig MO. Association of Circulating Serum miR-34a and miR-122 with Dyslipidemia among Patients with Non-Alcoholic Fatty Liver Disease. *PLoS One* 2016; **11**: e0153497 [PMID: 27077736 DOI: 10.1371/journal.pone.0153497]

82 **Tryndyak VP**, Latendresse JR, Montgomery B, Ross SA, Beland FA, Rusyn I, Pogribny IP. Plasma microRNAs are sensitive indicators of inter-strain differences in the severity of liver injury induced in mice by a choline- and folate-deficient diet. *Toxicol Appl Pharmacol* 2012; **262**: 52-59 [PMID: 22561871 DOI: 10.1016/j.taap.2012.04.018]

83 **Latorre J**, Moreno-Navarrete JM, Mercader JM, Sabater M, Rovira Ò, Gironès J, Ricart W, Fernández-Real JM, Ortega FJ. Decreased lipid metabolism but increased FA biosynthesis are coupled with changes in liver microRNAs in obese subjects with NAFLD. *Int J Obes* (Lond) 2017; **41**: 620-630 [PMID: 28119530 DOI: 10.1038/ijo.2017.21]

84 **Pirola CJ**, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, Flichman D, Mirshahi F, Sanyal AJ, Sookoian S. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. *Gut* 2015; **64**: 800-812 [PMID: 24973316 DOI: 10.1136/gutjnl-2014-306996]

85 **Braza-Boïls A**, Marí-Alexandre J, Molina P, Arnau MA, Barceló-Molina M, Domingo D, Girbes J, Giner J, Martínez-Dolz L, Zorio E. Deregulated hepatic microRNAs underlie the association between non-alcoholic fatty liver disease and coronary artery disease. *Liver Int* 2016; **36**: 1221-1229 [PMID: 26901384 DOI: 10.1111/liv.13097]

86 **Becker PP**, Rau M, Schmitt J, Malsch C, Hammer C, Bantel H, Müllhaupt B, Geier A. Performance of Serum microRNAs -122, -192 and -21 as Biomarkers in Patients with Non-Alcoholic Steatohepatitis. *PLoS One* 2015; **10**: e0142661 [PMID: 26565986 DOI: 10.1371/journal.pone.0142661]

87 **Yamada H**, Suzuki K, Ichino N, Ando Y, Sawada A, Osakabe K, Sugimoto K, Ohashi K, Teradaira R, Inoue T, Hamajima N, Hashimoto S. Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. *Clin Chim Acta* 2013; **424**: 99-103 [PMID: 23727030 DOI: 10.1016/j.cca.2013.05.021]

88 **Li J**, Ghazwani M, Zhang Y, Lu J, Li J, Fan J, Gandhi CR, Li S. miR-122 regulates collagen production via targeting hepatic stellate cells and suppressing P4HA1 expression. *J Hepatol* 2013; **58**: 522-528 [PMID: 23178710 DOI: 10.1016/j.jhep.2012.11.011]

89 **Li S**, Chen X, Zhang H, Liang X, Xiang Y, Yu C, Zen K, Li Y, Zhang CY. Differential expression of microRNAs in mouse liver under aberrant energy metabolic status. *J Lipid Res* 2009; **50**: 1756-1765 [PMID: 19372595 DOI: 10.1194/jlr.M800509-JLR200]

90 **Pogribny IP**, Starlard-Davenport A, Tryndyak VP, Han T, Ross SA, Rusyn I, Beland FA. Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice. *Lab Invest* 2010; **90**: 1437-1446 [PMID: 20548288 DOI: 10.1038/labinvest.2010.113]

91 **Wu T**, Liu YH, Fu YC, Liu XM, Zhou XH. Direct evidence of sirtuin downregulation in the liver of non-alcoholic fatty liver disease patients. *Ann Clin Lab Sci* 2014; **44**: 410-418 [PMID: 25361925]

92 **Ding J**, Li M, Wan X, Jin X, Chen S, Yu C, Li Y. Effect of miR-34a in regulating steatosis by targeting PPARα expression in nonalcoholic fatty liver disease. *Sci Rep* 2015; **5**: 13729 [PMID: 26330104 DOI: 10.1038/srep13729]

93 **Chen JQ**, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun Rev* 2016; **15**: 1171-1180 [PMID: 27639156 DOI: 10.1016/j.autrev.2016.09.003]

94 **Chen Y**, Siegel F, Kipschull S, Haas B, Fröhlich H, Meister G, Pfeifer A. miR-155 regulates differentiation of brown and beige adipocytes via a bistable circuit. *Nat Commun* 2013; **4**: 1769 [PMID: 23612310 DOI: 10.1038/ncomms2742]

95 **Virtue A**, Johnson C, Lopez-Pastraña J, Shao Y, Fu H, Li X, Li YF, Yin Y, Mai J, Rizzo V, Tordoff M, Bagi Z, Shan H, Jiang X, Wang H, Yang XF. MicroRNA-155 Deficiency Leads to Decreased Atherosclerosis, Increased White Adipose Tissue Obesity, and Non-alcoholic Fatty Liver Disease: A NOVEL MOUSE MODEL OF OBESITY PARADOX. *J Biol Chem* 2017; **292**: 1267-1287 [PMID: 27856635 DOI: 10.1074/jbc.M116.739839]

96 **Wang L**, Zhang N, Wang Z, Ai DM, Cao ZY, Pan HP. Decreased MiR-155 Level in the Peripheral Blood of Non-Alcoholic Fatty Liver Disease Patients may Serve as a Biomarker and may Influence LXR Activity. *Cell Physiol Biochem* 2016; **39**: 2239-2248 [PMID: 27832630 DOI: 10.1159/000447917]

97 **Csak T**, Bala S, Lippai D, Kodys K, Catalano D, Iracheta-Vellve A, Szabo G. MicroRNA-155 Deficiency Attenuates Liver Steatosis and Fibrosis without Reducing Inflammation in a Mouse Model of Steatohepatitis. *PLoS One* 2015; **10**: e0129251 [PMID: 26042593 DOI: 10.1371/journal.pone.0129251]

98 **Miller AM**, Gilchrist DS, Nijjar J, Araldi E, Ramirez CM, Lavery CA, Fernández-Hernando C, McInnes IB, Kurowska-Stolarska M. MiR-155 has a protective role in the development of non-alcoholic hepatosteatosis in mice. *PLoS One* 2013; **8**: e72324 [PMID: 23991091 DOI: 10.1371/journal.pone.0072324]

99 **Wang B**, Majumder S, Nuovo G, Kutay H, Volinia S, Patel T, Schmittgen TD, Croce C, Ghoshal K, Jacob ST. Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. *Hepatology* 2009; **50**: 1152-1161 [PMID: 19711427 DOI: 10.1002/hep.23100]

100 **Akuta N**, Kawamura Y, Suzuki F, Saitoh S, Arase Y, Kunimoto H, Sorin Y, Fujiyama S, Sezaki H, Hosaka T, Kobayashi M, Suzuki Y, Kobayashi M, Ikeda K, Kumada H. Impact of circulating miR-122 for histological features and hepatocellular carcinoma of nonalcoholic fatty liver disease in Japan. *Hepatol Int* 2016; **10**: 647-656 [PMID: 27074850 DOI: 10.1007/s12072-016-9729-2]

101 **Miyaaki H**, Ichikawa T, Kamo Y, Taura N, Honda T, Shibata H, Milazzo M, Fornari F, Gramantieri L, Bolondi L, Nakao K. Significance of serum and hepatic microRNA-122 levels in patients with non-alcoholic fatty liver disease. *Liver Int* 2014; **34**: e302-e307 [PMID: 24313922 DOI: 10.1111/liv.12429]

102 **Tan Y**, Ge G, Pan T, Wen D, Gan J. A pilot study of serum microRNAs panel as potential biomarkers for diagnosis of nonalcoholic fatty liver disease. *PLoS One* 2014; **9**: e105192 [PMID: 25141008 DOI: 10.1371/journal.pone.0105192]

103 **Yamada H**, Ohashi K, Suzuki K, Munetsuna E, Ando Y, Yamazaki M, Ishikawa H, Ichino N, Teradaira R, Hashimoto S. Longitudinal study of circulating miR-122 in a rat model of non-alcoholic fatty liver disease. *Clin Chim Acta* 2015; **446**: 267-271 [PMID: 25958847 DOI: 10.1016/j.cca.2015.05.002]

104 **Cermelli S**, Ruggieri A, Marrero JA, Ioannou GN, Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. *PLoS One* 2011; **6**: e23937 [PMID: 21886843 DOI: 10.1371/journal.pone.0023937]

105 **Liu XL**, Pan Q, Zhang RN, Shen F, Yan SY, Sun C, Xu ZJ, Chen YW, Fan JG. Disease-specific miR-34a as diagnostic marker of non-alcoholic steatohepatitis in a Chinese population. *World J Gastroenterol* 2016; **22**: 9844-9852 [PMID: 27956809 DOI: 10.3748/wjg.v22.i44.9844]

106 **Povero D**, Eguchi A, Li H, Johnson CD, Papouchado BG, Wree A, Messer K, Feldstein AE. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One* 2014; **9**: e113651 [PMID: 25470250 DOI: 10.1371/journal.pone.0113651]

107 **Vienberg S**, Geiger J, Madsen S, Dalgaard LT. MicroRNAs in metabolism. *Acta Physiol* (Oxf) 2017; **219**: 346-361 [PMID: 27009502 DOI: 10.1111/apha.12681]

108 **Rupaimoole R**, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017; **16**: 203-222 [PMID: 28209991 DOI: 10.1038/nrd.2016.246]

109 **Regulus Therapeutics I.** Regulus announces notice of allowance from US patent office related to microRNA- 103/107 Program in Metabolic Disorders. Press release, 2014

110 **Regulus Therapeutics I.** RG-125 (AZD4076), a microRNA therapeutic targeting microRNA 103/107 for the treatment of NASH in patients with type 2 diabetes/Pre-Diabetes, selected as clinical candidate by AstraZeneca. Press release 2015

111 Astra Zeneca Halts Development of Regulus NASH Candidate. GEN News Highlights, 2017

112 **Lagos-Quintana M**, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of Tissue-Specific MicroRNAs from Mouse. *Current Biology* 2002; **12**: 735-739 [DOI: 10.1016/S0960-9822(02)00809-6]

113 **Bandiera S**, Pfeffer S, Baumert TF, Zeisel MB. miR-122--a key factor and therapeutic target in liver disease. *J Hepatol* 2015; **62**: 448-457 [PMID: 25308172 DOI: 10.1016/j.jhep.2014.10.004]

114 **Wu GY**, Rui C, Chen JQ, Sho E, Zhan SS, Yuan XW, Ding YT. MicroRNA-122 Inhibits Lipid Droplet Formation and Hepatic Triglyceride Accumulation via Yin Yang 1. *Cell Physiol Biochem* 2017; **44**: 1651-1664 [PMID: 29216638 DOI: 10.1159/000485765]

115 **Su Q**, Kumar V, Sud N, Mahato RI. MicroRNAs in the pathogenesis and treatment of progressive liver injury in NAFLD and liver fibrosis. *Adv Drug Deliv Rev* 2018; **129**: 54-63 [PMID: 29391222 DOI: 10.1016/j.addr.2018.01.009]

116 **Guo XY**, Sun F, Chen JN, Wang YQ, Pan Q, Fan JG. circRNA_0046366 inhibits hepatocellular steatosis by normalization of PPAR signaling. *World J Gastroenterol* 2018; **24**: 323-337

[PMID: 29391755 DOI: 10.3748/wjg.v24.i3.323]
117 **Yang G**, Xiong G, Cao Z, Zheng S, You L, Zhang T, Zhao Y. miR-497 expression, function and clinical application in cancer. *Oncotarget* 2016; **7**: 55900-55911 [PMID: 27344185 DOI: 10.18632/oncotarget.10152]
118 **Hyun J**, Jung Y. MicroRNAs in liver fibrosis: Focusing on the interaction with hedgehog signaling. *World J Gastroenterol* 2016; **22**: 6652-6662 [PMID: 27547008 DOI: 10.3748/wjg.v22.i29.6652]
119 **Loosen SH**, Schueller F, Trautwein C, Roy S, Roderburg C. Role of circulating microRNAs in liver diseases. *World J Hepatol* 2017; **9**: 586-594 [PMID: 28515844 DOI: 10.4254/wjh.v9.i12.586]
120 **van Zandwijk N**, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, Huynh Y, Chrzanowska A, Fulham MJ, Bailey DL, Cooper WA, Kritharides L, Ridley L, Pattison ST, MacDiarmid J, Brahmbhatt H, Reid G. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol* 2017; **18**: 1386-1396 [PMID: 28870611 DOI: 10.1016/S1470-2045(17)30621-6]
121 **Hosseinahli N**, Aghapour M, Duijf PHG, Baradaran B. Treating cancer with microRNA replacement therapy: A literature review. *J Cell Physiol* 2018; **233**: 5574-5588 [PMID: 29521426 DOI: 10.1002/jcp.26514]
122 **Luck ME**, Muljo SA, Collins CB. Prospects for Therapeutic Targeting of MicroRNAs in Human Immunological Diseases. *J Immunol* 2015; **194**: 5047-5052 [PMID: 25980029 DOI: 10.4049/jimmunol.1403146]
123 **Lai EC**. Two decades of miRNA biology: lessons and challenges. *RNA* 2015; **21**: 675-677 [PMID: 25780186 DOI: 10.1261/rna.051193.115]
124 **Miranda RC**, Pietrzykowski AZ, Tang Y, Sathyan P, Mayfield D, Keshavarzian A, Sampson W, Hereld D. MicroRNAs: master regulators of ethanol abuse and toxicity? *Alcohol Clin Exp Res* 2010; **34**: 575-587 [PMID: 20102566 DOI: 10.1111/j.1530-0277.2009.01126.x]
125 **Ambade A**, Satishchandran A, Szabo G. Alcoholic hepatitis accelerates early hepatobiliary cancer by increasing stemness and miR-122-mediated HIF-1α activation. *Scienti Repo* 2016; **6**: 21340 [DOI: 10.1038/srep21340]
126 **Bala S**, Csak T, Saha B, Zatsiorsky J, Kodys K, Catalano D, Satishchandran A, Szabo G. The pro-inflammatory effects of miR-155 promote liver fibrosis and alcohol-induced steatohepatitis. *J Hepatol* 2016; **64**: 1378-1387 [PMID: 26867493 DOI: 10.1016/j.jhep.2016.01.035]
127 **Lippai D**, Bala S, Catalano D, Kodys K, Szabo G. Micro-RNA-155 deficiency prevents alcohol-induced serum endotoxin increase and small bowel inflammation in mice. *Alcohol Clin Exp Res* 2014; **38**: 2217-2224 [PMID: 25156614 DOI: 10.1111/acer.12483]
128 **Yeligar S**, Tsukamoto H, Kalra VK. Ethanol-induced expression of ET-1 and ET-BR in liver sinusoidal endothelial cells and human endothelial cells involves hypoxia-inducible factor-1alpha and microrNA-199. *J Immunol* 2009; **183**: 5232-5243 [PMID: 19783678 DOI: 10.4049/jimmunol.0901084]
129 **Ao R**, Wang Y, Tong J, Wang BF. Altered microRNA-9 Expression Level is Directly Correlated with Pathogenesis of Nonalcoholic Fatty Liver Disease by Targeting Onecut2 and SIRT1. *Med Sci Monit* 2016; **22**: 3804-3819 [PMID: 27756894]
130 **Zheng L**, Lv GC, Sheng J, Yang YD. Effect of miRNA-10b in regulating cellular steatosis level by targeting PPAR-alpha expression, a novel mechanism for the pathogenesis of NAFLD. *J Gastroenterol Hepatol* 2010; **25**: 156-163 [PMID: 19780876 DOI: 10.1111/j.1440-1746.2009.05949.x]
131 **Zhang Y**, Cheng X, Lu Z, Wang J, Chen H, Fan W, Gao X, Lu D. Upregulation of miR-15b in NAFLD models and in the serum of patients with fatty liver disease. *Diabetes Res Clin Pract* 2013; **99**: 327-334 [PMID: 23287814 DOI: 10.1016/j.diabres.2012.11.025]
132 **Du J**, Niu X, Wang Y, Kong L, Wang R, Zhang Y, Zhao S, Nan Y. MiR-146a-5p suppresses activation and proliferation of hepatic stellate cells in nonalcoholic fibrosing steatohepatitis through directly targeting Wnt1 and Wnt5a. *Sci Rep* 2015; **5**: 16163 [PMID: 26537990 DOI: 10.1038/srep16163]
133 **Rodrigues PM**, Afonso MB, Simão AL, Carvalho CC, Trindade A, Duarte A, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CM, Castro RE. miR-21 ablation and obeticholic acid ameliorate nonalcoholic steatohepatitis in mice. *Cell Death Dis* 2017; **8**: e2748 [PMID: 28406477 DOI: 10.1038/cddis.2017.172]
134 **Loyer X**, Paradis V, Hénique C, Vion AC, Colnot N, Guerin CL, Devue C, On S, Scetbun J, Romain M, Paul JL, Rothenberg ME, Marcellin P, Durand F, Bedossa P, Prip-Buus C, Baugé E, Staels B, Boulanger CM, Tedgui A, Rautou PE. Liver microRNA-21 is overexpressed in non-alcoholic steatohepatitis and contributes to the disease in experimental models by inhibiting PPARα expression. *Gut* 2016; **65**: 1882-1894 [PMID: 26338827 DOI: 10.1136/gutjnl-2014-308883]
135 **Dattaroy D**, Pourhoseini S, Das S, Alhasson F, Seth RK, Nagarkatti M, Michelotti GA, Diehl AM, Chatterjee S. Micro-RNA 21 inhibition of SMAD7 enhances fibrogenesis via leptin-mediated NADPH oxidase in experimental and human nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 2015; **308**: G298-G312 [PMID: 25501551 DOI: 10.1152/ajpgi.00346.2014]
136 **Wu H**, Ng R, Chen X, Steer CJ, Song G. MicroRNA-21 is a potential link between non-alcoholic fatty liver disease and hepatocellular carcinoma via modulation of the HBP1-p53-Srebp1c pathway. *Gut* 2016; **65**: 1850-1860 [PMID: 26282675 DOI: 10.1136/gutjnl-2014-308430]
137 **Sun C**, Huang F, Liu X, Xiao X, Yang M, Hu G, Liu H, Liao L. miR-21 regulates triglyceride and cholesterol metabolism in non-alcoholic fatty liver disease by targeting HMGCR. *Int J Mol Med* 2015; **35**: 847-853 [PMID: 25605429 DOI: 10.3892/ijmm.2015.2076]
138 **Ahn J**, Lee H, Jung CH, Ha T. Lycopene inhibits hepatic steatosis via microRNA-21-induced downregulation of fatty acid-binding protein 7 in mice fed a high-fat diet. *Mol Nutr Food Res* 2012; **56**: 1665-1674 [PMID: 22968990 DOI: 10.1002/mnfr.201200182]
139 **Ng R**, Wu H, Xiao H, Chen X, Willenbring H, Steer CJ, Song G. Inhibition of microRNA-24 expression in liver prevents hepatic lipid accumulation and hyperlipidemia. *Hepatology* 2014; **60**: 554-564 [PMID: 24677249 DOI: 10.1002/hep.27153]
140 **He Q**, Li F, Li J, Li R, Zhan G, Li G, Du W, Tan H. MicroRNA-26a-interleukin (IL)-6-IL-17 axis regulates the development of non-alcoholic fatty liver disease in a murine model. *Clin Exp Immunol* 2017; **187**: 174-184 [PMID: 27377869 DOI: 10.1111/cei.12838]
141 **Alisi A**, Da Sacco L, Bruscalupi G, Piemonte F, Panera N, De Vito R, Leoni S, Bottazzo GF, Masotti A, Nobili V. Mirnome analysis reveals novel molecular determinants in the pathogenesis of diet-induced nonalcoholic fatty liver disease. *Lab Invest* 2011; **91**: 283-293 [PMID: 20956972 DOI: 10.1038/labinvest.2010.166]
142 **Liu MX**, Gao M, Li CZ, Yu CZ, Yan H, Peng C, Li Y, Li CG, Ma ZL, Zhao Y, Pu MF, Miao LL, Qi XM, Ren J. Dicer1/miR-29/HMGCR axis contributes to hepatic free cholesterol accumulation in mouse non-alcoholic steatohepatitis. *Acta Pharmacol Sin* 2017; **38**: 660-671 [PMID: 28112179 DOI: 10.1038/aps.2016.158]
143 **Mattis AN**, Song G, Hitchner K, Kim RY, Lee AY, Sharma AD, Malato Y, McManus MT, Esau CC, Koller E, Koliwad S, Lim LP, Maher JJ, Raffai RL, Willenbring H. A screen in mice uncovers repression of lipoprotein lipase by microRNA-29a as a mechanism for lipid distribution away from the liver. *Hepatology* 2015; **61**: 141-152 [PMID: 25131933 DOI: 10.1002/hep.27379]
144 **Zarrinpar A**, Gupta S, Maurya MR, Subramaniam S, Loomba R. Serum microRNAs explain discordance of non-alcoholic fatty liver disease in monozygotic and dizygotic twins: a prospective study. *Gut* 2016; **65**: 1546-1554 [PMID: 26002934 DOI: 10.1136/gutjnl-2015-309456]
145 **Min HK**, Kapoor A, Fuchs M, Mirshahi F, Zhou H, Maher J, Kellum J, Warnick R, Contos MJ, Sanyal AJ. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab* 2012; **15**: 665-674 [PMID: 22560219 DOI: 10.1016/j.cmet.2012.04.004]
146 **Xu Y**, Zalzala M, Xu J, Li Y, Yin L, Zhang Y. A metabolic stress-

inducible miR-34a-HNF4α pathway regulates lipid and lipoprotein metabolism. *Nat Commun* 2015; **6**: 7466 [PMID: 26100857 DOI: 10.1038/ncomms8466]

147 **Castro RE**, Ferreira DM, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CM. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 119-125 [PMID: 22902550 DOI: 10.1016/j.jhep.2012.08.008]

148 **Derdak Z**, Villegas KA, Harb R, Wu AM, Sousa A, Wands JR. Inhibition of p53 attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 785-791 [PMID: 23211317 DOI: 10.1016/j.jhep.2012.11.042]

149 **Celikbilek M**, Baskol M, Taheri S, Deniz K, Dogan S, Zararsiz G, Gursoy S, Guven K, Ozbakır O, Dundar M, Yucesoy M. Circulating microRNAs in patients with non-alcoholic fatty liver disease. *World J Hepatol* 2014; **6**: 613-620 [PMID: 25232454 DOI: 10.4254/wjh.v6.i8.613]

150 **Zhang N**, Lei J, Lei H, Ruan X, Liu Q, Chen Y, Huang W. MicroRNA-101 overexpression by IL-6 and TNF-α inhibits cholesterol efflux by suppressing ATP-binding cassette transporter A1 expression. *Exp Cell Res* 2015; **336**: 33-42 [PMID: 26033364 DOI: 10.1016/j.yexcr.2015.05.023]

151 **Xu Q**, Li Y, Shang YF, Wang HL, Yao MX. miRNA-103: molecular link between insulin resistance and nonalcoholic fatty liver disease. *World J Gastroenterol* 2015; **21**: 511-516 [PMID: 25593466 DOI: 10.3748/wjg.v21.i2.511]

152 **Soronen J**, Yki-Järvinen H, Zhou Y, Sädevirta S, Sarin AP, Leivonen M, Sevastianova K, Perttilä J, Laurila PP, Sigruener A, Schmitz G, Olkkonen VM. Novel hepatic microRNAs upregulated in human nonalcoholic fatty liver disease. *Physiol Rep* 2016; **4** [PMID: 26733244 DOI: 10.14814/phy2.12661]

153 **Clarke JD**, Sharapova T, Lake AD, Blomme E, Maher J, Cherrington NJ. Circulating microRNA 122 in the methionine and choline-deficient mouse model of non-alcoholic steatohepatitis. *J Appl Toxicol* 2014; **34**: 726-732 [PMID: 24217942 DOI: 10.1002/jat.2960]

154 **Takaki Y**, Saito Y, Takasugi A, Toshimitsu K, Yamada S, Muramatsu T, Kimura M, Sugiyama K, Suzuki H, Arai E, Ojima H, Kanai Y, Saito H. Silencing of microRNA-122 is an early event during hepatocarcinogenesis from non-alcoholic steatohepatitis. *Cancer Sci* 2014; **105**: 1254-1260 [PMID: 25117675 DOI: 10.1111/cas.12498]

155 **Csak T**, Bala S, Lippai D, Satishchandran A, Catalano D, Kodys K, Szabo G. microRNA-122 regulates hypoxia-inducible factor-1 and vimentin in hepatocytes and correlates with fibrosis in diet-induced steatohepatitis. *Liver Int* 2015; **35**: 532-541 [PMID: 25040043 DOI: 10.1111/liv.12633]

156 **Zhang ZC**, Liu Y, Xiao LL, Li SF, Jiang JH, Zhao Y, Qian SW, Tang QQ, Li X. Upregulation of miR-125b by estrogen protects against non-alcoholic fatty liver in female mice. *J Hepatol* 2015; **63**: 1466-1475 [PMID: 26272872 DOI: 10.1016/j.jhep.2015.07.037]

157 **Li D**, Wang X, Lan X, Li Y, Liu L, Yi J, Li J, Sun Q, Wang Y, Li H, Zhong N, Holmdahl R, Lu S. Down-regulation of miR-144 elicits proinflammatory cytokine production by targeting toll-like receptor 2 in nonalcoholic steatohepatitis of high-fat-diet-induced metabolic syndrome E3 rats. *Mol Cell Endocrinol* 2015; **402**: 1-12 [PMID: 25534427 DOI: 10.1016/j.mce.2014.12.007]

158 **Feng YY**, Xu XQ, Ji CB, Shi CM, Guo XR, Fu JF. Aberrant hepatic microRNA expression in nonalcoholic fatty liver disease. *Cell Physiol Biochem* 2014; **34**: 1983-1997 [PMID: 25562147 DOI: 10.1159/000366394]

159 **Jiang W**, Liu J, Dai Y, Zhou N, Ji C, Li X. MiR-146b attenuates high-fat diet-induced non-alcoholic steatohepatitis in mice. *J Gastroenterol Hepatol* 2015; **30**: 933-943 [PMID: 25559563 DOI: 10.1111/jgh.12878]

160 **Xiao J**, Lv D, Zhao Y, Chen X, Song M, Liu J, Bei Y, Wang F, Yang W, Yang C. miR-149 controls non-alcoholic fatty liver by targeting FGF-21. *J Cell Mol Med* 2016; **20**: 1603-1608 [PMID: 27061435 DOI: 10.1111/jcmm.12848]

161 **Lin X**, Jia J, Du T, Li W, Wang X, Wei J, Lin X, Zeng H, Yao L, Chen X, Zhuang J, Weng J, Liu Y, Lin J, Wu Q, Wang W, Yao K, Xu K, Xiao D. Overexpression of miR-155 in the liver of transgenic mice alters the expression profiling of hepatic genes associated with lipid metabolism. *PLoS One* 2015; **10**: e0118417 [PMID: 25799309 DOI: 10.1371/journal.pone.0118417]

162 **Vella S**, Gnani D, Crudele A, Ceccarelli S, De Stefanis C, Gaspari S, Nobili V, Locatelli F, Marquez VE, Rota R, Alisi A. EZH2 down-regulation exacerbates lipid accumulation and inflammation in in vitro and in vivo NAFLD. *Int J Mol Sci* 2013; **14**: 24154-24168 [PMID: 24351808 DOI: 10.3390/ijms141224154]

163 **Li B**, Zhang Z, Zhang H, Quan K, Lu Y, Cai D, Ning G. Aberrant miR199a-5p/caveolin1/PPARα axis in hepatic steatosis. *J Mol Endocrinol* 2014; **53**: 393-403 [PMID: 25312970 DOI: 10.1530/JME-14-0127]

164 **Shpyleva S**, Pogribna M, Cozart C, Bryant MS, Muskhelishvili L, Tryndyak VP, Ross SA, Beland FA, Pogribny IP. Interstrain differences in the progression of nonalcoholic steatohepatitis to fibrosis in mice are associated with altered hepatic iron metabolism. *J Nutr Biochem* 2014; **25**: 1235-1242 [PMID: 25256357 DOI: 10.1016/j.jnutbio.2014.06.012]

165 **Xiao J**, Bei Y, Liu J, Dimitrova-Shumkovska J, Kuang D, Zhou Q, Li J, Yang Y, Xiang Y, Wang F, Yang C, Yang W. miR-212 downregulation contributes to the protective effect of exercise against non-alcoholic fatty liver via targeting FGF-21. *J Cell Mol Med* 2016; **20**: 204-216 [PMID: 26648452 DOI: 10.1111/jcmm.12733]

166 **Jin X**, Chen YP, Kong M, Zheng L, Yang YD, Li YM. Transition from hepatic steatosis to steatohepatitis: unique microRNA patterns and potential downstream functions and pathways. *J Gastroenterol Hepatol* 2012; **27**: 331-340 [PMID: 21793903 DOI: 10.1111/j.1440-1746.2011.06864.x]

167 **Hoekstra M**, van der Sluis RJ, Kuiper J, Van Berkel TJ. Nonalcoholic fatty liver disease is associated with an altered hepatocyte microRNA profile in LDL receptor knockout mice. *J Nutr Biochem* 2012; **23**: 622-628 [PMID: 21764575 DOI: 10.1016/j.jnutbio.2011.03.005]

168 **Meng X**, Guo J, Fang W, Dou L, Li M, Huang X, Zhou S, Man Y, Tang W, Yu L, Li J. Liver MicroRNA-291b-3p Promotes Hepatic Lipogenesis through Negative Regulation of Adenosine 5'-Monophosphate (AMP)-activated Protein Kinase α1. *J Biol Chem* 2016; **291**: 10625-10634 [PMID: 27013659 DOI: 10.1074/jbc.M116.713768]

169 **Cheng Y**, Mai J, Hou T, Ping J. MicroRNA-421 induces hepatic mitochondrial dysfunction in non-alcoholic fatty liver disease mice by inhibiting sirtuin 3. *Biochem Biophys Res Commun* 2016; **474**: 57-63 [PMID: 27107702 DOI: 10.1016/j.bbrc.2016.04.065]

170 **Hur W**, Lee JH, Kim SW, Kim JH, Bae SH, Kim M, Hwang D, Kim YS, Park T, Um SJ, Song BJ, Yoon SK. Downregulation of microRNA-451 in non-alcoholic steatohepatitis inhibits fatty acid-induced proinflammatory cytokine production through the AMPK/AKT pathway. *Int J Biochem Cell Biol* 2015; **64**: 265-276 [PMID: 25957914 DOI: 10.1016/j.biocel.2015.04.016]

171 **Ahn J**, Lee H, Chung CH, Ha T. High fat diet induced downregulation of microRNA-467b increased lipoprotein lipase in hepatic steatosis. *Biochem Biophys Res Commun* 2011; **414**: 664-669 [PMID: 21986524 DOI: 10.1016/j.bbrc.2011.09.120]

P- Reviewer: Abenavoli L, Gonzalez-Reimers E, Kharbanda KK
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4119-4131

DOI: 10.3748/wjg.v24.i36.4119

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

REVIEW

Calcium-sensing receptor in colorectal inflammation and cancer: Current insights and future perspectives

Luca Iamartino, Taha Elajnaf, Enikö Kallay, Martin Schepelmann

Luca Iamartino, Taha Elajnaf, Enikö Kallay, Martin Schepelmann, Institute of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna 1090, Austria

ORCID number: Luca Iamartino (0000-0003-0794-3565); Taha Elajnaf (0000-0002-7293-254X); Enikö Kallay (0000-0002-4996-0104); Martin Schepelmann (0000-0002-7017-5426).

Author contributions: Iamartino L and Elajnaf T contributed equally to this paper with literature review, tables, drafting, and revision of the manuscript; Iamartino L drew the figures; Kallay E contributed to this paper with conception, revision and editing of the manuscript; Schepelmann M contributed to this paper with literature review, conception, drafting, revision and editing of the manuscript; all authors approved the final version.

Supported by the European Union's Horizon 2020 research and innovation programme, No. 675228; and the Austrian Science Fund (FWF), No. P 29948-B28.

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Martin Schepelmann, PhD, Postdoctoral Fellow, Department of Pathophysiology and Allergy Research, Medical University of Vienna, Währinger Gürtel 18-20, Vienna 1090, Austria. martin.schepelmann@meduniwien.ac.at
Telephone: +61-743-14040051230
Fax: +61-743-14040051300

Received: May 30, 2018
Peer-review started: May 30, 2018
First decision: July 6, 2018
Revised: July 11, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 28, 2018

Abstract

The extracellular calcium-sensing receptor (CaSR) is best known for its action in the parathyroid gland and kidneys where it controls body calcium homeostasis. However, the CaSR has different roles in the gastrointestinal tract, where it is ubiquitously expressed. In the colon, the CaSR is involved in controlling multiple mechanisms, including fluid transport, inflammation, cell proliferation and differentiation. Although the expression pattern and functions of the CaSR in the colonic microenvironment are far from being completely understood, evidence has been accumulating that the CaSR might play a protective role against both colonic inflammation and colorectal cancer. For example, CaSR agonists such as dipeptides have been suggested to reduce colonic inflammation, while dietary calcium was shown to reduce the risk of colorectal cancer. CaSR expression is lost in colonic malignancies, indicating that the CaSR is a biomarker for colonic cancer progression. This dual anti-inflammatory and anti-tumourigenic role of the CaSR makes it especially interesting in colitis-associated colorectal cancer. In this review, we describe the clinical and experimental evidence for the role of the CaSR in colonic inflammation and colorectal cancer, the intracellular signalling pathways which are putatively involved in these actions, and the possibilities to exploit these actions of the CaSR for future therapies of colonic inflammation and cancer.

Key words: Calcium-sensing receptor; Colon; Cancer; Inflammation; Calcimimetics; Calcilytics

Core tip: The extracellular calcium-sensing receptor (CaSR) is best known for its roles in maintaining body calcium homeostasis, but it is also expressed in the intestines where it is assumed to be involved in pathologies such as inflammatory bowel disease and colorectal cancer. It has been suggested to act as a tumour suppressor in colorectal tumourigenesis. In this review we highlight the evidence for the anti-inflammatory and anti-tumourigenic roles of the CaSR, its signalling pathways, and its potential for future use as a drug target in the context of inflammatory bowel disease and colorectal cancer.

Iamartino L, Elajnaf T, Kallay E, Schepelmann M. Calcium-sensing receptor in colorectal inflammation and cancer: Current insights and future perspectives. *World J Gastroenterol* 2018; 24(36): 4119-4131 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4119.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4119

INTRODUCTION

Extracellular calcium-sensing receptor

The extracellular calcium-sensing receptor (CaSR) was first identified in bovine parathyroid cells. It is a G protein-coupled receptor (GPCR) that is activated by extracellular calcium (Ca^{2+}), which acts as a first messenger of the CaSR signalling cascade[1]. The main physiological role of the CaSR is to control serum Ca^{2+} levels through regulating the synthesis and secretion of parathyroid hormone (PTH), which acts directly on the kidneys, bones and indirectly on the intestines to maintain normocalcaemia[2]. Therefore, the CaSR acts as a "calciostat" which maintains serum Ca^{2+} concentration within a tight range (1.1-1.3 mmol/L free ionised Ca^{2+}) and is expressed in calcitropic tissues, such as parathyroid glands, kidneys and bone. In addition to its pivotal role in maintaining serum Ca^{2+} homeostasis, the CaSR also regulates non-calcitropic functions, such as gene expression, smooth muscle contraction, differentiation, proliferation, inflammation, and ion channel activity in other tissues, such as the colon, liver, vasculature, lung, pancreas, brain and the placenta[3-6]. The CaSR is also expressed along the entire gastrointestinal (GI) tract and regulates various functions in the intestines. These include dual regulation of fluid transport, where it stimulates Cl^- and short chain fatty acid-dependent HCO_3^- secretion, but inhibits cyclic adenosine monophosphate (cAMP)-dependent HCO_3^- secretion[7]. In addition, the CaSR is expressed in the myenteric plexi where it regulates gut motility[8]. It also acts as a nutrient sensor for digestion products[9], such as amino acids. Additionally, it plays a role in intestinal inflammation and in the maintenance of gut microbiota and immune homeostasis[10].

The CaSR is a multifaceted GPCR that couples to several heterotrimeric G proteins. It modulates signalling pathways downstream of $G_{q/11}$, $G_{i/o}$, $G_{12/13}$[11] and in specific cell contexts G_s[12]. Signalling output by the CaSR is also ligand-dependent as well as cell-type specific, thus adding to the diversity of the CaSR-mediated signalling pathways. Table 1[5,16-26] shows examples of both naturally occurring and synthetic CaSR ligands and their reported direct effects on inflammation and cancer *in vivo*.

Mutations in the *CASR* gene result in Ca^{2+} homeostasis-related diseases, including familial hypocalciuric hypercalcaemia (FHH1) and neonatal severe hyperparathyroidism (NSHPT), both of which are caused by inactivating mutations, as well as autosomal dominant hypocalcaemia (ADH1), which is caused by activating mutations (for review see[27]). Such disease causing mutations result in altered signalling output by the receptor and/or reduced cell surface expression[28]. In the intestines, more focus is directed towards the CaSR as a therapeutic target for intestinal diseases including diarrhoea, inflammatory bowel disease and colorectal cancer. In the colon, loss of CaSR expression is associated with colonic tumourigenesis[29]. In addition, clinical trials show that Ca^{2+} intake can favourably modulate normal colon tissue and circulating inflammation biomarkers for risk of colorectal neoplasms in sporadic colorectal adenoma patients[17]. This has led to the hypothesis that the CaSR plays a role in cancer prevention. In the following sections, we highlight the role of the CaSR in intestinal inflammation and colorectal cancer.

ROLE OF THE CaSR IN INFLAMMATION

The CaSR is expressed in a wide range of inflammation-associated cell types where it regulates various functions. It is expressed in immune cells including macrophages, eosinophils and monocytes[5,30,31]. In these CaSR-expressing human and murine circulating monocytes, extracellular Ca^{2+} induces a chemokinetic effect[32]. The CaSR is also implicated in immune regulation where it plays a dual role: as a responder to inflammatory cytokine release on the one hand, and as a promoter of inflammation on the other. The link between the CaSR and inflammation has been explored in several studies. *In vitro*, inflammatory cytokines upregulate the CaSR expression in various cell types through defined response elements on the *CASR* gene[33,34]. *In vivo* studies also suggest a link between inflammatory cytokines and the CaSR, as intraperitoneal injection of IL-1β and IL-6 reduced PTH and $1,25(OH)_2D_3$ levels followed by a decrease in serum Ca^{2+}[33,35]. Furthermore, clinical studies show that hypocalcaemia occurs in critically ill patients where plasma inflammatory cytokines levels are increased[36]. In addition, the expression of the CaSR is increased in monocytes from rheumatoid arthritis patients with severe coronary artery calcification[37].

Table 1 Examples of orthosteric agonists and allosteric modulators of the calcium-sensing receptor[13-15]

Ligand type	Class and examples	Reported effects on inflammation	Reported effects on cancer	Ref.
Orthosteric agonists	Inorganic divalent and trivalent cations: Zn^{2+} [1]Ca^{2+}; Mg^{2+}; Gd^{3+}	Reduces inflammation in mouse models of colitis Intake is correlated with reduced inflammation	High Ca^{2+} intake: Associated low risk for CRC	[16-18]
	Polyamines: Spermine spermidine, putrescine	Increase airway inflammation and hyperresponsiveness	Reduce pancreatic cancer growth in mice	[5,19]
	Aminoglycoside antibiotics: Neomycin, gentamycin, tobramycin	-	-	
	Basic polypeptides: poly-l-arginine, [1]poly-l-lysine, and amyloid β-peptides	Induces airway inflammation Reduces inflammation in mouse models of colitis	-	[5,20]
Combined orthosteric and allosteric modulators	D-amino-acid polypeptides: Etelcalcetide	-	-	
	L-amino acids: Phenylalanine, tryptophan	-	-	
	Glutamyl dipeptides: [1]γ-Glu-Val, [1]γ-Glu-Cys	Reduces inflammation in mouse models of colitis	-	[21]
Allosteric modulators (calcimimetics and calcilytics)	Small molecule calcimimetics: Sensipar ([1]Cinacalcet HCl), NPS-R568, GSK3004774	Increases airway hyperresponsiveness	Treatment of parathyroid tumours Inhibits neuroblastoma tumour growth Reduces hypercalcaemia of malignancy	[5,22-24]
	Small molecule calcilytics: [1]NPS-2143, Calhex, Ronacalaret, AXT-914	Reduces pulmonary inflammation and airway hyperresponsiveness in rodents	-	[5,25,26]

[1]Indicates the compounds for which the *in vivo* effects were reported. While many of these modulators have been reported to have *in vitro* effects on (cancer) cell lines, evidence of their *in vivo* activity has remained scarce. The table summarises their known (putatively) CaSR-mediated direct effects on inflammation and cancer in humans or animals. CRC: Colorectal cancer.

Inflammatory pathways regulated by the CaSR

The CaSR regulates diverse and intricate signalling networks and this regulation is tissue-, and ligand-dependent. In murine macrophages, the CaSR activates the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome through a mechanism that involves increased intracellular Ca^{2+} and decreased cAMP levels[38]. Moreover, the CaSR regulates polymorphonuclear neutrophil function through a mechanism that likely involves the NFκB pathway[39]. The mechanism by which L-tryptophan, L-valine and glutamyl dipeptides mediate the CaSR-dependent inhibition of pro-inflammatory cytokine secretion in colonocytes appears to require β-arrestin 2[20,21]. Moreover, in the thick ascending limb of the kidneys, the CaSR has been shown to induce TNF-α-dependent cyclooxygenase 2 expression and prostaglandin E_2 synthesis *via* a G_i-dependent mechanism[40]. However, the exact mechanism by which the CaSR regulates inflammation is still unclear and needs further investigation.

Tissue-specific roles of the CaSR in inflammation

Interestingly, regulation of inflammation by the CaSR appears to be tissue-dependent. One example is the pivotal role of the CaSR in airway hyperresponsiveness and inflammation in allergic asthma. Studies on mice show that the calcilytic NPS-2143 ameliorates the severity of allergen-induced airway hyperresponsiveness[5]. In agreement with that, NPS-2143 was also shown by an independent group to be protective against lipopolysaccharide-induced pulmonary inflammation[26] and against inflammation caused in cigarette smoke extract-stimulated airway epithelial cells[25]. The CaSR plays a pro-inflammatory role also in human adipose cells and adipose tissue, where it induced the expression of inflammatory cytokines[41]. Paradoxically, in the intestines the CaSR has been suggested by several studies to play an anti-inflammatory role. Below, we highlight the evidence for the anti-inflammatory effects of the CaSR in the intestines and the potential to exploit it for nutraceutical and pharmaceutical intervention.

CaSR IN INTESTINAL INFLAMMATION

In vivo anti-inflammatory effects of the intestinal CaSR

Evidence supporting the role of the CaSR in intestinal inflammation comes from a study in an intestinal epithelial cell-specific CaSR knock-out mouse model. This study showed that deletion of the CaSR from the intestinal epithelial cells diminished intestinal barrier integrity, altered the composition of the gut microbiota and induced stimulatory inflammatory responses[42]. These intestine specific CaSR knock-out mice were more susceptible to dextran sulphate sodium (DSS)-induced inflammation leading to colitis, which is a model for chemically induced inflammation in rodents. *Ex vivo* assessment of intestinal permeability revealed that in the knock-out mice the paracellular transport pathway

was impaired. Consistent with that observation, the colonic expression of tight junction proteins, particularly claudin-2, was reduced in knock-out mice, while the expression of myosin light-chain kinase-1, an enzyme that controls contractility of the perijunctional actomyosin rings and epithelial permeability, was significantly increased[42]. No significant differences were seen between the overall richness and diversity of the gut microbiota of knock-out and wild type littermates, yet the bacterial composition was significantly changed. Moreover, intestine specific CaSR knock-out mice had significantly lower epithelial expression of Reg3β and Reg3γ that encode secreted C-type lectins which bind and protect against translocation and dissemination of bacteria. Furthermore, gene array analysis revealed increased expression of inflammatory cytokines including IL-1R in the distal colons of the intestinal epithelium-specific CaSR knock-out mice, as well as in their colonic $CD4^+$ and $CD8^+$ T lymphocytes. In addition, a marked increase in NFκB-dependent genes was observed in the knock-out mice. The expression of programmed cell death protein 1 (PD-1) was significantly enhanced in colonic $CD4^+$ and $CD8^+$ T cells[42].

Similarly, recent studies support the anti-inflammatory role of the CaSR in a DSS-colitis mouse model, where poly-L-lysine and glutamyl dipeptides, orthosteric agonists of the CaSR, reduced inflammation. These anti-inflammatory effects were suggested to be dependent on the CaSR, as their effect was reduced by the intravenous administration of the calcilytic NPS-2143[20,21]. Whether this inhibition of the anti-inflammatory effects was due to the systemic actions of the calcilytic or due to a direct action of the drug at the inflamed tissue is yet unknown. Studies assessing whether the expression level of the CaSR is affected by the chronic inflammation of the intestine in human patients suffering from inflammatory bowel disease are still outstanding.

Mechanisms by which the CaSR putatively modulates colonic inflammation

Studies on colon cancer cell lines using CaSR agonists and allosteric modulators suggested that the CaSR influences the production of inflammatory cytokines induced by tumour necrosis factor α (TNF-α). L-tryptophan and L-valine inhibited interleukin 8 (IL-8) secretion in both Caco-2 and HT-29 colon cancer cell lines. This effect was reversed by the calcilytic NPS-2143[20]. In addition, glutamyl dipeptides inhibited pro-inflammatory cytokines and chemokines including IL-8, IL-6, and IL-1β, while increasing the expression of the anti-inflammatory IL-10 in Caco-2 cells[21]. However, it was reported that the CaSR is not detectable in colon cancer cell lines, such as HT-29, which is also supported by evidence from independent studies indicating the scarcity of the CaSR in colon cancer tissue and cell lines[43]. Therefore, further validation is needed to confirm whether these anti-inflammatory effects are actually mediated *via* the CaSR. Of note, inflammatory cytokines, such as TNF-α, IL-1β and IL-6, increased the expression of the CaSR at the mRNA and protein level in some colon cancer cell lines[34]. This was suggested to be a defence mechanism against inflammation in the intestines. However, this explanation will have to be carefully validated, as *e.g.*, in lung epithelium, CaSR expression is also increased in the inflamed tissue. There however, the increase (and indeed the CaSR itself) represent a rather pro-inflammatory mechanism, as inhibition of the CaSR markedly reduced airway inflammation and hyperresponsiveness[5].

Given that inflammation is a high risk factor for colorectal cancer, it is imperative to ask the following question: is there a causal relationship between activation of the CaSR, reduced inflammation and the prevention of colorectal cancer? As of yet, this question remains unanswered. It is unclear whether dietary or pharmacological activation of the CaSR in the GI tract prevents inflammation in humans. It is also still unclear whether loss of the CaSR in colorectal tumours correlates with loss of its proposed anti-inflammatory effects. Moreover, it is noteworthy that the presence of inflammatory cytokines in the GI tract and their effect on the expression and/or function of the CaSR add to the complexity of the scenario *in vivo*. Nonetheless, inflammation is a key risk factor for colorectal cancer[44,45], thus targeting the CaSR for mitigating inflammation may very well contribute to colorectal cancer prevention in one fell swoop. Below, we summarise the evidence for the involvement of the CaSR in cancer and specifically colorectal cancer as well as its potential as a therapeutic target.

ROLE OF THE CaSR IN CANCER

The CaSR plays a ying-yang role in tumours: while it is suggested to be an oncogene in breast and prostate tumours, in parathyroid, neuroblastoma and colorectal cancers it acts as a tumour-suppressor[46-48]. The CaSR signals *via* multiple signalling pathways and is sensitive to many ligands, the bioavailability of which varies among tissues. The different ligands and different signalling pathways can generate a tissue-specific CaSR response, justifying this dual behaviour during cancer development. Table 2[3,19,29,49-72] summarises the different roles of the CaSR in various types of cancer.

CaSR acts both as oncogene and tumour suppressor

The CaSR was implicated in the promotion of metastases from breast, prostate, and kidney tumours, thus acting as an oncogene in these tissues. Its oncogenic role is often mediated by parathyroid hormone related peptide (PTHrP).

Breast cancer has a tendency to form metastases in particular in the bones[73]. Metastases originated from breast tumours promote bone resorption which, in turn, causes the release of trophic factors (*e.g.*, TGF-β and IGF1) that stimulate tumour cell growth, thus forming a vicious cycle. Osteolysis is driven by osteoclasts that are activated by PTHrP, which is synthesised and

Table 2 Dual function of the calcium-sensing receptor as tumour suppressor and oncogene in various cancers and the affected calcium-sensing receptor-coupled signalling pathways

Cancer type	CaSR	Expression of the CaSR	Detection	Proposed signalling pathway	Ref.
Gastric	Oncogene	Increased	mRNA, protein	TRPV4	[49]
Prostate	Oncogene	Increased	mRNA, protein	PTHrP *via* trans-activation of the EGFR and ERK1/2 phosphorylation AKT phosphorylation	[50-52]
Breast	Oncogene	Increased in breast primary tumours and in bone metastases	mRNA, protein	PTHrP *via* cAMP ERK1/2 and TRPC1 Inhibition of OPG *via* epiregulin	[53-57]
Renal carcinoma	Oncogene	Increased in bone metastasising tumours	mRNA, protein	AKT phosphorylation	[58]
Colorectal	Tumour suppressor	Reduced	mRNA, protein	Canonical and non-canonical Wnt/β-catenin pathway and EMT	[3,29,59-62]
Endometrial	Tumour suppressor	Reduced	Protein	Apoptosis Wnt/β-catenin VEGFR3	[63]
Parathyroid	Tumour suppressor	Reduced	mRNA, protein	Caveolin-1 and Gαq Cyclin D1 and RGS5	[64-69]
Neuro-blastoma	Tumour suppressor	Reduced	mRNA, protein	Apoptosis *via* ERK1/2 Cancer testis antigens (CTAs)	[22,70,71]
Pancreatic	Unknown	Reduced	mRNA, protein	NCX1/Ca^{2+}/ β-catenin	[19,72]

released from breast cancer cells[3,74,75]. The CaSR, highly expressed in metastatic breast cancer cells[53], stimulates PTHrP release, contributing thereby to bone degradation[54]. A recent study revealed that cancer cells overexpressing the CaSR had a higher osteolytic potential compared with untransfected cells[57]. Therefore, the CaSR could be a predictive marker for bone metastasis and for the patient's poor prognosis.

Like breast cancers, prostate neoplastic lesions have a high capacity to form metastasis in the bone. Highly aggressive prostate cancer cells, such as PC-3, express the CaSR[50] while there is no evidence of CaSR expression in normal prostate tissue[3]. A cohort study, analysing 1241 prostate cancer patients, found that expression of the CaSR correlated positively with tumour lethality[76].

Although dietary calcium has been suggested to have beneficial effects on the digestive tract as being preventative against colorectal cancer, a recent study pointed out a controversial effect of calcium on gastric cancer development. Xie *et al*[49], have shown that calcium-activated CaSR promoted gastric cancer cell proliferation and metastasis. Thus, CaSR is suggested to act as an oncogene in the upper part of the gastro-intestinal tract, whereas it seems to act as a tumour suppressor in the lower gastro-intestinal tract (see below) although further studies are required to confirm this hypothesis.

In other cancers like parathyroid cancers, neuroblastoma and colorectal cancer the receptor acts as a tumour suppressor. In parathyroid tumours CaSR expression is inversely correlated with tumour development. CaSR mRNA expression is reduced in parathyroid adenomas and hyperplasias as compared with normal parathyroid tissue and it is lost in parathyroid carcinoma[65]. In the nervous system, the CaSR is expressed during the differentiation of neurons and glial cells[77,78]. In neuroblastoma, CaSR expression is positively correlated with neuroblast differentiation and low clinical risk, while undifferentiated and malignant neuroblastomas are CaSR-negative[79]. Indeed, ectopic re-expression of the CaSR in MYCN-amplified neuroblastoma cells, which are normally CaSR negative, reduced xenograft growth[71]. In addition, treatment with cinacalcet, a positive allosteric modulator of the CaSR, was able to induce the expression of differentiation markers, to inhibit cell proliferation *in vitro* and the growth of mouse tumour xenografts *in vivo*[22].

Another organ in which the CaSR acts as tumour suppressor is the colon.

CaSR IN COLORECTAL CANCER

The physiological role of the colon is to process and absorb undigested nutrients, absorb electrolytes and water, and to excrete waste products *via* the rectum. As it is a highly renewable tissue, it is prone to malignant transformation. Colorectal cancer (CRC) is one of the most recurrent types of malignancies in the western countries and accounts for over 1,2 million of new cases per year[80]. Colorectal tumourigenesis is a complex mechanism developing from the alteration of different molecular processes that control gene expression, cell cycle and apoptosis, which are affected by genetic (*e.g.*, APC mutation), environmental *(e.g.*, diet, alcohol abuse, cigarette smoking, *etc.*), microbial and inflammatory cues that either activate oncogenes or repress tumour suppressors leading then to tumour

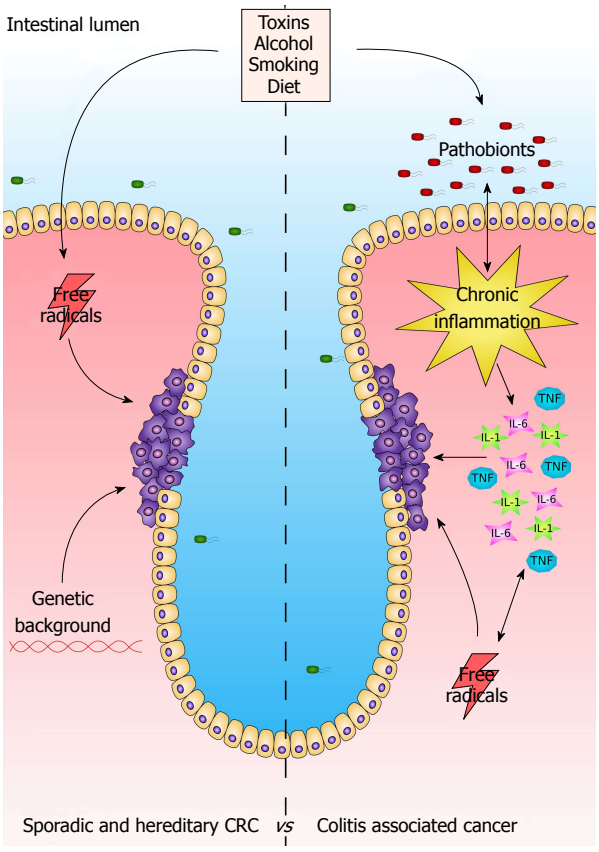


Figure 1 Scheme of colonic carcinogenesis. Left: Environmental cues such as toxins, alcohol, smoke and diet can produce free radicals (such as reactive oxygen and nitrogen species) that can damage genomic DNA. Accumulating mutations, in particular in genes that encode for mitogenic, cell cycle or apoptosis factors such as APC, BRAF, KRAS, EGF and p53 can then eventually lead to colon carcinogenesis. Genetic background such as inborn APC mutations (hereditary familial adenomatous polyposis) or other hereditary mutations also predispose towards colon tumourigenesis, although hereditary CRC is rare[81]. Right: In addition to their direct noxious effect on the tissue, environmental cues can also alter the microbiotic population of the intestine, promoting the proliferation of pathogenic bacteria (pathobionts). Pathobionts and chronic inflammation are closely related and both induce the expression of pro-inflammatory cytokines that accumulate in the mucosa. Persistent inflammation interferes with cell proliferation and apoptosis processes leading to tumourigenesis and in particular in colitis associated cancer. Inflammation itself also induces the production of free radicals that hamper genome stability and can thus cause tumour development[82]. TNF: Tumor necrosis factor; IL: Interleukin; CRC: Colorectal cancer.

development (Figure 1[81,82]).

As mentioned above, colonic inflammation is a risk factor for developing colorectal cancer. Chronic intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis often lead to colorectal cancer through a process called colitis-associated carcinogenesis (CAC). Similarly to (spontaneous) CRC, CAC leads to genome instability, targeting tumour suppressors and DNA repair mechanisms. However, CRC and CAC differ for prevalence and sequential-timing of the changes in biomarkers during their pathogenesis[83].

CAC is often accompanied by the alteration of the gut microbiota (dysbiosis). Commensal bacteria (eubionts) help to metabolise undigested food, modulating also the immune system of the digestive tract. On the other hand, pathogenic bacteria can trigger an immune response that, in the worst case, can lead to chronic colitis and other inflammatory bowel diseases[84]. As detailed above, the CaSR has been implicated in affecting gut microbiota, and the expression of inflammatory cytokines and thus might play a protective role against the development of CAC by protecting from the deleterious effects of inflammation.

Epidemiology

In 1985, a small trial demonstrated for the first time that calcium regulates colonocyte proliferation[85]. In the same year, Garland *et al*[16] published a retrospective study showing that diets with high calcium content lower the risk of developing colorectal tumours. In the following years, several cohort studies and animal experiments supported the theory that diets rich in calcium and vitamin D prevent the development of colon hyperplasia and cancer - in contrast to western diets with high fat and low calcium and fibre content[86-88]. Meta-analyses have since reported that high Ca^{2+} intake (more than 1400 mg/d), independent of its source, lowers the risk of CRC, in particular in the distal colon[89,90]. Indeed, the evidence for the protective actions of high levels of dietary calcium intake (dairy products) or calcium supplements was rated to be "probably strong" by the World Cancer Research Fund in its most recent update of 2017[91].

Numerous studies have suggested that there is a close interaction between the CaSR and calcium and its protective action against CRC. A randomised clinical trial found that dietary calcium supplementation increased the expression of the CaSR in the colonic mucosa[92]. In a meta-analysis, Yang *et al*[93] showed that while dietary calcium reduced the risk of developing CaSR positive tumours, the risk for CaSR negative ones remained unchanged, suggesting that dietary calcium exerts its anti-tumourigenic properties *via* CaSR. A recent study demonstrated that CaSR expression in the tumours correlated with a reduced risk of mortality, indicating that CaSR expression might be a biomarker for positive prognosis[94].

CaSR localisation in the intestine

A common agreement on the pattern of CaSR localization in the intestine is still missing. Whitfield suggested that the Ca^{2+} concentration is unevenly distributed along the colonic crypts, with low levels found at the bottom of the crypts and higher levels at the top. In this way, Ca^{2+} could exert its pro-differentiating and anti-proliferative effects only in the upper part of the crypts where the post-differentiated mature colonocytes are localised. CaSR activation would follow this concentration gradient along the crypts. Stronger activation of the CaSR at the top and weaker activation at the bottom could thus provide a physiological rationale for why the CaSR would inhibit proliferation on the differentiated top but allow proliferation at the rapidly dividing bottom of the

crypts[95]. It was also suggested that this Ca^{2+} gradient influences CaSR expression itself, in addition to the receptor's activation[46]. This theory is supported by the studies of Chakrabarty *et al*[96], who have found CaSR protein to be expressed only in the upper half of the crypts of human colon cancer biopsies. However, the actual expression pattern of the CaSR in the colon is still under debate. Contrary to the findings by Chakrabarty *et al*[96], Sheinin *et al*[97] have found CaSR expression only in the enteroendocrine cells of human colonic mucosa[97], whereas Cheng *et al*[8] have found the CaSR in the enteric nervous system and in the apical and basolateral side of the crypts of rat colons[8,98]. Further studies are therefore required to determine accurately the location of the CaSR in the colon and whether this expression pattern is dependent on factors like diet, age, *etc.*

We know that CaSR expression is lost in tumour cells. While it is still found in pre-neoplastic lesions, expression of the CaSR is lost in poorly differentiated tumours[29,96,99,100]. However, whether this loss is cause or effect of the tumourigenesis is still unknown.

CaSR down-regulation in CRC

Epigenetic aberrancies play a major role in tumour malignancy in general and thereby also in colorectal cancer[101]. CaSR expression is affected by repressive epigenetic marks in malignant colorectal lesions. The promoter region of the CaSR contains a large CpG island which is highly methylated in colorectal tumours. CaSR expression could be partially restored in colorectal cancer cell lines by the administration of 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation. This effect was further enhanced with the addition of histone deacetylase inhibitors, suggesting that in the CaSR promoter regions the acetylation of histones is reduced and, therefore, the chromatin has a less permissive structure that hinders the recruitment of the transcription machinery[29,102]. The level of CaSR methylation increases from hyperplastic polyps and adenomas to lymph node metastases in parallel with the reduction of the receptor's expression[102]. However, this is not a general mechanism, as in parathyroid tumours no hypermethylation of the CaSR locus was found[64,103].

Non-coding RNAs, such as miRNAs, also regulate CaSR expression in colorectal tumours. Different studies found that miR-21, miR-135a, miR-135b, miR-145, miR-146b and miR-503 inhibited CaSR expression in CRC cell lines and therefore constitute potential targets for restoring CaSR mRNA level[61,104,105].

So far, no CaSR mutations have been found that would promote tumour development in the intestine[48], although several SNPs (*e.g.*, Q1011E, A986S, R990G) might increase colorectal cancer susceptibility although their contribution is controversial[106-109].

It is important to fully understand the molecular mechanisms that drive CaSR loss during colorectal carcinogenesis and whether this loss could be reverted or prevented and whether such an action would be beneficial for patient prognosis, pointing towards the CaSR as potential therapeutic target for a novel anti-CRC therapy or prevention.

Evidence and molecular pathways for the anti-tumourigenic actions of the CaSR

Mouse models of systemic CaSR knock-out are not viable or die shortly after birth due to severe hyperparathyroidism and hypercalcemia[110]. However knocking out PTH rescues the lethal $CaSR^{-/-}$ phenotype in the PTH double knock-out ($PTH^{-/-}$ $CaSR^{-/-}$) mouse model[111]. The colonic mucosa of the $PTH^{-/-}$ $CaSR^{-/-}$ mice as well as that of the intestinal epithelium-specific CaSR knock-out mouse model show signs of hyperproliferation. These mice develop pre-malignant intestinal lesions and are highly susceptible to the carcinogen azoxymethane (AOM)[112].The intestines of these mice are often inflamed and express pro-inflammatory markers. Furthermore, $PTH^{-/-}$ $CaSR^{-/-}$ mice are highly sensitive to DSS induced inflammation as well, suggesting a possible role of the CaSR as an anti-inflammatory factor[42,112].

Overexpression of the exogenous CaSR in colon cancer cell lines induced cellular differentiation and apoptosis, and inhibited proliferation and invasion capacity in these transfected cells. Presence of the CaSR repressed expression of stem cells markers, re-established the expression of E-cadherin and inhibited epithelial to mesenchymal transition, a process exploited by cancer cells to form metastases[47,59].

Ca^{2+} exerts its anti-tumourigenic function not only by binding and precipitating toxic agents such as secondary bile acids and fatty acids but also by modulating different cellular mechanisms such as proliferation, differentiation and apoptosis, potentially *via* the CaSR[3,100,113,114]. The mechanism involves inhibition of c-myc, upregulation of E-cadherin and inhibition of the canonical wnt-signalling pathway[99,100,115]. A recent study reported that Ca^{2+} inhibited the expression of replication-licensing factors in a CaSR dependent manner[60].

Both colorectal cancer cell lines and CaSR-deficient mice show that loss of the CaSR causes a higher recruitment of β-catenin into the nucleus, thus sustaining a proliferative Wnt pathway[59,112,116]. However, some studies have discovered that the CaSR is able to activate the non-canonical Wnt pathway involving the interaction between Wnt5a and its receptor, Ror2 (receptor tyrosine kinase-like orphan receptor 2). Wnt5a/Ror2 counteracts the proliferative signalling of Wnt/β-catenin, recruiting the ubiquitin ligase Siah2, which, in turn, degrades β-catenin. In myofibroblasts, CaSR activation induces the secretion of Wnt5a, while, in colonic epithelia CaSR increases the expression of Ror2[62]. Thus, the CaSR might stimulate the Wnt5a/Ror2 paracrine pathway which inhibits colonic proliferation, interfering with Wnt/β-catenin, and seems to promote the expression of colonic differentiation markers such as sucrase-isomaltase, caudal type homeobox 2 and villin[62,117,118].

CaSR pathways could potentially interact with many cellular processes in preventing or counteracting

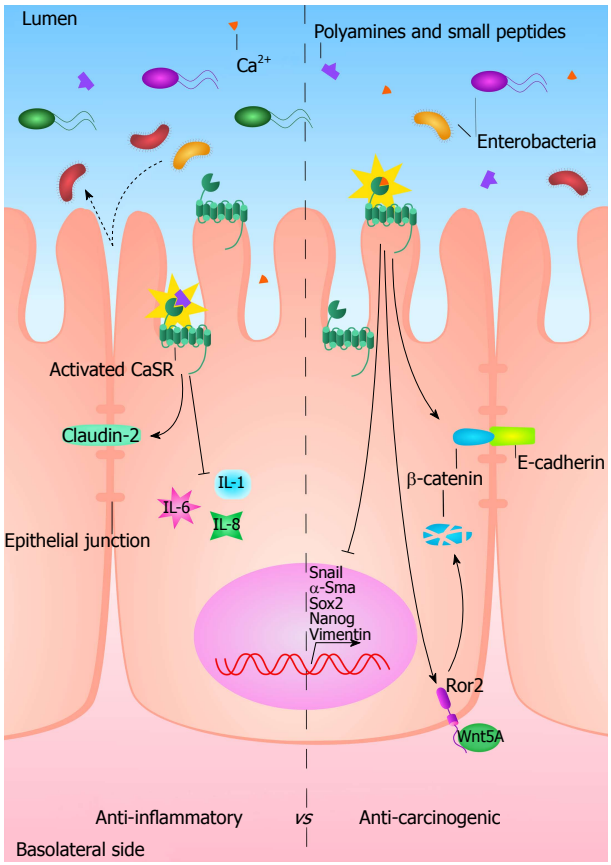


Figure 2 Protective function of the calcium-sensing receptor against inflammation and carcinogenesis in the colon. Left: The CaSR promotes intestinal barrier integrity, potentially by promoting claudin-2 expression, and inhibits the expression of pro-inflammatory cytokines, thus preventing inflammation. Right: The CaSR exerts an anti-tumourigenic effect by counteracting the mitogenic Wnt pathway, preventing β-catenin translocation into the nucleus, which is either sequestered by E-cadherin at the cell junctions or it is degraded by the non-canonical Wnt signalling (Ror2-Wnt5A) and inhibits the expression of mesenchymal and stem cells markers. IL: Interleukin; CaSR: Calcium-sensing receptor.

tumour development and progression. In this context, the existence of a cross talk between the CaSR and the vitamin D system has been suggested. It seems that both pathways converge in the modulation of the Wnt signalling to control colonocyte proliferation. Moreover, vitamin D seems to regulate CaSR transcription[119,120] through regulatory elements present in the CaSR promoter, which are recognised by the transcription factor vitamin D receptor. Indeed, a high vitamin D (2500 IU/kg) diet over 5 weeks more than doubled the expression of the CaSR in the colon mucosa of mice[121,122].

As of yet, a detailed description of CaSR signalling in the intestine is still missing. Given the fact that the CaSR is able to sense not only Ca^{2+}, but also polyamines and amino acids, which are highly abundant in the intestinal lumen through the food, and that ligand biased signalling is a known feature of the CaSR[3], it is possible that the CaSR could activate different down-stream signals depending on these specific ligands also in the colon. Potential mechanisms by which the CaSR could affect inflammation and CRC are summed up in Figure 2 but a detailed map of the molecular pathways that the CaSR activates in the gut is still missing. This would allow researchers to discover potential therapeutic targets for counteracting intestinal tumourigenesis.

FUTURE PERSPECTIVES - THE CaSR AS A DRUGGABLE TARGET IN THE COLON

The CaSR is considered to prevent or counteract intestinal carcinogenesis and inflammation. Thus, the CaSR might constitute a promising therapeutic target for the treatment of colorectal cancer and of inflammatory bowel diseases. Dietary Ca^{2+} supplementation reduces the risk for developing colorectal cancer and studies have shown the beneficial effects of CaSR agonists, such as dipeptides, polyamines for preventing colonic inflammation and cancer. As chronic inflammation is a risk factor for colorectal cancer, the CaSR might actually be a link that connects the beneficial effect of Ca^{2+} in preventing both inflammation and cancer in the colon. Indeed, these roles of the CaSR indicate that activating the CaSR, or in the case of CRC also restoring CaSR expression - or preventing its loss - might be an important way for treating or preventing colonic inflammation, CRC, and, especially, CAC. However, a direct pharmacological intervention study targeting the CaSR in colonic inflammation or colorectal cancer is still missing.

Further research will be required for finding and evaluating means to restore or prevent the loss of the expression of the CaSR during carcinogenesis. One such mean could be the use of pharmacological CaSR activators, the calcimimetics. In addition to their action as allosteric agonists of the CaSR, calcimimetics also act as so called "pharmacochaperones" for the CaSR. They stabilise the expression of the CaSR, preventing the receptor's degradation. At the same time, they increase trafficking of the CaSR from its intracellular reservoirs into the cell membrane[123,124]. As of yet, there are no data for the efficacy of calcimimetics for the prevention / treatment of CRC or CAC.

As chronic inflammation is posing a high risk for developing CAC, preventative measures should be administrable over long periods of time and should therefore ideally elicit few or no systemic side effects. Recently a novel calcimimetic, GSK3004774, which is non-resorbable and thus has gut restricted effects, has been published[125]. This compound could be useful for testing whether locally acting calcimimetics can elicit a preventive effect against intestinal inflammation, CRC and CAC without affecting systemic calcium homeostasis.

Known side effects of the FDA-approved calcimimetic cinacalcet treatment include hypocalcaemia and, notably, nausea[126]. Whether these gastrointestinal tract-related side effects are elicited *via* the systemic actions of the drug or a direct effect of the drug on the gastrointestinal

organs is unclear. In addition, calcimimetics have been shown to actually enhance inflammation in other epithelial tissues, *e.g.*, the lung, while calcilytics, antagonists of the CaSR, ameliorated the inflammation. In this context, the CaSR also promoted the activation of the immune system and showed a general pro-inflammatory action[5]. Whether these *in vivo* effects - in the complex context of immune-cells, inflamed tissue and cytokines - are tissue specific or related to a ubiquitous activation of CaSR-bearing lymphocytes is unclear. Taken together, these considerations do not allow a definite conclusion for a potential treatment of colonic inflammation or cancer with pharmacological CaSR modulators alone or in combination with conventional or targeted chemotherapies. Extensive future studies will be required to satisfactorily answer all these questions.

CONCLUSION

The CaSR emerges as a direct player in colonic inflammation and cancer development. Current evidence suggests that activation of the CaSR reduces the risk for both diseases, the strongest evidence being that dietary Ca^{2+} reduces the risk for CRC and that this effect is apparently mediated by the CaSR while expression of the CaSR is lost during tumourigenesis and progression of CRC. Making direct use of the CaSR as a drug target to reduce or prevent colonic inflammation and at the same time prevent colonic tumourigenesis seems a promising strategy, especially for CAC, where a dietary or pharmacological intervention could hit two birds with one stone, as it were. Future studies will be needed to address where exactly the receptor is expressed in the colonic microenvironment, which signalling pathways are mediated by the CaSR in the settings of inflammation and cancer *in vivo,* and whether these actions of the CaSR can be exploited for therapy and prevention.

REFERENCES

1 **Brown EM**, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* 1993; **366**: 575-580 [PMID: 8255296 DOI: 10.1038/366575a0]

2 **Conigrave AD**. The Calcium-Sensing Receptor and the Parathyroid: Past, Present, Future. *Front Physiol* 2016; **7**: 563 [PMID: 28018229 DOI: 10.3389/fphys.2016.00563]

3 **Brennan SC**, Thiem U, Roth S, Aggarwal A, Fetahu ISh, Tennakoon S, Gomes AR, Brandi ML, Bruggeman F, Mentaverri R, Riccardi D, Kallay E. Calcium sensing receptor signalling in physiology and cancer. *Biochim Biophys Acta* 2013; **1833**: 1732-1744 [PMID: 23267858 DOI: 10.1016/j.bbamcr.2012.12.011]

4 **Schepelmann M**, Yarova PL, Lopez-Fernandez I, Davies TS, Brennan SC, Edwards PJ, Aggarwal A, Graça J, Rietdorf K, Matchkov V, Fenton RA, Chang W, Krssak M, Stewart A, Broadley KJ, Ward DT, Price SA, Edwards DH, Kemp PJ, Riccardi D. The vascular Ca2+-sensing receptor regulates blood vessel tone and blood pressure. *Am J Physiol Cell Physiol* 2016; **310**: C193-C204 [PMID: 26538090 DOI: 10.1152/ajpcell.00248.2015]

5 **Yarova PL**, Stewart AL, Sathish V, Britt RD Jr, Thompson MA, P Lowe AP, Freeman M, Aravamudan B, Kita H, Brennan SC, Schepelmann M, Davies T, Yung S, Cholisoh Z, Kidd EJ, Ford WR, Broadley KJ, Rietdorf K, Chang W, Bin Khayat ME, Ward DT, Corrigan CJ, T Ward JP, Kemp PJ, Pabelick CM, Prakash YS, Riccardi D. Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. *Sci Transl Med* 2015; **7**: 284ra60 [PMID: 25904744 DOI: 10.1126/scitranslmed.aaa0282]

6 **Lopez-Fernandez I**, Schepelmann M, Brennan SC, Yarova PL, Riccardi D. The calcium-sensing receptor: one of a kind. *Exp Physiol* 2015; **100**: 1392-1399 [PMID: 26105576 DOI: 10.1113/EP085137]

7 **Tang L**, Peng M, Liu L, Chang W, Binder HJ, Cheng SX. Calcium-sensing receptor stimulates Cl(-)- and SCFA-dependent but inhibits cAMP-dependent HCO3(-) secretion in colon. *Am J Physiol Gastrointest Liver Physiol* 2015; **308**: G874-G883 [PMID: 25792563 DOI: 10.1152/ajpgi.00341.2014]

8 **Cheng SX**. Calcium-sensing receptor inhibits secretagogue-induced electrolyte secretion by intestine via the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G60-G70 [PMID: 22517767 DOI: 10.1152/ajpgi.00425.2011]

9 **Brennan SC**, Davies TS, Schepelmann M, Riccardi D. Emerging roles of the extracellular calcium-sensing receptor in nutrient sensing: control of taste modulation and intestinal hormone secretion. *Br J Nutr* 2014; **111** Suppl 1: S16-S22 [PMID: 24382107 DOI: 10.1017/S0007114513002250]

10 **Owen JL**, Cheng SX, Ge Y, Sahay B, Mohamadzadeh M. The role of the calcium-sensing receptor in gastrointestinal inflammation. *Semin Cell Dev Biol* 2016; **49**: 44-51 [PMID: 26709005 DOI: 10.1016/j.semcdb.2015.10.040]

11 **Conigrave AD**, Ward DT. Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. *Best Pract Res Clin Endocrinol Metab* 2013; **27**: 315-331 [PMID: 23856262 DOI: 10.1016/j.beem.2013.05.010]

12 **Mamillapalli R**, VanHouten J, Zawalich W, Wysolmerski J. Switching of G-protein usage by the calcium-sensing receptor reverses its effect on parathyroid hormone-related protein secretion in normal versus malignant breast cells. *J Biol Chem* 2008; **283**: 24435-24447 [PMID: 18621740 DOI: 10.1074/jbc.M801738200]

13 **Widler L**. Calcilytics: antagonists of the calcium-sensing receptor for the treatment of osteoporosis. *Future Med Chem* 2011; **3**: 535-547 [PMID: 21526895 DOI: 10.4155/fmc.11.17]

14 **Colella M**, Gerbino A, Hofer AM, Curci S. Recent advances in understanding the extracellular calcium-sensing receptor. *F1000Res* 2016; **5** [PMID: 27803801 DOI: 10.12688/f1000research.8963.1]

15 **Ward DT**, Riccardi D. New concepts in calcium-sensing receptor pharmacology and signalling. *Br J Pharmacol* 2012; **165**: 35-48 [PMID: 21627634 DOI: 10.1111/j.1476-5381.2011.01511.x]

16 **Heilbrun LK**, Nomura A, Hankin JH, Stemmermann GN. Dietary vitamin D and calcium and risk of colorectal cancer. *Lancet* 1985; 925-925 [PMID: 2858763]

17 **Bostick RM**. Effects of supplemental vitamin D and calcium on normal colon tissue and circulating biomarkers of risk for colorectal neoplasms. *J Steroid Biochem Mol Biol* 2015; **148**: 86-95 [PMID: 25597952 DOI: 10.1016/j.jsbmb.2015.01.010]

18 **Schepens MA**, Schonewille AJ, Vink C, van Schothorst EM, Kramer E, Hendriks T, Brummer RJ, Keijer J, van der Meer R, Bovee-Oudenhoven IM. Supplemental calcium attenuates the colitis-related increase in diarrhea, intestinal permeability, and extracellular matrix breakdown in HLA-B27 transgenic rats. *J Nutr* 2009; **139**: 1525-1533 [PMID: 19535420 DOI: 10.3945/jn.109.105205]

19 **Tang B**, Chow JY, Dong TX, Yang SM, Lu DS, Carethers JM, Dong H. Calcium sensing receptor suppresses human pancreatic tumorigenesis through a novel NCX1/Ca(2+)/β-catenin signaling pathway. *Cancer Lett* 2016; **377**: 44-54 [PMID: 27108064 DOI: 10.1016/j.canlet.2016.04.027]

20 **Mine Y**, Zhang H. Anti-inflammatory Effects of Poly-L-lysine in Intestinal Mucosal System Mediated by Calcium-Sensing Receptor Activation. *J Agric Food Chem* 2015; **63**: 10437-10447 [PMID: 26588227 DOI: 10.1021/acs.jafc.5b03812]

21 **Zhang H**, Kovacs-Nolan J, Kodera T, Eto Y, Mine Y. γ-Glutamyl cysteine and γ-glutamyl valine inhibit TNF-α signaling in intestinal epithelial cells and reduce inflammation in a mouse model of colitis via allosteric activation of the calcium-sensing receptor. *Biochim Biophys Acta* 2015; **1852**: 792-804 [PMID: 25558818 DOI: 10.1016/j.bbadis.2014.12.023]

22 **Rodríguez-Hernández CJ**, Mateo-Lozano S, García M, Casalà C, Briansó F, Castrejón N, Rodríguez E, Suñol M, Carcaboso AM, Lavarino C, Mora J, de Torres C. Cinacalcet inhibits neuroblastoma tumor growth and upregulates cancer-testis antigens. *Oncotarget* 2016; **7**: 16112-16129 [PMID: 26893368 DOI: 10.18632/oncotarget.7448]

23 **Asonitis N**, Kassi E, Kokkinos M, Giovanopoulos I, Petychaki F, Gogas H. Hypercalcemia of malignancy treated with cinacalcet. *Endocrinol Diabetes Metab Case Rep* 2017; **2017** [PMID: 29399364 DOI: 10.1530/EDM-17-0118]

24 **Rothe HM**, Liangos O, Biggar P, Petermann A, Ketteler M. Cinacalcet treatment of primary hyperparathyroidism. *Int J Endocrinol* 2011; **2011**: 415719 [PMID: 21461394 DOI: 10.1155/2011/415719]

25 **Lee JW**, Park JW, Kwon OK, Lee HJ, Jeong HG, Kim JH, Oh SR, Ahn KS. NPS2143 Inhibits MUC5AC and Proinflammatory Mediators in Cigarette Smoke Extract (CSE)-Stimulated Human Airway Epithelial Cells. *Inflammation* 2017; **40**: 184-194 [PMID: 27866297 DOI: 10.1007/s10753-016-0468-2]

26 **Lee JW**, Park HA, Kwon OK, Park JW, Lee G, Lee HJ, Lee SJ, Oh SR, Ahn KS. NPS 2143, a selective calcium-sensing receptor antagonist inhibits lipopolysaccharide-induced pulmonary inflammation. *Mol Immunol* 2017; **90**: 150-157 [PMID: 28800474 DOI: 10.1016/j.molimm.2017.07.012]

27 **Brown EM**, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev* 2001; **81**: 239-297 [PMID: 11152759 DOI: 10.1152/physrev.2001.81.1.239]

28 **Leach K**, Sexton PM, Christopoulos A, Conigrave AD. Engendering biased signalling from the calcium-sensing receptor for the pharmacotherapy of diverse disorders. *Br J Pharmacol* 2014; **171**: 1142-1155 [PMID: 24111791 DOI: 10.1111/bph.12420]

29 **Fetahu IS**, Höbaus J, Aggarwal A, Hummel DM, Tennakoon S, Mesteri I, Baumgartner-Parzer S, Kállay E. Calcium-sensing receptor silencing in colorectal cancer is associated with promoter hypermethylation and loss of acetylation on histone 3. *Int J Cancer* 2014; **135**: 2014-2023 [PMID: 24691920 DOI: 10.1002/ijc.28856]

30 **Yamaguchi T**, Kifor O, Chattopadhyay N, Bai M, Brown EM. Extracellular calcium (Ca2+o)-sensing receptor in a mouse monocyte-macrophage cell line (J774): potential mediator of the actions of Ca2+o on the function of J774 cells. *J Bone Miner Res* 1998; **13**: 1390-1397 [PMID: 9738511 DOI: 10.1359/jbmr.1998.13.9.1390]

31 **Yamaguchi T**, Olozak I, Chattopadhyay N, Butters RR, Kifor O, Scadden DT, Brown EM. Expression of extracellular calcium (Ca2+o)-sensing receptor in human peripheral blood monocytes. *Biochem Biophys Res Commun* 1998; **246**: 501-506 [PMID: 9610391 DOI: 10.1006/bbrc.1998.8648]

32 **Olszak IT**, Poznansky MC, Evans RH, Olson D, Kos C, Pollak MR, Brown EM, Scadden DT. Extracellular calcium elicits a chemokinetic response from monocytes in vitro and in vivo. *J Clin Invest* 2000; **105**: 1299-1305 [PMID: 10792005 DOI: 10.1172/JCI9799]

33 **Canaff L**, Hendy GN. Calcium-sensing receptor gene transcription is up-regulated by the proinflammatory cytokine, interleukin-1beta. Role of the NF-kappaB PATHWAY and kappaB elements. *J Biol Chem* 2005; **280**: 14177-14188 [PMID: 15684428 DOI: 10.1074/jbc.M408587200]

34 **Fetahu IS**, Hummel DM, Manhardt T, Aggarwal A, Baumgartner-Parzer S, Kállay E. Regulation of the calcium-sensing receptor expression by 1,25-dihydroxyvitamin D3, interleukin-6, and tumor necrosis factor alpha in colon cancer cells. *J Steroid Biochem Mol Biol* 2014; **144** Pt A: 228-231 [PMID: 24176760 DOI: 10.1016/j.jsbmb.2013.10.015]

35 **Canaff L**, Zhou X, Hendy GN. The proinflammatory cytokine, interleukin-6, up-regulates calcium-sensing receptor gene transcription via Stat1/3 and Sp1/3. *J Biol Chem* 2008; **283**: 13586-13600 [PMID: 18348986 DOI: 10.1074/jbc.M708087200]

36 **Zaloga GP**. Hypocalcemia in critically ill patients. *Crit Care Med* 1992; **20**: 251-262 [PMID: 1737459 DOI: 10.1097/00003246-199202000-00014]

37 **Paccou J**, Boudot C, Renard C, Liabeuf S, Kamel S, Fardellone P, Massy Z, Brazier M, Mentaverri R. Total calcium-sensing receptor expression in circulating monocytes is increased in rheumatoid arthritis patients with severe coronary artery calcification. *Arthritis Res Ther* 2014; **16**: 412 [PMID: 25134967 DOI: 10.1186/s13075-014-0412-5]

38 **Lee GS**, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP. *Nature* 2012; **492**: 123-127 [PMID: 23143333 DOI: 10.1038/nature11588]

39 **Zhai TY**, Cui BH, Zou L, Zeng JY, Gao S, Zhao Q, Wang Y, Xie WL, Sun YH. Expression and Role of the Calcium-Sensing Receptor in Rat Peripheral Blood Polymorphonuclear Neutrophils. *Oxid Med Cell Longev* 2017; **2017**: 3869561 [PMID: 29081886 DOI: 10.1155/2017/3869561]

40 **Ferreri NR**, Hao S, Pedraza PL, Escalante B, Vio CP. Eicosanoids and tumor necrosis factor-alpha in the kidney. *Prostaglandins Other Lipid Mediat* 2012; **98**: 101-106 [PMID: 22101002 DOI: 10.1016/j.prostaglandins.2011.11.002]

41 **Cifuentes M**, Fuentes C, Tobar N, Acevedo I, Villalobos E, Hugo E, Ben-Jonathan N, Reyes M. Calcium sensing receptor activation elevates proinflammatory factor expression in human adipose cells and adipose tissue. *Mol Cell Endocrinol* 2012; **361**: 24-30 [PMID: 22449852 DOI: 10.1016/j.mce.2012.03.006]

42 **Cheng SX**, Lightfoot YL, Yang T, Zadeh M, Tang L, Sahay B, Wang GP, Owen JL, Mohamadzadeh M. Epithelial CaSR deficiency alters intestinal integrity and promotes proinflammatory immune responses. *FEBS Lett* 2014; **588**: 4158-4166 [PMID: 24842610 DOI: 10.1016/j.febslet.2014.05.007]

43 **Rey O**, Young SH, Jacamo R, Moyer MP, Rozengurt E. Extracellular calcium sensing receptor stimulation in human colonic epithelial cells induces intracellular calcium oscillations and proliferation inhibition. *J Cell Physiol* 2010; **225**: 73-83 [PMID: 20648625 DOI: 10.1002/jcp.22198]

44 **Mármol I**, Sánchez-de-Diego C, Pradilla Dieste A, Cerrada E, Rodriguez Yoldi MJ. Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. *Int J Mol Sci* 2017; **18** [PMID: 28106826 DOI: 10.3390/ijms18010197]

45 **Lukas M**. Inflammatory bowel disease as a risk factor for colorectal cancer. *Dig Dis* 2010; **28**: 619-624 [PMID: 21088413 DOI: 10.1159/000320276]

46 **Saidak Z**, Mentaverri R, Brown EM. The role of the calcium-sensing receptor in the development and progression of cancer. *Endocr Rev* 2009; **30**: 178-195 [PMID: 19237714 DOI: 10.1210/er.2008-0041]

47 **Aggarwal A**, Prinz-Wohlgenannt M, Tennakoon S, Höbaus J, Boudot C, Mentaverri R, Brown EM, Baumgartner-Parzer S, Kállay E. The calcium-sensing receptor: A promising target for prevention of colorectal cancer. *Biochim Biophys Acta* 2015; **1853**: 2158-2167 [PMID: 25701758 DOI: 10.1016/j.bbamcr.2015.02.011]

48 **Tennakoon S**, Aggarwal A, Kállay E. The calcium-sensing receptor and the hallmarks of cancer. *Biochim Biophys Acta* 2016; **1863**: 1398-1407 [PMID: 26608608 DOI: 10.1016/j.bbamcr.2015.11.017]

49 **Xie R**, Xu J, Xiao Y, Wu J, Wan H, Tang B, Liu J, Fan Y, Wang S, Wu Y, Dong TX, Zhu MX, Carethers JM, Dong H, Yang S. Calcium Promotes Human Gastric Cancer via a Novel Coupling of Calcium-Sensing Receptor and TRPV4 Channel. *Cancer Res* 2017; **77**: 6499-6512 [PMID: 28951460 DOI: 10.1158/0008-5472.CAN-17-0360]

50 **Sanders JL**, Chattopadhyay N, Kifor O, Yamaguchi T, Brown EM. Ca(2+)-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells. *Am J Physiol Endocrinol Metab* 2001; **281**: E1267-E1274 [PMID: 11701443 DOI: 10.1152/ajpendo.2001.

281.6.E1267]
51 **Liao J**, Schneider A, Datta NS, McCauley LK. Extracellular calcium as a candidate mediator of prostate cancer skeletal metastasis. *Cancer Res* 2006; **66**: 9065-9073 [PMID: 16982748 DOI: 10.1158/0008-5472.CAN-06-0317]
52 **Bernichtein S**, Pigat N, Barry Delongchamps N, Boutillon F, Verkarre V, Camparo P, Reyes-Gomez E, Méjean A, Oudard SM, Lepicard EM, Viltard M, Souberbielle JC, Friedlander G, Capiod T, Goffin V. Vitamin D3 Prevents Calcium-Induced Progression of Early-Stage Prostate Tumors by Counteracting TRPC6 and Calcium Sensing Receptor Upregulation. *Cancer Res* 2017; **77**: 355-365 [PMID: 27879271 DOI: 10.1158/0008-5472.CAN-16-0687]
53 **Mihai R**, Stevens J, McKinney C, Ibrahim NB. Expression of the calcium receptor in human breast cancer--a potential new marker predicting the risk of bone metastases. *Eur J Surg Oncol* 2006; **32**: 511-515 [PMID: 16564154 DOI: 10.1016/j.ejso.2006.02.009]
54 **Lines C**, Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Butters RR, Brown EM. Extracellular Calcium-Sensing Receptor Expression and Its Potential Role in Regulating Parathyroid Hormone-Related Peptide Secretion in Human Breast Cancer. 2015; **141**: 0-7
55 **Saidak Z**, Boudot C, Abdoune R, Petit L, Brazier M, Mentaverri R, Kamel S. Extracellular calcium promotes the migration of breast cancer cells through the activation of the calcium sensing receptor. *Exp Cell Res* 2009; **315**: 2072-2080 [PMID: 19285978 DOI: 10.1016/j.yexcr.2009.03.003]
56 **El Hiani Y**, Lehen'kyi V, Ouadid-Ahidouch H, Ahidouch A. Activation of the calcium-sensing receptor by high calcium induced breast cancer cell proliferation and TRPC1 cation channel over-expression potentially through EGFR pathways. *Arch Biochem Biophys* 2009; **486**: 58-63 [PMID: 19332022 DOI: 10.1016/j.abb.2009.03.010]
57 **Boudot C**, Hénaut L, Thiem U, Geraci S, Galante M, Saldanha P, Saidak Z, Six I, Clézardin P, Kamel S, Mentaverri R. Overexpression of a functional calcium-sensing receptor dramatically increases osteolytic potential of MDA-MB-231 cells in a mouse model of bone metastasis through epiregulin-mediated osteoprotegerin downregulation. *Oncotarget* 2017; **8**: 56460-56472 [PMID: 28915604 DOI: 10.18632/oncotarget.16999]
58 **Joeckel E**, Haber T, Prawitt D, Junker K, Hampel C, Thüroff JW, Roos FC, Brenner W. High calcium concentration in bones promotes bone metastasis in renal cell carcinomas expressing calcium-sensing receptor. *Mol Cancer* 2014; **13**: 42 [PMID: 24576174 DOI: 10.1186/1476-4598-13-42]
59 **Aggarwal A**, Prinz-Wohlgenannt M, Gröschel C, Tennakoon S, Meshcheryakova A, Chang W, Brown EM, Mechtcheriakova D, Kállay E. The calcium-sensing receptor suppresses epithelial-to-mesenchymal transition and stem cell- like phenotype in the colon. *Mol Cancer* 2015; **14**: 61 [PMID: 25879211 DOI: 10.1186/s12943-015-0330-4]
60 **Aggarwal A**, Schulz H, Manhardt T, Bilban M, Thakker RV, Kallay E. Expression profiling of colorectal cancer cells reveals inhibition of DNA replication licensing by extracellular calcium. *Biochim Biophys Acta* 2017; **1864**: 987-996 [PMID: 28161520 DOI: 10.1016/j.bbamcr.2017.01.017]
61 **Fetahu IS**, Tennakoon S, Lines KE, Gröschel C, Aggarwal A, Mesteri I, Baumgartner-Parzer S, Mader RM, Thakker RV, Kállay E. miR-135b- and miR-146b-dependent silencing of calcium-sensing receptor expression in colorectal tumors. *Int J Cancer* 2016; **138**: 137-145 [PMID: 26178670 DOI: 10.1002/ijc.29681]
62 **MacLeod RJ**, Hayes M, Pacheco I. Wnt5a secretion stimulated by the extracellular calcium-sensing receptor inhibits defective Wnt signaling in colon cancer cells. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G403-G411 [PMID: 17463182 DOI: 10.1152/ajpgi.00119.2007]
63 **Xin X**, Zeng X, Feng D, Hua T, Liu S, Chi S, Hu Q, Wang H. The suppressive role of calcium sensing receptor in endometrial cancer. *Sci Rep* 2018; **8**: 1076 [PMID: 29348629 DOI: 10.1038/s41598-018-19286-1]
64 **Farnebo F**, Enberg U, Grimelius L, Bäckdahl M, Schalling M, Larsson C, Farnebo LO. Tumor-specific decreased expression of calcium sensing receptor messenger ribonucleic acid in sporadic primary hyperparathyroidism. *J Clin Endocrinol Metab* 1997; **82**: 3481-3486 [PMID: 9329389 DOI: 10.1210/jcem.82.10.4300]
65 **Haven CJ**, van Puijenbroek M, Karperien M, Fleuren GJ, Morreau H. Differential expression of the calcium sensing receptor and combined loss of chromosomes 1q and 11q in parathyroid carcinoma. *J Pathol* 2004; **202**: 86-94 [PMID: 14694525 DOI: 10.1002/path.1489]
66 **Corbetta S**, Eller-Vainicher C, Vicentini L, Lania A, Mantovani G, Beck-Peccoz P, Spada A. Modulation of cyclin D1 expression in human tumoral parathyroid cells: effects of growth factors and calcium sensing receptor activation. *Cancer Lett* 2007; **255**: 34-41 [PMID: 17482347 DOI: 10.1016/j.canlet.2007.03.014]
67 **Kifor O**, Kifor I, Moore FD Jr, Butters RR Jr, Cantor T, Gao P, Brown EM. Decreased expression of caveolin-1 and altered regulation of mitogen-activated protein kinase in cultured bovine parathyroid cells and human parathyroid adenomas. *J Clin Endocrinol Metab* 2003; **88**: 4455-4464 [PMID: 12970324 DOI: 10.1210/jc.2002-021427]
68 **Koh J**, Dar M, Untch BR, Dixit D, Shi Y, Yang Z, Adam MA, Dressman H, Wang X, Gesty-Palmer D, Marks JR, Spurney R, Druey KM, Olson JA Jr. Regulator of G protein signaling 5 is highly expressed in parathyroid tumors and inhibits signaling by the calcium-sensing receptor. *Mol Endocrinol* 2011; **25**: 867-876 [PMID: 21393447 DOI: 10.1210/me.2010-0277]
69 **Corbetta S**, Mantovani G, Lania A, Borgato S, Vicentini L, Beretta E, Faglia G, Di Blasio AM, Spada A. Calcium-sensing receptor expression and signalling in human parathyroid adenomas and primary hyperplasia. *Clin Endocrinol* (Oxf) 2000; **52**: 339-348 [PMID: 10718832]
70 **de Torres C**, Beleta H, Díaz R, Toran N, Rodríguez E, Lavarino C, García I, Acosta S, Suñol M, Mora J. The calcium-sensing receptor and parathyroid hormone-related protein are expressed in differentiated, favorable neuroblastic tumors. *Cancer* 2009; **115**: 2792-2803 [PMID: 19350667 DOI: 10.1002/cncr.24304]
71 **Casalà C**, Gil-Guiñón E, Ordóñez JL, Miguel-Queralt S, Rodríguez E, Galván P, Lavarino C, Munell F, de Alava E, Mora J, de Torres C. The calcium-sensing receptor is silenced by genetic and epigenetic mechanisms in unfavorable neuroblastomas and its reactivation induces ERK1/2-dependent apoptosis. *Carcinogenesis* 2013; **34**: 268-276 [PMID: 23108190 DOI: 10.1093/carcin/bgs338]
72 **Rácz GZ**, Kittel A, Riccardi D, Case RM, Elliott AC, Varga G. Extracellular calcium sensing receptor in human pancreatic cells. *Gut* 2002; **51**: 705-711 [PMID: 12377811 DOI: 10.1136/gut.51.5.705]
73 **Suva LJ**, Griffin RJ, Makhoul I. Mechanisms of bone metastases of breast cancer. *Endocr Relat Cancer* 2009; **16**: 703-713 [PMID: 19443538 DOI: 10.1677/ERC-09-0012]
74 **Thomas RJ**, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology* 1999; **140**: 4451-4458 [PMID: 10499498 DOI: 10.1210/endo.140.10.7037]
75 **Mundy GR**. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2002; **2**: 584-593 [PMID: 12154351 DOI: 10.1038/nrc867]
76 **Ahearn TU**, Tchrakian N, Wilson KM, Lis R, Nuttall E, Sesso HD, Loda M, Giovannucci E, Mucci LA, Finn S, Shui IM. Calcium-Sensing Receptor Tumor Expression and Lethal Prostate Cancer Progression. *J Clin Endocrinol Metab* 2016; **101**: 2520-2527 [PMID: 27115058 DOI: 10.1210/jc.2016-1082]
77 **Chattopadhyay N**, Ye CP, Yamaguchi T, Kifor O, Vassilev PM, Nishimura R, Brown EM. Extracellular calcium-sensing receptor in rat oligodendrocytes: expression and potential role in regulation of cellular proliferation and an outward K+ channel. *Glia* 1998; **24**: 449-458 [PMID: 9814825]
78 **Chattopadhyay N**, Espinosa-Jeffrey A, Tfelt-Hansen J, Yano S, Bandyopadhyay S, Brown EM, de Vellis J. Calcium receptor expression and function in oligodendrocyte commitment and

lineage progression: potential impact on reduced myelin basic protein in CaR-null mice. *J Neurosci Res* 2008; **86**: 2159-2167 [PMID: 18438915 DOI: 10.1002/jnr.21662]
79 **Mateo-Lozano S**, García M, Rodríguez-Hernández CJ, de Torres C. Regulation of Differentiation by Calcium-Sensing Receptor in Normal and Tumoral Developing Nervous System. *Front Physiol* 2016; **7**: 169 [PMID: 27242543 DOI: 10.3389/fphys.2016.00169]
80 **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]
81 **Raskov H**, Pommergaard HC, Burcharth J, Rosenberg J. Colorectal carcinogenesis--update and perspectives. *World J Gastroenterol* 2014; **20**: 18151-18164 [PMID: 25561783 DOI: 10.3748/wjg.v20.i48.18151]
82 **Terzić J**, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. *Gastroenterology* 2010; **138**: 2101-2114.e5 [PMID: 20420949 DOI: 10.1053/j.gastro.2010.01.058]
83 **Robles AI**, Traverso G, Zhang M, Roberts NJ, Khan MA, Joseph C, Lauwers GY, Selaru FM, Popoli M, Pittman ME, Ke X, Hruban RH, Meltzer SJ, Kinzler KW, Vogelstein B, Harris CC, Papadopoulos N. Whole-Exome Sequencing Analyses of Inflammatory Bowel Disease-Associated Colorectal Cancers. *Gastroenterology* 2016; **150**: 931-943 [PMID: 26764183 DOI: 10.1053/j.gastro.2015.12.036]
84 **Kamada N**, Seo SU, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* 2013; **13**: 321-335 [PMID: 23618829 DOI: 10.1038/nri3430]
85 **Lipkin M**, Newmark H. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects at high risk for familial colonic cancer. *N Engl J Med* 1985; **313**: 1381-1384 [PMID: 4058532 DOI: 10.1056/NEJM198511283132203]
86 **Yang K**, Kurihara N, Fan K, Newmark H, Rigas B, Bancroft L, Corner G, Livote E, Lesser M, Edelmann W, Velcich A, Lipkin M, Augenlicht L. Dietary induction of colonic tumors in a mouse model of sporadic colon cancer. *Cancer Res* 2008; **68**: 7803-7810 [PMID: 18829535 DOI: 10.1158/0008-5472.CAN-08-1209]
87 **Newmark HL**, Yang K, Kurihara N, Fan K, Augenlicht LH, Lipkin M. Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer. *Carcinogenesis* 2009; **30**: 88-92 [PMID: 19017685 DOI: 10.1093/carcin/bgn229]
88 **Lipkin M**. Preclinical and early human studies of calcium and colon cancer prevention. *Ann N Y Acad Sci* 1999; **889**: 120-127 [PMID: 10668488]
89 **Zhang X**, Keum N, Wu K, Smith-Warner SA, Ogino S, Chan AT, Fuchs CS, Giovannucci EL. Calcium intake and colorectal cancer risk: Results from the nurses' health study and health professionals follow-up study. *Int J Cancer* 2016; **139**: 2232-2242 [PMID: 27466215 DOI: 10.1002/ijc.30293]
90 **Keum N**, Aune D, Greenwood DC, Ju W, Giovannucci EL. Calcium intake and colorectal cancer risk: dose-response meta-analysis of prospective observational studies. *Int J Cancer* 2014; **135**: 1940-1948 [PMID: 24623471 DOI: 10.1002/ijc.28840]
91 Colorectal cancer. World Cancer Research Fund International. Available from: URL: https://www.wcrf.org/int/continuous-update-project/cup-findings-reports/colorectal-cancer
92 **Ahearn TU**, McCullough ML, Flanders WD, Long Q, Sidelnikov E, Fedirko V, Daniel CR, Rutherford RE, Shaukat A, Bostick RM. A randomized clinical trial of the effects of supplemental calcium and vitamin D3 on markers of their metabolism in normal mucosa of colorectal adenoma patients. *Cancer Res* 2011; **71**: 413-423 [PMID: 21084270 DOI: 10.1158/0008-5472.CAN-10-1560]
93 **Yang W**, Liu L, Masugi Y, Qian ZR, Nishihara R, Keum N, Wu K, Smith-Warner S, Ma Y, Nowak JA, Momen-Heravi F, Zhang L, Bowden M, Morikawa T, Silva AD, Wang M, Chan AT, Fuchs CS, Meyerhardt JA, Ng K, Giovannucci E, Ogino S, Zhang X. Calcium intake and risk of colorectal cancer according to expression status of calcium-sensing receptor (CASR). *Gut* 2018; **67**: 1475-1483 [PMID: 28676564 DOI: 10.1136/gutjnl-2017-314163]
94 **Momen-Heravi F**, Masugi Y, Qian ZR, Nishihara R, Liu L, Smith-Warner SA, Keum N, Zhang L, Tchrakian N, Nowak JA, Yang W, Ma Y, Bowden M, da Silva A, Wang M, Fuchs CS, Meyerhardt JA, Ng K, Wu K, Giovannucci E, Ogino S, Zhang X. Tumor expression of calcium sensing receptor and colorectal cancer survival: Results from the nurses' health study and health professionals follow-up study. *Int J Cancer* 2017; **141**: 2471-2479 [PMID: 28856682 DOI: 10.1002/ijc.31021]
95 **Whitfield JF**. Calcium, calcium-sensing receptor and colon cancer. *Cancer Lett* 2009; **275**: 9-16 [PMID: 18725175 DOI: 10.1016/j.canlet.2008.07.001]
96 **Chakrabarty S**, Wang H, Canaff L, Hendy GN, Appelman H, Varani J. Calcium sensing receptor in human colon carcinoma: interaction with Ca(2+) and 1,25-dihydroxyvitamin D(3). *Cancer Res* 2005; **65**: 493-498 [PMID: 15695391]
97 **Sheinin Y**, Kállay E, Wrba F, Kriwanek S, Peterlik M, Cross HS. Immunocytochemical localization of the extracellular calcium-sensing receptor in normal and malignant human large intestinal mucosa. *J Histochem Cytochem* 2000; **48**: 595-602 [PMID: 10769043 DOI: 10.1177/002215540004800503]
98 **Cheng SX**, Okuda M, Hall AE, Geibel JP, Hebert SC. Expression of calcium-sensing receptor in rat colonic epithelium: evidence for modulation of fluid secretion. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G240-G250 [PMID: 12065312 DOI: 10.1152/ajpgi.00500.2001]
99 **Chakrabarty S**, Radjendirane V, Appelman H, Varani J. Extracellular calcium and calcium sensing receptor function in human colon carcinomas: promotion of E-cadherin expression and suppression of beta-catenin/TCF activation. *Cancer Res* 2003; **63**: 67-71 [PMID: 12517779]
100 **Kállay E**, Kifor O, Chattopadhyay N, Brown EM, Bischof MG, Peterlik M, Cross HS. Calcium-dependent c-myc proto-oncogene expression and proliferation of Caco-2 cells: a role for a luminal extracellular calcium-sensing receptor. *Biochem Biophys Res Commun* 1997; **232**: 80-83 [PMID: 9125156 DOI: 10.1006/bbrc.1997.6225]
101 **Robertson KD**. DNA methylation and human disease. *Nat Rev Genet* 2005; **6**: 597-610 [PMID: 16136652 DOI: 10.1038/nrg816]
102 **Hizaki K**, Yamamoto H, Taniguchi H, Adachi Y, Nakazawa M, Tanuma T, Kato N, Sukawa Y, Sanchez JV, Suzuki H, Sasaki S, Imai K, Shinomura Y. Epigenetic inactivation of calcium-sensing receptor in colorectal carcinogenesis. *Mod Pathol* 2011; **24**: 876-884 [PMID: 21317879 DOI: 10.1038/modpathol.2011.10]
103 **Cetani F**, Pinchera A, Pardi E, Cianferotti L, Vignali E, Picone A, Miccoli P, Viacava P, Marcocci C. No evidence for mutations in the calcium-sensing receptor gene in sporadic parathyroid adenomas. *J Bone Miner Res* 1999; **14**: 878-882 [PMID: 10352095 DOI: 10.1359/jbmr.1999.14.6.878]
104 **Singh N**, Chakrabarty S. Induction of CaSR expression circumvents the molecular features of malignant CaSR null colon cancer cells. *Int J Cancer* 2013; **133**: 2307-2314 [PMID: 23674327 DOI: 10.1002/ijc.28270]
105 **Noguchi T**, Toiyama Y, Kitajima T, Imaoka H, Hiro J, Saigusa S, Tanaka K, Inoue Y, Mohri Y, Toden S, Kusunoki M. miRNA-503 Promotes Tumor Progression and Is Associated with Early Recurrence and Poor Prognosis in Human Colorectal Cancer. *Oncology* 2016; **90**: 221-231 [PMID: 26999740 DOI: 10.1159/000444493]
106 **Peters U**, Chatterjee N, Yeager M, Chanock SJ, Schoen RE, McGlynn KA, Church TR, Weissfeld JL, Schatzkin A, Hayes RB. Association of genetic variants in the calcium-sensing receptor with risk of colorectal adenoma. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 2181-2186 [PMID: 15598778]
107 **Zhu Y**, Wang PP, Zhai G, Bapat B, Savas S, Woodrow JR, Sharma I, Li Y, Zhou X, Yang N, Campbell PT, Dicks E, Parfrey PS, Mclaughlin JR. Vitamin D receptor and calcium-sensing receptor polymorphisms and colorectal cancer survival in the Newfoundland population. *Br J Cancer* 2017; **117**: 898-906 [PMID: 28765616 DOI: 10.1038/bjc.2017.242]
108 **Mahmoudi T**, Karimi K, Arkani M, Farahani H, Nobakht H, Dabiri R, Asadi A, Zali MR. Parathyroid hormone gene rs6256 and calcium sensing receptor gene rs1801725 variants are not

associated with susceptibility to colorectal cancer in Iran. *Asian Pac J Cancer Prev* 2014; **15**: 6035-6039 [PMID: 25124570]

109 **Jenab M**, McKay J, Bueno-de-Mesquita HB, van Duijnhoven FJ, Ferrari P, Slimani N, Jansen EH, Pischon T, Rinaldi S, Tjønneland A, Olsen A, Overvad K, Boutron-Ruault MC, Clavel-Chapelon F, Engel P, Kaaks R, Linseisen J, Boeing H, Fisher E, Trichopoulou A, Dilis V, Oustoglou E, Berrino F, Vineis P, Mattiello A, Masala G, Tumino R, Vrieling A, van Gils CH, Peeters PH, Brustad M, Lund E, Chirlaque MD, Barricarte A, Suárez LR, Molina E, Dorronsoro M, Sala N, Hallmans G, Palmqvist R, Roddam A, Key TJ, Khaw KT, Bingham S, Boffetta P, Autier P, Byrnes G, Norat T, Riboli E. Vitamin D receptor and calcium sensing receptor polymorphisms and the risk of colorectal cancer in European populations. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2485-2491 [PMID: 19706842 DOI: 10.1158/1055-9965.EPI-09-0319]

110 **Ho C**, Conner DA, Pollak MR, Ladd DJ, Kifor O, Warren HB, Brown EM, Seidman JG, Seidman CE. A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Nat Genet* 1995; **11**: 389-394 [PMID: 7493018 DOI: 10.1038/ng1295-389]

111 **Kos CH**, Karaplis AC, Peng JB, Hediger MA, Goltzman D, Mohammad KS, Guise TA, Pollak MR. The calcium-sensing receptor is required for normal calcium homeostasis independent of parathyroid hormone. *J Clin Invest* 2003; **111**: 1021-1028 [PMID: 12671051 DOI: 10.1172/JCI17416]

112 **MacLeod RJ**. Extracellular calcium-sensing receptor/PTH knockout mice colons have increased Wnt/β-catenin signaling, reduced non-canonical Wnt signaling, and increased susceptibility to azoxymethane-induced aberrant crypt foci. *Lab Invest* 2013; **93**: 520-527 [PMID: 23545937 DOI: 10.1038/labinvest.2013.51]

113 **Fedirko V**, Bostick RM, Flanders WD, Long Q, Sidelnikov E, Shaukat A, Daniel CR, Rutherford RE, Woodard JJ. Effects of vitamin d and calcium on proliferation and differentiation in normal colon mucosa: a randomized clinical trial. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2933-2941 [PMID: 19861511 DOI: 10.1158/1055-9965.EPI-09-0239]

114 **Fedirko V**, Bostick RM, Flanders WD, Long Q, Shaukat A, Rutherford RE, Daniel CR, Cohen V, Dash C. Effects of vitamin D and calcium supplementation on markers of apoptosis in normal colon mucosa: a randomized, double-blind, placebo-controlled clinical trial. *Cancer Prev Res* (Phila) 2009; **2**: 213-223 [PMID: 19258546 DOI: 10.1158/1940-6207.CAPR-08-0157]

115 **Bhagavathula N**, Kelley EA, Reddy M, Nerusu KC, Leonard C, Fay K, Chakrabarty S, Varani J. Upregulation of calcium-sensing receptor and mitogen-activated protein kinase signalling in the regulation of growth and differentiation in colon carcinoma. *Br J Cancer* 2005; **93**: 1364-1371 [PMID: 16278666 DOI: 10.1038/sj.bjc.6602852]

116 **Rey O**, Chang W, Bikle D, Rozengurt N, Young SH, Rozengurt E. Negative cross-talk between calcium-sensing receptor and β-catenin signaling systems in colonic epithelium. *J Biol Chem* 2012; **287**: 1158-1167 [PMID: 22094462 DOI: 10.1074/jbc.M111.274589]

117 **Pacheco II**, Macleod RJ. CaSR stimulates secretion of Wnt5a from colonic myofibroblasts to stimulate CDX2 and sucrase-isomaltase using Ror2 on intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G748-G759 [PMID: 18703641 DOI: 10.1152/ajpgi.00560.2007]

118 **Cheung R**, Kelly J, Macleod RJ. Regulation of villin by wnt5a/ror2 signaling in human intestinal cells. *Front Physiol* 2011; **2**: 58 [PMID: 21949508 DOI: 10.3389/fphys.2011.00058]

119 **Canaff L**, Hendy GN. Human calcium-sensing receptor gene. Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D. *J Biol Chem* 2002; **277**: 30337-30350 [PMID: 12036954 DOI: 10.1074/jbc.M201804200]

120 **Singh N**, Aslam MN, Varani J, Chakrabarty S. Induction of calcium sensing receptor in human colon cancer cells by calcium, vitamin D and aquamin: Promotion of a more differentiated, less malignant and indolent phenotype. *Mol Carcinog* 2015; **54**: 543-553 [PMID: 26076051 DOI: 10.1002/mc.22123]

121 **Aggarwal A**, Höbaus J, Tennakoon S, Prinz-Wohlgenannt M, Graça J, Price SA, Heffeter P, Berger W, Baumgartner-Parzer S, Kállay E. Active vitamin D potentiates the anti-neoplastic effects of calcium in the colon: A cross talk through the calcium-sensing receptor. *J Steroid Biochem Mol Biol* 2016; **155**: 231-238 [PMID: 25758239 DOI: 10.1016/j.jsbmb.2015.02.006]

122 **Aggarwal A**, Kállay E. Cross Talk between the Calcium-Sensing Receptor and the Vitamin D System in Prevention of Cancer. *Front Physiol* 2016; **7**: 451 [PMID: 27803671 DOI: 10.3389/fphys.2016.00451]

123 **Huang Y**, Cavanaugh A, Breitwieser GE. Regulation of stability and trafficking of calcium-sensing receptors by pharmacologic chaperones. *Adv Pharmacol* 2011; **62**: 143-173 [PMID: 21907909 DOI: 10.1016/B978-0-12-385952-5.00007-5]

124 **Cook AE**, Mistry SN, Gregory KJ, Furness SG, Sexton PM, Scammells PJ, Conigrave AD, Christopoulos A, Leach K. Biased allosteric modulation at the CaS receptor engendered by structurally diverse calcimimetics. *Br J Pharmacol* 2015; **172**: 185-200 [PMID: 25220431 DOI: 10.1111/bph.12937]

125 **Sparks SM**, Spearing PK, Diaz CJ, Cowan DJ, Jayawickreme C, Chen G, Rimele TJ, Generaux C, Harston LT, Roller SG. Identification of potent, nonabsorbable agonists of the calcium-sensing receptor for GI-specific administration. *Bioorg Med Chem Lett* 2017; **27**: 4673-4677 [PMID: 28916340 DOI: 10.1016/j.bmcl.2017.09.008]

126 **Palmer SC**, Nistor I, Craig JC, Pellegrini F, Messa P, Tonelli M, Covic A, Strippoli GF. Cinacalcet in patients with chronic kidney disease: a cumulative meta-analysis of randomized controlled trials. *PLoS Med* 2013; **10**: e1001436 [PMID: 23637579 DOI: 10.1371/journal.pmed.1001436]

P- Reviewer: Shi YJ, Camara NOS, Gupta R **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4132-4151

DOI: 10.3748/wjg.v24.i36.4132

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

REVIEW

Production of extracellular lysophosphatidic acid in the regulation of adipocyte functions and liver fibrosis

Fang Yang, Guo-Xun Chen

Fang Yang, School of Laboratory Medicine, Hubei University of Chinese Medicine, Wuhan 430065, Hubei Province, China

Guo-Xun Chen, Department of Nutrition, University of Tennessee at Knoxville, Knoxville, TN 37996, United States

ORCID number: Fang Yang (0000-0002-7092-2532); Guo-Xun Chen (0000-0001-6226-4050).

Author contributions: Yang F searched the publications, summarized data in tables, drew figures, discussed the literature and wrote the review; Chen GX designed the structure of the review and modified the review; all authors approved the final version of this review.

Supported by the National Natural Science Foundation of China, No. 31401510.

Conflict-of-interest statement: We declare that we have no conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Guo-Xun Chen, MD, PhD, Associate Professor, Research Scientist, Department of Nutrition, University of Tennessee at Knoxville, Room 229, Jessie Harris Building, 1215 West Cumberland Avenue, Knoxville, TN 37996, United States. gchen6@utk.edu
Telephone: +1-865-9746254
Fax: +1-865-9743491

Received: March 24, 2018
Peer-review started: March 25, 2018
First decision: April 18, 2018
Revised: April 24, 2018
Accepted: May 6, 2018
Article in press: May 6, 2018
Published online: September 28, 2018

Abstract

Lysophosphatidic acid (LPA), a glycerophospholipid, consists of a glycerol backbone connected to a phosphate head group and an acyl chain linked to sn-1 or sn-2 position. In the circulation, LPA is in sub-millimolar range and mainly derived from hydrolysis of lysophosphatidylcholine, a process mediated by lysophospholipase D activity in proteins such as autotaxin (ATX). Intracellular and extracellular LPAs act as bioactive lipid mediators with diverse functions in almost every mammalian cell type. The binding of LPA to its receptors LPA_{1-6} activates multiple cellular processes such as migration, proliferation and survival. The production of LPA and activation of LPA receptor signaling pathways in the events of physiology and pathophysiology have attracted the interest of researchers. Results from studies using transgenic and gene knockout animals with alterations of ATX and LPA receptors genes, have revealed the roles of LPA signaling pathways in metabolic active tissues and organs. The present review was aimed to summarize recent progresses in the studies of extracellular and intracellular LPA production pathways. This includes the functional, structural and biochemical properties of ATX and LPA receptors. The potential roles of LPA production and LPA receptor signaling pathways in obesity, insulin resistance and liver fibrosis are also discussed.

Key words: Autotaxin; Lysophosphatidic acid receptors; Obesity; Lysophosphatidic acid; Insulin resistance; Liver fibrosis

Core tip: Lysophosphatidic acid (LPA) is mainly derived from hydrolysis of lysophosphatidylcholine, a process mediated by lysophospholipase D activity in proteins such as autotaxin (ATX). The binding of LPA to its receptors LPA_{1-6} activates multiple cellular signaling pathways and leads to changes. Studies using genetically modified animals have begun to reveal the roles of LPA pathways in metabolic active tissues and organs. The present review summarized recent progresses in the studies of extracellular and intracellular LPA production pathways; the functions, structural and biochemical properties of ATX and LPA receptors. Furthermore, the potential roles of LPA production and LPA receptor signaling pathways in obesity, insulin resistance and liver fibrosis are discussed.

Yang F, Chen GX. Production of extracellular lysophosphatidic acid in the regulation of adipocyte functions and liver fibrosis. *World J Gastroenterol* 2018; 24(36): 4132-4151 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4132.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4132

INTRODUCTION

Lysophosphatidic acid (1- or 2-acyl-sn-glycerol 3-phosphate/radyl-glycerol-phosphate, LPA) is one type of water-soluble glycerophospholipid with molecular mass about 430-480 Da. All LPA molecules contain a glycerol backbone linked to a phosphate head group at sn-3 position and an acyl chain esterified to sn-1 or sn-2 position. Due to variation of the fatty acyl chain, LPA molecules are in different forms and derived from multiple sources, such as membrane lipids[1]. The LPA molecules produced extracellularly exert a variety of physiological responses after binding to their receptors as shown in Figure 1.

LPA was first identified as an active ingredient of Darmstoff by Vogt in 1957[2,3]. The term Darmstoff was used to describe a smooth-muscle-stimulating substance which was first observed with bath fluid of isolated intestine preparations[4]. The substance was acidic and soluble in many organic solvents, properties that distinguish Darmstoff from amines and polypeptides[5]. Results of acidic hydrolysis and paper chromatography showed that the smooth-muscle-stimulating activity of Darmstoff was due to a mixture of acidic phospholipids (PLs), one of which is an acetal phosphatidic acid[2,3]. In the 1960s, studies on smooth muscle and blood pressure suggested that LPA had biological activities[6,7]. Later on, various molecules of LPA species were isolated and identified from soy beans. It was shown that intravenous injection of LPA from crude soybean lecithin caused hypertension in rats and guinea pigs, but hypotension in cats and rabbits[8]. This raised intriguing questions regarding the activation mechanism of this lipid specie. Since then, the myriad biological effects of LPA have drawn attention of biomedical scientists.

Subsequently, LPA in incubated serum at 36 ℃ for 18-24 h was shown to cause aggregation of feline and human platelets[9]. Whether LPA acts through its detergent-like physical property or its interaction with a specific receptor remained a critical question. Later, LPA was shown to stimulate cell proliferation in a pertussis toxin-sensitive manner[10]. This finding suggested that LPA acts through G protein-coupled receptor (GPCR). This information led to the cloning and identification of a GPCR, which is now known as LPA receptor 1 (LPA_1)[11]. It is known now that as a bioactive lipid mediator, LPA activates at least 6 specific GPCRs, named as LPA_{1-6}. These GPCRs are coupled with several G_α proteins such as $G_{\alpha 12/13}$, $G_{\alpha q/11}$, $G_{\alpha i/o}$, and $G_{\alpha s}$. The binding of LPA to these receptors stimulates the activations of small GTPases, Ras, Rho, and Rac, and induces downstream actions[12].

The existence of extracellular LPA indicates its production outside a cell. Since autotaxin (ATX) was first identified from human plasma and found to be a lysophosphatidic acid-producing enzyme in 2002[13], the ATX-LPA receptor signaling pathway has been implicated in a variety of disease processes including the vascular and neural development, hair follicle development, tumor progression, lymphocyte trafficking, bone development, pulmonary fibrosis, fat mass regulation, cholestatic pruritus, neuropathic pain, embryo implantation, obesity and glucose homeostasis, spermatogenesis, fetal hydrocephalus, chronic inflammation, cellular proliferation, and smooth muscle contraction during development[14-18]. Both ATX and LPA have attracted the interest of researchers in an effort to understand their roles in pathophysiology and to develop new agents to treat above-mentioned pathological conditions.

EXTRACELLULAR AND INTRACELLULAR PRODUCTION AND DEGRADATION OF LPA

LPA and its common precursor lysophosphatidylcholine (LPC) can be found both extracellular and intracellular as signaling mediators and membrane components, respectively. Structurally, LPA is an acyl group esterified to the sn-1 or sn-2 position of the glycerol backbone. Due to the differences of acyl chain length, saturation and backbone position, various LPA chemical forms can be found in tissues and cells. Extracellular LPA is thought to mediate bioactive effects through LPA receptors[19]. Intracellular LPA is an important intermediate for the *de novo* biosynthesis of complex glycerolipids, including mono-, di-, and triglycerides, as well as PLs[20]. In addition, it has been thought that LPA can function as a ligand for transcription factor peroxisome proliferator-activated receptor γ (PPARγ)[21]. This indicates that LPA may play important roles in the regulation of gene expression.

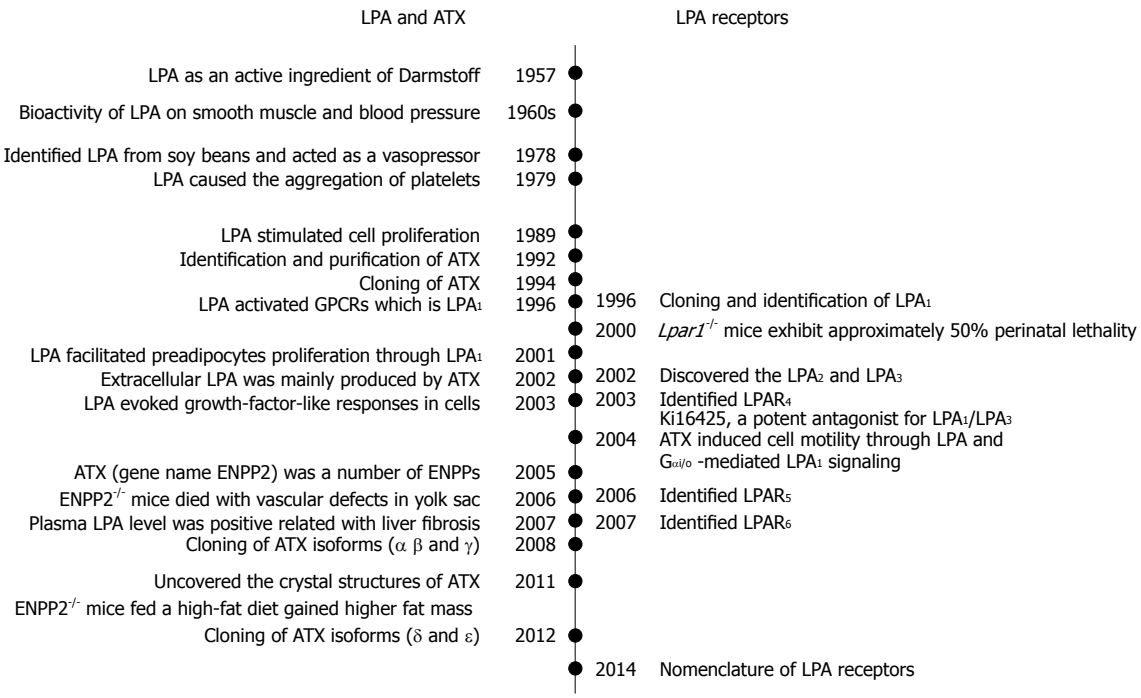


Figure 1 Chronological events related the identifications of lysophosphatidic acid, ecto-nucleotide pyrophosphatase/phosphodiesterase/autotaxin and lysophosphatidic acid receptors. On the left side, it shows the events associated with the identifications of LPA molecules and ATX for its production. On the right side, it shows the cloning events of LPAR1-6. LPA: Lysophosphatidic acid; ATX: Autotaxin; LPA1-6: Lysophosphatidic acid receptor 1-6; ENPP2: Ectonucleotide pyrophosphatase/phosphodiesterase family member 2.

Pathways for LPA production

As shown in Figure 2, there are five major pathways for LPA production, (1) the lysophospholipids-ATX (LPLs-ATX) pathway, (2) the phosphatidic acid - phospholipase A_1 or A_2 (PA-PLA$_1$/PLA$_2$) pathway, (3) the *de novo* glycerophosphate acyltransferase (GPAT) synthesis pathway, (4) the monoacylglycerol kinase (MAGK) pathway, and (5) the oxidative modification of low-density lipoprotein (LDL) pathway. Despite recent advances in the identification of the enzymes responsible for LPA production, the regulation of these enzymes still remains obscure.

LPLs-ATX pathway

In the first pathway, LPLs generated from PLs by PLA$_1$ or PLA$_2$ are converted to LPA by a plasma enzyme ATX[22,13], which we will describe in later part of this article. A major source of extracellular LPA is LPC, other LPLs such as lysophosphatidylserine and lysophosphatidylethanolamine can also be enzymatically processed to produce LPA. This pathway accounts for the majority of circulating LPA.

PA-PLA$_1$/PLA$_2$ pathway

LPA is also produced intracellularly as an intermediate for the synthesis of other glycerolipids[20]. LPA can be produced enzymatically from intracellular organelles such as mitochondria and endoplasmic reticulum. Phosphatidic acid (PA) is first generated from PLs or diacylglycerol by phospholipase D enzymes (PLD$_1$ and PLD$_2$) and diacylglycerol kinase (DGK) activities, respectively. Then, one acyl group is removed from the sn-1 position by PLA$_1$ or at the sn-2 position by PLA$_2$ enzymes to generate LPA. This pathway may be more important in specific tissues with expression of DGK such as the brain and skin[23].

De novo GPAT synthesis pathway

GPATs catalyze the first step in glycerolipid synthesis, *i.e.*, the conversion of glycerol-3-phosphate (G3P) to LPA by the transfer of fatty acids from acyl-CoA. Since GPAT exhibits the lowest specific activity of enzymes in the *de novo* triacylglycerol (TAG) and PLs synthesis pathways, it has been considered to be the rate limiting enzyme for them[24]. Many studies have been published on the regulation of TAG synthesis and its relevance to obesity and insulin resistance. GPAT activity in mitochondria was shown to be regulated by fatty acid-binding protein (FABP)[25,26]. It has been shown that mitochondrial GPAT activity was inhibited by LPA. FABP reversed the inhibition of LPA through the binding and extracting LPA from the mitochondrial outer membrane. The extracted LPA was converted to PA by microsomes, where acylglycerophosphate acyltransferases (AGPATs) are located[25,26]. These results suggested that FABP regulated the *de novo* synthesis of PA through the stimulation of mitochondrial GPAT and transport of LPA from mitochondria to microsomes.

MAGK pathway

Lipid phosphate phosphatases (LPPs) are also involved in the LPA turnover. LPPs can be found extracellularly

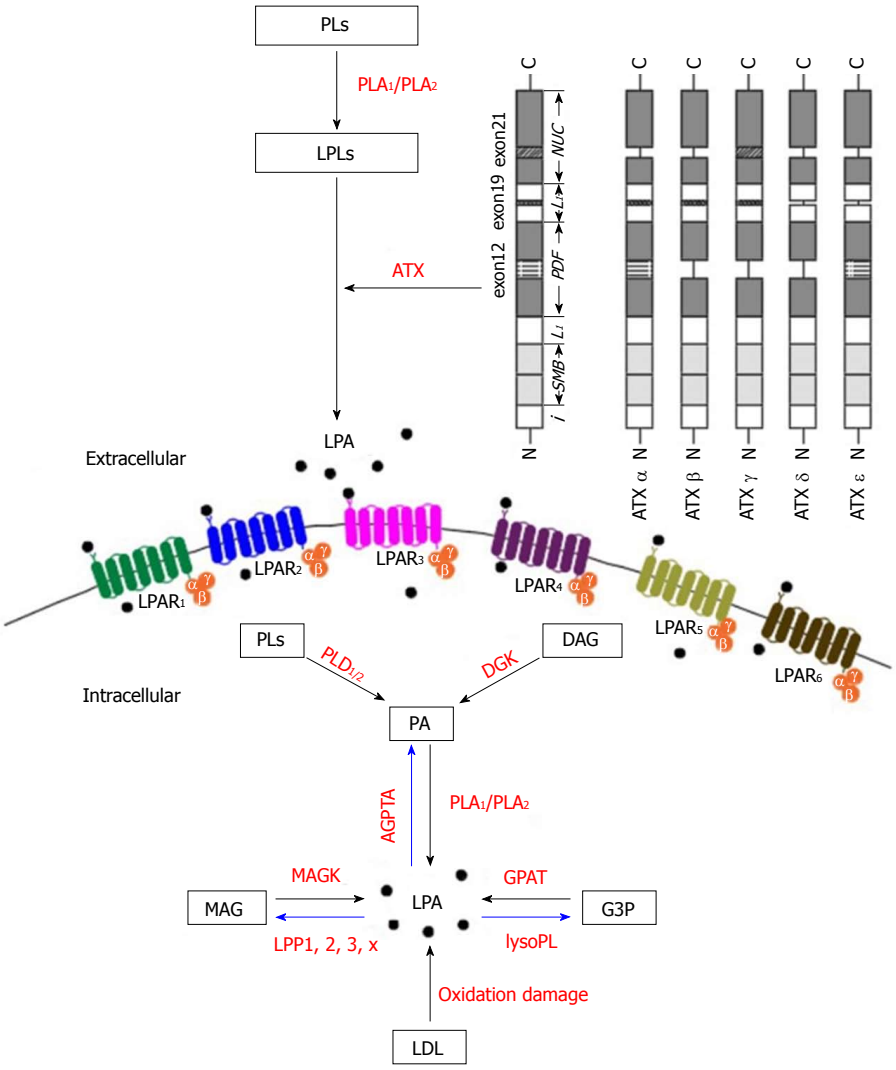


Figure 2 Biochemical pathways of lysophosphatidic acid synthesis and degradation. LPA can be produced extracellularly and intracellularly as signaling mediators and membrane components, respectively. There are five major pathways for LPA production, (1) the lysophospholipids-ATX (LPLs-ATX) pathway, (2) the phosphatidic acid - phospholipase A_1 or A_2 (PA-PLA_1/PLA_2) pathway, (3) the *de novo* glycerophosphate acyltransferase (GPAT) synthesis pathway, (4) the monoacylglycerol kinase (MAGK) pathway, and (5) the oxidative modification of low-density lipoprotein (LDL) pathway. In the upper right corner of the figure, there are catalytically active isoforms (ATXα, ATXβ, ATXγ, ATXδ and ATXε), which are expressed in different tissues. PLs: Phospholipids; PLA_1/PLA_2: Phospholipase $A_{1/2}$; LPLs: Lysophospholipids; ATX: Autotaxin; ATXα-δ: Protein structure scheme of the domains of ATX; LPA: Lysophosphatidic acid; DAG: Diacylglycerol; DGK: Diacylglycerol kinase; $PLD_{1/2}$: Phospholipase $D_{1/2}$; PA: Phosphatidic acid; AGPAT: Acylglycerophosphate acyltransferase; MAG: Monoacylglycerol; MAGK: Monoacylglycerol kinase; LPP: Lipid phosphate phosphatase; G3P: Glycerol-3-phosphate; lyso PL: Lysophospholipase; GPAT: Glycerophosphate acyltransferase; LDL: Low-density lipoprotein; i: Intramembrane domain; SMB: N-terminal somatomedin B-like domains; L_1: L_1 linker region; PDF: Phosphodiesterase domain; L_2: L_2 linker region; NUC: C-terminal nuclease-like domain; LPA_{1-6}: Lysophosphatidic acid receptor 1-6.

or intracellularly in endoplasmic reticulum or Golgi, where they dephosphorylate LPA, which leads to the formation of monoacylglycerol (MAG)[27]. MAG may then be phosphorylated by MAGK and thus participate in another round of LPA signaling[20]. Thus, the production of LPA is regulated by the availability of precursors as well as the expression of catalytic enzymes.

Oxidative modification of LDL

LPA was found as an active molecule on oxidized and modified LDL, in where it may contribute to platelet activation, endothelial cell stress-fiber and gap formation[28,29]. LPAs on these lipoproteins activate platelets through G-protein coupled LPA receptors and a Rho/Rho kinase signaling pathway, which leads to platelet shape change and subsequent aggregation. The biologically active LPA-like products generated by this non-enzymatic oxidation co-migrated with an authentic LPA standard in thin layer chromatography. LPA was found to be accumulated in atherosclerotic plaques, which might act to activate platelets. The level of LPA is very high in the human carotid atherosclerotic lesion, suggesting the roles in thrombogenesis and rupture[28,29]. An alternative explanation to the generation of LPA from oxidized LDL is that ATX might be activated. The acyl/alkyl composition, the precursor of LPA and the mechanism responsible for LPA generation in the oxidized LDL remain to be addressed in the future.

Pathways for the degradation of LPA

There are three major pathways that degrade LPA as shown in Figure 2. The first is the removal of phosphate to form MAG by LPPs[30]. LPA has a half-life of 3 min when it is added to cells expressing LPP[31]. Four isoforms of LPP have been cloned and characterized in mammals, LPP1/PAP-2α/PAP-2α1[32], LPP1α/PAP-2α2[33], LPP2/PAP-2c/PAP-2α[34] and LPP3/PAP-2β/PAP-2β[32]. The second LPA degradation pathway involves the action of AGPAT enzymes, also known as lysophosphatidic acid acyltransferase. These microsomal enzymes catalyze the transfer of an acyl group from acyl-CoA to LPA to form PA. Proteins with AGPAT activity include a family of transmembrane enzymes[35], and membrane associated proteins involved in membrane fission such as endophilin[36] and C-terminal-binding protein/brefeldin A-ADP ribosylated substrate[37]. The third pathway for LPA degradation involves the hydrolysis of the acyl group from the G3P head group by the action of lysophospholipases. The majority of characterized lysophospholipases act on LPC[38].

ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASES

The ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) family contains seven members with structurally similar catalytic domains that hydrolyze phosphodiester bonds in various substrates, including nucleoside triphosphates, LPLs, and choline phosphate esters[39,40]. ATX, or ENPP2, is the best-characterized member of ENPP family. ENPPs are defined by their ability to hydrolyze phosphodiester bonds of various nucleotides *in vitro*[41-43]. ATX/ENPP2 was originally identified as a tumor cell-motility-stimulating factor from the conditional medium of A2058 human melanoma cells[44]. Since the addition of pertussis toxin reduced cellular motility, ATX's effects were thought to involve $G_{i/o}$-mediated signaling[39,44]. ATX can be secreted as a 100 kDa glycoprotein. It is produced by multiple tissues including adipose tissue[45,46]. It is believed that the circulated ATX/ENPP2 is degraded by the liver[47]. Extracellular LPA was found to be present in sub-micromolar ranges. The responsible enzyme was identified to be ATX[13,22]. ATX-mediated autocrine signaling induces cell motility through LPA production and $G_{i/o}$-mediated LPA receptor signaling[48].

From both a structural and evolutionary point of view, ENPP family members have been categorized into two subgroups, ENPP1-3 and ENPP4-7[39]. ENPP1-3 all have two N-terminal somatomedin B-like (SMB) domains, a central phosphodiesterase (PDE) domain and a C-terminal nuclease (NUC)-like domain as shown in Figure 2. ENPP4-7 only have similarity in the PDE domain. The crystal structures of mouse[49] and rat[50] ATX show loops on both sides of the catalytic domain, which may help to determine the binding specificity. The SMB and PDE domains are connected by the first loop (L_1 linker region), whereas the PDE and NUC domains are connected by the second loop (L_2 linker region)[49].

The secreted ATX is a constitutively active glycoprotein with a N-terminal signal peptide sequence containing a furin cleavage site[46]. The other ENPPs are transmembrane or anchored proteins. In addition to its pyrophosphatase/phosphodiesterase activities, ATX has lysophospholipase D (lysoPLD) activity. The N-terminal signal peptide of the ATX precursor is removed first, and then, the remaining part is cleaved by proprotein convertases before the active ATX with lysoPLD activity is released into the extracellular environment, which converts LPC into LPA and choline[47]. The structure of PDE domain has a lipid binding pocket and a nearby tunnel allowing entry of substrates and release of products[51]. The NUC domain has been thought to maintain the rigidity of the PDE domain, and the two N-terminal SMB domains mediate binding of ATX to integrin[52]. This binding brings ATX to the cell membrane, which allows the production of LPA in a location close to its receptors[51,53,54].

The structures of ATX in complex with diverse LPAs show distinct conformations after different acyl chains occupy the binding pocket[49]. LPAs with saturated chains bind in the hydrophobic pocket in a more elongated fashion, whereas LPAs with unsaturated chains have a bent conformation due to the presence of carbon-carbon double bond(s). For LPA (22:6), the acyl chain shows a U-shaped conformation in the binding pocket[49]. ATX prefers LPC species with shorter and unsaturated acyl chain as substrates, and the rank order is 14:0 > 16:0 > 18:3 > 18:1 > 18:0. All these show that ATX is able to hydrolyze LPCs with different lengths and saturations of acyl chains to produce the corresponding LPAs.

The cDNA of ATX/ENPP2 was cloned in 1994[55]. After that, its homology with phosphodiesterases was revealed, and the cloning and tissue distribution of the three human and mouse isoforms (α, β and γ) were determined in 2008[50]. Two more isoforms (δ and ε) were identified in 2012[56]. The ATX gene is located on mouse chromosome 15 and on human chromosome 8. The human and mouse ATX gene structures are conserved[50]. The mouse ATX gene spans more than 80 kb and contains at least 27 exons. The three splicing sites in exons 12, 19 and 21 can theoretically result in eight isoforms, in which five were detected. These isoforms are catalytically active (ATX α-δ) and expressed in different tissues. They are ATXα (ATXm), ATXβ (ATXt), ATXγ (PD-Iα)[50], ATXδ and ATXε[56]. ATXβ and ATXδ, which are the most and second most abundant isoforms, respectively, share similar biochemical characteristics (Figure 2). Houben *et al*[53] characterized that a 52-residue polybasic insertion corresponding to exon 12 in ATXα isoform confers specific binding to heparan sulfate proteoglycans thereby targeting LPA production to the plasma membrane. This is another potential mechanism for localizing ATXα to cell membranes and for LPA production in close proximity to LPA receptors. Exon 12 encodes a 52-amino acid insertion of the mouse ATXα and ATXε

isoforms (amino acids 324-375), whereas exon 21 encodes an additional 25-amino acid of the murine ATXγ isoform (amino acids 593-617). Novel isoforms ATXδ and ATXε have a 4-amino acid deletion on exon 19. This complex way of exon arrangement has been maintained through evolution. Human ATX exhibit 93% sequence identity with rodent ATX while all important residues are highly conserved[49].

ATX has a broad profile of tissue expression, with relatively high levels in the blood, brain, kidney, and lymphoid organs[57-59]. Secretion of ATX leads to high concentration in cerebrospinal fluid and in the endothelial venules of lymphoid tissues[60-62]. The cellular sources of plasma ATX are incompletely understood. Nevertheless, adipocytes may be a source[63,64]. ATX is also stored in platelets and released during their activation[65,66]. Circulating ATX is rapidly taken up by the scavenger receptors of liver sinusoidal endothelial cells, and then degraded by the liver[46]. Thus, like insulin, ATX is largely removed from the circulation through first passage by the liver. For the ATX isoforms, high expression levels of ATXβ and ATXγ mRNA were detected in peripheral tissues and the brain, whereas ATXα was shown the lowest expression level in both the central nervous system and peripheral tissues among the three isoforms in human. In mice, ATXβ is widely expressed in the brain and peripheral tissues, and ATXγ and ATXα showed little variation in their distribution[50]. Human brain and retina showed relatively higher expression level of ATXα than that of ATXβ and ATXγ, whereas the expression levels of ATXδ and ATXε in the small intestine and spleen are higher than that in other tissues[56].

LPA has been quantified in a variety of species, tissues, and fluids, including neural tissue, cerebrospinal fluid, fertilized hen white, seminal fluid, tears, plasma, serum, urine, saliva, and aqueous humor[67-69]. The formation of LPA species depends on the precursor PLs, which can vary by acyl chain length and degree of saturation. The term LPA most often refers to 18:1 oleoyl-LPA (1-acyl-2-hydroxy-sn-glycero-3-phosphate), as it is the most commonly one. Other chemical forms of LPA can be observed in various biological systems that have concentrations ranging from low nanomolar to micromolar levels[67,70]. LPA concentrations in human and rat blood can range from 0.1 μmol/L in plasma and up to 10 μmol/L in serum, which is well over the apparent nanomolar kDa of LPA_{1-6}[71-74]. The LPA molecules containing 18:2, 20:4, 16:1, 16:0, and 18:1 acyl chains are particularly abundant in plasma[75-77]. Current methods to detect LPA include indirect enzymatic assays[73], TLC-GC, LC-MS, and LC-MS/MS[78-80].

LPA RECEPTORS-MEDIATED LPA SIGNALING

LPA acts as a potent mitogen, which was previously known as "ventricular zone gene-1 (vzg-1)" due its high level in the embryonic neuroproliferative layer of the cerebral cortex[11,12]. The cloning and functional identification of LPA_1 led to determination of other receptor genes based upon sequence homology[81-83]. This is particularly true for the "endothelial differentiation gene" (EDG) members[84] that include LPA and sphingosine 1-phosphate receptors. Then, two other LPA receptors, LPA_2 and LPA_3 (also known as EDG4, and EDG7), were subsequently discovered based on shared homology with LPA_1(EDG2)[85]. Later on, LPA_4 (P2RY9, GPR23)[86], LPA_5 (GPR92)[87] and LPA_6 (P2RY5, GPR87)[88] were identified. They share 35% amino acid homology to the purinergic (P2Y) family of GPCRs, as compared to less than 20% homology to LPA_1, suggesting that LPA_{4-6} are more closely related to the P2Y receptors[52]. Here, LPA_1-LPA_6 are for proteins, and their gene symbols are *LPAR1-LPAR6* for human and *Lpar1-Lpar6* for non-human[89].

All LPA receptors signal through at least one of the four heterotrimeric G_α proteins ($G_{\alpha 12/13}$, $G_{\alpha q/11}$, $G_{\alpha i/o}$, and $G_{\alpha s}$)[12,90], resulting in downstream signals that produce diverse physiological and pathophysiological effects (Figure 3). $G_{\alpha 12/13}$-mediated LPA signaling regulates cytoskeletal remodeling, cell migration and invasion through activation of Rho pathway proteins[91]. Rho signals to c-jun N-terminal kinase (JNK) and p38 through Rho-associated kinase (ROCK) and protein kinase N. The LPA-coupled $G_{\alpha q/11}$ protein primarily regulates Ca^{2+} homeostasis through phospholipase C (PLC), which generates the second messengers IP_3 and diacylglycerol (DAG)[92-94]. $G_{\beta\gamma}$ and $G_{\alpha i/o}$ subunits mediate the activation of phosphatidylinositol 3-kinase (PI3K) which results in the stimulation of the Akt pathway and increase of protein translation after the activation of the mammalian target of rapamycin (mTOR) signaling pathway. Activation of PI3K by $G_{\beta\gamma}$ subunits also stimulates the activity of Rac, leading to cell migration and JNK regulation of pro-inflammatory gene expression, and Ras activity, leading to the stimulation of Raf- mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway to promote the expression of genes involved in proliferation and invasion. $G_{\alpha i/o}$, besides PI3K, also stimulates the Ras-Raf-MEK-ERK pathway promoting cell survival and other functions[95,96]. $G_{\alpha s}$ can activate adenylyl cyclase and increase cAMP concentration upon LPA stimulation[97]. However, the same enzyme is also inhibited by $G_{\alpha i/o}$, showing the complexity of signaling pathways after the activation of LPA receptors[98].

All six LPA receptors can be stimulated by 1-acyl-LPAs, which show different potencies. LPA_3 and LPA_6 prefer unsaturated 2-acyl-LPA, while LPA_5 likes ether-linked 1-alkyl-LPA species[99,100]. In addition, lysophosphatidylserine, lysophosphatidylinositol, and lysophosphatidylethanolamine, have been thought to activate these receptors as well[101]. Different LPA molecules may have preference to different subtypes of LPA receptors[102]. Table 1 summarizes PLA receptors expression profiles and their known physiological functions in humans and mice.

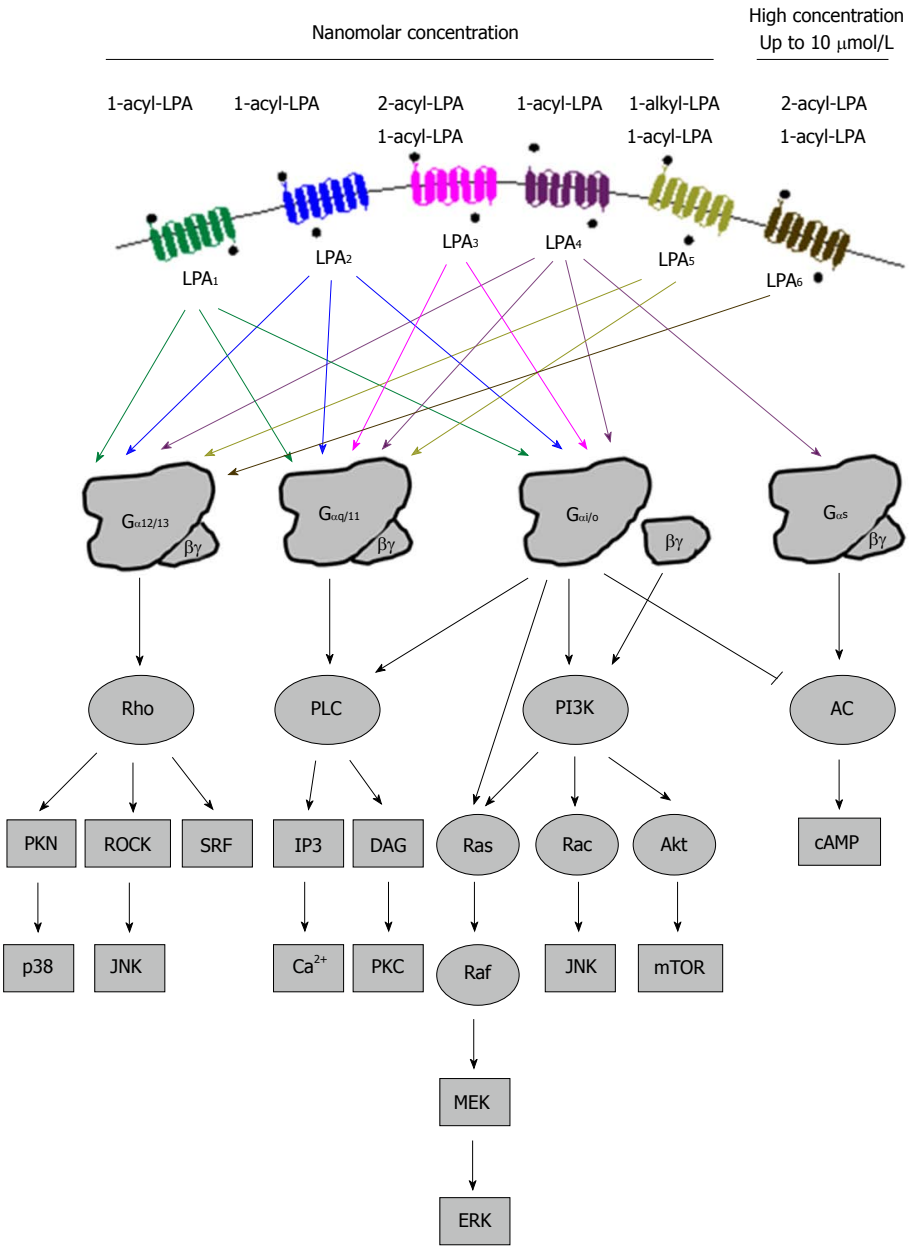


Figure 3 Summary of lysophosphatidic acid activated intracellular signaling pathways *via* the six cognate lysophosphatidic acid receptors. PLC: Phospholipase C; PI3K: Phosphatidylinositol 3-kinase; AC: Denylyl cyclase; PNK: Polynucleotide 5'-hydroxyl-kinase; ROCK: Rho-associated kinase; JNK: c-jun N-terminal kinase; SRF: Serum response factor; IP3: Inositol 1,4,5-triphosphate; DAG: Diacylglyerol; PKC: Protein kinase C; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; Akt: Protein kinase B; Mtor: Mammalian target of rapamycin.

LPA1

LPA1 is the first LPA receptor identified based on studies of LPA in the brain[11]. LPA1 couples to three Gα proteins - Gα12/13, Gαq/11, and Gαi/o, which can result in the activation of downstream pathways including Akt, Rho, Ras, and PLC (Figure 3). These pathways mediate many cellular responses initiated by LPA1 such as neurodevelopment regulation, cell proliferation, differentiation, apoptosis and survival, cell-cell contact through a variety of mechanisms[68,84,103-106]. *Lpar1*$^{-/-}$ mice exhibit about 50% perinatal lethality, which was attributed to the defective development of olfaction. The survived ones had reduction of body size, craniofacial dysmorphism, and loss of Schwann cells[107]. Dysregulation at glutamatergic synapses was observed in *Lpar1*$^{-/-}$ mice[108]. When the original *Lpar1*$^{-/-}$ mouse line was expanded, a spontaneous variant named "Málaga LPA1" arose. They showed more severe brain defects than the original *Lpar1*$^{-/-}$ line mice did[109]. The loss of LPA1 in animals seems to modulate the development of several diseases including cancer, obesity, neuropathic pain, fibrosis and male infertility[110].

LPA2

The amino acid sequence of LPA2 is about 50% identical to that of LPA1, and it associates with Gαi/o, Gαq/11, and Gα12/13, the same as LPA1[106] (Figure 3). These G proteins use Ras, PI3K/Rac, PLC/DGA and Rho to mediate their

Table 1 Expression pattern of lysophosphatidic acid receptors and their known physiological functions in humans and mice

Name	Information	Previous orphan names	Major expression tissue (high to low level)	Knockout effects in mouse	Biological functions	Ref.
LPA_1	Human chromosome locus 9q31.3; 41.1 kDa[1]; 364 aa[2]; Identity[3] 97.3% Mouse chromosome locus 4, 32.2 cM; 41.1 kDa; 364 aa	*vzg-1*, *edg-2*, 39.4 kDa; 344 aa *mrec1.3*, *lpA1*	Brain, placenta, urinary bladder, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, and skeletal muscle. Brain, heart, lungs, stomach, intestine, placenta, kidneys, spleen, uterus, testes.	Perinatal lethality, retarded growth, defective olfaction, reduced body size, craniofacial dysmorphism with blunted snouts, and increased apoptosis in sciatic nerve Schwann cells.	Neurodevelopment regulation, cell proliferation, differentiation, apoptosis and survival, cell-cell contact through serum-response element activation, cell migration and cytoskeletal organization, Ca^{2+} homeostasis, cAMP-regulated cellular processes and adenylyl cyclase inhibition	Yung *et al*[68], 2014; Archbold *et al*[138], 2014; Choi *et al*[139], 2008; Anliker *et al*[103], 2013; Sakai *et al*[104], 2013; Wittpoth *et al*[98], 1999; An *et al*[82], 1998; Contos *et al*[107], 2000; Contos *et al*[106], 2000; Fukushima *et al*[84], 2001.
LPA_2	Human chromosome 19p13.11; 39.1 kDa; 351 aa; Identity 83.5% Mouse chromosome 8, 33.91 cM; 38.7 kDa; 348 aa	*edg-4*, *lpA2*	Leukocytes, testis, prostate, spleen, thymus and pancreas. Kidney, testis, uterus, lung, stomach, spleen, thymus, postnatal brain, and heart.	Normal	Cell migration, viable and healthy, nervous system development and immune system regulation.	Yung *et al*[68], 2014; An *et al*[82], 1998; Contos *et al*[106], 2000b; Archbold *et al*[138], 2014; Ohuchi *et al*[111], 2008; Choi *et al*[110], 2010; Valentine *et al*[140], 2008; Xu *et al*[113], 2004; Lai *et al*[112], 2005; Contos *et al*[115], 2002; Choi *et al*[139], 2008.
LPA_3	Human chromosomal locus 1p22.3; 40.1 kDa; 353 aa; Identity 91.2% Mouse chromosome locus 3, 71.03 cM; 40.3 kDa; 354 aa	*edg-7*, *lpA3*	Heart, testis, prostate, pancreas, lung, ovary, and brain. Lung, kidney, uterus, testis, small intestine, brain, heart, stomach, placenta, spleen, and thymus.	Delayed embryo implantation, embryo crowding, and reduced litter size for female null mutants.	Male and female reproductive physiology, inflammation, cell Ca^{2+} homeostasis and cAMP regulation, vertebrate left-right patterning during embryogenesis.	Yung *et al*[68], 2014; Bandoh *et al*[83], 1999; Im *et al*[116], 2000; Contos *et al*[107], 2000a; Zhao *et al*[117], 2015; Ye *et al*[118], 2010; Hama *et al*[119], 2010; Lai *et al*[120], 2012
LPA_4	Human chromosome Xq21.1; 41.9 kDa; 370 aa; Identity 98.4% Mouse chromosome X region D; 41.9 kDa; 370 aa	*P2Y9/GPR23*	Ovaries, thymus, pancreas, brain, heart, small intestine, testis, prostate, colon, and spleen. Heart, ovary, skin, thymus, and bone Marrow.	Inhibition of its differentiation into osteoblasts in human mesenchymal stem cell line; For mouse: increased trabecular bone volume, number, and thickness; pericardial effusions, severe edema and hemorrhage, abnormally dilated blood and lymphatic vessels and lymph sacs, and impaired pericyte recruitment.	ROCK-dependent cell aggregation and N-cadherin-dependent cell adhesion, cAMP accumulation, differentiation of immortalized hippocampal progenitor cells, negatively cell motility regulation and osteogenesis.	Yung *et al*[68], 2014; Ohuchi *et al*[111], 2008; Choi *et al*[110], 2010; Liu *et al*[123], 2010; Mansell *et al*[124], 2010; Liu *et al*[125], 2009; Sumida *et al*[126], 2010; Yanagida *et al*[71]; Lee *et al*[97], 2007; Rhee *et al*[121], 2006; Lee *et al*[122], 2008
LPA_5	Human chromosome 12p13.31; 41.3 kDa; 372 aa; Identity 79.0% Mouse chromosome 6, 59.21 cM; 41.4 kDa; 372 aa	*GPR92*	Spleen, heart, small intestine, placenta, colon, and liver. Small intestine, lung, heart, stomach, colon, spleen, thymus, skin, liver, platelets, mast cells, gastrointestinal lymphocytes, and dorsal root ganglia.	Reduced lung metastasis by melanoma cells.	Neurite retraction, stress fiber formation, receptor internalization, water absorption, Ca^{2+} mobilization and cAMP accumulation, LPA-induced release of chemokine ligand 4 in mast cells.	Yung *et al*[68], 2014; Lee *et al*[87], 2006; Lee *et al*[141], 2015; Amisten *et al*[142], 2008; Lundequist *et al*[129], 2011; Araki *et al*[130], 2014; Lin *et al*[128], 2010; Yanagida *et al*[143], 2013

LPA$_6$	Human chromosome 13q14.2; 39.4 kDa; 344 aa; Identity 93.0% Mouse chromosome 14, region D3; 39.4 kDa; 344 aa	*P2Y5*	Hair, skin. Hair, immune cells.	Hypotrichosis	Hair development, increased intracellular Ca^{2+}, reduced forskolin-stimulated cAMP accumulation, and ERK1/2 activation	Yanagida *et al*[144], 2011; Yanagida *et al*[143], 2013; Raza *et al*[135], 2014; Dong *et al*[145], 2014; Lee *et al*[141], 2015; Lee *et al*[133], 2009

[1]Molecular mass were obtained from UniProt[146]; [2]aa means amino acids; [3]Identities between human and mouse lysophospholipid receptors were calculated in UniProt[146]. *vzg-1*: Ventricular zone gene-1; *edg*: Endothelial differentiation gene.

down-stream signals, which may regulate cell survival and migration[107]. LPA$_2$ regulates cell survival and cell migration in the development of nervous system and functions of immune system[68,90,106,110,111]. The focal adhesion molecule thyroid receptor-interacting protein 6[112,113] and several PDZ-domain and zinc finger proteins[114] interact with LPA$_2$. The PDZ-binding domain of LPA$_2$ regulates Na^+/H^+ exchanger regulatory factor 2 activity, and activates PLC-3 and Akt/ERK signaling pathways. These pathways stimulate cell migration, enhance survival, and alter gene expression, accounting for the functions attributed to LPA$_2$. *Lpar2*$^{-/-}$ mice are viable and healthy, while those null for both *Lpar1* and *Lpar2* show features essentially consistent with those of *Lpar1*$^{-/-}$[115]. These data suggest functional redundancy of LPA$_2$ with LPA$_1$.

LPA$_3$

LPAR3/Lpar3 was cloned based upon homology to already identified LPA receptor genes using degenerated primers in a PCR-based cloning strategy[83,116]. LPA$_3$ couples with $G_{\alpha q/11}$ and $G_{\alpha i/o}$ to mediate adenylyl cyclase inhibition, PLC activation and Ca^{2+} mobilization, and Ras activation[105] (Figure 3). LPA$_3$ prefers 2-acyl-LPAs containing unsaturated fatty acids[83]. It mediates the activation of a series of physiological processes such as male and female reproductive physiology, inflammation, cell Ca^{2+} homeostasis and cAMP regulation[107,117-119]. LPA$_3$ appears to determine vertebrate left-right patterning during embryogenesis as downregulation of *Lpar3* or inhibition of LPA$_3$ activity disrupted patterning process in zebrafish[120]. *Lpar3*$^{-/-}$ mice are viable with no reported neural deficits, even though LPA$_3$ is found in the frontal cortex, hippocampus, and amygdala[83,116]. On the other hand, female *Lpar3*$^{-/-}$ mice have a delayed embryo implantation, and reduced litter size[117].

LPA$_4$

The first so-called non-EDG LPA receptor was identified in 2003, and named as LPA$_4$. It shares homology (approximately 20%) with LPA$_{1-3}$, and it is more closer to the P2Y receptor family[86]. LPA$_4$ was identified by screening orphan receptors using calcium mobilization as a readout for ligand-induced signals. LPA4 couples with $G_{\alpha 12/13}$, $G_{\alpha q/11}$, $G_{\alpha i/o}$ and $G_{\alpha s}$[97], and activates Rho/ROCK to induce neurite retraction and stress fiber formation[71,97] (Figure 3). It induces ROCK-dependent cell aggregation and N-cadherin-dependent cell adhesion[71]. LPA$_4$ is only LPA receptor that activates $G_{\alpha s}$ to induce cAMP level[97]. The activation of LPA$_4$ was thought to regulate the differentiation of immortalized hippocampal progenitor cells[121]. In addition, the activation of LPA$_4$ could inhibit LPA-induced cell migration, but LPA exposure increased lamellipodia formation and transwell movement of LPA$_4$ null cells, indicating an increased sensitivity[122]. It shows the ability of LPA$_4$ to negatively regulate cell motility and indicates that differential effects may be achieved by simultaneously expressing multiple LPA receptors. *LPAR4*-deficient human mesenchymal stem cells lost ability to differentiate into osteoblasts[123]. While adult *Lpar4*$^{-/-}$ mice appear grossly normal[122], they exhibit increased trabecular bone volume, number, and thickness[124,125]. LPA$_4$ pathway seems to inhibit osteogenesis. *Lpar4*$^{-/-}$ mice had reduction of prenatal survival rate during embryo development, which is accompanied by changes such as pericardial effusions, severe edema and hemorrhage[126].

LPA$_5$

LPA$_5$, the fifth LPA receptor, was identified in 2006[87,127]. It shares about 35% homology with *LPAR4*, and 22% homology with *LPAR1-3*[87]. LPA$_5$ couples with $G_{\alpha 12/13}$ and $G_{\alpha q/11}$, which mediate neurite retraction, stress fiber formation, and receptor internalization in LPA$_5$-expressing cell lines[87] (Figure 3). It also activates $G_{\alpha q/11}$ to increase intracellular calcium mobilization, and cAMP accumulation *via* a non-$G_{\alpha s}$ mechanism, suggesting the involvement of other G-proteins[87,127]. LPA$_5$ signaling may also affect intestinal water absorption[128]. This is achieved through the LPA-induced recruitment of Na^+/H^+ exchanger 3 to the microvilli mediated by the interaction between LPA$_5$ and Na^+/H^+ exchanger regulatory factor 2. Additionally, LPA$_5$ is the main LPA receptor responsible for LPA-induced release of chemokine ligand 4 in mast cells[129]. Interestingly, LPA$_5$ in B16 melanoma cells, prefers alkyl-LPA (18:1) to acyl-LPA (18:1)[99]. *Lpar5*$^{-/-}$ null mice exhibit reduced lung metastasis by melanoma cells compared with wild type ones[130].

LPA$_6$

The most recently identified LPA receptor is LPA$_6$. It was first isolated from a chicken T cell library and named receptor 6H1 in 1993[131], and then, renamed to P2Y5 because of sequence homology with P2Y receptors in

1996[132]. LPA_6 couples with G_α-protein $G_{\alpha 12/13}$ (Figure 3). Its activation by LPA causes cAMP accumulation, changes in cell morphology, and guanosine 5′-3-*O*-(thio) triphosphate binding[71]. When LPA_6 was expressed together with a G_α protein, LPA stimulation increased intracellular Ca^{2+} level, and decreased forskolin-induced cAMP level and ERK activation in intestinal cells[133]. LPA_6 has been thought to be involved in familial hair loss[134,135]. Mutations of lipase member H and LPA_6 in patients with hypotrichosis are respectively associated with a decrease in LPA production and abnormal LPA_6 activation in cells[134,136,137]. These findings demonstrate the roles of LPA_6 and LPA signaling may be therapeutic targets for the treatment or prevention of human hair loss[138-146].

LPA RECEPTOR SIGNALING IN OBESITY AND INSULIN RESISTANCE

Recently, obesity has become major public health concern, particularly in the United States. According to 2015 Center of Disease Control and Prevention estimates, more than one-third of adults (34.9% or 78.6 million) and 17% of youth in the United States were obese in 2011-2014[147]. Obesity is associated with the development of chronic metabolic diseases including diabetes, heart disease, stroke, and some types of cancer. The long-term effects of being overweight correlate with premature death, cardiovascular disease, metabolic morbidities, and asthma, among other problems[148]. Both environmental factors and genetic factors contribute to the obesity development. Many factors modulate the propensity to accumulate fat in cells, including an increased ratio of adipocyte precursor cells to differentiated adipocytes[149].

LPA receptor signaling regulates adipogenesis

Obesity is associated with adipocyte hypertrophy and hyperplasia. Hypertrophy results in excessive TAG accumulation in adipocytes. Hyperplasia results in recruitment of new adipocytes *via* proliferation and differentiation. LPA was found to induce proliferation of 3T3F442A preadipocytes, indicating the role of LPA signaling in fat storage[150]. LPA stimulation increases the growth of 3T3F442A cells *via* LPA_1, which activates the Ras-Raf-MEK-ERK pathway, and of the focal adhesion kinase[20,151].

It has been reported that *Lpar1*$^{-/-}$ mice exhibited greater adiposity than the control mice without alteration of feeding behavior, despite of lowered body weight[107]. Interestingly, *Lpar1*$^{-/-}$ mice were resistant to diet-induced obesity that may result at least in part from alterations in leptin production[64]. Mature adipocytes express more ATX than preadipocytes. When secreted from adipose tissue, ATX may promote preadipocyte proliferation. Its expression was up-regulated during adipocyte differentiation, and in *db/db* mice[44,45].

The serum levels of LPC, the precursors of LPA, increases gradually in rabbits fed a high-cholesterol diet for 12 wk. The levels of individual LPAs formed after the incubation of serum for 24 h elevated with the increase of the length of time that rabbits were fed a high cholesterol diet[152]. These studies indicate that feeding of a high-fat diet can cause an increase in the circulating level of LPA. Preadipocytes mainly express LPA_1[153], and the mRNA level of *Lpar1* expression in preadipocytes is higher than that in mature 3T3-L1 adipocytes[154]. However, in human adipose tissue, obesity does not influence on *LPAR1* expression[155]. This discrepancy of LPA_1 expression levels between human and mouse adipose tissues suggest that obesity promotes LPA synthesis rather than activation in adipose tissue.

The LPA-induced proliferation of preadipocytes[20,153,154] has been thought to be mediated through LPA_1 and the activation of the Ras-Raf-MEK-ERK pathway[154,156,157]. LPA inhibits differentiation of white and brown preadipocyte cell lines, which include porcine preadipocyte cell line; mouse preadipocyte cell line, 3T3-L1 and 3T3F442A; and human Simpson-Golabi-Behmel Syndrome preadipocyte cells[153,154,158,159]. This inhibition is mediated by LPA_1 *via* the Rho-ROCK pathway[160,161]. All these result in a down-regulation of PPARγ, and impaired responses of PPARγ-targeted genes to its ligands, which leads to reduced TAG accumulation, and expression levels of adipogenic genes[153,154].

The activation of Rho-ROCK pathway delayed the activation of the Wnt-signaling pathway, which has been partially attributed to the inhibited PPARγ expression and adipogenesis. When mice with the adipocyte-specific knockout of ATX gene (FATX-KO) were fed a high-fat diet, they had more fat mass and larger adipocyte size, but not adipocyte number, than the control mice did in the absence of any change of food intake. The deletion of ATX in mice appeared to lead sensitivity to diet-induced obesity, which might be due to elevated expression levels of PPARγ and its down-stream adipogenic genes in subcutaneous white adipose tissue. Interestingly, those knockout mice had improved glucose tolerance and less systemic insulin resistance than the control mice fed the same diet[63,161]. LPA stimulation seems to have anti-adipogenic effect in white adipocytes[153] and in brown preadipocytes[159]. Aforementioned experiments seem to indicate that ATX-LPA receptor signaling pathway may inhibit the development of adipose tissue (Figure 4).

On the other hand, others reported that ATX promotes preadipocytes proliferation and differentiation into adipocytes, thereby promoting adipocyte hyperplasia and obesity. It was showed that deletion of ATX results in smaller body weight gain, smaller fat pad weights and adipocyte numbers, less insulin resistance and glucose tolerance in heterozygous *Enpp2*$^{+/-}$ mice and adipocyte-specific FATX-KO mice fed a high-fat diet than their littermates controls[162]. Moreover, the FATX-KO improved brown adipose tissue function,

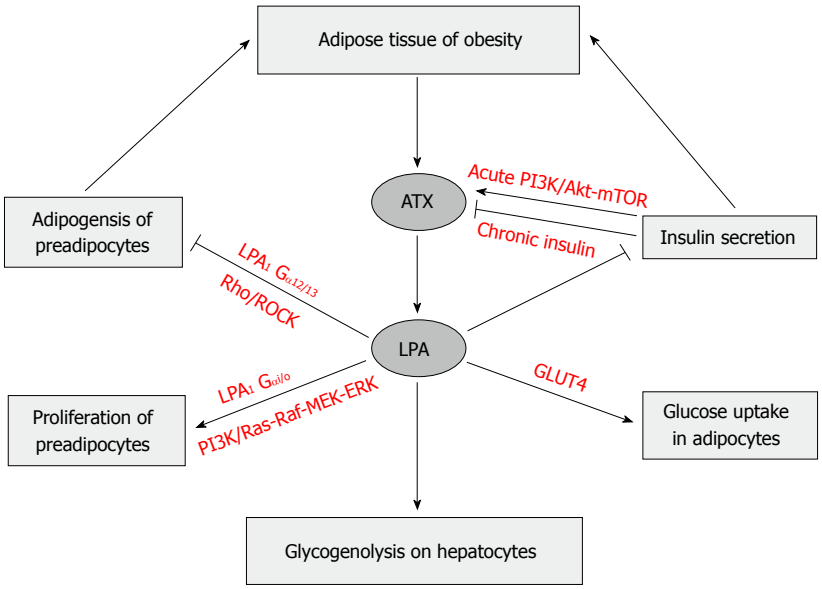


Figure 4 Autotaxin-lysophosphatidic acid signaling axis regulates adipose tissue development and glucose homeostasis in obesity. In adipose tissue, especially in mature adipocytes, the elevated expression of AXT leads to production of LPA and then induced proliferation of preadipocytes *via* LPA_1 through Ras-Raf-MEK-ERK pathway. On the other hand, LPA inhibits differentiation of white and brown preadipocytes, which is mediated by LPA_1 *via* the Rho-ROCK pathway. Short-term insulin treatment increases ATX secretion in adipocytes *via* PI3K/Akt-mTOR pathway, whereas long-term insulin treatment reduces ATX activity. LPA produced by ATX in obesity has a tonic inhibitory effect on glucose homeostasis through inhibition of insulin secretion in isolated pancreas islets, increase of glucose transport in myocyte and adipocytes *via* GLUT4 translocation in a PI3K dependent manner, and elevation of glycogenolysis in hepatocytes. ROCK: Rho-associated kinase; PI3K: Phosphatidylinositol 3-kinase; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin; GLUT4: Glucose transporter type 4.

increased energy expenditure, and improved systemic metabolism. Transgenic mice expressing the human ATX/ENPP2 gene under the control of α1 antitrypsin gene promoter became sensitive to diet-induced obesity due to reduced expression of brown adipose tissue-related genes in peripheral white adipose tissue and accumulated significantly more fat without any change of locomotor activities, thermogenic profiles, and systemic metabolism[159]. In mice, ATX is highly expressed in visceral white adipose tissue and brown adipose tissue and is downregulated in adipose tissue hypertrophy[162]. In human, ATX expression is higher in subcutaneous than in visceral fat, and the latter fat pad in obese subjects has higher ATX expression level than that in non-obese subjects, which is correlated with leptin expression[155]. The circulating ATX levels correlated negatively with body mass index, and mRNA levels of ATX were reduced in subcutaneous fat from obese subjects[162]. Moreover, ATX expression in adipose tissues may be negatively regulated by LPA through a feedback regulatory mechanism, which may involves inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β[163].

ATX-LPA receptor signaling axis exerts a negative effect on glucose homeostasis in obesity

Earlier studies found that increases in adipocyte size correlated with insulin resistance, and increased risk of type 2 diabetes[164,165]. The expression of ATX is increased in the adipose tissue of obese and insulin-resistant subjects and mice[44,155,166]. It has been shown that LPA also regulates glucose metabolism[167,168]. LPA was found to enhance glucose uptake in a dose-dependent manner in both GLUT4myc L6 myotubes and 3T3-L1 adipocytes, a process that was attributed to the increase of GLUT4 translocation in a PI3K dependent manner. Moreover, the effect of LPA on glucose uptake was completely inhibited by pretreating cells with $LPA_{1/3}$ receptor antagonist Ki16425 and Gi inhibitor pertussis toxin[169]. LPA significantly lowered blood glucose levels in normal mice and streptozotocin-induced diabetic mice, suggesting the promotion of glucose usage, but not stimulation of insulin secretion[169].

The elevation of ATX expression in adipocytes of *db/db* mice occurred simultaneously with the development of hyperglycemia, and only 3 wk after the emergence of hyperinsulinemia in them[166]. ATX expression was up-regulated by treatment with TNFα, and down-regulated by rosiglitazone in 3T3F442A adipocytes[166]. The upregulation of ATX expression in adipocytes of *db/db* mice seems to be associated with the emergence of hyperglycemia rather than fat accumulation or hyperinsulinemia[166].

The plasma levels of LPC, as the precursor of LPA, are reduced in obese and type 2 diabetic mice, suggesting that it may regulate blood glucose level. This reduction may contribute to the impairment of glucose homeostasis[170]. Interestingly, adipocyte specific ATX knockout mice fed with a high-fat diet showed greater adiposity and better tolerance to glucose challenge than control mice[63], suggesting a negative effect of LPA on glucose homeostasis. Similarly, LPA production appears to impair glucose disposal probably through a reduction

of plasma insulin as pharmacological inhibition of $LPAR_{1/3}$ activation improves glucose homeostasis in obese and prediabetic mice[171]. Another possibility is that the progression of diabetes affects ATX expression in adipose tissue[162]. It has been shown that treatments with high concentrations of glucose and insulin led to ATX secretion in adipocytes. Short-term insulin treatment increased ATX activity, whereas long-term insulin treatment reduced the levels of ATX mRNA and protein, and its activity[172].

In humans, ATX expression in adipose tissue significantly increased in diabetes patients in contrast with obese-only subjects[158,166]. Its expression in subcutaneous fat is higher than that in visceral fat. Nevertheless, ATX in visceral, but not subcutaneous, fat of obese subjects is higher than that in non-obese patients[155]. Interestingly, the circulating ATX levels in the blood were reduced in obese subjects[162]. The females have higher blood ATX level than males[173].

The variations of ATX expression were correlated with some clinical parameters. In obese patients, visceral fat ATX was positively correlated with diastolic arterial blood pressure, plasma leptin level, and expression levels of inducible nitric oxide synthase and apelin receptor[155]. In older and obese humans, plasma ATX correlated with fasting glucose, fasting insulin, and glucose level 2 h after an oral glucose tolerance test, and body mass index[173]. LPA produced by ATX in obesity has a tonic inhibitory effect on glucose homeostasis through inhibition of insulin secretion in isolated pancreas islets, increase of glucose transport in myocyte and adipocytes, and elevation of glycogenolysis in hepatocytes[164]. LPA was reported to activate glycogenolysis in hepatocytes *in vitro*[174], suggesting that LPA's effects on glucose homeostasis may be mediated by the liver. All these indicate that LPA production *via* ATX and its receptors activation may impact glucose homeostasis (Figure 4).

POSSIBLE ROLE OF LPA SIGNALING IN LIVER FIBROSIS

The liver plays a critical role in the control of glucose and lipid homeostasis. The disturbance of this homeostasis may lead to development of metabolic diseases such as type 2 diabetes[175] and nonalcoholic fatty liver disease (NAFLD)[176]. Liver fibrosis is a process that leads to the alteration of the hepatic architecture marked by the accumulation of proteins such as collagen in extracellular matrix. This is generally associated with the development of liver diseases such as NAFLD and hepatitis. If left untreated, the further development of these diseases and liver fibrosis will lead to cirrhosis, and liver failure, which needs liver transplantation for the treatment. Factors causing damages of hepatocytes result in activation of hepatic stellate cells (HSCs) and production of pro-inflammatory and pro-fibrotic factors, which will stimulate formation of accumulation of proteins in extracellular matrix[177].

The injuries caused by nutritional and environmental factors alter liver structures and functions, which may lead to the liver fibrosis[178]. The hepatic matrix is remodeled by the inflammatory responses after liver injury. Upon the stimulation, the generation of the liver matrix such as collagen, elastin, hyaluronan, proteoglycans and fibronectin is elevated, which is followed by remodeling processes. All these are associated with the activation of HSCs, and the change of local architecture and the reduction of liver functions. Excessive production and accumulation of extracellular matrix in the liver results in fibrosis, which can lead to liver cirrhosis[178]. In addition to HSCs, other cells responsible for the fibrosis include fibrocytes from hematopoietic stem cells, portal fibroblasts, bone marrow derived mesenchymal cells, epithelial-mesenchymal transition and endothelial to mesenchymal transition[178].

The excessive accumulation of lipids and alterations of their metabolism have been used to explain the etiology of type 2 diabetes, which is associated with profound changes of hepatic gene expression[175,179]. This alteration of hepatic lipid metabolism may cause the development of fibrosis. For example, the elevation of lipid peroxidation in zone 3 hepatocytes has been suggested with the development of fibrosis[180]. On the other hand, changes of fatty acid compositions in plasma phospholipids have been observed in subjects with fibrosis[181,182]. All these show that the alterations of plasma phospholipids in patients with metabolic diseases may play a role in the development of fibrosis.

It has been shown that serum ATX activity and LPA level increase with the development of liver fibrosis in patients with chronic hepatitis C[74,183-185], and with cholestasis and pruritus[186,187]. The association of elevated plasma ATX level with chronic liver disease (CLD) in patients suggests a shorter overall survival in a 10-year follow-up study[185]. Moreover, the increased expression level of hepatic ATX mRNA was found in the majority of publically available CLD and hepatocellular carcinoma (HCC) microarray data sets, suggesting an association of ATX with liver pathophysiology[185]. ATX and LPA levels increased in the plasma of patients with hepatitis C virus (HCV) infection, and positively associate with liver fibrosis stages[183,184,188,189]. HCV infection may stabilize the activity of hypoxia inducible factor in a PI3K dependent manner, which may increase ATX expression, and in turn induce liver fibrosis[190].

It has been shown that serum ATX level correlated with fibrosis grade, and is useful as its marker in liver fibrosis[191]. The higher expression level of *LPA2* mRNA has been associated with the poorer differentiation of HCC cells, and a higher *LPA6* mRNA level is associated with microvascular invasion of HCC. The high expression levels of *LPA2* and *LPA6* mRNA in HCC predict a high potential for malignancy. The elevated levels of *LPA6* and *LPA6* mRNA in conjunction with plasma ATX predict higher rate of recurrence after surgical removal of the tumors[192]. In addition, the plasma level of ATX

has been considered as a potential pathogenic factor and/or biomarker for nonalcoholic fatty liver disease in nondiabetic and obese women[193]. Moreover, the plasma ATX levels correlated with prognosis of cirrhosis (Child-Pugh score), showing the link of ATX and the severity of cirrhosis in patients with CLD[194].

In rats, plasma LPA level and serum ATX activity were increased in liver injury and were correlated with severity of the damage; the former in relation to the extent of fibrosis, and the latter in relation to the extent of hepatocyte damage[186,195]. In mice, different hepatotoxic stimuli linked with the development of different forms of CLD were shown to stimulate hepatocyte ATX expression, leading to increased LPA production, HSCs activation, and signals for fibrosis development[185].

LPA was first shown to stimulate rat HSCs proliferation through MAP kinase activation in 1998[196]. Then, LPA was shown to enhance HSCs contractility through modulation of cellular morphology and attachment to extracellular matrices *via* Rho-kinase[197,198]. LPA also inhibits the apoptosis of those cells through Rho/Rho kinase activation[199], suggesting its involvement in the pathogenesis of liver fibrosis. Moreover, LPA was shown to induce nuclear translocation of inducible nitric-oxide synthase in hepatocytes[200]. These findings demonstrate the possible involvement of LPA in the development of liver fibrosis.

CONCLUSION

LPA is a highly bioactive lipid mediator with a number of cellular sources and exerts its actions through a family of receptors coupling with GPCRs in various cell types. Here, we have discussed recent advances in pathways for extracellular and intracellular production of LPA, the functions as well as structural and biochemical properties of ATX and LPA receptors. For the past 20-30 years, the cloning and identification of proteins mediating LPA production and signal transduction pathways open a new field for us to understand relevance of these proteins in physiology and disease development. The association of LPA production and signal pathways with chronic metabolic diseases has been gradually realized. We have highlighted the roles of LPA signaling pathways in the obesity, insulin resistance and liver fibrosis.

The realization of the importance of LPA-mediated functions leads to more open questions begging for answers. (1) The regulations of enzymes involved in LPA synthesis and degradation pathways remain to be further investigated. Whether intracellularly produced LPA can cross the plasma membrane into the extracellular compartment is currently unclear. Additional enzymes or pathways for the production of LPA are still worth exploring. For example, phosphatidylglycerol was shown to be converted to LPA under the catalytic action of GPAT as reported[201]. (2) For ATX, an important player for the extracellular LPA production, how its activity is regulated, and how the newly produced LPA is released remain to be addressed. Future analysis will undoubtedly shed some light on these. (3) There are many factors contributing to the pathophysiology of obesity and metabolic diseases. Therefore, the precise role of LPA signaling pathways in these diseases remains to be investigated further. In addition, mechanisms by which the LPA and its receptor signaling pathways in the differentiation of both white and brown adipocytes remain to be clarified. This may help for the control of lipid metabolism. (4) LPA seems to have a negative effect on glucose homeostasis in obesity. This was observed only in obese patients, but not in non-obese subjects. So future human studies should focus on more heathy subjects and compare with those parameters of obese patients. (5) LPA appears to inhibit insulin secretion. Whether this inhibitory effect is due to a direct action of LPA on pancreas islets or a possible regulation of liver glycogen mobilization and/or muscle glucose oxidation remains to be clarified. And (6) It was shown that the plasma LPA level and serum ATX activity both were increased in association with liver fibrosis. The underlying mechanism remains to be determined.

Taken together, the LPA signaling pathways contain multiple points that potentially involve in the development of obesity, liver fibrosis and related pathologies. The development of novel pharmacological modulators targeting intervention points may open new research fields and provide potential medicinal therapies to reduce human suffering. The prospects are bright for expanding insights and contributions in LPA biology.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Elizabeth (Betsy) Anderson Steeves (Department of Nutrition at the University of Tennessee, Knoxville, TN, United States) for her critical reading and editing of this manuscript. In addition, we also would like to thank Dr. Jianan Qi (Southeast University, China) for figures editorial assistance.

REFERENCES

1 **van Meer G**, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* 2008; **9**: 112-124 [PMID: 18216768 DOI: 10.1038/nrm2330]
2 **Vogt W**. The chemical nature of Darmstoff. *J Physiol* 1957; **137**: 154-167 [PMID: 13439591]
3 **Vogt W**. Pharmacologically active lipidsoluble acids of natural occurrence. *Nature* 1957; **179**: 300-304; passim [PMID: 13407704]
4 **Vogt W**. [Not Available]. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 1949; **206**: 1-11 [PMID: 18115712]
5 **Vogt W**. [Properties and preparation of Darmstoff]. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 1955; **227**: 224-233 [PMID: 13309417]
6 **Vogt W**. Pharamacologically active acidic phospholipids and glycolipids. *Biochem Pharmacol* 1963; **12**: 415-420 [PMID: 13997687]
7 **Sen S**, Smeby RR, Bumpus FM. Antihypertensive effect of an isolated phospholipid. *Am J Physiol* 1968; **214**: 337-341 [PMID: 4295739 DOI: 10.1152/ajplegacy.1968.214.2.337]
8 **Tokumura A**, Fukuzawa K, Tsukatani H. Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. *Lipids* 1978; **13**: 572-574 [PMID: 703535]

9 **Schumacher KA**, Classen HG, Späth M. Platelet aggregation evoked in vitro and in vivo by phosphatidic acids and lysoderivatives: identity with substances in aged serum (DAS). *Thromb Haemost* 1979; **42**: 631-640 [PMID: 116384]

10 **van Corven EJ**, Groenink A, Jalink K, Eichholtz T, Moolenaar WH. Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* 1989; **59**: 45-54 [PMID: 2551506]

11 **Hecht JH**, Weiner JA, Post SR, Chun J. Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J Cell Biol* 1996; **135**: 1071-1083 [PMID: 8922387]

12 **Chun J**, Hla T, Lynch KR, Spiegel S, Moolenaar WH. International Union of Basic and Clinical Pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. *Pharmacol Rev* 2010; **62**: 579-587 [PMID: 21079037 DOI: 10.1124/pr.110.003111]

13 **Tokumura A**, Majima E, Kariya Y, Tominaga K, Kogure K, Yasuda K, Fukuzawa K. Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J Biol Chem* 2002; **277**: 39436-39442 [PMID: 12176993 DOI: 10.1074/jbc.M205623200]

14 **Aikawa S**, Hashimoto T, Kano K, Aoki J. Lysophosphatidic acid as a lipid mediator with multiple biological actions. *J Biochem* 2015; **157**: 81-89 [PMID: 25500504 DOI: 10.1093/jb/mvu077]

15 **Rancoule C**, Dusaulcy R, Tréguer K, Grès S, Attané C, Saulnier-Blache JS. Involvement of autotaxin/lysophosphatidic acid signaling in obesity and impaired glucose homeostasis. *Biochimie* 2014; **96**: 140-143 [PMID: 23639740 DOI: 10.1016/j.biochi.2013.04.010]

16 **Barbayianni E**, Kaffe E, Aidinis V, Kokotos G. Autotaxin, a secreted lysophospholipase D, as a promising therapeutic target in chronic inflammation and cancer. *Prog Lipid Res* 2015; **58**: 76-96 [PMID: 25704398 DOI: 10.1016/j.plipres.2015.02.001]

17 **Sheng X**, Yung YC, Chen A, Chun J. Lysophosphatidic acid signalling in development. *Development* 2015; **142**: 1390-1395 [PMID: 25852197 DOI: 10.1242/dev.121723]

18 **Velasco M**, O'Sullivan C, Sheridan GK. Lysophosphatidic acid receptors (LPARs): Potential targets for the treatment of neuropathic pain. *Neuropharmacology* 2017; **113**: 608-617 [PMID: 27059127 DOI: 10.1016/j.neuropharm.2016.04.002]

19 **Okudaira S**, Yukiura H, Aoki J. Biological roles of lysophosphatidic acid signaling through its production by autotaxin. *Biochimie* 2010; **92**: 698-706 [PMID: 20417246 DOI: 10.1016/j.biochi.2010.04.015]

20 **Pagès C**, Simon MF, Valet P, Saulnier-Blache JS. Lysophosphatidic acid synthesis and release. *Prostaglandins Other Lipid Mediat* 2001; **64**: 1-10 [PMID: 11324699]

21 **McIntyre TM,** Pontsler AV, Silva AR, St Hilaire A, Xu Y, Hinshaw JC, Zimmerman GA, Hama K, Aoki J, Arai H, Prestwich GD. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. *Proc Natl Acad Sci USA* 2003; **100**: 131-136 [PMID: 12502787 DOI: 10.1073/pnas.0135855100]

22 **Umezu-Goto M**, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J Cell Biol* 2002; **158**: 227-233 [PMID: 12119361 DOI: 10.1083/jcb.200204026]

23 **Nakamura K**, Kishimoto T, Ohkawa R, Okubo S, Tozuka M, Yokota H, Ikeda H, Ohshima N, Mizuno K, Yatomi Y. Suppression of lysophosphatidic acid and lysophosphatidylcholine formation in the plasma in vitro: proposal of a plasma sample preparation method for laboratory testing of these lipids. *Anal Biochem* 2007; **367**: 20-27 [PMID: 17568554]

24 **Coleman RA**, Lee DP. Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res* 2004; **43**: 134-176 [PMID: 14654091]

25 **Vancura A**, Carroll MA, Haldar D. A lysophosphatidic acid-binding cytosolic protein stimulates mitochondrial glycerophosphate acyltransferase. *Biochem Biophys Res Commun* 1991; **175**: 339-343 [PMID: 1998517]

26 **Vancura A**, Haldar D. Regulation of mitochondrial and microsomal phospholipid synthesis by liver fatty acid-binding protein. *J Biol Chem* 1992; **267**: 14353-14359 [PMID: 1629224]

27 **Brindley DN**, Pilquil C. Lipid phosphate phosphatases and signaling. *J Lipid Res* 2009; **50** Suppl: S225-S230 [PMID: 19066402 DOI: 10.1194/jlr.R800055-JLR200]

28 **Siess W**, Zangl KJ, Essler M, Bauer M, Brandl R, Corrinth C, Bittman R, Tigyi G, Aepfelbacher M. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc Natl Acad Sci U S A* 1999; **96**: 6931-6936 [PMID: 10359816]

29 **Siess W**. Platelet interaction with bioactive lipids formed by mild oxidation of low-density lipoprotein. *Pathophysiol Haemost Thromb* 2006; **35**: 292-304 [PMID: 16877878]

30 **Pyne S**, Kong KC, Darroch PI. Lysophosphatidic acid and sphingosine 1-phosphate biology: the role of lipid phosphate phosphatases. *Semin Cell Dev Biol* 2004; **15**: 491-501 [PMID: 15271294]

31 **Jasinska R**, Zhang QX, Pilquil C, Singh I, Xu J, Dewald J, Dillon DA, Berthiaume LG, Carman GM, Waggoner DW, Brindley DN. Lipid phosphate phosphohydrolase-1 degrades exogenous glycerolipid and sphingolipid phosphate esters. *Biochem J* 1999; **340**: 677-686 [PMID: 10359651]

32 **Kai M**, Wada I, Imai S, Sakane F, Kanoh H. Identification and cDNA cloning of 35-kDa phosphatidic acid phosphatase (type 2) bound to plasma membranes. Polymerase chain reaction amplification of mouse H2O2-inducible hic53 clone yielded the cDNA encoding phosphatidic acid phosphatase. *J Biol Chem* 1996; **271**: 18931-18938 [PMID: 8702556]

33 **Leung DW**, Tompkins CK, White T. Molecular cloning of two alternatively spliced forms of human phosphatidic acid phosphatase cDNAs that are differentially expressed in normal and tumor cells. *DNA Cell Biol* 1998; **17**: 377-385 [PMID: 9570154 DOI: 10.1089/dna.1998.17.377]

34 **Roberts R**, Sciorra VA, Morris AJ. Human type 2 phosphatidic acid phosphohydrolases. Substrate specificity of the type 2a, 2b, and 2c enzymes and cell surface activity of the 2a isoform. *J Biol Chem* 1998; **273**: 22059-22067 [PMID: 9705349]

35 **Aguado B**, Campbell RD. Characterization of a human lysophosphatidic acid acyltransferase that is encoded by a gene located in the class III region of the human major histocompatibility complex. *J Biol Chem* 1998; **273**: 4096-4105 [PMID: 9461603]

36 **Modregger J**, Schmidt AA, Ritter B, Huttner WB, Plomann M. Characterization of Endophilin B1b, a brain-specific membrane-associated lysophosphatidic acid acyl transferase with properties distinct from endophilin A1. *J Biol Chem* 2003; **278**: 4160-4167 [PMID: 12456676]

37 **Weigert R**, Silletta MG, Spanò S, Turacchio G, Cericola C, Colanzi A, Senatore S, Mancini R, Polishchuk EV, Salmona M, Facchiano F, Burger KN, Mironov A, Luini A, Corda D. CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature* 1999; **402**: 429-433 [PMID: 10586885 DOI: 10.1038/46587]

38 **Wang A**, Dennis EA. Mammalian lysophospholipases. *Biochim Biophys Acta* 1999; **1439**: 1-16 [PMID: 10395961]

39 **Stefan C**, Jansen S, Bollen M. NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem Sci* 2005; **30**: 542-550 [PMID: 16125936]

40 **Zimmermann H,** Zebisch M, Sträter N. Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 2012; **8**: 437-502 [PMID: 26811110 DOI: 10.1007/s11302-012-9309-4]

41 **Boutin JA**, Ferry G. Autotaxin. *Cell Mol Life Sci* 2009; **66**: 3009-3021 [PMID: 19506801 DOI: 10.1007/s00018-009-0056-9]

42 **Nakanaga K**, Hama K, Aoki J. Autotaxin--an LPA producing enzyme with diverse functions. *J Biochem* 2010; **148**: 13-24 [PMID: 20495010 DOI: 10.1093/jb/mvq052]

43 **Yuelling LM**, Fuss B. Autotaxin (ATX): a multi-functional and multi-modular protein possessing enzymatic lysoPLD activity and matricellular properties. *Biochim Biophys Acta* 2008; **1781**: 525-530 [PMID: 18485925 DOI: 10.1016/j.bbalip.2008.04.009]

44 **Stracke ML**, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E, Liotta LA. Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J Biol Chem* 1992; **267**: 2524-2529 [PMID: 1733949]

45 **Ferry G**, Tellier E, Try A, Grés S, Naime I, Simon MF, Rodriguez M, Boucher J, Tack I, Gesta S, Chomarat P, Dieu M, Raes M, Galizzi JP, Valet P, Boutin JA, Saulnier-Blache JS. Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity. *J Biol Chem* 2003; **278**: 18162-18169 [PMID: 12642576]

46 **Gesta S**, Simon MF, Rey A, Sibrac D, Girard A, Lafontan M, Valet P, Saulnier-Blache JS. Secretion of a lysophospholipase D activity by adipocytes: involvement in lysophosphatidic acid synthesis. *J Lipid Res* 2002; **43**: 904-910 [PMID: 12032165]

47 **Jansen S**, Andries M, Vekemans K, Vanbilloen H, Verbruggen A, Bollen M. Rapid clearance of the circulating metastatic factor autotaxin by the scavenger receptors of liver sinusoidal endothelial cells. *Cancer Lett* 2009; **284**: 216-221 [PMID: 19482419 DOI: 10.1016/j.canlet.2009.04.029]

48 **Lee HY**, Clair T, Mulvaney PT, Woodhouse EC, Aznavoorian S, Liotta LA, Stracke ML. Stimulation of tumor cell motility linked to phosphodiesterase catalytic site of autotaxin. *J Biol Chem* 1996; **271**: 24408-24412 [PMID: 8798697]

49 **Nishimasu H**, Okudaira S, Hama K, Mihara E, Dohmae N, Inoue A, Ishitani R, Takagi J, Aoki J, Nureki O. Crystal structure of autotaxin and insight into GPCR activation by lipid mediators. *Nat Struct Mol Biol* 2011; **18**: 205-212 [PMID: 21240269 DOI: 10.1038/nsmb.1998]

50 **Giganti A**, Rodriguez M, Fould B, Moulharat N, Cogé F, Chomarat P, Galizzi JP, Valet P, Saulnier-Blache JS, Boutin JA, Ferry G. Murine and human autotaxin alpha, beta, and gamma isoforms: gene organization, tissue distribution, and biochemical characterization. *J Biol Chem* 2008; **283**: 7776-7789 [PMID: 18175805 DOI: 10.1074/jbc.M708705200]

51 **Moolenaar WH**, Perrakis A. Insights into autotaxin: how to produce and present a lipid mediator. *Nat Rev Mol Cell Biol* 2011; **12**: 674-679 [PMID: 21915140 DOI: 10.1038/nrm3188]

52 **Hausmann J**, Kamtekar S, Christodoulou E, Day JE, Wu T, Fulkerson Z, Albers HM, van Meeteren LA, Houben AJ, van Zeijl L, Jansen S, Andries M, Hall T, Pegg LE, Benson TE, Kasiem M, Harlos K, Kooi CW, Smyth SS, Ovaa H, Bollen M, Morris AJ, Moolenaar WH, Perrakis A. Structural basis of substrate discrimination and integrin binding by autotaxin. *Nat Struct Mol Biol* 2011; **18**: 198-204 [PMID: 21240271 DOI: 10.1038/nsmb.1980]

53 **Houben AJ**, van Wijk XM, van Meeteren LA, van Zeijl L, van de Westerlo EM, Hausmann J, Fish A, Perrakis A, van Kuppevelt TH, Moolenaar WH. The polybasic insertion in autotaxin α confers specific binding to heparin and cell surface heparan sulfate proteoglycans. *J Biol Chem* 2013; **288**: 510-519 [PMID: 23150666 DOI: 10.1074/jbc.M112.358416]

54 **Fulkerson Z**, Wu T, Sunkara M, Kooi CV, Morris AJ, Smyth SS. Binding of autotaxin to integrins localizes lysophosphatidic acid production to platelets and mammalian cells. *J Biol Chem* 2011; **286**: 34654-34663 [PMID: 21832043 DOI: 10.1074/jbc.M111.276725]

55 **Murata J**, Lee HY, Clair T, Krutzsch HC, Arestad AA, Sobel ME, Liotta LA, Stracke ML. cDNA cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterases. *J Biol Chem* 1994; **269**: 30479-30484 [PMID: 7982964]

56 **Hashimoto T**, Okudaira S, Igarashi K, Hama K, Yatomi Y, Aoki J. Identification and biochemical characterization of a novel autotaxin isoform, ATXδ, with a four-amino acid deletion. *J Biochem* 2012; **151**: 89-97 [PMID: 21994952 DOI: 10.1093/jb/mvr126]

57 **Bächner D**, Ahrens M, Betat N, Schröder D, Gross G. Developmental expression analysis of murine autotaxin (ATX). *Mech Dev* 1999; **84**: 121-125 [PMID: 10473125]

58 **Nimitphong H**, Holick MF. Vitamin D status and sun exposure in southeast Asia. *Dermatoendocrinol* 2013; **5**: 34-37 [PMID: 24494040 DOI: 10.4161/derm.24054]

59 **Savaskan NE**, Rocha L, Kotter MR, Baer A, Lubec G, van Meeteren LA, Kishi Y, Aoki J, Moolenaar WH, Nitsch R, Bräuer AU. Autotaxin (NPP-2) in the brain: cell type-specific expression and regulation during development and after neurotrauma. *Cell Mol Life Sci* 2007; **64**: 230-243 [PMID: 17192809]

60 **Brock K**, Cant R, Clemson L, Mason RS, Fraser DR. Effects of diet and exercise on plasma vitamin D (25(OH)D) levels in Vietnamese immigrant elderly in Sydney, Australia. *J Steroid Biochem Mol Biol* 2007; **103**: 786-792 [PMID: 17215122 DOI: 10.1016/j.jsbmb.2006.12.048]

61 **Nakasaki T**, Tanaka T, Okudaira S, Hirosawa M, Umemoto E, Otani K, Jin S, Bai Z, Hayasaka H, Fukui Y, Aozasa K, Fujita N, Tsuruo T, Ozono K, Aoki J, Miyasaka M. Involvement of the lysophosphatidic acid-generating enzyme autotaxin in lymphocyte-endothelial cell interactions. *Am J Pathol* 2008; **173**: 1566-1576 [PMID: 18818380 DOI: 10.2353/ajpath.2008.071153]

62 **Bai Z**, Cai L, Umemoto E, Takeda A, Tohya K, Komai Y, Veeraveedu PT, Hata E, Sugiura Y, Kubo A, Suematsu M, Hayasaka H, Okudaira S, Aoki J, Tanaka T, Albers HM, Ovaa H, Miyasaka M. Constitutive lymphocyte transmigration across the basal lamina of high endothelial venules is regulated by the autotaxin/lysophosphatidic acid axis. *J Immunol* 2013; **190**: 2036-2048 [PMID: 23365076 DOI: 10.4049/jimmunol.1202025]

63 **Dusaulcy R**, Rancoule C, Grès S, Wanecq E, Colom A, Guigné C, van Meeteren LA, Moolenaar WH, Valet P, Saulnier-Blache JS. Adipose-specific disruption of autotaxin enhances nutritional fattening and reduces plasma lysophosphatidic acid. *J Lipid Res* 2011; **52**: 1247-1255 [PMID: 21421848 DOI: 10.1194/jlr.M014985]

64 **Dusaulcy R**, Daviaud D, Pradère JP, Grès S, Valet P, Saulnier-Blache JS. Altered food consumption in mice lacking lysophosphatidic acid receptor-1. *J Physiol Biochem* 2009; **65**: 345-350 [PMID: 20358347 DOI: 10.1007/BF03185929]

65 **Pamuklar Z**, Federico L, Liu S, Umezu-Goto M, Dong A, Panchatcharam M, Fulkerson Z, Berdyshev E, Natarajan V, Fang X, van Meeteren LA, Moolenaar WH, Mills GB, Morris AJ, Smyth SS. Autotaxin/lysopholipase D and lysophosphatidic acid regulate murine hemostasis and thrombosis. *J Biol Chem* 2009; **284**: 7385-7394 [PMID: 19139100 DOI: 10.1074/jbc.M807820200]

66 **Leblanc R**, Lee SC, David M, Bordet JC, Norman DD, Patil R, Miller D, Sahay D, Ribeiro J, Clézardin P, Tigyi GJ, Peyruchaud O. Interaction of platelet-derived autotaxin with tumor integrin αVβ3 controls metastasis of breast cancer cells to bone. *Blood* 2014; **124**: 3141-3150 [PMID: 25277122 DOI: 10.1182/blood-2014-04-568683]

67 **Aoki J**. Mechanisms of lysophosphatidic acid production. *Semin Cell Dev Biol* 2004; **15**: 477-489 [PMID: 15271293]

68 **Yung YC**, Stoddard NC, Chun J. LPA receptor signaling: pharmacology, physiology, and pathophysiology. *J Lipid Res* 2014; **55**: 1192-1214 [PMID: 24643338 DOI: 10.1194/jlr.R046458]

69 **Sugiura T**, Nakane S, Kishimoto S, Waku K, Yoshioka Y, Tokumura A. Lysophosphatidic acid, a growth factor-like lipid, in the saliva. *J Lipid Res* 2002; **43**: 2049-2055 [PMID: 12454265]

70 **Sugiura T**, Nakane S, Kishimoto S, Waku K, Yoshioka Y, Tokumura A, Hanahan DJ. Occurrence of lysophosphatidic acid and its alkyl ether-linked analog in rat brain and comparison of their biological activities toward cultured neural cells. *Biochim Biophys Acta* 1999; **1440**: 194-204 [PMID: 10521703]

71 **Yanagida K**, Masago K, Nakanishi H, Kihara Y, Hamano F, Tajima Y, Taguchi R, Shimizu T, Ishii S. Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. *J Biol Chem* 2009; **284**: 17731-17741 [PMID: 19386608 DOI: 10.1074/jbc.M808506200]

72 **Aoki J**, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T,

Mizuno K, Saku K, Taguchi R, Arai H. Serum lysophosphatidic acid is produced through diverse phospholipase pathways. *J Biol Chem* 2002; **277**: 48737-48744 [PMID: 12354767 DOI: 10.1074/jbc.M206812200]

73 **Hosogaya S**, Yatomi Y, Nakamura K, Ohkawa R, Okubo S, Yokota H, Ohta M, Yamazaki H, Koike T, Ozaki Y. Measurement of plasma lysophosphatidic acid concentration in healthy subjects: strong correlation with lysophospholipase D activity. *Ann Clin Biochem* 2008; **45**: 364-368 [PMID: 18583620 DOI: 10.1258/acb.2008.007242]

74 **Watanabe N**, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, Hama K, Okudaira S, Tanaka M, Tomiya T, Yanase M, Tejima K, Nishikawa T, Arai M, Arai H, Omata M, Fujiwara K, Yatomi Y. Both plasma lysophosphatidic acid and serum autotaxin levels are increased in chronic hepatitis C. *J Clin Gastroenterol* 2007; **41**: 616-623 [PMID: 17577119]

75 **Sano T**, Baker D, Virag T, Wada A, Yatomi Y, Kobayashi T, Igarashi Y, Tigyi G. Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. *J Biol Chem* 2002; **277**: 21197-21206 [PMID: 11929870 DOI: 10.1074/jbc.M201289200]

76 **Yatomi Y,** Igarashi K, Nakamura K, Ohkawa R, Masuda A, Suzuki A, Kishimoto T, Ikeda H, Aoki J. Clinical introduction of lysophosphatidic acid (LPA) and autotaxin assays. In Lysophospholipid Receptors Signaling and Biochemistry. Wiley: Hoboken Pub, 2013: 709-735

77 **Scherer M**, Schmitz G, Liebisch G. High-throughput analysis of sphingosine 1-phosphate, sphinganine 1-phosphate, and lysophosphatidic acid in plasma samples by liquid chromatography-tandem mass spectrometry. *Clin Chem* 2009; **55**: 1218-1222 [PMID: 19325012 DOI: 10.1373/clinchem.2008.113779]

78 **Smyth SS**, Cheng HY, Miriyala S, Panchatcharam M, Morris AJ. Roles of lysophosphatidic acid in cardiovascular physiology and disease. *Biochim Biophys Acta* 2008; **1781**: 563-570 [PMID: 18586114 DOI: 10.1016/j.bbalip.2008.05.008]

79 **Aaltonen N**, Laitinen JT, Lehtonen M. Quantification of lysophosphatidic acids in rat brain tissue by liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010; **878**: 1145-1152 [PMID: 20381434 DOI: 10.1016/j.jchromb.2010.03.030]

80 **Lee JW**, Nishiumi S, Yoshida M, Fukusaki E, Bamba T. Simultaneous profiling of polar lipids by supercritical fluid chromatography/tandem mass spectrometry with methylation. *J Chromatogr A* 2013; **1279**: 98-107 [PMID: 23380365 DOI: 10.1016/j.chroma.2013.01.020]

81 **An S**, Bleu T, Huang W, Hallmark OG, Coughlin SR, Goetzl EJ. Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett* 1997; **417**: 279-282 [PMID: 9409733]

82 **An S**, Bleu T, Hallmark OG, Goetzl EJ. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* 1998; **273**: 7906-7910 [PMID: 9525886]

83 **Bandoh K**, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H, Inoue K. Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* 1999; **274**: 27776-27785 [PMID: 10488122]

84 **Fukushima N**, Ishii I, Contos JJ, Weiner JA, Chun J. Lysophospholipid receptors. *Annu Rev Pharmacol Toxicol* 2001; **41**: 507-534 [PMID: 11264467]

85 **Lynch KR**. Lysophospholipid receptor nomenclature. *Biochim Biophys Acta* 2002; **1582**: 70-71 [PMID: 12069811]

86 **Noguchi K**, Ishii S, Shimizu T. Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* 2003; **278**: 25600-25606 [PMID: 12724320 DOI: 10.1074/jbc.M302648200]

87 **Lee CW**, Rivera R, Gardell S, Dubin AE, Chun J. GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* 2006; **281**: 23589-23597 [PMID: 16774927 DOI: 10.1074/jbc.M603670200]

88 **Tabata K**, Baba K, Shiraishi A, Ito M, Fujita N. The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* 2007; **363**: 861-866 [PMID: 17905198 DOI: 10.1016/j.bbrc.2007.09.063]

89 **Kihara Y**, Maceyka M, Spiegel S, Chun J. Lysophospholipid receptor nomenclature review: IUPHAR Review 8. *Br J Pharmacol* 2014; **171**: 3575-3594 [PMID: 24602016 DOI: 10.1111/bph.12678]

90 **Fukushima N**, Ishii S, Tsujiuchi T, Kagawa N, Katoh K. Comparative analyses of lysophosphatidic acid receptor-mediated signaling. *Cell Mol Life Sci* 2015; **72**: 2377-2394 [PMID: 25732591 DOI: 10.1007/s00018-015-1872-8]

91 **Xiang SY**, Dusaban SS, Brown JH. Lysophospholipid receptor activation of RhoA and lipid signaling pathways. *Biochim Biophys Acta* 2013; **1831**: 213-222 [PMID: 22986288 DOI: 10.1016/j.bbalip.2012.09.004]

92 **Hildebrandt JP**. Lysophosphatidic acid induces inositol phosphate and calcium signals in exocrine cells from the avian nasal salt gland. *J Membr Biol* 1995; **144**: 49-58 [PMID: 7595941]

93 **Gennero I**, Xuereb JM, Simon MF, Girolami JP, Bascands JL, Chap H, Boneu B, Sié P. Effects of lysophosphatidic acid on proliferation and cytosolic Ca++ of human adult vascular smooth muscle cells in culture. *Thromb Res* 1999; **94**: 317-326 [PMID: 10379820]

94 **Litosch I**. Phosphatidic acid potentiates G(alpha)q stimulation of phospholipase C-beta1 signaling. *Biochem Biophys Res Commun* 2009; **390**: 603-607 [PMID: 19818737 DOI: 10.1016/j.bbrc.2009.10.013]

95 **Dorsam RT**, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer* 2007; **7**: 79-94 [PMID: 17251915]

96 **McCudden CR**, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signaling: back to the future. *Cell Mol Life Sci* 2005; **62**: 551-577 [PMID: 15747061]

97 **Lee CW**, Rivera R, Dubin AE, Chun J. LPA(4)/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing G(s)-, G(q)/G(i)-mediated calcium signaling and G(12/13)-mediated Rho activation. *J Biol Chem* 2007; **282**: 4310-4317 [PMID: 17166850 DOI: 10.1074/jbc.M610826200]

98 **Wittpoth C**, Scholich K, Yigzaw Y, Stringfield TM, Patel TB. Regions on adenylyl cyclase that are necessary for inhibition of activity by beta gamma and G(ialpha) subunits of heterotrimeric G proteins. *Proc Natl Acad Sci U S A* 1999; **96**: 9551-9556 [PMID: 10449730]

99 **Jongsma M**, Matas-Rico E, Rzadkowski A, Jalink K, Moolenaar WH. LPA is a chemorepellent for B16 melanoma cells: action through the cAMP-elevating LPA5 receptor. *PLoS One* 2011; **6**: e29260 [PMID: 22195035 DOI: 10.1371/journal.pone.0029260]

100 **Williams JR**, Khandoga AL, Goyal P, Fells JI, Perygin DH, Siess W, Parrill AL, Tigyi G, Fujiwara Y. Unique ligand selectivity of the GPR92/LPA5 lysophosphatidate receptor indicates role in human platelet activation. *J Biol Chem* 2009; **284**: 17304-17319 [PMID: 19366702 DOI: 10.1074/jbc.M109.003194]

101 **Makide K**, Kitamura H, Sato Y, Okutani M, Aoki J. Emerging lysophospholipid mediators, lysophosphatidylserine, lysophosphatidylthreonine, lysophosphatidylethanolamine and lysophosphatidylglycerol. *Prostaglandins Other Lipid Mediat* 2009; **89**: 135-139 [PMID: 19427394 DOI: 10.1016/j.prostaglandins.2009.04.009]

102 **Kano K**, Arima N, Ohgami M, Aoki J. LPA and its analogs-attractive tools for elucidation of LPA biology and drug development. *Curr Med Chem* 2008; **15**: 2122-2131 [PMID: 18781939]

103 **Anliker B**, Choi JW, Lin ME, Gardell SE, Rivera RR, Kennedy G, Chun J. Lysophosphatidic acid (LPA) and its receptor, LPA1 , influence embryonic schwann cell migration, myelination, and cell-to-axon segregation. *Glia* 2013; **61**: 2009-2022 [PMID: 24115248 DOI: 10.1002/glia.22572]

104 **Sakai N**, Chun J, Duffield JS, Wada T, Luster AD, Tager AM. LPA1-induced cytoskeleton reorganization drives fibrosis through CTGF-dependent fibroblast proliferation. *FASEB J* 2013; **27**: 1830-1846 [PMID: 23322166 DOI: 10.1096/fj.12-219378]

105 **Ishii I**, Fukushima N, Ye X, Chun J. Lysophospholipid receptors:

signaling and biology. *Annu Rev Biochem* 2004; **73**: 321-354 [PMID: 15189145]
106 **Contos JJ**, Ishii I, Chun J. Lysophosphatidic acid receptors. *Mol Pharmacol* 2000; **58**: 1188-1196 [PMID: 11093753]
107 **Contos JJ**, Fukushima N, Weiner JA, Kaushal D, Chun J. Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc Natl Acad Sci USA* 2000; **97**: 13384-13389 [PMID: 11087877]
108 **Musazzi L**, Di Daniel E, Maycox P, Racagni G, Popoli M. Abnormalities in α/β-CaMKII and related mechanisms suggest synaptic dysfunction in hippocampus of LPA1 receptor knockout mice. *Int J Neuropsychopharmacol* 2011; **14**: 941-953 [PMID: 20942999 DOI: 10.1017/S1461145710001240]
109 **Estivill-Torrús G**, Llebrez-Zayas P, Matas-Rico E, Santín L, Pedraza C, De Diego I, Del Arco I, Fernández-Llebrez P, Chun J, De Fonseca FR. Absence of LPA1 signaling results in defective cortical development. *Cereb Cortex* 2008; **18**: 938-950 [PMID: 17656621 DOI: 10.1093/cercor/bhm132]
110 **Choi JW**, Herr DR, Noguchi K, Yung YC, Lee CW, Mutoh T, Lin ME, Teo ST, Park KE, Mosley AN, Chun J. LPA receptors: subtypes and biological actions. *Annu Rev Pharmacol Toxicol* 2010; **50**: 157-186 [PMID: 20055701 DOI: 10.1146/annurev.pharmtox.010909.105753]
111 **Ohuchi H**, Hamada A, Matsuda H, Takagi A, Tanaka M, Aoki J, Arai H, Noji S. Expression patterns of the lysophospholipid receptor genes during mouse early development. *Dev Dyn* 2008; **237**: 3280-3294 [PMID: 18924241 DOI: 10.1002/dvdy.21736]
112 **Lai YJ**, Chen CS, Lin WC, Lin FT. c-Src-mediated phosphorylation of TRIP6 regulates its function in lysophosphatidic acid-induced cell migration. *Mol Cell Biol* 2005; **25**: 5859-5868 [PMID: 15988003 DOI: 10.1128/MCB.25.14.5859-5868.2005]
113 **Xu J**, Lai YJ, Lin WC, Lin FT. TRIP6 enhances lysophosphatidic acid-induced cell migration by interacting with the lysophosphatidic acid 2 receptor. *J Biol Chem* 2004; **279**: 10459-10468 [PMID: 14688263 DOI: 10.1074/jbc.M311891200]
114 **Lin FT**, Lai YJ. Regulation of the LPA2 receptor signaling through the carboxyl-terminal tail-mediated protein-protein interactions. *Biochim Biophys Acta* 2008; **1781**: 558-562 [PMID: 18501721 DOI: 10.1016/j.bbalip.2008.04.013]
115 **Contos JJ**, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J. Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Biol* 2002; **22**: 6921-6929 [PMID: 12215548]
116 **Im DS**, Heise CE, Harding MA, George SR, O'Dowd BF, Theodorescu D, Lynch KR. Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol Pharmacol* 2000; **57**: 753-759 [PMID: 10727522]
117 **Zhao C**, Sardella A, Davis L, Poubelle PE, Bourgoin SG, Fernandes MJ. A transgenic mouse model for the in vivo bioluminescence imaging of the expression of the lysophosphatidic acid receptor 3: relevance for inflammation and uterine physiology research. *Transgenic Res* 2015; **24**: 625-634 [PMID: 25982332 DOI: 10.1007/s11248-015-9882-8]
118 **Ye X**, Chun J. Lysophosphatidic acid (LPA) signaling in vertebrate reproduction. *Trends Endocrinol Metab* 2010; **21**: 17-24 [PMID: 19836970 DOI: 10.1016/j.tem.2009.08.003]
119 **Hama K**, Aoki J. LPA(3), a unique G protein-coupled receptor for lysophosphatidic acid. *Prog Lipid Res* 2010; **49**: 335-342 [PMID: 20230855 DOI: 10.1016/j.plipres.2010.03.001]
120 **Lai SL**, Yao WL, Tsao KC, Houben AJ, Albers HM, Ovaa H, Moolenaar WH, Lee SJ. Autotaxin/Lpar3 signaling regulates Kupffer's vesicle formation and left-right asymmetry in zebrafish. *Development* 2012; **139**: 4439-4448 [PMID: 23095890 DOI: 10.1242/dev.081745]
121 **Rhee HJ**, Nam JS, Sun Y, Kim MJ, Choi HK, Han DH, Kim NH, Huh SO. Lysophosphatidic acid stimulates cAMP accumulation and cAMP response element-binding protein phosphorylation in immortalized hippocampal progenitor cells. *Neuroreport* 2006; **17**: 523-526 [PMID: 16543818 DOI: 10.1097/01.wnr.0000209011.16718.68]
122 **Lee Z**, Cheng CT, Zhang H, Subler MA, Wu J, Mukherjee A, Windle JJ, Chen CK, Fang X. Role of LPA4/p2y9/GPR23 in negative regulation of cell motility. *Mol Biol Cell* 2008; **19**: 5435-5445 [PMID: 18843048 DOI: 10.1091/mbc.E08-03-0316]
123 **Liu YB**, Kharode Y, Bodine PV, Yaworsky PJ, Robinson JA, Billiard J. LPA induces osteoblast differentiation through interplay of two receptors: LPA1 and LPA4. *J Cell Biochem* 2010; **109**: 794-800 [PMID: 20069565 DOI: 10.1002/jcb.22471]
124 **Mansell JP**, Barbour M, Moore C, Nowghani M, Pabbruwe M, Sjostrom T, Blom AW. The synergistic effects of lysophosphatidic acid receptor agonists and calcitriol on MG63 osteoblast maturation at titanium and hydroxyapatite surfaces. *Biomaterials* 2010; **31**: 199-206 [PMID: 19796809 DOI: 10.1016/j.biomaterials.2009.09.035]
125 **Liu S**, Umezu-Goto M, Murph M, Lu Y, Liu W, Zhang F, Yu S, Stephens LC, Cui X, Murrow G, Coombes K, Muller W, Hung MC, Perou CM, Lee AV, Fang X, Mills GB. Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases. *Cancer Cell* 2009; **15**: 539-550 [PMID: 19477432 DOI: 10.1016/j.ccr.2009.03.027]
126 **Sumida H**, Noguchi K, Kihara Y, Abe M, Yanagida K, Hamano F, Sato S, Tamaki K, Morishita Y, Kano MR, Iwata C, Miyazono K, Sakimura K, Shimizu T, Ishii S. LPA4 regulates blood and lymphatic vessel formation during mouse embryogenesis. *Blood* 2010; **116**: 5060-5070 [PMID: 20713964 DOI: 10.1182/blood-2010-03-272443]
127 **Kotarsky K**, Boketoft A, Bristulf J, Nilsson NE, Norberg A, Hansson S, Owman C, Sillard R, Leeb-Lundberg LM, Olde B. Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. *J Pharmacol Exp Ther* 2006; **318**: 619-628 [PMID: 16651401 DOI: 10.1124/jpet.105.098848]
128 **Lin S**, Yeruva S, He P, Singh AK, Zhang H, Chen M, Lamprecht G, de Jonge HR, Tse M, Donowitz M, Hogema BM, Chun J, Seidler U, Yun CC. Lysophosphatidic acid stimulates the intestinal brush border Na(+)/H(+) exchanger 3 and fluid absorption via LPA(5) and NHERF2. *Gastroenterology* 2010; **138**: 649-658 [PMID: 19800338 DOI: 10.1053/j.gastro.2009.09.055]
129 **Lundequist A**, Boyce JA. LPA5 is abundantly expressed by human mast cells and important for lysophosphatidic acid induced MIP-1β release. *PLoS One* 2011; **6**: e18192 [PMID: 21464938 DOI: 10.1371/journal.pone.0018192]
130 **Araki M**, Kitayoshi M, Dong Y, Hirane M, Ozaki S, Mori S, Fukushima N, Honoki K, Tsujiuchi T. Inhibitory effects of lysophosphatidic acid receptor-5 on cellular functions of sarcoma cells. *Growth Factors* 2014; **32**: 117-122 [PMID: 24798396 DOI: 10.3109/08977194.2014.911294]
131 **Kaplan MH**, Smith DI, Sundick RS. Identification of a G protein coupled receptor induced in activated T cells. *J Immunol* 1993; **151**: 628-636 [PMID: 8393036]
132 **Webb TE**, Kaplan MG, Barnard EA. Identification of 6H1 as a P2Y purinoceptor: P2Y5. *Biochem Biophys Res Commun* 1996; **219**: 105-110 [PMID: 8619790]
133 **Lee M**, Choi S, Halldén G, Yo SJ, Schichnes D, Aponte GW. P2Y5 is a G(alpha)i, G(alpha)12/13 G protein-coupled receptor activated by lysophosphatidic acid that reduces intestinal cell adhesion. *Am J Physiol Gastrointest Liver Physiol* 2009; **297**: G641-G654 [PMID: 19679818 DOI: 10.1152/ajpgi.00191.2009]
134 **Pasternack SM**, von Kügelgen I, Al Aboud K, Lee YA, Rüschendorf F, Voss K, Hillmer AM, Molderings GJ, Franz T, Ramirez A, Nürnberg P, Nöthen MM, Betz RC. G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. *Nat Genet* 2008; **40**: 329-334 [PMID: 18297070 DOI: 10.1038/ng.84]
135 **Raza SI**, Muhammad D, Jan A, Ali RH, Hassan M, Ahmad W, Rashid S. In silico analysis of missense mutations in LPAR6 reveals abnormal phospholipid signaling pathway leading to hypotrichosis. *PLoS One* 2014; **9**: e104756 [PMID: 25119526 DOI: 10.1371/journal.pone.0104756]

136 **Shimomura Y**, Garzon MC, Kristal L, Shapiro L, Christiano AM. Autosomal recessive woolly hair with hypotrichosis caused by a novel homozygous mutation in the P2RY5 gene. *Exp Dermatol* 2009; **18**: 218-221 [PMID: 18803659 DOI: 10.1111/j.1600-0625.2008.00788.x]

137 **Nahum S**, Morice-Picard F, Taieb A, Sprecher E. A novel mutation in LPAR6 causes autosomal recessive hypotrichosis of the scalp. *Clin Exp Dermatol* 2011; **36**: 188-194 [PMID: 21070332 DOI: 10.1111/j.1365-2230.2010.03944.x]

138 **Archbold JK**, Martin JL, Sweet MJ. Towards selective lysophospholipid GPCR modulators. *Trends Pharmacol Sci* 2014; **35**: 219-226 [PMID: 24746475 DOI: 10.1016/j.tips.2014.03.004]

139 **Choi JW**, Lee CW, Chun J. Biological roles of lysophospholipid receptors revealed by genetic null mice: an update. *Biochim Biophys Acta* 2008; **1781**: 531-539 [PMID: 18407842 DOI: 10.1016/j.bbalip.2008.03.004]

140 **Valentine WJ**, Fells JI, Perygin DH, Mujahid S, Yokoyama K, Fujiwara Y, Tsukahara R, Van Brocklyn JR, Parrill AL, Tigyi G. Subtype-specific residues involved in ligand activation of the endothelial differentiation gene family lysophosphatidic acid receptors. *J Biol Chem* 2008; **283**: 12175-12187 [PMID: 18316373 DOI: 10.1074/jbc.M708847200]

141 **Lee SC**, Fujiwara Y, Liu J, Yue J, Shimizu Y, Norman DD, Wang Y, Tsukahara R, Szabo E, Patil R, Banerjee S, Miller DD, Balazs L, Ghosh MC, Waters CM, Oravecz T, Tigyi GJ. Autotaxin and LPA1 and LPA5 receptors exert disparate functions in tumor cells versus the host tissue microenvironment in melanoma invasion and metastasis. *Mol Cancer Res* 2015; **13**: 174-185 [PMID: 25158955 DOI: 10.1158/1541-7786.MCR-14-0263]

142 **Amisten S**, Braun OO, Bengtsson A, Erlinge D. Gene expression profiling for the identification of G-protein coupled receptors in human platelets. *Thromb Res* 2008; **122**: 47-57 [PMID: 17920662]

143 **Yanagida K**, Kurikawa Y, Shimizu T, Ishii S. Current progress in non-Edg family LPA receptor research. *Biochim Biophys Acta* 2013; **1831**: 33-41 [PMID: 22902318 DOI: 10.1016/j.bbalip.2012.08.003]

144 **Yanagida K**, Ishii S. Non-Edg family LPA receptors: the cutting edge of LPA research. *J Biochem* 2011; **150**: 223-232 [PMID: 21746769 DOI: 10.1093/jb/mvr087]

145 **Dong Y**, Hirane M, Araki M, Fukushima N, Tsujiuchi T. Lysophosphatidic acid receptor-5 negatively regulates cellular responses in mouse fibroblast 3T3 cells. *Biochem Biophys Res Commun* 2014; **446**: 585-589 [PMID: 24632199 DOI: 10.1016/j.bbrc.2014.03.016]

146 **UniProt Consortium**. Update on activities at the Universal Protein Resource (UniProt) in 2013. *Nucleic Acids Res* 2013; **41**: D43-D47 [PMID: 23161681 DOI: 10.1093/nar/gks1068]

147 **Ogden CL**, Carroll MD, Fryar CD, Flegal KM. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. *NCHS Data Brief* 2015; : 1-8 [PMID: 26633046]

148 **Reilly JJ**, Kelly J. Long-term impact of overweight and obesity in childhood and adolescence on morbidity and premature mortality in adulthood: systematic review. *Int J Obes* (Lond) 2011; **35**: 891-898 [PMID: 20975725 DOI: 10.1038/ijo.2010.222]

149 **Isakson P**, Hammarstedt A, Gustafson B, Smith U. Impaired preadipocyte differentiation in human abdominal obesity: role of Wnt, tumor necrosis factor-alpha, and inflammation. *Diabetes* 2009; **58**: 1550-1557 [PMID: 19351711 DOI: 10.2337/db08-1770]

150 **Valet P**, Pagès C, Jeanneton O, Daviaud D, Barbe P, Record M, Saulnier-Blache JS, Lafontan M. Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes. A paracrine signal for preadipocyte growth. *J Clin Invest* 1998; **101**: 1431-1438 [PMID: 9525986 DOI: 10.1172/JCI806]

151 **Pagès G**, Girard A, Jeanneton O, Barbe P, Wolf C, Lafontan M, Valet P, Saulnier-Blache JS. LPA as a paracrine mediator of adipocyte growth and function. *Ann N Y Acad Sci* 2000; **905**: 159-164 [PMID: 10818451]

152 **Tokumura A**, Kanaya Y, Kitahara M, Miyake M, Yoshioka Y, Fukuzawa K. Increased formation of lysophosphatidic acids by lysophospholipase D in serum of hypercholesterolemic rabbits. *J Lipid Res* 2002; **43**: 307-315 [PMID: 11861673]

153 **Simon MF**, Daviaud D, Pradère JP, Grès S, Guigné C, Wabitsch M, Chun J, Valet P, Saulnier-Blache JS. Lysophosphatidic acid inhibits adipocyte differentiation via lysophosphatidic acid 1 receptor-dependent down-regulation of peroxisome proliferator-activated receptor gamma2. *J Biol Chem* 2005; **280**: 14656-14662 [PMID: 15710620 DOI: 10.1074/jbc.M412585200]

154 **Nobusue H**, Kondo D, Yamamoto M, Kano K. Effects of lysophosphatidic acid on the in vitro proliferation and differentiation of a novel porcine preadipocyte cell line. *Comp Biochem Physiol B Biochem Mol Biol* 2010; **157**: 401-407 [PMID: 20826223 DOI: 10.1016/j.cbpb.2010.08.010]

155 **Rancoule C**, Dusaulcy R, Tréguer K, Grès S, Guigné C, Quilliot D, Valet P, Saulnier-Blache JS. Depot-specific regulation of autotaxin with obesity in human adipose tissue. *J Physiol Biochem* 2012; **68**: 635-644 [PMID: 22644624 DOI: 10.1007/s13105-012-0181-z]

156 **Holmström TE**, Mattsson CL, Wang Y, Iakovleva I, Petrovic N, Nedergaard J. Non-transactivational, dual pathways for LPA-induced Erk1/2 activation in primary cultures of brown pre-adipocytes. *Exp Cell Res* 2010; **316**: 2664-2675 [PMID: 20576526 DOI: 10.1016/j.yexcr.2010.05.029]

157 **Mattsson CL**, Andersson ER, Nedergaard J. Differential involvement of caveolin-1 in brown adipocyte signaling: impaired beta3-adrenergic, but unaffected LPA, PDGF and EGF receptor signaling. *Biochim Biophys Acta* 2010; **1803**: 983-989 [PMID: 20381543 DOI: 10.1016/j.bbamcr.2010.03.015]

158 **Saulnier-Blache JS**. Secretion and role of autotaxin and lysophosphatidic acid in adipose tissue. *J Soc Biol* 2006; **200**: 77-81 [PMID: 17144165]

159 **Federico L**, Ren H, Mueller PA, Wu T, Liu S, Popovic J, Blalock EM, Sunkara M, Ovaa H, Albers HM, Mills GB, Morris AJ, Smyth SS. Autotaxin and its product lysophosphatidic acid suppress brown adipose differentiation and promote diet-induced obesity in mice. *Mol Endocrinol* 2012; **26**: 786-797 [PMID: 22474126 DOI: 10.1210/me.2011-1229]

160 **Noguchi M**, Hosoda K, Fujikura J, Fujimoto M, Iwakura H, Tomita T, Ishii T, Arai N, Hirata M, Ebihara K, Masuzaki H, Itoh H, Narumiya S, Nakao K. Genetic and pharmacological inhibition of Rho-associated kinase II enhances adipogenesis. *J Biol Chem* 2007; **282**: 29574-29583 [PMID: 17681946 DOI: 10.1074/jbc.M705972200]

161 **Li L**, Tam L, Liu L, Jin T, Ng DS. Wnt-signaling mediates the anti-adipogenic action of lysophosphatidic acid through cross talking with the Rho/Rho associated kinase (ROCK) pathway. *Biochem Cell Biol* 2011; **89**: 515-521 [PMID: 21999426 DOI: 10.1139/o11-048]

162 **Nishimura S**, Nagasaki M, Okudaira S, Aoki J, Ohmori T, Ohkawa R, Nakamura K, Igarashi K, Yamashita H, Eto K, Uno K, Hayashi N, Kadowaki T, Komuro I, Yatomi Y, Nagai R. ENPP2 contributes to adipose tissue expansion and insulin resistance in diet-induced obesity. *Diabetes* 2014; **63**: 4154-4164 [PMID: 24969110 DOI: 10.2337/db13-1694]

163 **Benesch MG**, Zhao YY, Curtis JM, McMullen TP, Brindley DN. Regulation of autotaxin expression and secretion by lysophosphatidate and sphingosine 1-phosphate. *J Lipid Res* 2015; **56**: 1134-1144 [PMID: 25896349 DOI: 10.1194/jlr.M057661]

164 **Lundgren M**, Svensson M, Lindmark S, Renström F, Ruge T, Eriksson JW. Fat cell enlargement is an independent marker of insulin resistance and 'hyperleptinaemia'. *Diabetologia* 2007; **50**: 625-633 [PMID: 17216279]

165 **Arner E**, Westermark PO, Spalding KL, Britton T, Rydén M, Frisén J, Bernard S, Arner P. Adipocyte turnover: relevance to human adipose tissue morphology. *Diabetes* 2010; **59**: 105-109 [PMID: 19846802 DOI: 10.2337/db09-0942]

166 **Boucher J**, Quilliot D, Pradères JP, Simon MF, Grès S, Guigné C, Prévot D, Ferry G, Boutin JA, Carpéné C, Valet P, Saulnier-Blache JS. Potential involvement of adipocyte insulin resistance in obesity-associated up-regulation of adipocyte lysophospholipase D/autotaxin expression. *Diabetologia* 2005; **48**: 569-577 [PMID: 15700135]

167 **Coy PE**, Taneja N, Lee I, Hecquet C, Bryson JM, Robey RB. LPA is a novel lipid regulator of mesangial cell hexokinase activity and HKII isoform expression. *Am J Physiol Renal Physiol* 2002; **283**: F271-F279 [PMID: 12110510 DOI: 10.1152/ajprenal.00093.2001]

168 **Keller JN**, Steiner MR, Mattson MP, Steiner SM. Lysophosphatidic acid decreases glutamate and glucose uptake by astrocytes. *J Neurochem* 1996; **67**: 2300-2305 [PMID: 8931461]

169 **Yea K**, Kim J, Lim S, Park HS, Park KS, Suh PG, Ryu SH. Lysophosphatidic acid regulates blood glucose by stimulating myotube and adipocyte glucose uptake. *J Mol Med* (Berl) 2008; **86**: 211-220 [PMID: 17924084 DOI: 10.1007/s00109-007-0269-z]

170 **Barber MN**, Risis S, Yang C, Meikle PJ, Staples M, Febbraio MA, Bruce CR. Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes. *PLoS One* 2012; **7**: e41456 [PMID: 22848500 DOI: 10.1371/journal.pone.0041456]

171 **Rancoule C**, Attané C, Grès S, Fournel A, Dusaulcy R, Bertrand C, Vinel C, Tréguer K, Prentki M, Valet P, Saulnier-Blache JS. Lysophosphatidic acid impairs glucose homeostasis and inhibits insulin secretion in high-fat diet obese mice. *Diabetologia* 2013; **56**: 1394-1402 [PMID: 23508306 DOI: 10.1007/s00125-013-2891-3]

172 **D'Souza K**, Kane DA, Touaibia M, Kershaw EE, Pulinilkunnil T, Kienesberger PC. Autotaxin Is Regulated by Glucose and Insulin in Adipocytes. *Endocrinology* 2017; **158**: 791-803 [PMID: 28324037 DOI: 10.1210/en.2017-00035]

173 **Reeves VL**, Trybula JS, Wills RC, Goodpaster BH, Dubé JJ, Kienesberger PC, Kershaw EE. Serum Autotaxin/ENPP2 correlates with insulin resistance in older humans with obesity. *Obesity* (Silver Spring) 2015; **23**: 2371-2376 [PMID: 26727116 DOI: 10.1002/oby.21232]

174 **Im DS**, Fujioka T, Katada T, Kondo Y, Ui M, Okajima F. Characterization of sphingosine 1-phosphate-induced actions and its signaling pathways in rat hepatocytes. *Am J Physiol* 1997; **272**: G1091-G1099 [PMID: 9176218 DOI: 10.1152/ajpgi.1997.272.5.G1091]

175 **McGarry JD**. Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 2002; **51**: 7-18 [PMID: 11756317]

176 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

177 **Chen G**. The link between Hepatic Vitamin A Metabolism and Nonalcoholic Fatty Liver Disease. *Curr Drug Targets* 2015; **16**: 1281-1292 [PMID: 25808650]

178 **Elpek GÖ**. Cellular and molecular mechanisms in the pathogenesis of liver fibrosis: An update. *World J Gastroenterol* 2014; **20**: 7260-7276 [PMID: 24966597 DOI: 10.3748/wjg.v20.i23.7260]

179 **Brown MS**, Goldstein JL. Selective versus total insulin resistance: a pathogenic paradox. *Cell Metab* 2008; **7**: 95-96 [PMID: 18249166 DOI: 10.1016/j.cmet.2007.12.009]

180 **MacDonald GA**, Bridle KR, Ward PJ, Walker NI, Houglum K, George DK, Smith JL, Powell LW, Crawford DH, Ramm GA. Lipid peroxidation in hepatic steatosis in humans is associated with hepatic fibrosis and occurs predominately in acinar zone 3. *J Gastroenterol Hepatol* 2001; **16**: 599-606 [PMID: 11422610]

181 **Drzymała-Czyż S**, Szczepanik M, Krzyżanowska P, Duś-Żuchowska M, Pogorzelski A, Sapiejka E, Juszczak P, Lisowska A, Koletzko B, Walkowiak J. Serum Phospholipid Fatty Acid Composition in Cystic Fibrosis Patients with and without Liver Cirrhosis. *Ann Nutr Metab* 2017; **71**: 91-98 [PMID: 28738321 DOI: 10.1159/000477913]

182 **Puri P**, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min HK, Contos MJ, Sterling RK, Fuchs M, Zhou H, Watkins SM, Sanyal AJ. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* 2009; **50**: 1827-1838 [PMID: 19937697 DOI: 10.1002/hep.23229]

183 **Cooper AB**, Wu J, Lu D, Maluccio MA. Is autotaxin (ENPP2) the link between hepatitis C and hepatocellular cancer? *J Gastrointest Surg* 2007; **11**: 1628-34; discussion 1634-5 [PMID: 17902023]

184 **Schlatzer DM**, Sugalski JM, Chen Y, Barnholtz-Sloan J, Davitkov P, Hazlett FE, Funderburg N, Rodriguez B, Lederman MM, Sieg SF, Chance MR, Anthony DD. Plasma proteome analysis reveals overlapping, yet distinct mechanisms of immune activation in chronic HCV and HIV infections. *J Acquir Immune Defic Syndr* 2013; **63**: 563-571 [PMID: 23507661 DOI: 10.1097/QAI.0b013e3182909847]

185 **Kaffe E**, Katsifa A, Xylourgidis N, Ninou I, Zannikou M, Harokopos V, Foka P, Dimitriadis A, Evangelou K, Moulas AN, Georgopoulou U, Gorgoulis VG, Dalekos GN, Aidinis V. Hepatocyte autotaxin expression promotes liver fibrosis and cancer. *Hepatology* 2017; **65**: 1369-1383 [PMID: 27981605 DOI: 10.1002/hep.28973]

186 **Kremer AE**, Martens JJ, Kulik W, Ruëff F, Kuiper EM, van Buuren HR, van Erpecum KJ, Kondrackiene J, Prieto J, Rust C, Geenes VL, Williamson C, Moolenaar WH, Beuers U, Oude Elferink RP. Lysophosphatidic acid is a potential mediator of cholestatic pruritus. *Gastroenterology* 2010; **139**: 1008-1018, 1018.e1 [PMID: 20546739 DOI: 10.1053/j.gastro.2010.05.009]

187 **Kremer AE**, van Dijk R, Leckie P, Schaap FG, Kuiper EM, Mettang T, Reiners KS, Raap U, van Buuren HR, van Erpecum KJ, Davies NA, Rust C, Engert A, Jalan R, Oude Elferink RP, Beuers U. Serum autotaxin is increased in pruritus of cholestasis, but not of other origin, and responds to therapeutic interventions. *Hepatology* 2012; **56**: 1391-1400 [PMID: 22473838 DOI: 10.1002/hep.25748]

188 **Wu JM**, Xu Y, Skill NJ, Sheng H, Zhao Z, Yu M, Saxena R, Maluccio MA. Autotaxin expression and its connection with the TNF-alpha-NF-kappaB axis in human hepatocellular carcinoma. *Mol Cancer* 2010; **9**: 71 [PMID: 20356387 DOI: 10.1186/1476-4598-9-71]

189 **Kondo M**, Ishizawa T, Enooku K, Tokuhara Y, Ohkawa R, Uranbileg B, Nakagawa H, Tateishi R, Yoshida H, Kokudo N, Koike K, Yatomi Y, Ikeda H. Increased serum autotaxin levels in hepatocellular carcinoma patients were caused by background liver fibrosis but not by carcinoma. *Clin Chim Acta* 2014; **433**: 128-134 [PMID: 24642343 DOI: 10.1016/j.cca.2014.03.006]

190 **Farquhar MJ**, Humphreys IS, Rudge SA, Wilson GK, Bhattacharya B, Ciaccia M, Hu K, Zhang Q, Mailly L, Reynolds GM, Ashcroft M, Balfe P, Baumert TF, Roessler S, Wakelam MJO, McKeating JA. Autotaxin-lysophosphatidic acid receptor signalling regulates hepatitis C virus replication. *J Hepatol* 2017; **66**: 919-929 [PMID: 28126468 DOI: 10.1016/j.jhep.2017.01.009]

191 **Nakagawa H**, Ikeda H, Nakamura K, Ohkawa R, Masuzaki R, Tateishi R, Yoshida H, Watanabe N, Tejima K, Kume Y, Iwai T, Suzuki A, Tomiya T, Inoue Y, Nishikawa T, Ohtomo N, Tanoue Y, Omata M, Igarashi K, Aoki J, Koike K, Yatomi Y. Autotaxin as a novel serum marker of liver fibrosis. *Clin Chim Acta* 2011; **412**: 1201-1206 [PMID: 21419756 DOI: 10.1016/j.cca.2011.03.014]

192 **Enooku K**, Uranbileg B, Ikeda H, Kurano M, Sato M, Kudo H, Maki H, Koike K, Hasegawa K, Kokudo N, Yatomi Y. Higher LPA2 and LPA6 mRNA Levels in Hepatocellular Carcinoma Are Associated with Poorer Differentiation, Microvascular Invasion and Earlier Recurrence with Higher Serum Autotaxin Levels. *PLoS One* 2016; **11**: e0161825 [PMID: 27583415 DOI: 10.1371/journal.pone.0161825]

193 **Rachakonda VP**, Reeves VL, Aljammal J, Wills RC, Trybula JS, DeLany JP, Kienesberger PC, Kershaw EE. Serum autotaxin is independently associated with hepatic steatosis in women with severe obesity. *Obesity* (Silver Spring) 2015; **23**: 965-972 [PMID: 25865747 DOI: 10.1002/oby.20960]

194 **Pleli T**, Martin D, Kronenberger B, Brunner F, Köberle V, Grammatikos G, Farnik H, Martinez Y, Finkelmeier F, Labocha S, Ferreirós N, Zeuzem S, Piiper A, Waidmann O. Serum autotaxin is a parameter for the severity of liver cirrhosis and overall survival in patients with liver cirrhosis--a prospective cohort study. *PLoS One* 2014; **9**: e103532 [PMID: 25062038 DOI: 10.1371/journal.pone.0103532]

195 **Watanabe N**, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Tomiya T, Tejima K, Nishikawa T, Arai M, Yanase M, Aoki J, Arai H, Omata M, Fujiwara K, Yatomi Y. Plasma lysophosphatidic acid level and serum autotaxin activity are increased in liver injury in rats in relation to its severity. *Life Sci* 2007; **81**: 1009-1015 [PMID:

17850827 DOI: 10.1016/j.lfs.2007.08.013]
196 **Ikeda H**, Yatomi Y, Yanase M, Satoh H, Nishihara A, Kawabata M, Fujiwara K. Effects of lysophosphatidic acid on proliferation of stellate cells and hepatocytes in culture. *Biochem Biophys Res Commun* 1998; **248**: 436-440 [PMID: 9675156]
197 **Yanase M**, Ikeda H, Matsui A, Maekawa H, Noiri E, Tomiya T, Arai M, Yano T, Shibata M, Ikebe M, Fujiwara K, Rojkind M, Ogata I. Lysophosphatidic acid enhances collagen gel contraction by hepatic stellate cells: association with rho-kinase. *Biochem Biophys Res Commun* 2000; **277**: 72-78 [PMID: 11027642 DOI: 10.1006/bbrc.2000.3634]
198 **Yanase M**, Ikeda H, Ogata I, Matsui A, Noiri E, Tomiya T, Arai M, Inoue Y, Tejima K, Nagashima K, Nishikawa T, Shibata M, Ikebe M, Rojkind M, Fujiwara K. Functional diversity between Rho-kinase- and MLCK-mediated cytoskeletal actions in a myofibroblast-like hepatic stellate cell line. *Biochem Biophys Res Commun* 2003; **305**: 223-228 [PMID: 12745062]
199 **Ikeda H**, Nagashima K, Yanase M, Tomiya T, Arai M, Inoue Y, Tejima K, Nishikawa T, Omata M, Kimura S, Fujiwara K. Involvement of Rho/Rho kinase pathway in regulation of apoptosis in rat hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G880-G886 [PMID: 12829436]
200 **Gobeil F Jr**, Zhu T, Brault S, Geha A, Vazquez-Tello A, Fortier A, Barbaz D, Checchin D, Hou X, Nader M, Bkaily G, Gratton JP, Heveker N, Ribeiro-da-Silva A, Peri K, Bard H, Chorvatova A, D' Orléans-Juste P, Goetzl EJ, Chemtob S. Nitric oxide signaling via nuclearized endothelial nitric-oxide synthase modulates expression of the immediate early genes iNOS and mPGES-1. *J Biol Chem* 2006; **281**: 16058-16067 [PMID: 16574649 DOI: 10.1074/jbc.M602219200]
201 **Tigyi G**, Parrill AL. Molecular mechanisms of lysophosphatidic acid action. *Prog Lipid Res* 2003; **42**: 498-526 [PMID: 14559069]

P- Reviewer: Bourgoin SG, Tajiri K, Yun CC **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4152-4163

DOI: 10.3748/wjg.v24.i36.4152

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

MINIREVIEWS

Ten years of sorafenib in hepatocellular carcinoma: Are there any predictive and/or prognostic markers?

Giorgia Marisi, Alessandro Cucchetti, Paola Ulivi, Matteo Canale, Giuseppe Cabibbo, Leonardo Solaini, Francesco G Foschi, Serena De Matteis, Giorgio Ercolani, Martina Valgiusti, Giovanni L Frassineti, Mario Scartozzi, Andrea Casadei Gardini

Giorgia Marisi, Paola Ulivi, Matteo Canale, Serena De Matteis, Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola 47014, Italy

Alessandro Cucchetti, Leonardo Solaini, Giorgio Ercolani, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, Bologna 40126, Italy

Alessandro Cucchetti, Leonardo Solaini, Giorgio Ercolani, General and Oncologic Surgery, Morgagni-Pierantoni Hospital, Forlì 47121, Italy

Giuseppe Cabibbo, Section of Gastroenterology, DI.BI.M.I.S., University of Palermo, Palermo 35628, Italy

Francesco G Foschi, Department of Internal Medicine, Degli Infermi Hospital, Faenza 48018, Italy

Martina Valgiusti, Giovanni L Frassineti, Andrea Casadei Gardini, Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRCCS, Meldola 47014, Italy

Mario Scartozzi, Department of Medical Oncology, University of Cagliari, Cagliari 45698, Italy

ORCID number: Giorgia Marisi (0000-0003-4012-0042); Alessandro Cucchetti (0000-0001-5269-1964); Paola Ulivi (0000-0002-4334-9662); Matteo Canale (0000-0002-9768-7149); Giuseppe Cabibbo (0000-0002-0946-3859); Leonardo Solaini (0000-0002-5031-9285); Francesco G Foschi (0000-0002-8216-0918); Serena De Matteis (0000-0001-9219-4081); Giorgio Ercolani (0000-0003-4334-5167); Martina Valgiusti (0000-0002-9843-3538); Giovanni L Frassineti (0000-0001-9376-0057); Mario Scartozzi (0000-0001-5977-5546); Andrea Casadei Gardini (0000-0001-6289-7202).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of final version.

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors contributed their efforts in this manuscript.

Manuscript source: Invited manuscript

Correspondence to: Alessandro Cucchetti, MD, PhD, Adjunct Professor, Surgeon, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, Via Albertoni 15, Granarolo Dell'Emilia, Bologna 40126, Italy. aleqko@libero.it
Telephone: +39-543-731111
Fax: +39-543-739123

Received: July 3, 2018
Peer-review started: July 3, 2018
First decision: July 17, 2018
Revised: August 6, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 28, 2018

Abstract

Sorafenib has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma (HCC) since 2007 and numerous studies

have investigated the role of markers involved in the angiogenesis process at both the expression and genetic level and clinical aspect. What results have ten years of research produced? Several clinical and biological markers are associated with prognosis. The most interesting clinical parameters are adverse events, Barcelona Clinic Liver Cancer stage, and macroscopic vascular invasion, while several single nucleotide polymorphisms and plasma angiopoietin-2 levels represent the most promising biological biomarkers. A recent pooled analysis of two phase III randomized trials showed that the neutrophil-to-lymphocyte ratio, etiology and extra-hepatic spread are predictive factors of response to sorafenib, but did not identify any predictive biological markers. After 10 years of research into sorafenib there are still no validated prognostic or predictive factors of response to the drug in HCC. The aim of the present review was to summarize 10 years of research into sorafenib, looking in particular at the potential of associated clinical and biological markers to predict its efficacy in patients with advanced HCC.

Key words: Biomarker; Angiopoietin; Neutrophil-to-lymphocyte ratio; Polymorphisms; Sorafenib; MicroRNA; Adverse events; Hepatocellular carcinoma; Vascular endothelial growth factor

Core tip: Sorafenib has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma, but after 10 years of research into sorafenib response or resistance, there are still no validated prognostic or predictive factors of response.

Marisi G, Cucchetti A, Ulivi P, Canale M, Cabibbo G, Solaini L, Foschi FG, De Matteis S, Ercolani G, Valgiusti M, Frassineti GL, Scartozzi M, Casadei Gardini A. Ten years of sorafenib in hepatocellular carcinoma: Are there any predictive and/or prognostic markers? *World J Gastroenterol* 2018; 24(36): 4152-4163 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4152.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4152

INTRODUCTION

Sorafenib, an oral multikinase inhibitor, has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma (HCC) since 2007[1]. It works by inhibiting the activity of several tyrosine kinases involved in tumor angiogenesis and progression, including vascular endothelial growth factor receptor (VEGFR-2/3), platelet-derived growth factor receptor (PDGF-R), Flt3 and c-Kit, and also targets Raf kinases involved in the MAPK/ERK pathway[2] (Figure 1). The molecular mechanisms by which sorafenib exerts its activity have still not been fully elucidated, and both Raf/MEK/ERK-dependent and -independent mechanisms have been observed[3].

Sorafenib is expensive and associated with adverse events (AEs). Furthermore, a proportion of treated patients show no response to the drug. It would thus be useful to have predictive markers capable of identifying those who are more likely to benefit from therapy. The availability of more accurate predictive or prognostic factors would also help to spare potentially resistant patients from unnecessary toxicity.

Ten years have passed since sorafenib was first commercialized and about 2800 studies have been published on the kinase inhibitor. But how many associated prognostic and/or predictive markers have been identified? Numerous studies have focused on the role of markers involved in the angiogenesis process at both the expression and genetic levels. The largest biomarker study conducted to date is the SHARP trial[4], which included an adequate number of participants and a placebo-controlled group. Smaller single-arm studies exploring predictive or prognostic markers for sorafenib have also been conducted, but the results of these have yet to be validated.

The aim of the present review was to summarize 10 years of research into sorafenib, looking in particular at the potential of associated clinical and biological markers to predict its efficacy in patients with advanced HCC (Tables 1 and 2).

CLINICAL PARAMETERS

Alpha-fetoprotein

Alpha-fetoprotein (AFP) is secreted by about 50% of all HCCs and is the main serological marker used for the diagnosis of the tumor[5]. The SHARP trial[4] showed that high baseline AFP plasma levels (> 200 ng/mL) had a negative impact on overall survival (OS), a finding recently confirmed in a pooled analysis of the SHARP trial and the Asia Pacific trial by Bruix *et al*[6]. High baseline serum AFP levels (≥ 400 ng/mL) also appear to be associated with shorter time-to-progression (TTP). Notably, in an analysis of six prospective phase II trials evaluating systemic therapies for patients with advanced HCC, no association was observed between baseline AFP levels and prognosis[7].

Several studies[8-10] have highlighted a consistent correlation between an early decrease of > 20% in AFP levels following sorafenib and objective response and better outcome in advanced HCC patients. Shao *et al*[8] evaluated for the first time this aspect and they observed that patients with early AFP response had an improved progression-free survival (PFS) (7.5 mo *vs* 1.9 mo) and OS (15.3 mo *vs* 4.1 mo). This data was confirmed by Personeni *et al*[10] a few years later. They reported that early responders had a significantly better median OS and TTP than non-responders (13.8 mo *vs* 8.2 mo, P = 0.022 and 7.9 mo *vs* 2.4 mo, P = 0.004;

Table 1 Predictive and/or prognostic value of clinical markers in hepatocellular carcinoma patients

Clinical markers	Predictive value	Prognostic value	Ref.
Alpha-fetoprotein	No	Yes	[6]
Adverse events			
Hand-foot skin reaction	No	Yes	[13]
Hypertension	No	Uncertain	[16,19,20]
Diarrhea	No	Yes	[21]
Child-Pugh A *vs* B	No	Yes	[27-29]
Macroscopic vascular invasion	No	Yes	[6]
BCLC B *vs* C	No	Yes	[6,29,32]
Starting dose and dose reduction	No	Yes	[29,32]
Etiology HCV *vs* HBV	Yes	Yes	[6]
Chronic treatment with metformin	No	Yes	[35,36]
Neutrophil-to-lymphocyte ratio	Yes	Yes	[6,41,44]
Extra hepatic spread	Yes	Yes	[6]

HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

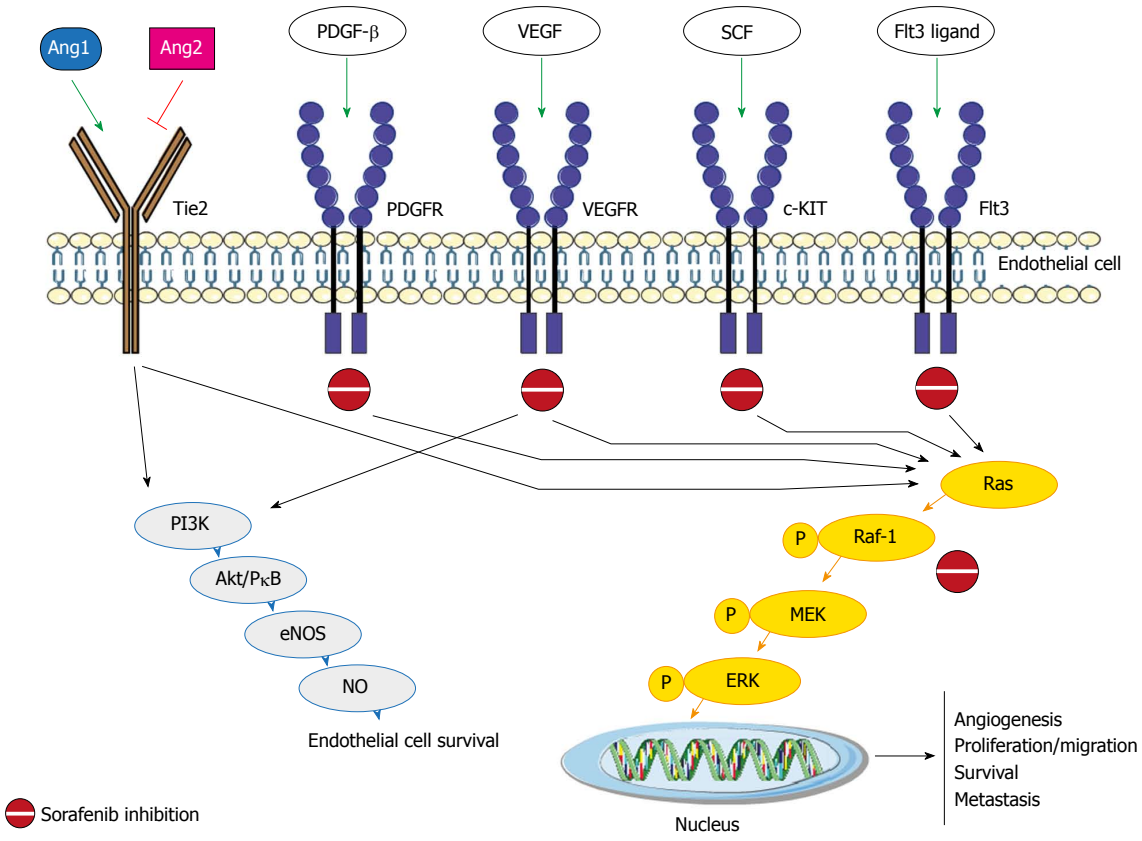


Figure 1 Sorafenib pathaway and the main molecular factors. Ang: Angiopoietin; Tie2: Tyrosine-protein kinase receptor; PDGFR: Platelet-derived growth factor receptors; VEGFR: Vascular endothelial growth factor receptor; SCF: Stem cell factor; PI3K: PhosphatidylInositol 3-Kinase; Akt/PKB: Protein-chinasi B; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; P: Phospho-; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal–regulated kinase.

respectively). Conversely, Nakazawa *et al*[11] did not observe such an association.

Adverse events

The main AEs of Sorafenib are hand-foot skin reaction (HFSR), hypertension and diarrhea. Several papers have highlighted a consistent correlation between AEs and survival in patients treated with Sorafenib.

Vincenzi *et al*[12] evaluated for the first time the correlation between HSFR and outcome. They showed, in a small series of patients treated with sorafenib, that patients with HSFR had a significantly higher disease control rate with respect to patients without HSFR. This data was confirmed in a prospective study of 147 patients by Reig *et al*[13]. They reported different OS when patients were subdivided according to the presence or not of skin toxicity during the first 60 d of treatment (18.2 mo *vs* 10.1 mo, respectively)[13] . A

Table 2 Predictive and/or prognostic value of biological markers in hepatocellular carcinoma patients

Biological markers	Predictive value	Prognostic value	Ref.
Serum and plasma proteins			
VEGF-A	No	Uncertain	[4,57]
Ang-2	No	Yes	[4]
IGF-1	No	No	[55]
Single nucleotide polymorphisms			
VEGF-A rs2010963	No	Yes	[65]
VEGF-C rs4604006	No	Yes	[65]
eNOS (*eNOS*-786/*eNOS* VNTR)	No	Yes	[66]
Ang-2 rs55633437	No	Yes	[67]
HIF-1 alpha rs12434438	No	Yes	[68]
Amplifications			
VEGF	No	Uncertain	[70]
FGF3/FGF4	No	Uncertain	[71]
miRNAs			
miR-425-3p	No	Yes	[74]
miR-224	No	Yes	[75]
miR-181a-5p	No	Yes	[77]
miR-339-5p	No	Yes	[77]
miR-423-5p	No	Yes	[78]
miR-10b-3p	No	Yes	[79]
miR-221	No	Uncertain	[76]
Tissue biomarker expression			
Phospho-ERK	Uncertain	Uncertain	[81,82]
PDGFR-b	No	Yes	[84]
c-Met	No	No	[84]
VEGFR	No	No	[84]
p-c-Jun	No	Yes	[85]

Ang-2: Angiopoietin-2; IGF-1: Insulin-like growth factor-1; VEGF-A: Vascular endothelial growth factor A; HIF-1: Hypoxia-inducible factor 1; FGF: Fibroblast growth factor; miRNAs: MicroRNAs; eNOS: Endothelial nitric oxide synthase; PDGFR: Platelet-derived growth factor receptors; VEGFR: Vascular endothelial growth factor receptor; ERK: Extracellular signal–regulated kinase.

recently meta-analysis confirmed that HSFR was a good indicator of outcome for OS and TTP in HCC patients receiving sorafenib[14]

Hypertension (HTN) is frequently associated with the use of angiogenesis inhibitors[15]. Casadei Gardini *et al*[16] showed that early HTN (15 d after the start of treatment) rather than later onset HTN *vs* patients without HTN was associated with better PFS (6.0 mo *vs* 2.5 mo; $P < 0.001$) and OS (14.6 mo *vs* 3.9 mo; $P = 0.003$). This finding has been confirmed in some studies[17,18] but not in others[19,20].

Bettinger *et al*[21] reported for the first time that diarrhea was an independent positive prognostic factor (HR = 0.41; $P = 0.001$) in 112 patients with advanced HCC, a finding also confirmed by Koschny *et al*[22].

Finally, other authors showed that the number of AEs was associated with predict survival in patients treated with sorafenib. In particular, Di Costanzo *et al*[23] evaluated the potential of pretreatment clinical variables to predict survival. Three groups of patients were taken into account: patients without AEs (group 0), patients with one AE (group 1) and patients with two to three AEs (group 2). The study reported a strong correlation between this classification and disease progression at 3 mo (41.9%, 25.9% and 12.7% of patients in groups 0, 1 and 2, respectively; $P = 0.014$). These data were subsequently confirmed in the validation cohort[24]. A recent meta-analysis by Abdel-Rahman *et al*[25] revealed an association between specific side-effects (hypertension, HFSR and diarrhea) and patient outcome (HR = 0.38; 95%CI: 0.30-0.48; $P < 0.00001$).

Stage, liver functionality and etiology

Child-Pugh A *vs* Child-Pugh B: In the SHARP trial[4] and the Asia Pacific trial[26], more than 95% of patients were classified as having Child-Pugh A cirrhosis, thus preventing the investigation of the potential benefits of sorafenib in Child-Pugh B patients.

Hollebecque *et al*[27] reported for the first time the results from a prospective study on sorafenib efficacy in 120 advanced HCC patients, 20 of whom Child-Pugh B cirrhosis. OS was 11.1 mo, with a significantly longer median survival in Child-Pugh A patients than Child-Pugh B patients (13 mo *vs* 4.5 mo, $P = 0.0008$). A few years later, Pressiani *et al*[28] studied clinical outcome in a population of 300 consecutive patients; PFS in the Child-Pugh A group was 4.3 mo *vs* 2.1 mo in the Child-Pugh B arm (HR = 3.23; 95%CI: 2.38-4.39; $P < 0.001$), TTP was 4.2 mo *vs* 3.8 mo and OS was 10.0 mo *vs* 3.8 mo, respectively ($P < 0.001$).

The most important work on the use of sorafenib in Child-Pugh subgroups was the GIDEON study published in 2016[29]. This study observed that median OS was significantly longer in patients with Child-Pugh A (13.6 mo) than in those with Child Pugh B (5.2 mo) or Child-Pugh C (2.6 mo).

Macroscopic vascular invasion: It is widely acknowledged that the presence of macroscopic vascular invasion leads to a poorer prognosis[26,30]. The meta-analysis by Peng *et al*[31] confirmed its prognostic value and the pooled analysis by Bruix *et al*[6] affirmed the importance of macroscopic vascular invasion as a predictor of survival but not of response to treatment.

BCLC stage: In the SHARP trial, patients with Barcelona Clinic Liver Cancer (BCLC) B had a median OS of 14.5 mo compared to 9.7 mo for those with BCLC C[4]. Later, SOFIA[32] and GIDEON study[29] confirmed this data. In the SOFIA trial[32] the OS was 8.4 mo in BCLC C *vs* 20.6 mo in BCLC B patients ($P < 0.0001$), but the time to radiologic progression did not differ significantly between the 2 groups. In the GIDEON study[29], median OS according to BCLC by Child-Pugh cross-classification followed a similar trend, *i.e.* patients with Child-Pugh A and BCLC stage B showed longer OS than those with Child-Pugh B and BCLC B (19.5 mo *vs* 10.0 mo); and patients with Child-Pugh A and BCLC stage C had longer OS than those with Child-Pugh B and BCLC stage C (11.2 mo *vs* 3.8 mo).

Recently, Bruix *et al*[6]'s pooled analysis confirmed that BCLC C patients had a poorer prognosis than those with BCLC B HCC (HR = 1.59; $P = 0.02$).

Sorafenib starting dose and dose escalation/reduction: The two most important studies that evaluated sorafenib starting dose and dose escalation/reduction are SOFIA[32] and GIDEON trial[29].

In the SOFIA trial[32] sorafenib was down-dosed in 161 (54%) patients because of AEs (133 patients, 83%) and a reduction in liver function (28 patients, 17%). Median OS of the 77 patients receiving a half-dose of sorafenib for 70% of the treatment period was 21.6 mo (95%CI: 13.6-29.6) compared with 9.6 mo (95%CI: 6.9-12.3) for the remaining 219 patients who had a dose reduction for < 70% of the treatment period or who maintained the full dosage.

A sub-analysis of the GIDEON study[29] evaluated the starting dose of sorafenib with respect to clinical outcome and toxicity. Patients starting on 400 mg/d were slightly older, had baseline characteristics indicative of greater disease progression and had a higher incidence of AEs than those with a starting dose of 800 mg/d (96% *vs* 88%). Treatment duration (18.0 wk *vs* 13.0 wk) and median OS (12.1 mo *vs* 9.4 mo) were longer in patients receiving 800 mg/d.

Etiology: In the subgroup analysis of the SHARP study[4], the HR for OS was 0.76 in HBV-positive patients (95%CI: 0.38-1.50, P = not significant) and 0.50 (95%CI: 0.32-0.77) in HCV-positive patients. Results were similar for TTP (HR = 1.03 and 0.43 for HBV-positive and HCV-positive patients, respectively). Similar data were obtained for HBV-positive HCC patients in the phase III randomized Asia Pacific trial, *i.e.* the HR for OS was 0.74 (95%CI: 0.51-1.06, not significant) with respect to patients with the other etiology, for which the HR was 0.57 (95%CI: 0.29-1.33)[26]. Bruix *et al*[6]'s pooled analysis of the SHARP/Asia Pacific trial results showed that the absence of HCV was a potential prognostic factor for poorer OS (HR = 0.7, $P = 0.02$). The same authors revealed that HBV-positive patients did not show a significant difference in treatment response with respect to their HBV-negative counterparts (HR = 0.78; 95%CI: 0.57-1.06) and OS (HR = 1.128, $P = 0.4538$). We believe that the 2 etiologic groups respond differently to sorafenib and that further investigation is warranted in specific studies[33].

Metformin treatment: Type 2 diabetes is a significant risk factor for the development of malignancies, including HCC[34]. Casadei Gardini *et al*[35] published findings of reduced sorafenib efficacy in HCC patients treated chronically with or without metformin for type II diabetes mellitus (PFS 2.6 mo *vs* 5.0 mo, respectively; and OS 10.4 mo *vs* 15.1 mo, respectively). The same authors validated these data in a series of more than 250 cases[36], also highlighting a possible role of sirtuin-3 in resistance to sorafenib[37]. Di Costanzo *et al*[38] recently reported an increase in TTP and OS in diabetic with respect to non-diabetic HCC patients. However, no distinction was made between the different hypoglycemic therapies administered.

Immune inflammation indicators

Systemic inflammatory responses have been shown to reflect the promotion of angiogenesis, DNA damage and tumor invasion through an upregulation of cytokines[39]. Previous research revealed that lymphocytes play a crucial role in tumor defense by inducing cytotoxic cell death and inhibiting tumor cell proliferation and migration[40]. Consequently, several inflammation and immune-based prognostic scores, such as lymphocyte count, neutrophil-lymphocyte ratio (NLR), and systemic immune-inflammation index (SII), have been developed to predict survival and recurrence in cancers, including HCC. Casadei Gardini *et al*[41] evaluated for the first time SII, NLR and platelet-lymphocyte ratio (PLR) in a small case series, observing that SII were independent prognostic factors for OS. Other studies showed that NLR was a significant independent risk factor for shorter survival[42,43]. NLR was also found to be an independent prognostic factor for both response and survival in Bruix *et al*[6]'s pooled analysis and Lue *et al*[44]'s retrospective

study on Spanish patients.

IMAGING EXAMINATIONS

The response to sorafenib does not correlate with a change in lesion dimension, but it is more correlate with intralesional vascularization. For this reason, the RECIST criteria[45,46] usually used for tumor response evaluation is inappropriate to evaluate the response to sorafenib in patients with advanced HCC. The modified RECIST (mRECIST) appear more indicate for evalutation the response. They include vascularization and tumor arterial enhancement changes of the target lesion on computed tomographic (CT). Several studies have demonstrated the superiority of the mRECIST criteria with respect to the RECIST criteria in assessing the response to treatment with sorafenib[47]. Various functional imaging tools were proposed to evaluate the antiangiogenic effects, but none of these has entered normal clinical practice[48-52]. Finally, a recently study showed that texture features on pretreatment contrast material-enhanced CT images can help predict OS and TTP in these patients[53] .

BIOLOGICAL PARAMETERS

Serum and plasma proteins

Although plasma biomarkers are the best candidates for evaluating sorafenib efficacy, only the SHARP trial produced results with borderline significance[4]. Baseline angiopoietin-2 (Ang-2) and vascular endothelial growth factor-A (VEGF-A) plasma levels independently predicted survival in both the entire patient population and the placebo cohort. Conversely, none of the tested biomarkers significantly predicted response to sorafenib[4]. Insulin-like growth factor (IGF)-1 levels have been found to decrease in patients with cirrhosis of the liver or HCC[54], and high pretreatment levels of IGF-1 predict better PFS and OS in advanced HCC patients receiving first-line antiangiogenic therapy[55].

The role of serum cytokines as biomarkers for the prediction of sorafenib responses is interesting, in particular Kim *et al*[56] developed a new prediction model for sorafenib response that combines relevant serum markers, tumor related factors, and cirrhosis-related factors in a scoring system.

VEGF-A: Llovet *et al*[4] showed that, although baseline plasma VEGF-A concentrations did not exhibit a predictive value, low plasma VEGF-A was associated with improved prognosis (HR = 1.48, 95%CI: 1.08-2.03, P = 0.015). However, other authors did not find any association between VEGF-A and prognosis in patients treated with sorafenib[57]. Tsuchiya *et al*[58]'s analysis of plasma VEGF concentrations during sorafenib treatment revealed that a decrease in the protein 8 wk after the start of therapy predicted better overall survival in advanced HCC patients (30.9 mo *vs* 14.4 mo; P = 0.038).

Ang-2: In the presence of VEGF, Ang-2 destabilizes blood vessels, promotes vascular sprouting, and is associated with an invasive and metastatic cancer phenotype[59]. Llovet *et al*[4] demonstrated that high baseline Ang-2 levels were correlated with more aggressive disease (HR = 1.58, 95%CI: 1.20-2.07, P = 0.001). Moreover, levels of the protein increased during treatment in the placebo group, suggestive of poor outcome related to disease progression in this cohort, whereas they remained constant during treatment with sorafenib, reflecting the generally more favorable outcome of this group. Overall increased Ang-2 expression levels were associated with poorer outcome in both groups, suggesting that this marker could be useful in monitoring treatment response. In agreement with Llovet's study, Miyahara *et al*[57] reported that high baseline Ang-2 serum levels were associated with poor outcome in advanced HCC patients receiving sorafenib (HR = 2.51, 95%CI: 1.01-6.57, P = 0.048). Although these results indicate the potential prognostic value of Ang-2 in HCC, its role in predicting response to sorafenib remains to be verified.

IGF-1: Shao *et al*[55] found that high-pretreatment serum levels of IGF-1 were associated with a better DCR and improved PFS and OS in patients undergoing antiangiogenic therapy. Although the study did not have a control arm, the substantial significant difference in DCR between patients with high and low levels of IGF-1 (71% *vs* 39%) denotes the potential usefulness of IGF-1 as a predictive biomarker of response to antiangiogenic therapy.

Multiple-factor analyses: By using baseline serum basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) levels as covariates together etiology (B-viral), platelet count, BCLC stage and protein induced by vitamin K absence-II, Kim *et al*[56] reported that a total score of < 6 could be a relevant cutoff value for selecting patients who are most likely to benefit from sorafenib therapy. Moreover, Hayashi *et al*[60] found that serum interleukin (IL)-5, IL-8, CXCL9, PDGF-BB, TGF-α, and VEGF-A were elevated in the long survivors group among HCC patients who received sorafenib, potentially reflecting the activation of stromal signaling in the tumor microenvironment.

Genetic markers

Molecular and genomic analyses from tumor and non-tumor tissue have proven useful in evaluating prognosis and could open up new avenues for tailoring treatment[61]. Genetic alterations, such as single nucleotide polymorphisms (SNPs) in genes encoding for proteins involved in the angiogenic process,

have been studied as potential biomarkers for anti-angiogenic therapy. SNP evaluation would seem to be more advantageous than protein or gene expression analyses as it can be performed at any time during the course of the disease, is not substantially influenced by laboratory biases, and is relatively inexpensive. Some authors have focused on molecular profiling in formalin-fixed paraffin-embedded (FFPE) samples, comparing the mutation profiles of HCC biopsy samples and the response to sorafenib treatment[62]. Gene amplification, gene mutations and expression profiling of tumors have now become a research priority and are expected to lead to personalized treatment for HCC patients[63,64].

SNPs: Specific SNPs in *VEGF* and *VEGFR* genes have been found to be correlated with PFS and OS in HCC patients treated with sorafenib. In multivariate analysis, *VEGF-A* rs2010963 and *VEGF-C* rs4604006 were found to be independent factors influencing outcome in terms of PFS (HR = 0.25, 95%CI: 0.19-1.02, *P* = 0.0376 and HR = 0.22, 95%CI: 0.14-0.81, *P* = 0.004, respectively) and OS (HR = 0.28, 95%CI: 0.23-0.96, *P* = 0.02 and HR = 0.25, 95%CI: 0.17-0.99, *P* = 0.04, respectively)[65]. In the Italian multicenter, retrospective ePHAS [endothelial nitric oxide synthase (*eNOS*) polymorphisms in HCC and sorafenib] study, *eNOS* polymorphisms were analyzed in relation to PFS and OS. In univariate analysis, training cohort patients homozygous for *eNOS* haplotype (HT1:T-4b at *eNOS*-786/*eNOS* VNTR) showed a lower median PFS (2.6 mo *vs* 5.8 mo, HR = 5.43, 95%CI: 2.46-11.98, *P* < 0.0001) and OS (3.2 mo *vs* 14.6 mo, HR = 2.35, 95%CI: 1.12-4.91, *P* = 0.024) than those with other haplotypes. These results were confirmed in a validation set and multivariate analysis further substantiated this haplotype as the only independent prognostic factor[66]. More recently, evidence emerged that patients homozygous for *ANGPT2* (Ang2 gene) rs55633437 GG genotype showed significantly longer PFS (*P* < 0.001) and OS (*P* < 0.001) than those with the other genotypes (GT+TT)[67].

In the ALICE-2 study, Faloppi *et al*[68] investigated the role of hypoxia-inducible factor 1-alpha (*HIF-1*α) SNPs, confirming the results of the ALICE-1 study[65]. In multivariate analysis, rs12434438 of *HIF-1*α, rs2010963 of *VEGF-A* and rs4604006 of *VEGF-C* were confirmed as independent factors and may help to identify patients who are more likely to respond to sorafenib[68]. The prospective INNOVATE study is ongoing to validate the role of *VEGF*, *eNOS, Ang-2* and *HIF-1*α SNPs in relation to clinical outcome in advanced HCC patients treated with sorafenib (NCT02786342)[69].

Gene amplification, gene mutations and RNA expression: A relation between *VEGF-A* gene amplification and response to sorafenib was observed in a study performed on a mouse model of HCC[70]. The authors found that HCC patients with tumor *VEGF-A* amplification showed markedly better survival than those with non-amplified tumors, highlighting that *VEGFA* amplification is a potential biomarker of response to *VEGF-A*-blocking drugs in HCC[70]. Arao *et al*[71] observed that FGF3/FGF4 amplification and multiple lung metastases were frequently observed in responders to sorafenib, although the sample size was relatively small.

Sakai *et al*[62] used targeted DNA and RNA sequencing in FFPE specimens from fine-needle biopsy to identify candidate biomarkers of response to sorafenib in 46 HCC patients. A significant difference was observed in the number of oncogene mutations between progressing and non-progressing patients (*P* = 0.045), suggesting that tumor mutational burden may be predictive of sorafenib effectiveness. Tumor gene expression of NRG1, TGFa, and PECAM1 would also seem to be a marker of treatment response and PFS.

MicroRNAs

MicroRNAs (miRNAs) affect drug response directly or indirectly by regulating the expression of genes involved in drug transportation, metabolism, and downstream signaling pathways. The deregulation of various miRNAs has been reported in *in vitro*, *in vivo* and population studies[72,73], confirming its correlation with response to sorafenib. Some authors have evaluated the predictive role of miRNA expression in HCC tissue[74,75], while others have studied circulating miRNA levels prior to sorafenib treatment[76]. To date, the most interesting tissue miRNAs are miR-425-3p[74] and miR-224[75]. High levels of miR-425-3p have been associated with longer TTP and PFS (HR = 0.4, 95%CI: 0.2-0.7, *P* = 0.0008 and HR = 0.5, 95%CI: 0.3-0.9, *P* = 0.007, respectively), and elevated miR-224 expression have been correlated with increased PFS and OS (HR = 0.28, 95%CI: 0.09-0.92, *P* = 0.029 and HR = 0.0.24, 95%CI: 0.07-0.79, *P* = 0.012, respectively). Circulating miRNAs have also been studied in the serum of HCC patients to predict early response to sorafenib treatment, with miR-181a-5p and miR-339-5p associated with partial response and disease progression[77], miR-423-5p with stable disease or partial response[78] and miR-10b-3p with shorter survival[79].

Another potentially interesting circulating miRNA is miR-221, which was studied by Fornari *et al*[76] in both animal models and in a patient population. Patients with radiologic disease progression after 2-mo treatment had higher pretreatment miR-221 levels than responders (*P* = 0.007).

Larger confirmatory studies are needed before miRNAs can be considered valid biomarkers for clinical practice.

Tissue biomarker expression

A number of studies have analyzed tissue biomarkers

that may be very specific to the disease of interest[80]. In a phase II study of sorafenib in advanced HCC, Abou-Alfa *et al*[81] showed that patients whose tumors expressed higher baseline phospho-ERK levels had a longer TTP. However, other studies reported conflicting results[82,83]. With regard to the expression of angiogenic markers in tumor tissue, it has been observed that high platelet-derived growth factor receptor beta expression is correlated with poor OS but not with PFS in HCC patients receiving sorafenib. High expression of the proto-oncogene c-Met may predict the therapeutic effectiveness of sorafenib in HCC patients, but no differences in terms of outcome have been seen with respect to VEGFR-2 expression[84]. Hagiwara *et al*[85] studied another interesting tissue biomarker, phospho-c-Jun, reporting a significantly higher expression ($P < 0.001$) in non-responding compared to responding patients treated with sorafenib.

CONCLUSION

After 10 years of research into sorafenib, there are still no validated prognostic or predictive markers of response to sorafenib in hepatocellular carcinoma. Furthermore, the main results obtained to date come from 2 important randomized trials and from different subanalyses and pooled analyses rather than from normal clinical practice. The fact of there being only one drug for the treatment of these patients has certainly done nothing to stimulate research into identifying and validating predictors of response and prognosis. However, given the recent publication of a positive phase III trial[86] and the ongoing NCT01658878 immunotherapy study, the race is now on to see who will be the first to identify a prognostic and predictive factor for sorafenib and/or new drugs in this setting. In conclusion, the use of metabolomic profiling and whole genome analysis to examine the association between patient outcome and response to sorafenib could become alternative approaches to the search for new biomarkers in HCC.

ACKNOWLEDGMENTS

The authors thank Gráinne Tierney and Cristiano Verna for editorial assistance.

REFERENCES

1 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

2 **Cervello M**, Bachvarov D, Lampiasi N, Cusimano A, Azzolina A, McCubrey JA, Montalto G. Molecular mechanisms of sorafenib action in liver cancer cells. *Cell Cycle* 2012; **11**: 2843-2855 [PMID: 22801548 DOI: 10.4161/cc.21193]

3 **Tai WT**, Cheng AL, Shiau CW, Huang HP, Huang JW, Chen PJ, Chen KF. Signal transducer and activator of transcription 3 is a major kinase-independent target of sorafenib in hepatocellular carcinoma. *J Hepatol* 2011; **55**: 1041-1048 [PMID: 21354226 DOI: 10.1016/j.jhep.2011.01.047]

4 **Llovet JM**, Peña CE, Lathia CD, Shan M, Meinhardt G, Bruix J; SHARP Investigators Study Group. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012; **18**: 2290-2300 [PMID: 22374331 DOI: 10.1158/1078-0432.CCR-11-2175]

5 **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]

6 **Bruix J**, Cheng AL, Meinhardt G, Nakajima K, De Sanctis Y, Llovet J. Prognostic factors and predictors of sorafenib benefit in patients with hepatocellular carcinoma: Analysis of two phase III studies. *J Hepatol* 2017; **67**: 999-1008 [PMID: 28687477 DOI: 10.1016/j.jhep.2017.06.026]

7 **Lin ZZ**, Hsu C, Hu FC, Shao YY, Chang DY, Yang CH, Hong RL, Hsu CH, Cheng AL. Factors impacting prognosis prediction in BCLC stage C and Child-Pugh class A hepatocellular carcinoma patients in prospective clinical trials of systemic therapy. *Oncologist* 2012; **17**: 970-977 [PMID: 22673633 DOI: 10.1634/theoncologist.2011-0411]

8 **Shao YY**, Lin ZZ, Hsu C, Shen YC, Hsu CH, Cheng AL. Early alpha-fetoprotein response predicts treatment efficacy of antiangiogenic systemic therapy in patients with advanced hepatocellular carcinoma. *Cancer* 2010; **116**: 4590-4596 [PMID: 20572033 DOI: 10.1002/cncr.25257]

9 **Yau T**, Yao TJ, Chan P, Wong H, Pang R, Fan ST, Poon RT. The significance of early alpha-fetoprotein level changes in predicting clinical and survival benefits in advanced hepatocellular carcinoma patients receiving sorafenib. *Oncologist* 2011; **16**: 1270-1279 [PMID: 21885876 DOI: 10.1634/theoncologist.2011-0105]

10 **Personeni N**, Bozzarelli S, Pressiani T, Rimassa L, Tronconi MC, Sclafani F, Carnaghi C, Pedicini V, Giordano L, Santoro A. Usefulness of alpha-fetoprotein response in patients treated with sorafenib for advanced hepatocellular carcinoma. *J Hepatol* 2012; **57**: 101-107 [PMID: 22414760 DOI: 10.1016/j.jhep.2012.02.016]

11 **Nakazawa T**, Hidaka H, Takada J, Okuwaki Y, Tanaka Y, Watanabe M, Shibuya A, Minamino T, Kokubu S, Koizumi W. Early increase in α-fetoprotein for predicting unfavorable clinical outcomes in patients with advanced hepatocellular carcinoma treated with sorafenib. *Eur J Gastroenterol Hepatol* 2013; **25**: 683-689 [PMID: 23395995 DOI: 10.1097/MEG.0b013e32835d913b]

12 **Vincenzi B**, Santini D, Russo A, Addeo R, Giuliani F, Montella L, Rizzo S, Venditti O, Frezza AM, Caraglia M, Colucci G, Del Prete S, Tonini G. Early skin toxicity as a predictive factor for tumor control in hepatocellular carcinoma patients treated with sorafenib. *Oncologist* 2010; **15**: 85-92 [PMID: 20051477 DOI: 10.1634/theoncologist.2009-0143]

13 **Reig M**, Torres F, Rodriguez-Lope C, Forner A, LLarch N, Rimola J, Darnell A, Ríos J, Ayuso C, Bruix J. Early dermatologic adverse events predict better outcome in HCC patients treated with sorafenib. *J Hepatol* 2014; **61**: 318-324 [PMID: 24703956 DOI: 10.1016/j.jhep.2014.03.030]

14 **Wang P**, Tan G, Zhu M, Li W, Zhai B, Sun X. Hand-foot skin reaction is a beneficial indicator of sorafenib therapy for patients with hepatocellular carcinoma: a systemic review and meta-analysis. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 1-8 [PMID: 28847184 DOI: 10.1080/17474124.2017.1373018]

15 **Scartozzi M**, Galizia E, Chiorrini S, Giampieri R, Berardi R, Pierantoni C, Cascinu S. Arterial hypertension correlates with clinical outcome in colorectal cancer patients treated with first-line bevacizumab. *Ann Oncol* 2009; **20**: 227-230 [PMID: 18842611 DOI: 10.1093/annonc/mdn637]

16 **Casadei Gardini A**, Scarpi E, Marisi G, Foschi FG, Donati G, Giampalma E, Faloppi L, Scartozzi M, Silvestris N, Bisulli M, Corbelli J, Gardini A, La Barba G, Veneroni L, Tamberi S, Cascinu

S, Frassineti GL. Early onset of hypertension and serum electrolyte changes as potential predictive factors of activity in advanced HCC patients treated with sorafenib: results from a retrospective analysis of the HCC-AVR group. *Oncotarget* 2016; **7**: 15243-15251 [PMID: 26893366 DOI: 10.18632/oncotarget.7444]

17 **Zhong BY**, Ni CF, Chen L, Zhu HD, Teng GJ. Early Sorafenib-related Biomarkers for Combination Treatment with Transarterial Chemoembolization and Sorafenib in Patients with Hepatocellular Carcinoma. *Radiology* 2017; **284**: 583-592 [PMID: 28263701 DOI: 10.1148/radiol.2017161975]

18 **Howell J**, Pinato DJ, Ramaswami R, Bettinger D, Arizumi T, Ferrari C, Yen C, Gibbin A, Burlone ME, Guaschino G, Sellers L, Black J, Pirisi M, Kudo M, Thimme R, Park JW, Sharma R. On-target sorafenib toxicity predicts improved survival in hepatocellular carcinoma: a multi-centre, prospective study. *Aliment Pharmacol Ther* 2017; **45**: 1146-1155 [PMID: 28252185 DOI: 10.1111/apt.13977]

19 **Shin SY**, Lee YJ. Correlation of skin toxicity and hypertension with clinical benefit in advanced hepatocellular carcinoma patients treated with sorafenib. *Int J Clin Pharmacol Ther* 2013; **51**: 837-846 [PMID: 24075093 DOI: 10.5414/CP201907]

20 **Otsuka T**, Eguchi Y, Kawazoe S, Yanagita K, Ario K, Kitahara K, Kawasoe H, Kato H, Mizuta T; Saga Liver Cancer Study Group. Skin toxicities and survival in advanced hepatocellular carcinoma patients treated with sorafenib. *Hepatol Res* 2012; **42**: 879-886 [PMID: 22469363 DOI: 10.1111/j.1872-034X.2012.00991.x]

21 **Bettinger D**, Schultheiss M, Knüppel E, Thimme R, Blum HE, Spangenberg HC. Diarrhea predicts a positive response to sorafenib in patients with advanced hepatocellular carcinoma. *Hepatology* 2012; **56**: 789-790 [PMID: 22307848 DOI: 10.1002/hep.25637]

22 **Koschny R**, Gotthardt D, Koehler C, Jaeger D, Stremmel W, Ganten TM. Diarrhea is a positive outcome predictor for sorafenib treatment of advanced hepatocellular carcinoma. *Oncology*2013; **84**: 6-13 [PMID: 23075905 DOI: 10.1159/000342425]

23 **Di Costanzo GG**, de Stefano G, Tortora R, Farella N, Addario L, Lampasi F, Lanza AG, Cordone G, Imparato M, Caporaso N. Sorafenib off-target effects predict outcomes in patients treated for hepatocellular carcinoma. *Future Oncol* 2015; **11**: 943-951 [PMID: 25760975 DOI: 10.2217/fon.14.291]

24 **Di Costanzo GG**, Casadei Gardini A, Marisi G, Foschi FG, Scartozzi M, Granata R, Faloppi L, Cascinu S, Silvestris N, Brunetti O, Palmieri VO, Ercolani G, Tortora R. Validation of a Simple Scoring System to Predict Sorafenib Effectiveness in Patients with Hepatocellular Carcinoma. *Target Oncol* 2017; **12**: 795-803 [PMID: 28770532 DOI: 10.1007/s11523-017-0522-5]

25 **Abdel-Rahman O**, Lamarca A. Development of sorafenib-related side effects in patients diagnosed with advanced hepatocellular carcinoma treated with sorafenib: a systematic-review and meta-analysis of the impact on survival. *Expert Rev Gastroenterol Hepatol* 2017; **11**: 75-83 [PMID: 27882800 DOI: 10.1080/17474124.2017.1264874]

26 **Cheng AL**, Guan Z, Chen Z, Tsao CJ, Qin S, Kim JS, Yang TS, Tak WY, Pan H, Yu S, Xu J, Fang F, Zou J, Lentini G, Voliotis D, Kang YK. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma according to baseline status: subset analyses of the phase III Sorafenib Asia-Pacific trial. *Eur J Cancer* 2012; **48**: 1452-1465 [PMID: 22240282 DOI: 10.1016/j.ejca.2011.12.006]

27 **Hollebecque A**, Cattan S, Romano O, Sergent G, Mourad A, Louvet A, Dharancy S, Boleslawski E, Truant S, Pruvot FR, Hebbar M, Ernst O, Mathurin P. Safety and efficacy of sorafenib in hepatocellular carcinoma: the impact of the Child-Pugh score. *Aliment Pharmacol Ther* 2011; **34**: 1193-1201 [PMID: 21958438 DOI: 10.1111/j.1365-2036.2011.04860.x]

28 **Pressiani T**, Boni C, Rimassa L, Labianca R, Fagiuoli S, Salvagni S, Ferrari D, Cortesi E, Porta C, Mucciarini C, Latini L, Carnaghi C, Banzi M, Fanello S, De Giorgio M, Lutman FR, Torzilli G, Tommasini MA, Ceriani R, Covini G, Tronconi MC, Giordano L, Locopo N, Naimo S, Santoro A. Sorafenib in patients with Child-Pugh class A and B advanced hepatocellular carcinoma: a prospective feasibility analysis. *Ann Oncol* 2013; **24**: 406-411 [PMID: 23041587 DOI: 10.1093/annonc/mds343]

29 **Marrero JA**, Kudo M, Venook AP, Ye SL, Bronowicki JP, Chen XP, Dagher L, Furuse J, Geschwind JH, de Guevara LL, Papandreou C, Takayama T, Sanyal AJ, Yoon SK, Nakajima K, Lehr R, Heldner S, Lencioni R. Observational registry of sorafenib use in clinical practice across Child-Pugh subgroups: The GIDEON study. *J Hepatol* 2016; **65**: 1140-1147 [PMID: 27469901 DOI: 10.1016/j.jhep.2016.07.020]

30 **Lee S**, Kang JH, Kim DY, Ahn SH, Park JY, Kim BK, Kim SU, Han KH. Prognostic factors of sorafenib therapy in hepatocellular carcinoma patients with failure of transarterial chemoembolization. *Hepatol Int* 2017; **11**: 292-299 [PMID: 28324324 DOI: 10.1007/s12072-017-9792-3]

31 **Peng S**, Zhao Y, Xu F, Jia C, Xu Y, Dai C. An updated meta-analysis of randomized controlled trials assessing the effect of sorafenib in advanced hepatocellular carcinoma. *PLoS One*2014; **9**: e112530 [PMID: 25460347 DOI: 10.1371/journal.pone.0112530]

32 **Iavarone M**, Cabibbo G, Piscaglia F, Zavaglia C, Grieco A, Villa E, Cammà C, Colombo M; SOFIA (SOraFenib Italian Assessment) study group. Field-practice study of sorafenib therapy for hepatocellular carcinoma: a prospective multicenter study in Italy. *Hepatology* 2011; **54**: 2055-2063 [PMID: 21898496 DOI: 10.1002/hep.24644]

33 **Casadei Gardini A**, Frassineti GL, Foschi FG, Ercolani G, Ulivi P. Sorafenib and Regorafenib in HBV- or HCV-positive hepatocellular carcinoma patients: Analysis of RESORCE and SHARP trials. *Dig Liver Dis* 2017; **49**: 943-944 [PMID: 28546063 DOI: 10.1016/j.dld.2017.04.022]

34 **Donadon V**, Balbi M, Mas MD, Casarin P, Zanette G. Metformin and reduced risk of hepatocellular carcinoma in diabetic patients with chronic liver disease. *Liver Int* 2010; **30**: 750-758 [PMID: 20331505 DOI: 10.1111/j.1478-3231.2010.02223.x]

35 **Casadei Gardini A**, Marisi G, Scarpi E, Scartozzi M, Faloppi L, Silvestris N, Masi G, Vivaldi C, Brunetti O, Tamberi S, Foschi FG, Tamburini E, Tenti E, Ricca Rosellini S, Ulivi P, Cascinu S, Nanni O, Frassineti GL. Effects of metformin on clinical outcome in diabetic patients with advanced HCC receiving sorafenib. *Expert Opin Pharmacother* 2015; **16**: 2719-2725 [PMID: 26513009 DOI: 10.1517/14656566.2015.1102887]

36 **Casadei Gardini A**, Faloppi L, De Matteis S, Foschi FG, Silvestris N, Tovoli F, Palmieri V, Marisi G, Brunetti O, Vespasiani-Gentilucci U, Perrone G, Valgiusti M, Granato AM, Ercolani G, Negrini G, Tamburini E, Aprile G, Passardi A, Santini D, Cascinu S, Frassineti GL, Scartozzi M. Metformin and insulin impact on clinical outcome in patients with advanced hepatocellular carcinoma receiving sorafenib: Validation study and biological rationale. *Eur J Cancer* 2017; **86**: 106-114 [PMID: 28985579 DOI: 10.1016/j.ejca.2017.09.003]

37 **De Matteis S**, Granato AM, Napolitano R, Molinari C, Valgiusti M, Santini D, Foschi FG, Ercolani G, Vespasiani Gentilucci U, Faloppi L, Scartozzi M, Frassineti GL, Casadei Gardini A. Interplay Between SIRT-3, Metabolism and Its Tumor Suppressor Role in Hepatocellular Carcinoma. *Dig Dis Sci* 2017; **62**: 1872-1880 [PMID: 28527050 DOI: 10.1007/s10620-017-4615-x]

38 **Di Costanzo GG**, Tortora R, Morisco F, Addario L, Guarino M, Cordone G, Falco L, Caporaso N. Impact of Diabetes on Outcomes of Sorafenib Therapy for Hepatocellular Carcinoma. *Target Oncol* 2017; **12**: 61-67 [PMID: 27503006 DOI: 10.1007/s11523-016-0454-5]

39 **Ferrucci PF**, Gandini S, Battaglia A, Alfieri S, Di Giacomo AM, Giannarelli D, Cappellini GC, De Galitiis F, Marchetti P, Amato G, Lazzeri A, Pala L, Cocorocchio E, Martinoli C. Baseline neutrophil-to-lymphocyte ratio is associated with outcome of ipilimumab-treated metastatic melanoma patients. *Br J Cancer* 2015; **112**: 1904-1910 [PMID: 26010413 DOI: 10.1038/bjc.2015.180]

40 **Hu B**, Yang XR, Xu Y, Sun YF, Sun C, Guo W, Zhang X, Wang

WM, Qiu SJ, Zhou J, Fan J. Systemic immune-inflammation index predicts prognosis of patients after curative resection for hepatocellular carcinoma. *Clin Cancer Res* 2014; **20**: 6212-6222 [PMID: 25271081 DOI: 10.1158/1078-0432.CCR-14-0442]

41 **Casadei Gardini A**, Scarpi E, Faloppi L, Scartozzi M, Silvestris N, Santini D, de Stefano G, Marisi G, Negri FV, Foschi FG, Valgiusti M, Ercolani G, Frassineti GL. Immune inflammation indicators and implication for immune modulation strategies in advanced hepatocellular carcinoma patients receiving sorafenib. *Oncotarget* 2016; **7**: 67142-67149 [PMID: 27613839 DOI: 10.18632/oncotarget.11565]

42 **Howell J**, Pinato DJ, Ramaswami R, Arizumi T, Ferrari C, Gibbin A, Burlone ME, Guaschino G, Toniutto P, Black J, Sellers L, Kudo M, Pirisi M, Sharma R. Integration of the cancer-related inflammatory response as a stratifying biomarker of survival in hepatocellular carcinoma treated with sorafenib. *Oncotarget* 2017; **8**: 36161-36170 [PMID: 28212535 DOI: 10.18632/oncotarget.15322]

43 **da Fonseca LG**, Barroso-Sousa R, Bento Ada S, Blanco BP, Valente GL, Pfiffer TE, Hoff PM, Sabbaga J. Pre-treatment neutrophil-to-lymphocyte ratio affects survival in patients with advanced hepatocellular carcinoma treated with sorafenib. *Med Oncol* 2014; **31**: 264 [PMID: 25273866 DOI: 10.1007/s12032-014-0264-5]

44 **Lué A**, Serrano MT, Bustamante FJ, Iñarrairaegui M, Arenas JI, Testillano M, Lorente S, Gil C, de la Torre M, Gomez A, Sangro B. Neutrophil-to-lymphocyte ratio predicts survival in European patients with hepatocellular carcinoma administered sorafenib. *Oncotarget* 2017; **8**: 103077-103086 [PMID: 29262546 DOI: 10.18632/oncotarget.21528]

45 **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]

46 **Eisenhauer EA**, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228-247 [PMID: 19097774 DOI: 10.1016/j.ejca.2008.10.026]

47 **Ronot M**, Bouattour M, Wassermann J, Bruno O, Dreyer C, Larroque B, Castera L, Vilgrain V, Belghiti J, Raymond E, Faivre S. Alternative Response Criteria (Choi, European association for the study of the liver, and modified Response Evaluation Criteria in Solid Tumors [RECIST]) Versus RECIST 1.1 in patients with advanced hepatocellular carcinoma treated with sorafenib. *Oncologist* 2014; **19**: 394-402 [PMID: 24652387 DOI: 10.1634/theoncologist.2013-0114]

48 **Sugimoto K**, Moriyasu F, Saito K, Rognin N, Kamiyama N, Furuichi Y, Imai Y. Hepatocellular carcinoma treated with sorafenib: early detection of treatment response and major adverse events by contrast-enhanced US. *Liver Int* 2013; **33**: 605-615 [PMID: 23305331 DOI: 10.1111/liv.12098]

49 **Zocco MA**, Garcovich M, Lupascu A, Di Stasio E, Roccarina D, Annicchiarico BE, Riccardi L, Ainora ME, Ponziani F, Caracciolo G, Rapaccini GL, Landolfi R, Siciliano M, Pompili M, Gasbarrini A. Early prediction of response to sorafenib in patients with advanced hepatocellular carcinoma: the role of dynamic contrast enhanced ultrasound. *J Hepatol* 2013; **59**: 1014-1021 [PMID: 23811306 DOI: 10.1016/j.jhep.2013.06.011]

50 **Zhou C**, Liu J, Li Y, Liu L, Zhang X, Ma CY, Hua SC, Yang M, Yuan Q. microRNA-1274a, a modulator of sorafenib induced a disintegrin and metalloproteinase 9 (ADAM9) down-regulation in hepatocellular carcinoma. *FEBS Lett* 2011; **585**: 1828-1834 [PMID: 21530512 DOI: 10.1016/j.febslet.2011.04.040]

51 **Hsu CY**, Shen YC, Yu CW, Hsu C, Hu FC, Hsu CH, Chen BB, Wei SY, Cheng AL, Shih TT. Dynamic contrast-enhanced magnetic resonance imaging biomarkers predict survival and response in hepatocellular carcinoma patients treated with sorafenib and metronomic tegafur/uracil. *J Hepatol* 2011; **55**: 858-865 [PMID: 21338641 DOI: 10.1016/j.jhep.2011.01.032]

52 **Lee JH**, Park JY, Kim DY, Ahn SH, Han KH, Seo HJ, Lee JD, Choi HJ. Prognostic value of 18F-FDG PET for hepatocellular carcinoma patients treated with sorafenib. *Liver Int* 2011; **31**: 1144-1149 [PMID: 21745288 DOI: 10.1111/j.1478-3231.2011.02541.x]

53 **Mulé S**, Thiefin G, Costentin C, Durot C, Rahmouni A, Luciani A, Hoeffel C. Advanced Hepatocellular Carcinoma: Pretreatment Contrast-enhanced CT Texture Parameters as Predictive Biomarkers of Survival in Patients Treated with Sorafenib. *Radiology* 2018; **288**: 445-455 [PMID: 29584597 DOI: 10.1148/radiol.2018171320]

54 **Su WW**, Lee KT, Yeh YT, Soon MS, Wang CL, Yu ML, Wang SN. Association of circulating insulin-like growth factor 1 with hepatocellular carcinoma: one cross-sectional correlation study. *J Clin Lab Anal* 2010; **24**: 195-200 [PMID: 20486202 DOI: 10.1002/jcla.20320]

55 **Shao YY**, Huang CC, Lin SD, Hsu CH, Cheng AL. Serum insulin-like growth factor-1 levels predict outcomes of patients with advanced hepatocellular carcinoma receiving antiangiogenic therapy. *Clin Cancer Res* 2012; **18**: 3992-3997 [PMID: 22623732 DOI: 10.1158/1078-0432.CCR-11-2853]

56 **Kim HY**, Lee DH, Lee JH, Cho YY, Cho EJ, Yu SJ, Kim YJ, Yoon JH. Novel biomarker-based model for the prediction of sorafenib response and overall survival in advanced hepatocellular carcinoma: a prospective cohort study. *BMC Cancer* 2018; **18**: 307 [PMID: 29558905 DOI: 10.1186/s12885-018-4211-2]

57 **Miyahara K**, Nouso K, Tomoda T, Kobayashi S, Hagihara H, Kuwaki K, Toshimori J, Onishi H, Ikeda F, Miyake Y, Nakamura S, Shiraha H, Takaki A, Yamamoto K. Predicting the treatment effect of sorafenib using serum angiogenesis markers in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 2011; **26**: 1604-1611 [PMID: 22011296 DOI: 10.1111/j.1440-1746.2011.06887.x]

58 **Tsuchiya K**, Asahina Y, Matsuda S, Muraoka M, Nakata T, Suzuki Y, Tamaki N, Yasui Y, Suzuki S, Hosokawa T, Nishimura T, Ueda K, Kuzuya T, Nakanishi H, Itakura J, Takahashi Y, Kurosaki M, Enomoto N, Izumi N. Changes in plasma vascular endothelial growth factor at 8 weeks after sorafenib administration as predictors of survival for advanced hepatocellular carcinoma. *Cancer* 2014; **120**: 229-237 [PMID: 24122122 DOI: 10.1002/cncr.28384]

59 **Hu B**, Cheng SY. Angiopoietin-2: development of inhibitors for cancer therapy. *Curr Oncol Rep* 2009; **11**: 111-116 [PMID: 19216842]

60 **Hayashi T**, Yamashita T, Terashima T, Suda T, Okada H, Asahina Y, Hayashi T, Hara Y, Nio K, Sunagozaka H, Takatori H, Arai K, Sakai Y, Yamashita T, Mizukoshi E, Honda M, Kaneko S. Serum cytokine profiles predict survival benefits in patients with advanced hepatocellular carcinoma treated with sorafenib: a retrospective cohort study. *BMC Cancer* 2017; **17**: 870 [PMID: 29258450 DOI: 10.1186/s12885-017-3889-x]

61 **Villanueva A**, Minguez B, Forner A, Reig M, Llovet JM. Hepatocellular carcinoma: novel molecular approaches for diagnosis, prognosis, and therapy. *Annu Rev Med* 2010; **61**: 317-328 [PMID: 20059340 DOI: 10.1146/annurev.med.080608.100623]

62 **Sakai K**, Takeda H, Nishijima N, Orito E, Joko K, Uchida Y, Izumi N, Nishio K, Osaki Y. Targeted DNA and RNA sequencing of fine-needle biopsy FFPE specimens in patients with unresectable hepatocellular carcinoma treated with sorafenib. *Oncotarget* 2015; **6**: 21636-21644 [PMID: 26046304 DOI: 10.18632/oncotarget.4270]

63 **Kan Z**, Zheng H, Liu X, Li S, Barber TD, Gong Z, Gao H, Hao K, Willard MD, Xu J, Hauptschein R, Rejto PA, Fernandez J, Wang G, Zhang Q, Wang B, Chen R, Wang J, Lee NP, Zhou W, Lin Z, Peng Z, Yi K, Chen S, Li L, Fan X, Yang J, Ye R, Ju J, Wang K, Estrella H, Deng S, Wei P, Qiu M, Wulur IH, Liu J, Ehsani ME, Zhang

C, Loboda A, Sung WK, Aggarwal A, Poon RT, Fan ST, Wang J, Hardwick J, Reinhard C, Dai H, Li Y, Luk JM, Mao M. Whole-genome sequencing identifies recurrent mutations in hepatocellular carcinoma. *Genome Res* 2013; **23**: 1422-1433 [PMID: 23788652 DOI: 10.1101/gr.154492.113]

64 **Cleary SP**, Jeck WR, Zhao X, Chen K, Selitsky SR, Savich GL, Tan TX, Wu MC, Getz G, Lawrence MS, Parker JS, Li J, Powers S, Kim H, Fischer S, Guindi M, Ghanekar A, Chiang DY. Identification of driver genes in hepatocellular carcinoma by exome sequencing. *Hepatology* 2013; **58**: 1693-1702 [PMID: 23728943 DOI: 10.1002/hep.26540]

65 **Scartozzi M**, Faloppi L, Svegliati Baroni G, Loretelli C, Piscaglia F, Iavarone M, Toniutto P, Fava G, De Minicis S, Mandolesi A, Bianconi M, Giampieri R, Granito A, Facchetti F, Bitetto D, Marinelli S, Venerandi L, Vavassori S, Gemini S, D'Errico A, Colombo M, Bolondi L, Bearzi I, Benedetti A, Cascinu S. VEGF and VEGFR genotyping in the prediction of clinical outcome for HCC patients receiving sorafenib: the ALICE-1 study. *Int J Cancer* 2014; **135**: 1247-1256 [PMID: 24510746 DOI: 10.1002/ijc.28772]

66 **Casadei Gardini A**, Marisi G, Faloppi L, Scarpi E, Foschi FG, Iavarone M, Lauletta G, Corbelli J, Valgiusti M, Facchetti F, Della Corte C, Neri LM, Tamberi S, Cascinu S, Scartozzi M, Amadori D, Nanni O, Tenti E, Ulivi P, Frassineti GL. eNOS polymorphisms and clinical outcome in advanced HCC patients receiving sorafenib: final results of the ePHAS study. *Oncotarget* 2016; **7**: 27988-27999 [PMID: 27058899 DOI: 10.18632/oncotarget.8569]

67 **Marisi G,** Scartozzi M, Faloppi L, Foschi GF, Lauletta G, Valgiusti M, Aprile G, Fornaro L, Iavarone M, Ercolani G, Frassineti GL, Amadori D, Casadei Gardini A. Ang-2 polymorphisms in relation to outcome in advanced HCC patients receiving sorafenib. *J Clin Oncol* 2017; **35** Suppl 15: e15666 [DOI: 10.1200/JCO.2017.35.15_suppl.e15666]

68 **Faloppi L,** Casadei Gardini A, Masi G, Silvestris N, Loretelli C, Ulivi P, Vivaldi C, Bianconi M, Giampieri R, Bittoni A, Andrikou K, Del Prete M, Scartozzi M, Cascinu S. Angiogenesis polymorphisms profile in the prediction of clinical outcome of advanced HCC patients receiving sorafenib: Combined analysis of VEGF and HIF-1α. Final results of the ALICE-2 study. *J Clin Oncol* 2016; **34** Suppl 4: 280 [DOI: 10.1200/jco.2016.34.4_suppl.280]

69 **Casadei Gardini A**, Faloppi L, Aprile G, Brunetti O, Caparello C, Corbelli J, Chessa L, Bruno D, Ercolani G, Leonetti A, de Stefano G, Farella N, Foschi FG, Lanzi A, Dadduzio V, Marisi G, Masi G, Negri FV, Pagan F, Santini D, Scarpi E, Silletta M, Silvestris N, Tamburini E, Tassinari D, Vivaldi C, Gentilucci UV, Zagonel V, Calvetti L, Cascinu S, Frassineti GL, Scartozzi M. Multicenter Prospective Study of Angiogenesis Polymorphism Validation in HCC Patients Treated with Sorafenib. An INNOVATE Study Protocol. *Tumori* 2017; : tj5000704 Epub ahead of print [PMID: 29739298 DOI: 10.5301/tj.5000704]

70 **Horwitz E**, Stein I, Andreozzi M, Nemeth J, Shoham A, Pappo O, Schweitzer N, Tornillo L, Kanarek N, Quagliata L, Zreik F, Porat RM, Finkelstein R, Reuter H, Koschny R, Ganten T, Mogler C, Shibolet O, Hess J, Breuhahn K, Grunewald M, Schirmacher P, Vogel A, Terracciano L, Angel P, Ben-Neriah Y, Pikarsky E. Human and mouse VEGFA-amplified hepatocellular carcinomas are highly sensitive to sorafenib treatment. *Cancer Discov* 2014; **4**: 730-743 [PMID: 24687604 DOI: 10.1158/2159-8290.CD-13-0782]

71 **Arao T**, Ueshima K, Matsumoto K, Nagai T, Kimura H, Hagiwara S, Sakurai T, Haji S, Kanazawa A, Hidaka H, Iso Y, Kubota K, Shimada M, Utsunomiya T, Hirooka M, Hiasa Y, Toyoki Y, Hakamada K, Yasui K, Kumada T, Toyoda H, Sato S, Hisai H, Kuzuya T, Tsuchiya K, Izumi N, Arii S, Nishio K, Kudo M. FGF3/FGF4 amplification and multiple lung metastases in responders to sorafenib in hepatocellular carcinoma. *Hepatology* 2013; **57**: 1407-1415 [PMID: 22890726 DOI: 10.1002/hep.25956]

72 **He C**, Dong X, Zhai B, Jiang X, Dong D, Li B, Jiang H, Xu S, Sun X. MiR-21 mediates sorafenib resistance of hepatocellular carcinoma cells by inhibiting autophagy via the PTEN/Akt pathway. *Oncotarget* 2015; **6**: 28867-28881 [PMID: 26311740 DOI: 10.18632/oncotarget.4814]

73 **Xu Y**, Huang J, Ma L, Shan J, Shen J, Yang Z, Liu L, Luo Y, Yao C, Qian C. MicroRNA-122 confers sorafenib resistance to hepatocellular carcinoma cells by targeting IGF-1R to regulate RAS/RAF/ERK signaling pathways. *Cancer Lett* 2016; **371**: 171-181 [PMID: 26655273 DOI: 10.1016/j.canlet.2015.11.034]

74 **Vaira V**, Roncalli M, Carnaghi C, Faversani A, Maggioni M, Augello C, Rimassa L, Pressiani T, Spagnuolo G, Di Tommaso L, Fagiuoli S, Rota Caremoli E, Barberis M, Labianca R, Santoro A, Bosari S. MicroRNA-425-3p predicts response to sorafenib therapy in patients with hepatocellular carcinoma. *Liver Int* 2015; **35**: 1077-1086 [PMID: 25040368 DOI: 10.1111/liv.12636]

75 **Gyöngyösi B**, Végh É, Járay B, Székely E, Fassan M, Bodoky G, Schaff Z, Kiss A. Pretreatment MicroRNA Level and Outcome in Sorafenib-treated Hepatocellular Carcinoma. *J Histochem Cytochem* 2014; **62**: 547-555 [PMID: 24804874 DOI: 10.1369/0022155414537277]

76 **Fornari F**, Pollutri D, Patrizi C, La Bella T, Marinelli S, Casadei Gardini A, Marisi G, Baron Toaldo M, Baglioni M, Salvatore V, Callegari E, Baldassarre M, Galassi M, Giovannini C, Cescon M, Ravaioli M, Negrini M, Bolondi L, Gramantieri L. In Hepatocellular Carcinoma miR-221 Modulates Sorafenib Resistance through Inhibition of Caspase-3-Mediated Apoptosis. *Clin Cancer Res*2017; **23**: 3953-3965 [PMID: 28096271 DOI: 10.1158/1078-0432.CCR-16-1464]

77 **Nishida N**, Arizumi T, Hagiwara S, Ida H, Sakurai T, Kudo M. MicroRNAs for the Prediction of Early Response to Sorafenib Treatment in Human Hepatocellular Carcinoma. *Liver Cancer*2017; **6**: 113-125 [PMID: 28275578 DOI: 10.1159/000449475]

78 **Stiuso P**, Potenza N, Lombardi A, Ferrandino I, Monaco A, Zappavigna S, Vanacore D, Mosca N, Castiello F, Porto S, Addeo R, Prete SD, De Vita F, Russo A, Caraglia M. MicroRNA-423-5p Promotes Autophagy in Cancer Cells and Is Increased in Serum From Hepatocarcinoma Patients Treated With Sorafenib. *Mol Ther Nucleic Acids* 2015; **4**: e233 [PMID: 25782064 DOI: 10.1038/mtna.2015.8]

79 **Yoon EL**, Yeon JE, Ko E, Lee HJ, Je JH, Yoo YJ, Kang SH, Suh SJ, Kim JH, Seo YS, Yim HJ, Byun KS. An Explorative Analysis for the Role of Serum miR-10b-3p Levels in Predicting Response to Sorafenib in Patients with Advanced Hepatocellular Carcinoma. *J Korean Med Sci* 2017; **32**: 212-220 [PMID: 28049231 DOI: 10.3346/jkms.2017.32.2.212]

80 **Carvajal-Hausdorf DE**, Schalper KA, Neumeister VM, Rimm DL. Quantitative measurement of cancer tissue biomarkers in the lab and in the clinic. *Lab Invest* 2015; **95**: 385-396 [PMID: 25502176 DOI: 10.1038/labinvest.2014.157]

81 **Abou-Alfa GK**, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, De Greve J, Douillard JY, Lathia C, Schwartz B, Taylor I, Moscovici M, Saltz LB. Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2006; **24**: 4293-4300 [PMID: 16908937 DOI: 10.1200/JCO.2005.01.3441]

82 **Personeni N**, Rimassa L, Pressiani T, Destro A, Ligorio C, Tronconi MC, Bozzarelli S, Carnaghi C, Di Tommaso L, Giordano L, Roncalli M, Santoro A. Molecular determinants of outcome in sorafenib-treated patients with hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2013; **139**: 1179-1187 [PMID: 23568548 DOI: 10.1007/s00432-013-1429-x]

83 **Chen D**, Zhao P, Li SQ, Xiao WK, Yin XY, Peng BG, Liang LJ. Prognostic impact of pERK in advanced hepatocellular carcinoma patients treated with sorafenib. *Eur J Surg Oncol* 2013; **39**: 974-980 [PMID: 23845703 DOI: 10.1016/j.ejso.2013.06.018]

84 **Chu JS**, Ge FJ, Zhang B, Wang Y, Silvestris N, Liu LJ, Zhao CH, Lin L, Brunetti AE, Fu YL, Wang J, Paradiso A, Xu JM. Expression and prognostic value of VEGFR-2, PDGFR-β, and c-Met in advanced hepatocellular carcinoma. *J Exp Clin Cancer Res* 2013; **32**: 16 [PMID: 23552472 DOI: 10.1186/1756-9966-32-16]

85 **Hagiwara S**, Kudo M, Nagai T, Inoue T, Ueshima K, Nishida N, Watanabe T, Sakurai T. Activation of JNK and high expression level of CD133 predict a poor response to sorafenib

in hepatocellular carcinoma. *Br J Cancer* 2012; **106**: 1997-2003 [PMID: 22596232 DOI: 10.1038/bjc.2012.145]

86 **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

P- Reviewer: Lin ZY, Sun XY, Yao DF **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4164-4177

DOI: 10.3748/wjg.v24.i36.4164

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

ORIGINAL ARTICLE

Basic Study

Differential expression of mucin 1 and mucin 2 in colorectal cancer

Aldona Kasprzak, Elżbieta Siodła, Małgorzata Andrzejewska, Jacek Szmeja, Agnieszka Seraszek-Jaros, Szczepan Cofta, Witold Szaflarski

Aldona Kasprzak, Elżbieta Siodła, Małgorzata Andrzejewska, Witold Szaflarski, Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan 60-781, Poland

Jacek Szmeja, Chair and Department of General Surgery, Endocrinological and Gastroenterological Oncology, Poznan University of Medical Sciences, Poznan 60-355, Poland

Agnieszka Seraszek-Jaros, Department of Bioinformatics and Computational Biology, Chair of Clinical Pathomorphology, Poznan University of Medical Sciences, Poznan 60-529, Poland

Szczepan Cofta, Department of Pulmonology, Allergology and Respiratory Oncology, Poznan University of Medical Science, Poznan 60-569, Poland

ORCID number: Aldona Kasprzak (0000-0002-1773-3038); Elżbieta Siodła (0000-0003-2614-219X); Małgorzata Andrzejewska (0000-0003-4980-1725); Jacek Szmeja (0000-0003-0289-3511); Agnieszka Seraszek-Jaros (0000-0003-4787-1033); Szczepan Cofta (0000-0002-8849-5487); Witold Szaflarski (0000-0001-5059-268X).

Author contributions: Kasprzak A contributed to conception, design of the study, analyzed data and coordinated the research; Siodła E and Andrzejewska M performed the majority of immunohistochemical and molecular investigations; Szmeja J, Cofta S and Szaflarski W interpreted the data, analyzed the results, and performed a critical revision of the manuscript; Szmeja J provided samples of cancer patients and clinical data; Seraszek-Jaros A performed biostatistics and analyzed the data; Kasprzak A and Szaflarski W drafted the manuscript.

Supported by National Science Center in Poland, No. 2015/17/B/NZ7/03043.

Institutional review board statement: This study was approved by Committee on Bioethics of Poznan University of Medical Sciences, No. 924/14.

Conflict-of-interest statement: The authors have no conflicts of interest, including no conflicts concerning employment, consultancies, stock ownership, honoraria, paid expert testimonies, patent applications/registrations, grants or other funding.

Manuscript source: Unsolicited manuscript

Correspondence to: Aldona Kasprzak, MD, PhD, Professor, Department of Histology and Embryology, Poznan University of Medical Sciences, Swiecicki Street 6, Poznan 60-781, Poland. akasprza@ump.edu.pl
Telephone: +48-61-8546441
Fax: +48-61-8546440

Received: July 16, 2018
Peer-review started: July 16, 2018
First decision: July 24, 2018
Revised: August 15, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 28, 2018

Abstract

AIM

To determine tissue expression (mRNA, protein) of two types of mucins [mucin 1 (MUC1) and mucin 2 (MUC2)] in patients with colorectal cancer (CRC).

METHODS

Expression of membrane-bound mucin (MUC1) and secretory mucin (MUC2) in CRC (mRNA, protein) were analyzed in tissue material including fragments of tumors

obtained from CRC patients (n = 34), and fragments of normal colorectal tissue from the same patients (control). The analysis was conducted using real-time quantitative polymerase chain reaction (RT-qPCR) (transcripts), immunohistochemistry (IHC) (apomucins), and the modern approach for morphometric analysis of IHC reaction (HSV filter software). Results on tissue expression of both mucins (mRNA, protein) were compared to histological alterations in colorectal cancer samples and correlated with selected clinical data in the patients. The statistical analysis was conducted using Statistica PL v. 12.0 software.

RESULTS

Significantly higher expression of the MUC1 mRNA in the CRC, compared with the control and the borderline correlation of mRNA expression with MUC1 protein levels in colorectal samples was observed. The expression of apomucins concerned cell membranes (MUC1) and cytoplasm (MUC2) and occurred both in control tissues and in most cancerous samples. There were no significant relationships between MUC1 (mRNA, protein) and the clinicopathological data of patients. MUC2 protein expression was significantly lower as compared to the control, while MUC2 mRNA expression was comparable in both groups. The MUC1/MUC2 ratio was significantly higher in CRC tissues than in the control. The higher expression of MUC2 was a feature of mucinous CRC subtypes, and characterized higher histological stage of tumors. Negative correlations have been obtained between MUC2 and the Ki-67 antigen, as well as between MUC2 and p53 protein expressions in CRC.

CONCLUSION

A combination of tissue overexpression of MUC1, reduced MUC2 expression, and high ratio of MUC1/MUC2 is a factor of poor prognosis in CRC patients. MUC2 tissue expression allows to differentiate mucinous and nonmucinous CRC subtypes.

Key words: Mucins; Real-time quantitative polymerase chain reaction; Colorectal cancer; Immunohistochemistry; HSV filter program

Core tip: Colorectal cancers (CRC) represent the second most widely manifested malignant tumor worldwide in women and third in men. The evident expression of two mucins [mucin 1 (MUC1) and mucin 2 (MUC2)] occurs in a normal and cancerous large intestine. Using RT-qPCR analysis and immunohistochemistry we confirmed higher expression of the MUC1 mRNA, lower MUC2 protein, and higher MUC1/MUC2 expression ratio in CRC samples as compared to the control. MUC2 protein expression correlates with increased cellular proliferation. A combination of tissue overexpression of MUC1, reduced MUC2 expression, and high ratio of MUC1/MUC2 may be a useful factor of poor prognosis in CRC patients.

Kasprzak A, Siodła E, Andrzejewska M, Szmeja J, Seraszek-Jaros A, Cofta S, Szaflarski W. Differential expression of mucin 1 and mucin 2 in colorectal cancer. *World J Gastroenterol* 2018; 24(36): 4164-4177 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4164.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4164

INTRODUCTION

Colorectal cancer (CRC) is diagnosed in more than 1.3 million people worldwide, annually, with the number steadily increasing. Currently globally, this cancer is the third most common cancer in men and second most common in women[1]. In Poland CRC is the second most common cancer in men and woman, with the third leading causes of cancer deaths in Greater Poland Region[2]. While genetic factors play a major role in etiopathogenesis of CRC, the basis of most the cases of that cancer is unclear. Considering mutation source, CRC is classified as sporadic (70%), hereditary (25%) and congenital (3%-5%)[1,3]. Among the main pathologic alterations in CRC are quantitative and qualitative changes in glycoproteins called mucins[4-6]. Qualitative alterations of mucins include carbohydrate groups, as well as apomucin molecules[4,7-9]. The majority of CRC are nonmucinous adenocarcinomas (approximately 80%). A mucinous adenocarcinoma is a histological subtype of CRC with poorer prognosis than aforementioned. Quantitative changes identified in nonmucinous adenocarcinomas concern a reduction in total mucus output. In contrast, mucinous carcinomas are hypersecretory for mucus[4].

According to modern proteomics, the secreted mucin, mucin 2 (MUC2) is the main constituent of intestinal mucus, produced mainly by the goblet cells of the small and large intestine and playing a critical protective role[4,10-12]. Membrane-associated mucin 1 (MUC1) (episialin), in contrast, is widely expressed by normal glandular epithelial cells, with its high expression in malignant cells[4,6]. Structural changes of the MUC1, observed in the course of carcinogenesis, lead to the activation of signaling pathways such as: MAPK, PI3K/Akt, and Wnt[6,13]. In the blood serum of cancer patients, the MUC1-N subunits, CA 15.3 and CA 19.9 antigens can be detected, while the MUC1 itself was second among the top 75 Tumor-Associated antigens[6].

In the carcinogenesis initiation and CRC progression, overexpression of MUC1 and the decline in MUC2 expression is most commonly described[14-20]. These observations are also confirmed by meta-analysis[21-23]. However, knowledge of the role of tissue mucins expression, at various stages of the colon carcinogenesis is incomplete. Poorly known is the prognostic role of mucins in the mucinous subtypes of CRC, which generally have a worse clinical course and a worse response to chemotherapy[24,25]. Sporadic mucinous CRC had a worse survival rate than its nonmucinous counterpart[26], and mucinous differentiation results in a 2%-8% increased hazard of death, which persists after correction for

stage[27]. Unlike the nonmucinous CRC, the mucinous subtype is correlated with higher MUC2 and lower MUC1 expression[4,21,24,28]. Research into the role of mucins in pathogenesis and CRC clinical studies (especially in mucinous subtypes) are also current topics from a methodological point of view. The lack of standardized methods of quantitative evaluation of mucins expression (especially at tissue level) and/or frequent lack of control groups, are a great difficulty in comparative analysis[15,29,30].

The goal of the present work was the verification of the hypothesis, that the examination of the tissue expression of selected mucins (mRNA, protein), using modern methods of quantitative assessment [real-time quantitative polymerase chain reaction (RT-qPCR), HSV filter software], could improve the diagnostic/prognostic usefulness of these markers of CRC. The specific aim of the study was to evaluate tissue expression (mRNA, protein) of two mucins (MUC1 and MUC2) in patients with colorectal carcinoma, and to assess the relationship between tissue expression of mucins and selected clinicopathological data.

MATERIALS AND METHODS

Patients and tissue samples

The examined CRC group included 34 patients (27 men, 7 women) from Greater Poland Region, 32 to 89 years of age from the Chair and Department of General Surgery, Endocrinological and Gastroenterological Oncology, Poznan University of Medical Sciences, who were diagnosed and subjected to surgery between 2010-2015. We arbitrarily selected patients with CRC only from the Greater Poland Region, not treated before (radio- or chemotherapy), without significant additional systemic diseases, from whom consent was obtained, the perioperative tissue material met the requirements for scientific research and with available clinicopathological data.

Patients affected by diabetes, active chronic organ diseases (heart, kidney, liver), including autoimmune diseases and other cancers, have been excluded from the study. In three patients, hyperglycemia was observed in fasting, four patients were in hypertension treatment. There have been mild premalignant lesions (mainly adenomatous colon), that have been surgically removed as a preventive measure in the past, in 14/34 (41%) of patients.

The available clinical data for the study group, than was taken into account, included: descriptive histopathological diagnosis, histologic grade and stage on Dukes, Astler and Coller's modified Dukes' scales, and TNM system classification[31,32], age, patient sex and basic laboratory studies (complete blood count, number of leukocytes and platelets, as well as glucose levels). Seven patients (21%) of the entire study group died during the analysis period. Duration of patient's survival reflected the time between the date of operation for colorectal cancer and the establishing diagnosis (October 1, 2010), and October 1, 2015.

Locations of the colorectal tumors were divided into proximal (right) colon (caecum, ascending, transverse colon) and distal (left) colon (descending, sigmoid colon and rectum). Macroscopic types were divided into protruded type (height of tumor $\geqslant$ 3 mm) and flat type (height of tumor < 3 mm).

Thirty-four paired specimens of colorectal tumor and non-tumor tissues were obtained during surgical treatment. For the CRC, colon mucosa and, depending on the depth of tumor invasion, submucosal layers approximately 15 cm from the tumor site, served as control tissues. In no case was tissue additional to that which would be removed normally during a particular surgical procedure.

The tissue samples were stored in RNA Stabilization Solution (RNA*later*®, Applied Biosystems) at -80 ℃ until use. Additionally, formalin-fixed paraffin-embedded tumor specimens of 34 colorectal carcinomas and fragments of the confirmed control specimens were obtained from patients.

Informed consent was obtained from every subject, and the institutional review committee approved this study (No. 924/14).

RT-qPCR

CRC tumoral fragments and control tissues from 23 patients were qualified for the experiments that used the RT-qPCR technique as previously described[33].

One microliter of given cDNA or DNA was added to the reaction mixture, composed of 12.5 μL 2 × Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas), 1 μL specific primer pair (*f.c.* 0.3 μmol/L) and 10.5 μL H_2O. Primers for studies on expression of *MUC1* and *MUC2* mRNA expression are indicated in Table 1. β-*actin*, glycerylaldehyde-3-phosphate dehydrogenase (*GADPH*), and hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) served as the housekeeping genes (geometric mean) for the gene expression analysis. All the primers were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw (Table 1).

The reactions were driven in twin.tec real-time PCR plates with PCR Film (Eppendorf) using Mastercycler ep realplex^2 (Eppendorf). The PCR program was as followed: (1) Initial denaturation, 95 ℃, 10 min; (2) Denaturation, 95 ℃, 15 s; (3) Annealing, 60 ℃, 30 s; (4) Extension, 72 ℃, 30 s. The number of cycles was 40-50. Melting curves were made and 2% agarose gel electrophoresis was used to verify the amplification product specificity and size, respectively. All samples were amplified in duplicate or triplicate, and in case when results varied by more than 15%, the reactions were repeated.

Absolute quantitation method was used to quantify mRNA copy numbers of MUC1 and MUC2. Absolute quantification determines the exact copy concentration

Table 1 Primer sequences used for real-time quantitative polymerase chain reaction analysis

Transcript	Sequence (5'-3' direction)	ENST number http://www.ensembl.org	Product size
MUC1	TCCAATATTAAGTTCAGGCCAGGA CACATCACTCACGCTGACGT	00000185499.16	768 bp
MUC2	TGAAGACCTGCGGCTGTGT CAGTCGAACTCGAAGTGCTCC	00000198788.8	3108 bp
β-*actin*	TCTGGCACCACACCTTCTAC GATAGCACAGCCTGGATAGC	00000298556	169 bp
GADPH	GAAGGTGAAGGTCGGAGTCA GACAAGCTTCCCGTTCTCAG	00000229239	199 bp
HRPT1	CTGAGGATTTGGAAAGGGTG AATCCAGCAGGTCAGCAAAC	00000298556	156 bp

of a target gene by relating the *C*t value to a standard curve. Prior to absolute quantification, the *C*t values were normalized by comparison to the average of *C*t's obtained for three housekeeping genes (β-*actin, GAPDH,* and *HPRT1*).

Evaluation of alterations in expression of MUC1 and MUC2 mRNA, involved a comparison of mRNA copy numbers for those mucins per microgram of RNA, between the tumor and control samples from the same patient.

Immunocytochemistry

Tissue sections, 5 μm thick, were deposited onto SuperFrost/Plus microscope slides. In order to qualify the material for the study, routine staining of the sections with hematoxylin and eosin (HE) was performed. Anti-human mouse monoclonal antibodies (mAbs) specific for human Ki-67 antigen (clone MIB-1) (Dako Denmark A/S, Glostrup, Denmark, ready to use), anti-p53 (clone DO-7) (Dako), as well as the anti-MUC1 (clone Ma552) and anti-MUC2 (clone Ccp58) (both from Novocastra™, both in 1:100 dilution) antibodies were used. The sections were incubated with these primary mAbs through the night, at 4 ℃, and afterwards with dextran backbone, to which horseradish peroxidase (HRP) was attached, and with secondary biotinylated link anti-rabbit and anti-mouse IgG (Dako REAL™ EnVision™ Detection System peroxidase/DAB+, Rabbit/Mouse, Dako), with microwave-oven pre-treatment for antigen retrieval. Positive reaction manifested, in at least three sequential sections, as a dark brown or black precipitate in the cell nucleus (Ki-67, p53) and cell membrane/cytoplasm (MUC1 and MUC2). The preparations were counterstained using hematoxylin. Every test was accompanied by a negative control, in which specific antibodies were supplemented by a normal serum of a respective species in 0.05 mol/L Tris-HCl, pH approximately 7.6, supplemented with 0.1% bovine serum albumin (BSA) and 15 mmol/L sodium azide (internal negative control). All the steps of immunocytochemistry (IHC) technique were previously described[34]. Histological slides with IHC expression were examined under the optical Olympus BH-2 microscope, coupled to a digital camera. Color microscope images were recorded and archived using a 40 × objective (at least 10 fields in every microscope slide with an IHC positive reaction), with the use of LUCIA Image 5.0 computer software.

Semiquantitative evaluation of Ki-67 antigen and p53 expression

Expression of Ki-67 antigen and p53 (only clearly labelled cell nuclei were considered), was calculated, taking mean proportion of immunopositive cells in 10 light microscope fields into account. Expression was evaluated using the modified semi-quantitative scale[35], in which the score of 1 corresponded to up to 10% positive cells; the scores of 2, 3 and 4 corresponded to 11%-25%, 26%-50% and ⩾ 51% positive cells, respectively.

Morphometric evaluation of MUC1 and MUC2 tissue expression

The images with positive IHC reaction, 2560 × 1920 pixels in size, recorded in the LUCIA Image 5.0 software, were subjected to morphometric analysis, using the quantitative morphometric HSV Filter software, originally developed in the Department of Bioinformatics and Computational Biology, Poznan University of Medical Sciences, according to the following formula: (area of positive IHC reaction/area studied) × 100%.

In the Results section, values of average IHC expression of both mucins were presented, expressed in percentages, manifested by the IHC reactions per field of colorectal cancer/control sample area.

Statistical analysis

At the first stage of statistical analysis, consistency of all of the results with normal distribution of Gauss was verified using the Shapiro-Wilk test. Parameters of descriptive statistics (mean value, median value, SD, and minimum and maximum value) were calculated.

Data related to quantitative mucin expression (mRNA, protein), in CRC group, were compared with the data obtained for the control samples of the same patients (linked variables) with the Wilcoxon test. In cases of unlinked variables in two groups, the non-parametric Mann-Whitney's test was applied. The *t*-Student test was applied in case of consistency of the results with normal Gaussian distribution.

Table 2 Clinicopathologic features of colorectal carcinoma patients *n* (%)

Variable		CRC (*n* = 34)
Age (yr)	< 50	2 (6)
	⩾ 50	32 (94)
Sex	male	27 (79)
	female	7 (21)
Tumor location	Right colon	10 (29)
	Left colon	21 (62)
	Rectum	3 (9)
Mucin content	Nonmucinous	24 (71)
	Mucinous	10 (29)
Histologic grade (G)	Carcinoma *in situ*	1 (3)
	Well differentiated (G1)	1 (3)
	Moderately differentiated (G2)	23 (68)
	Poorly differentiated (G3)	9 (26)
Gross morphology	Protruded	21 (62)
	Flat	13 (38)
Dukes/Astler and Coller stage	Carcinoma *in situ*	1 (3)
	A/B1	4 (12)
	B/B2, B3	10 (29)
	C/C1, C2, C3	14 (41)
	D	5 (15)
TNM classification system	Carcinoma *in situ*	1 (3)
	Ⅰ and Ⅱ	4 (12)
	Ⅲ and Ⅳ	29 (85)
Status	Survival	27 (79)
	Death	7 (21)

CRC: Colorectal carcinoma.

The percentage shares of IHC positivity of both mucins were evaluated, using the difference test between two proportions.

Correlations between data rows were determined employing Spearman's rank correlation index. The Kaplan-Meier survival curves and Log-rank test were used to compare overall survival rates. The results were accepted to be significant at the level of *P* value less than 0.05. The statistical analysis was conducted using Statistica PL v. 12.0 software (StatSoft Inc., Tulsa, OK, United States). The statistical analysis of the study was performed by biomedical statistician (AS-J). The statistical method of the study was reviewed by a statistician (Kaczmarek E) from the Department of Bioinformatics and Computational Biology, Chair of Pathology, Poznan University of Medical Science.

RESULTS

Clinicopathological data in CRC patients

Patients over 50 years of age were predominant in the Study Group (94%). The cancer was primarily located in the distal part of the colon (left colon) (62%). In 3 patients, the tumor was localized in the rectum. In 21 patients (62%), protruded type of tumor was observed, while the flat type was seen in 13 patients (38%).

The majority of patients were diagnosed with tubular adenocarcinoma located in the colon or rectum, and nonmucinous subtype of CRC (71%) prevailed. Among these patients one had a mixed-type tumor with the neuroendocrine component, the other was diagnosed as adenocarcinoma *in situ*. Mucinous subtype of CRC was diagnosed in 10/34 patients.

The histopathological study showed a majority of moderately differentiated adenocarcinoma of the colon or rectum [grade 2 (G2)] (68%) compared to other grades. On a Dukes scale and in its modified form (Astler and Coller scale), most tumors were assessed at Stage C/C1-C3. In five patients from the whole Study Group (15%), there were distant metastases present (all to the liver). The vast majority of patients (85%) was classified stage Ⅲ and Ⅳ on the TNM classification system.

The clinicopathological characteristics of CRC patients were collected in Table 2.

MUC1 and MUC2 expression analysis at mRNA level

The expression of the MUC1 and MUC2 transcripts was present in all control and cancerous tissue samples. Our study showed that the expression of the MUC1 mRNA in the CRC tissues (75095 ± 72149 copies/μg RNA) was significantly higher when compared with the control tissue (32413 ± 44486 copies/μg RNA) ($P = 0.004$), and the expression of MUC2 mRNA was comparable in the study and control group (350227 ± 529270 *vs* 219744 ± 324252 copies/μg RNA) ($P = 0.274$).

The MUC1/MUC2 transcripts ratio in the test group, although higher (1.56 ± 4.50), did not differ significantly from the one obtained in the control tissue (0.28 ± 0.40) ($P = 0.128$) (Table 3).

MUC1 and MUC2 mRNA expression and pathological data

No significant differences could be disclosed in the amount of MUC1 and MUC2 transcripts on one hand and

Table 3 Tissue expression of mRNA and proteins of both mucins, mucin 1/mucin 2 ratio in colorectal carcinoma and in unaltered colorectal tissue

		Group	Number	Mean	Median	Min	Max	SD	[a]*P* value
MUC1	mRNA	CRC	23	75095	49309	5648	267473	72149	0.004
		Control	23	32413	20075	2	199681	44486	
	Protein	CRC	34	2.57	1.64	0.33	9.80	2.24	0.627
		Control	32	2.16	1.72	0.00	6.54	1.64	
MUC2	mRNA	CRC	23	350227	191457	2806	2399156	529270	0.274
		Control	23	219744	130691	1	1509936	324252	
	Protein	CRC	34	4.94	2.15	0.20	32.30	7.24	0.035
		Control	32	7.40	5.15	0.73	31.60	6.77	
MUC1/MUC2 ratio	mRNA	CRC	23	1.56	0.25	0.06	21.30	4.50	0.128
		Control	23	0.28	0.18	0.04	2.00	0.40	
	Protein	CRC	34	1.84	0.80	0.04	10.35	2.54	0.003
		Control	32	0.52	0.37	0.00	1.95	0.51	

Control: Unaltered colorectal tissue; [a]*P*: Comparing colorectal carcinoma and control. MUC1: Mucin 1; MUC2: Mucin 2; SD: Standard deviation; CRC: Colorectal carcinoma.

CRC subtype (mucinous *vs* nonmucinous), colon tumor size, anatomical location of the CRC (proximal *vs* distal section of the colon), histologic grade or stage in the Dukes, or Astler and Coller scale, on the other (data not shown).

The comparison of the mRNA expression of both mucins, depending on the parameters in the TNM classification system was possible only for patients with N0 and N1, with no significant differences observed in this case as well (data not shown).

MUC1 and MUC2 expression at protein level

Using immunohistochemistry, a positive MUC1 immunoexpression was detected in all CRC samples (100%) and in 29/32 control colorectal samples (91%), thus the detectability of the positive expression of both mucins was similar. The immunoexpression of MUC2 was present in all CRC samples and in all samples of the colorectal control.

Tissue localization of MUC1 and MUC2 immunoexpression

MUC1 tissue expression in CRC was pronounced and related mostly to cell membranes on the apical surface of the neoplastic cells lining the glandular structures and in the lumen of altered intestinal crypts (extracellular mucins fields) (Figure 1A and B). In the control tissue of large intestine, membranous expression of MUC1 prevailed and was observed mainly on the surface of normal intestinal crypts (Figure 1C).

In contrast, MUC2 expression was mainly related to the cytoplasm of neoplastic cells with differentiated expression of this mucin, from single immunopositive cells (Figure 1D) to intense reaction in the cytoplasm of numerous cancer cells and/or localized extracellularly (Figure 1E). In the normal intestinal mucosa (control), cytoplasmic expression of MUC2 prevailed and was observed in normal intestinal crypts (Figure 1F).

No preferred detection sites were observed for both mucins (MUC1 and MUC2) within the evaluated area of the colorectal tumor (center, periphery).

Quantitative analysis of MUC1 and MUC2 immunoexpression

The mean expression of MUC1 in colorectal tumors (2.57% ± 2.24 % of IHC reaction) and in the normal large intestine (2.16% ± 1.64%) was comparable (P = 0.627). In the case of MUC2, significantly lower expression of this glycoprotein in CRC (4.94% ± 7.24%) than in the control (7.40% ± 6.77%) has been shown (P = 0.035) (Table 3).

The MUC1/MUC2 expression ratio was significantly higher in the CRC tissues (1.84 ± 2.54), than in the control (0.52 ± 0.51) (P = 0.003) (Table 3).

MUC1 and MUC2 immunoexpression and pathological data

A significantly higher expression of MUC2 in mucinous CRC (10.97% ± 11.17% of IHC reaction), compared to the rest of the CRC (2.40% ± 2.00%), was shown (P = 0.018). No such differences were observed with MUC1 expression (Figure 2). No significant differences could be disclosed in the expression of both mucins from one hand and tumor size, the anatomical location of the CRC, histologic grade (G2 *vs* G3) and in patients with N0 and N1 in the TNM classification system (data not shown).

In the case of MUC2 expression, significantly higher expression of this mucins in colorectal tumors in Stage C was shown (5.54% ± 7.57%), compared with Stage B (2.12% ± 2.64%) (P = 0.044) (Figure 3). The analysis of mucins expression in tumors of Stage B2 and C2 on Astler and Coller scale, confirmed these results, although only a borderline statistical significance (P = 0.066) was obtained for MUC2 expression (data not shown).

MUC1 and MUC2 transcript vs protein expression

High positive Spearman's correlation was observed in patients affected by CRC, between mutual expression of both analyzed mucin transcripts (r = 0.602; P < 0.05), but not between protein expression itself (r = 0.046)

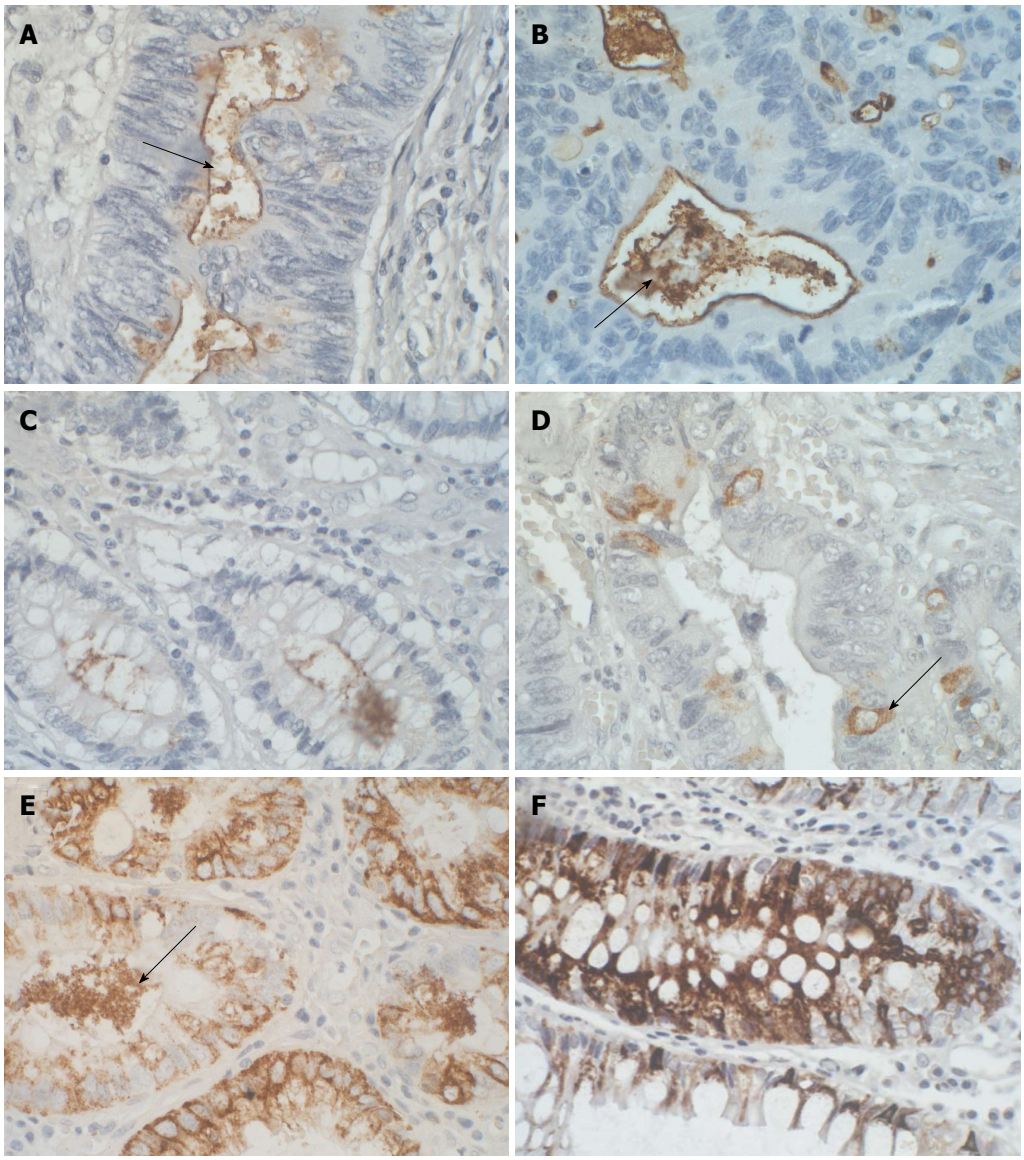


Figure 1 Immunohistochemical illustrations of colorectal carcinoma and control colon with mucin 1 and mucin 2 positive expression. A: Representative IHC expression of MUC1 in luminal surface epithelium (arrow) of tumor-changed colon crypt; B: Membranous and extracellular pattern (arrow) of MUC1 expression in neoplastic cells lining the glandular structures of CRC; C: Representative image of MUC1 membranous localization in normal colon crypts; D: Cytoplasmic expression of MUC2 in scattered epithelial cells of the tumor-changed colon crypt (arrow); E: IHC intense reaction of MUC2 expression in the cytoplasm of numerous cancer cells and/or localized in the lumen of the colon crypts (arrow); F: Cytoplasmic expression of MUC2 in majority of goblet cells in normal colon epithelium. Sections were counterstained with hematoxylin. Objective × 40. MUC1: Mucin 1; MUC2: Mucin 2; IHC: Immunohistochemical; CRC: Colorectal cancer.

(Table 4). Additionally, in CRC tumor tissues, borderline positive Spearman's correlation, between mRNA and protein expression of MUC1 (r = 0.405; P = 0.055). MUC2 didn't show a statistically significant correlation between mRNA and protein expression (Table 4).

MUC1 and MUC2 expression (mRNA and proteins) and clinical data

Negative correlation between MUC1 mRNA expression and the age of patients was observed (r = -0.481; P < 0.05). Furthermore, positive correlations considered the expression of both mucins (MUC1 and MUC2) and the blood leukocyte count (r = 0.465 and r = 0.474 respectively; P < 0.05 in both cases). Additionally, the expression of MUC1 protein was positively correlated with thrombocyte numbers in patients affected by CRC (r = 0.474; P < 0.05) (Table 5).

Mean survival time of patients affected by CRC was 52 ± 3 mo. The Kaplan-Meier analysis shows that neither MUC1, nor MUC2 apomucins expression were significantly associated with survival probability in patients with CRC (Figure 4A and B). Survival curves of 34 patients with CRC showed that also expression of mRNA for both mucins in tissue samples was not associated with the prognosis of CRC (data not shown).

Ki-67 proliferating antigen and p53 immunoexpression

The positive expression of Ki-67 proliferating antigen was detected in 28/34 (82%) of CRC tissue samples. Additionally, a significantly higher expression of Ki-67 in

Table 4 Values of Spearman's coefficient for correlation between both mucins (mRNA, protein) and Ki-67 and/or p53 protein expressions in colorectal carcinoma samples

	MUC1	MUC2	mRNA MUC1	mRNA MUC2	Ki-67	p53
MUC1	-	0.046	0.405[a]	0.199	0.015	-0.106
MUC2	0.046	-	0.457	0.126	-0.428[1]	-0.389[1]
mRNA MUC1	0.405[a]	0.457	-	0.602[1]	-0.121	-0.215
mRNA MUC2	0.199	0.126	0.602[1]	-	0.033	-0.145
Ki-67	0.015	-0.428[1]	-0.121	0.033	-	0.602[1]
p53	-0.106	-0.389[1]	-0.215	-0.145	0.602[1]	-

[1]Indicate values of *r* coefficient for which $P < 0.05$; [a]$P = 0.055$. MUC1: Mucin 1; MUC2: Mucin 2.

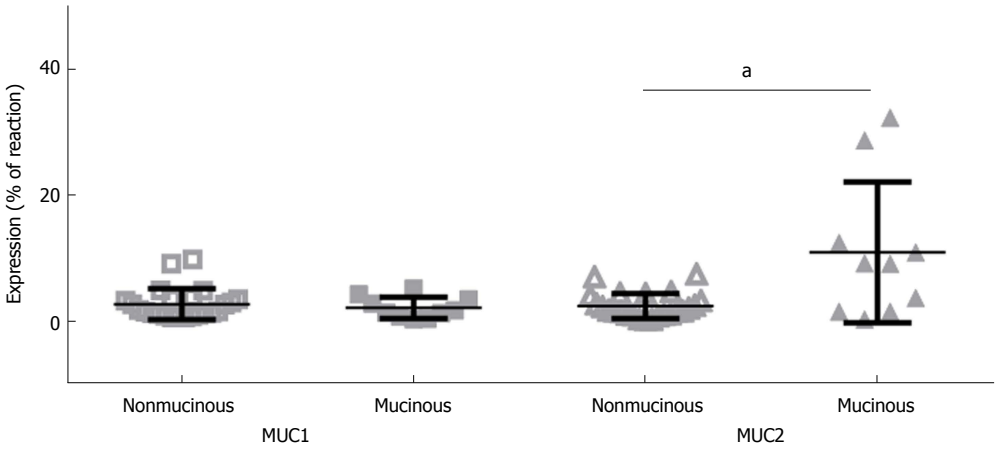


Figure 2 Comparative immunoexpression of mucin 1 and mucin 2 in nonmucinous and mucinous subtypes of colorectal carcinoma. Mean ± SD. [a]*P* (level of significance) value < 0.05. MUC1: Mucin 1; MUC2: Mucin 2.

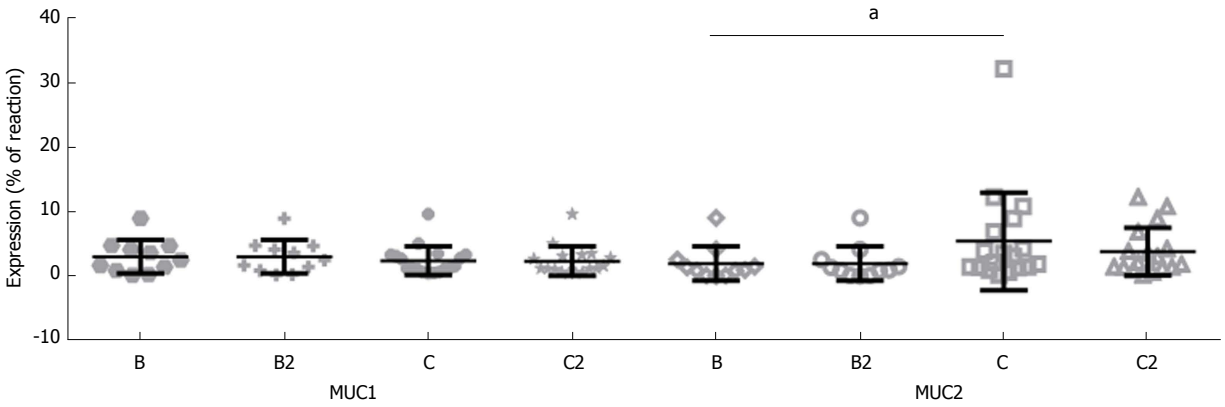


Figure 3 Tissue expression of mucin 1 and mucin 2 in colorectal carcinoma as related to Dukes and Astler and Coller staging system. Mean ± SD. B, C: Dukes staging system; B2, C2: Astler and Coller staging system. [a]*P* (level of significance) value < 0.05. MUC1: Mucin 1; MUC2: Mucin 2.

CRC as compared with control was demonstrated ($P < 0.001$) (data not shown).

Only the nuclear location of Ki-67 within different percentages of immunopositive tumor cells was observed (Figure 5A and B). The Ki-67 antigen expression in the control samples was evident mainly in the individual basally located nuclei of the goblet cells lining the unaltered intestinal crypts (Figure 5C).

Positive expression of p53 was demonstrated in 19/34 (56%) patients. Similar to Ki-67 antigen, only the nuclear location was observed and, as a rule, a very intense IHC reaction concerning the majority of polymorphic cell nuclei in the evaluated samples (Figure 5D). 44% of CRC patients did not show the presence of the protein in tumor samples (Figure 5E). In the healthy colorectal samples (control), p53 expression was not detected in any specimen (Figure 5F).

MUC1 and MUC2 expression (mRNA and proteins) vs Ki-67 and p53 expression

A significant, relatively high, negative Spearman's correlation, between the expression of MUC2 apomucin and

Table 5 Values of Spearman's coefficient for correlation between mucins expression (mRNA/protein) in colorectal carcinoma and selected clinical data

	Age (yr)	Hemoglobin (g/dL)	WBC ($\times 10^9$/L)	Thrombocytes (g/L)	Glucose (mg/dL)
MUC1	-0.322	-0.175	0.067	0.474[1]	-0.277
MUC2	0.053	-0.123	-0.097	-0.085	-0.346
mRNA MUC1	-0.481[1]	0.189	0.465[1]	0.203	-0.325
mRNA MUC2	-0.412	-0.098	0.474[1]	-0.145	-0.306

[1]Indicate values of *r* coefficient for which $P < 0.05$. WBC: White blood cell; MUC1: Mucin 1; MUC2: Mucin 2.

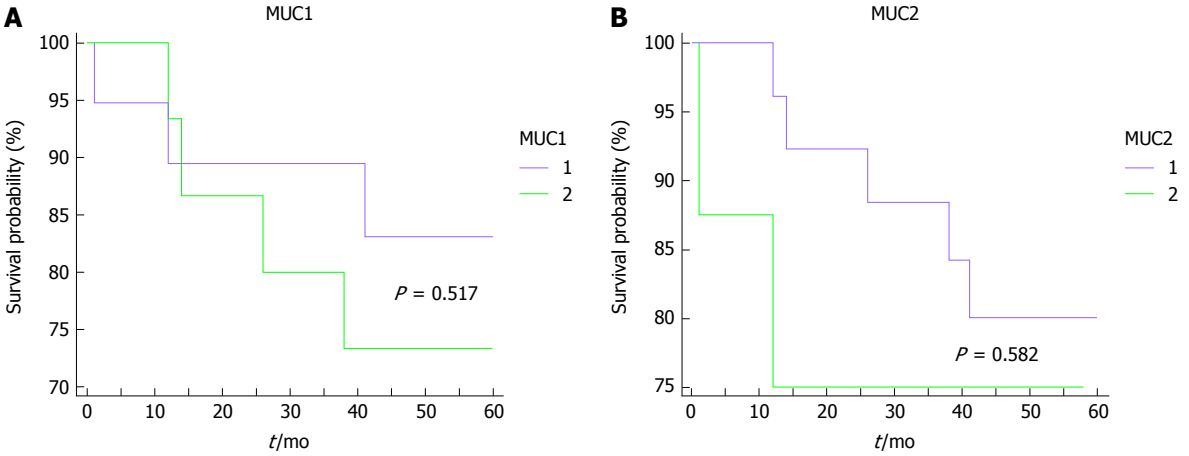


Figure 4 Kaplan-Meier survival curves for colorectal carcinoma patients, as related to tissue expression of mucin 1 and mucin 2 showing that expression of both mucins in tissue samples are not associated with survival time. A: Kaplan Meier survival curve related to tissue expression of MUC1; B: Kaplan Meier survival curve related to tissue expression of MUC2. 1: Under mean tissue expression; 2: Above mean tissue expression. MUC1: Mucin 1; MUC2: Mucin 2.

Ki-67 (r = -0.428; $P < 0.05$) was observed (Figure 6). In contrast, relatively weak, negative correlation between MUC2 protein and p53 expression (r = -0.389; $P < 0.05$), was shown in CRC tissues (data not shown). Additionally, a high positive correlation was observed for Ki-67 antigen and p53 expression in study group (r = 0.603; $P < 0.05$) (data not shown).

DISCUSSION

Some discrepancies are present between the results of MUC1 expression detection, in healthy colon and rectum, with the use of immunohistochemistry. Some scientific publications, notably the recent ones, document lack of MUC1 expression in control large intestine in adults[18-20], or emphasize low detectability (10%) of this apomucin[36].

In this study, positive MUC1 expression (mRNA, protein) was observed in almost all of the control samples of large intestinal tissue. These results are coherent with the findings of other authors, conducted with the use of light and electron microscopy[37,38]. Descriptions of clear, membranous expression of MUC1, on the luminal surface of glandular cells of normal colon epithelium, are available also in interactive databases[39]. Therefore, our own research using IHC technique confirms both, detectability and evident membranous expression of this mucin, in tissues of healthy large intestine.

In neoplastic CRC tissues collected in this study, detectability of MUC1 expression *via* IHC was 100%, being much higher than those achieved by other authors, which note it from below 20%[40], through 32%-40%[14,18,41], approximately 55%[20], to 70%-80%[29]. According to some publications, MUC1 expression was more commonly detected in CRC patients with lymph node metastases discovered during surgical procedures, than those without such metastases (84.2% *vs* 34.6%)[20], which is not confirmed by the current study. However, our results are similar to those obtained in CRC tissue microarrays, in which the authors also did not observe correlation between MUC1 expression and histologic grade, stage, vascular invasion, or cancer type[14,30]. The results of research by Matsuda *et al*[41], concerning more common expression of MUC1 in CRC of more severe histologic stages, were not confirmed in our studies. However, similarly to the authors[41], we have also not found any correlation between MUC1 and p53 expression. As in our studies, Kesari *et al*[19] did not observe differences in MUC1 expression depending on the histologic stage, but a higher incidence of expression of this mucin was described in G2, than in G1 of this cancer (55% *vs* 11%)[19]. The positive relationship between MUC1 expression and histologic grade and stage can also be found in other publications, with the appreciation of this expression as a high risk factor of death in Caucasian population (HR: 2.03; P = 0.038)[29,37]. The co-expression of MUC1 and p53 was a bad prognostic factor for the overall survival (OS) of these patients[29]. Our own

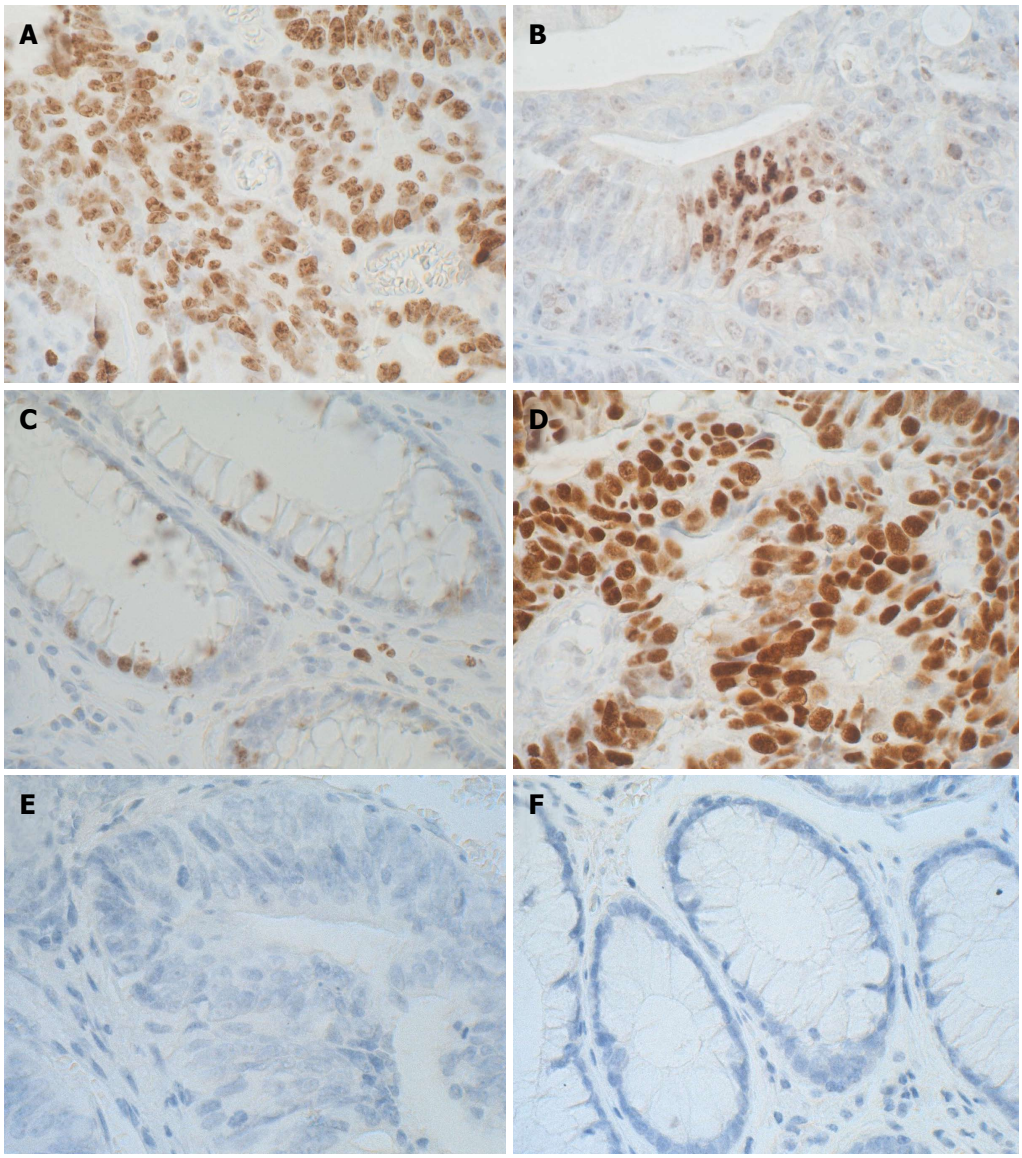


Figure 5 Immunohistochemical illustrations of colorectal carcinoma and control colon with Ki-67 antigen and p53 expression. A: Representative IHC expression of Ki-67 proliferating antigen in the majority of tumor cell nuclei; B: An intense nuclear pattern of Ki-67 expression in focally located tumor cells; C: Representative image of Ki-67 proliferating antigen immunoexpression in the individual basally located nuclei of the goblet cells lining the unaltered intestinal crypts; D: A pronounced p53 nuclear pattern of IHC reaction in glandular structures of CRC; E: Negative IHC reaction for p53 in tumor of other CRC patient; F: Negative IHC reaction for p53 in normal colon. Sections were counterstained with hematoxylin. Objective × 40. IHC: Immunohistochemical; CRC: Colorectal cancer.

research cannot confirm the above observations, as well as the results of other authors, where MUC1 expression was shown to be an independent marker of prognosis (HR: 1.339, 95%CI: 1.002-1.790; P = 0.048)[14]. The lack of dependence between MUC1 expression and histologic grade or stage, in the current study probably results from the very homogenous group of patients with CRC in the range of histologically assessed parameters [68% with grade 2; 56% with stage C (C1-C3)/D in Dukes/Astler and Coller scale; 85% with stage Ⅲ and Ⅳ in TNM classification system]. Furthermore, it should be stressed, that the results of multiple authors are mainly based on the analysis of the detection (incidence) of MUC1 expression, rather than a reliable quantitative assessment[19,29]. In several works, MUC1 expression was admittedly evaluated, using semi-quantitative methods[20,30,36], but some of them did not have control groups[14,19,30]. There are publications that intensify the IHC reaction, and introduce the division into the so-called high, low and negative MUC1 expression, occurring in a different percentage of patients (12%, 52%, 36%, respectively)[30]. Hence, the overexpression of the MUC1 protein was observed in varying percentages in different patients, from 12%[30] to approximately 40%[19].

Most of the cited researchers, in their studies, used monoclonal, primary Ma695 antibody (Novocastra), in 1:100 dilution[18,19,30,40]. In the current study, antibodies from the same company have been used, also in 1:100 dilution. However, we have chosen another clone (Ma552). Furthermore, the use of a reliable, repeatable method of quantitative evaluation of IHC expression (HSV filter program), may explain the discrepancies obtained in the

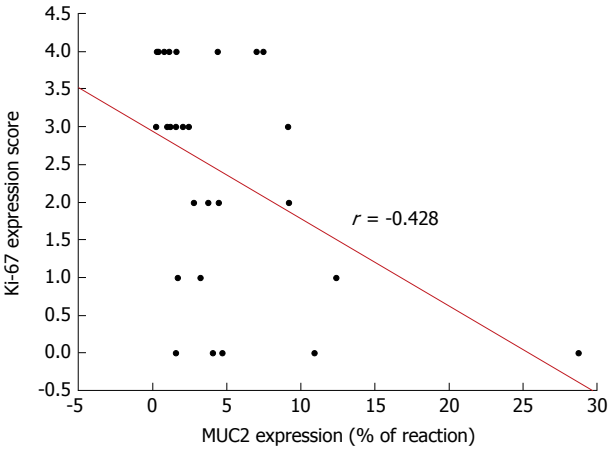


Figure 6 Spearman's correlation between the expression of mucin 2 protein and of Ki-67 proliferating antigen in colorectal carcinoma. MUC2: Mucin 2.

study results, at least partially, including the increased MUC1 expression in both control, and cancerous large intestine, presented in our study.

Current studies, employing the RT-qPCR method, have been shown a significantly higher mean expression of MUC1 mRNA in CRC, compared to the healthy tissue. This expression showed borderline correlation (P = 0.055) with MUC1 protein expression. It is difficult to relate this result to the literature data.

The results of correlation analysis, between the MUC1 expression (mRNA, protein) and the available patient clinical data, are not as spectacular as those obtained by other authors. In the case of MUC1, a negative correlation is shown between the mRNA (but not the protein itself) expression and the age of the CRC affected patients. The data on that specific relation was not found in literature. In addition, we have observed positive correlations between the MUC1 mRNA and the number of leukocytes, while the expression of the MUC1 apomucin itself, correlated with the number of platelets in the CRC. There are also no exact references to these results in literature. However, in mouse model, it was shown that carcinoma mucins (fragments from human colonic adenocarcinoma LS180 cells) initiate thrombosis through adhesion-dependent, reciprocal activation of neutrophils and platelets. These studies provide insights into mucin-dependent, thrombin-independent thrombosis in patients with Trousseau syndrome[42].

Similar to MUC1 expression, the detectability of MUC2 (mRNA, protein), obtained in the current, study was higher, than that presented in the literature data, which cite approximately 30%[14], approximately 50%-64%[20,30,43] and 92% of positive CRC cases[44]. Some sources document a higher incidence of MUC2 expression detection (72%-100%), only in the case of mucinous CRC subtypes[29,40]. The production of MUC2 mRNA in the healthy colorectal tissues is documented by numerous researchers[18,45,46], although some of the authors describe it in just 20% of the control tissues[44].

The cytoplasmic pattern of MUC2 expression, demonstrated in the present work, confirms previous observations in the healthy and cancer-altered large intestine[39,46,47]. We have described similar amounts of MUC2 mRNA in CRC and control, but a lower expression of the MUC2 protein in patients with CRC, compared to control. Confronting this with literature, the results of the IHC study are consistent with those obtained by many researchers[14-20,29,41,43,48], while in the case of MUC2 transcripts detection, few works record reduced expression of MUC2 mRNA in the CRC, compared to the normal tissue[45]. In the current work, higher expression of MUC2 in the mucinous CRC, as compared with nonmucinous subtypes of cancer, has been shown, which also confirms the results of other authors' research[24,47,49].

In CRC patients gathered in this work, a higher expression of MUC2 was also observed in the more advanced histologic stages of the tumor. In addition, there have been significant negative correlations between MUC2 with Ki-67 and p53 expressions. This could indicate a significant relation between the decrease in the expression of this glycoprotein in the course of colon carcinogenesis, and the pro-proliferative activity (Ki-67), or deregulation of the tumor suppressive *P53* signaling. This result is confirmed by the research of other authors[50,51]. However, similarly to MUC1, there was no significant correlation between MUC2 expression and histologic grade, size, or location of the tumor, which is also consistent with the literature data[43,52].

In the current study, it was also not possible to find statistically significant relationships between mucin expression (mRNA, protein) and survival of patients with CRC. In the 5-year period evaluated, seven people died of cancer (21%), the average survival was 52 mo from the time of surgery. The small number of patients analyzed, including the deceased, did not allow to draw binding conclusions on the predictive role of MUC1 and MUC2 tissue expression, in the CRC patients of the Greater Poland Region.

Research by other authors points to a link between MUC1 overexpression and poorer survival, especially in mucinous tumors. These authors prove, that higher frequency of MUC1 immunoreactivity in the mucinous subtype of CRC was independently related to greater rate of cancer death in colorectal patients[26]. In the case of MUC2, however, other studies have also shown important correlations between MUC2 expression reduction/loss, shorter survival time (OS), shorter progression-free/disease-free (PFS) in patients with stage Ⅱ and Ⅲ colorectal carcinomas[30,53] and longer disease-free (DFS) and disease-specific survival (DSS) in patients with positive MUC2 expression. The loss of expression of this mucin was correlated with the recurrence of cancer[52]. In some studies, the relationship between MUC2 expression and survival was not spectacular, but only borderline, and more often concerned well-to-moderately differentiated adenocarcinomas [P = 0.064 for recurrence/metastasis-free survival (RFS) and P = 0.172 for OS] but not for poorly differentiated adenocarcinomas[43].

Although the current study is based on a relatively small group of patients (n = 34), with the predominance of nonmucinous subtype of CRC, it can be assumed, that the expression of both mucins (MUC1 and MUC2), at the level of mRNA and protein, occurs in a normal and tumor-altered colon. Lower tissue expression of MUC2 in CRC, as compared with control, correlates with increased cellular proliferation and could become a marker of cancer progression. The intensity of MUC2 expression allows to differentiate mucinous and nonmucinous CRC subtypes.

The clinical limitations of the current study can be summarized as follows: (1) Most likely due to the homogeneous study group in the range of histologically assessed parameters (68% patients with G2 and 85% with stage Ⅲ and Ⅳ in TNM classification), not all differences in MUC1 and MUC2 expression or correlations with clinical data have reached statistical significance. And (2) The small number of deceased patients (n = 7) analyzed in the current study, did not allow to draw binding conclusions on the predictive role of MUC1 and MUC2 tissue expression for the survival time of patients with CRC of the Greater Poland Region.

Future study is required and a larger number of patients should be evaluated to confirm our findings. Better characterization of the role of mucins in molecular mechanisms in colorectal carcinogenesis requires further testing, also on an *in vitro* model.

In conclusion, a combination of tissue overexpression of MUC1, reduced MUC2 expression, and high ratio of MUC1/MUC2 is a factor of poor prognosis in CRC patients. MUC2 tissue expression allows to differentiate mucinous and nonmucinous CRC subtypes.

ARTICLE HIGHLIGHTS

Research background

In Poland, colorectal carcinoma (CRC) is the second most common cancer in men and woman, with the third leading causes of cancer deaths in Greater Poland Region. Altered mucin expression is correlated with the prognosis of this cancer. *In vivo* as well as *in vitro* studies on the expression of mucins may have also therapeutic implications.

Research motivation

The role of mucin expression at various stages of the colon carcinogenesis is incomplete. The prognostic role of mucins in the mucinous subtypes of CRC is poorly known. Research into the role of mucins in pathogenesis and CRC clinical studies (especially in mucinous subtypes) are also current topics from a methodological point of view. The lack of standardized methods of quantitative evaluation of mucins expression (especially at tissue level) and/or frequent lack of control groups, are a great difficulty in comparative analysis.

Research objectives

Current research determines tissue expression (mRNA, protein) of membrane-bound mucin [mucin 1 (MUC1)] and secretory mucin [mucin 2 (MUC2)] in healthy and colorectal cancer tissue samples and evaluates the relationship between tissue expression of both mucins and selected clinicopathological data of the patients with CRC.

Research methods

The research on tissue expression of two types of mucins (MUC1 and MUC2) in cancerous and normal colorectal tissue samples was performed using real-time quantitative polymerase chain reaction (RT-qPCR) to evaluate expression of transcripts, immunohistochemistry (IHC) for demonstrating apomucins localization, and the morphometric analysis of intensity of IHC reaction using modern HSV filter software.

Research results

Significantly higher expression of the MUC1 mRNA in the CRC, while MUC2 transcript expression was comparable with the control colorectal samples. Using immunohistochemistry, we observed lower MUC2 protein as compared to control tissue. MUC2 protein expression correlated negatively with cellular proliferation (Ki-67 antigen expression) and expression of mutated form of p53. In neoplastic tissue of CRC it was observed also higher MUC1/MUC2 ratio as compared with healthy colorectal tissue. Higher expression of MUC2 was a feature of mucinous CRC subtypes, and characterized higher histological stage of tumors. Future study is required to explain molecular mechanisms of CRC carcinogenesis including mucins and *TP53* pathway.

Research conclusions

Our study confirmed that the colorectal carcinogenesis is closely related to overexpression of MUC1 and the decline in MUC2 expression. The use of increasingly repetitive and reliable method for the quantitative evaluation of mucins expression may prove useful to evaluate different patterns of IHC reaction (membranous, cytoplasmic, *etc*.) and can be useful in various subtype of colorectal cancer, as our research shows (mucinous *vs* nonmucinous CRC). Both the microscopic demonstration of evident MUC1 expression, especially in healthy colorectal tissue (control), and morphometric quantitative evaluation (Filter HSV program) of membranous (MUC1) and secreted (MUC2) expression is the novelty of the present work. The quantitative method used in the current study, can be used for further comparative research and to evaluate tissue expression of other types of mucins in CRC. The use of quantitative methods in immunocytochemistry can improve the detection of tissue markers in CRC and assess their true value in daily medical practice.

Research perspectives

This study proposed, that the examination of the tissue expression of MUC1 and MUC2 (mRNA, protein), should use modern methods of quantitative assessment of transcripts (*e.g.* RT-qPCR), and more reliable morphometric methods (*e.g.* HSV filter software). Only these methods could improve the diagnostic/prognostic usefulness of mucins as tissue biomarkers in CRC patients. A better explanation of molecular mechanisms in the colorectal carcinogenesis with mucin involvement requires further testing, also on an *in vitro* model. These results could be the basis for further studies to understand the carcinogenesis of colorectal cancer.

REFERENCES

1 **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]
2 **Dyzmann-Sroka A**, Malicki J. Cancer incidence and mortality in the Greater Poland Region-Analysis of the year 2010 and future trends. *Rep Pract Oncol Radiother* 2014; **19**: 296-300 [PMID: 25184053 DOI: 10.1016/j.rpor.2014.04.001]
3 **Mármol I**, Sánchez-de-Diego C, Pradilla Dieste A, Cerrada E, Rodriguez Yoldi MJ. Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. *Int J Mol Sci* 2017; **18**: [PMID: 28106826 DOI: 10.3390/ijms18010197]
4 **Corfield AP**, Carroll D, Myerscough N, Probert CS. Mucins in the gastrointestinal tract in health and disease. *Front Biosci* 2001; **6**: D1321-D1357 [PMID: 11578958 DOI: 10.2741/A684]
5 **Sheng YH**, Hasnain SZ, Florin TH, McGuckin MA. Mucins in inflammatory bowel diseases and colorectal cancer. *J Gastroenterol Hepatol* 2012; **27**: 28-38 [PMID: 21913981 DOI: 10.1111/j.1440-1746.2011.06909.x]
6 **Nath S**, Mukherjee P. MUC1: a multifaceted oncoprotein with a key role in cancer progression. *Trends Mol Med* 2014; **20**: 332-342 [PMID: 24667139 DOI: 10.1016/j.molmed.2014.02.007]
7 **Mastrodonato M**, Mentino D, Liquori GE, Ferri D. Histochemical

characterization of the sialic acid residues in mouse colon mucins. *Microsc Res Tech* 2013; **76**: 156-162 [PMID: 23109168 DOI: 10.1002/jemt.22146]

8 **Jain P**, Mondal SK, Sinha SK, Mukhopadhyay M, Chakraborty I. Diagnostic and prognostic significance of different mucin expression, preoperative CEA, and CA-125 in colorectal carcinoma: A clinicopathological study. *J Nat Sci Biol Med* 2014; **5**: 404-408 [PMID: 25097424 DOI: 10.4103/0976-9668.136207]

9 **Mastrodonato M**, Mentino D, Portincasa P, Calamita G, Liquori GE, Ferri D. High-fat diet alters the oligosaccharide chains of colon mucins in mice. *Histochem Cell Biol* 2014; **142**: 449-459 [PMID: 24770665 DOI: 10.1007/s00418-014-1221-2]

10 **Van der Sluis M**, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 2006; **131**: 117-129 [PMID: 16831596 DOI: 10.1053/j.gastro.2006.04.020]

11 **Kim YS**, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep* 2010; **12**: 319-330 [PMID: 20703838 DOI: 10.1007/s11894-010-0131-2]

12 **Pelaseyed T**, Bergström JH, Gustafsson JK, Ermund A, Birchenough GM, Schütte A, van der Post S, Svensson F, Rodríguez-Piñeiro AM, Nyström EE, Wising C, Johansson ME, Hansson GC. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol Rev* 2014; **260**: 8-20 [PMID: 24942678 DOI: 10.1111/imr.12182]

13 **Niv Y**. MUC1 and colorectal cancer pathophysiology considerations. *World J Gastroenterol* 2008; **14**: 2139-2141 [PMID: 18407586 DOI: 10.3748/wjg.14.2139]

14 **Duncan TJ**, Watson NF, Al-Attar AH, Scholefield JH, Durrant LG. The role of MUC1 and MUC3 in the biology and prognosis of colorectal cancer. *World J Surg Oncol* 2007; **5**: 31 [PMID: 17349047 DOI: 10.1186/1477-7819-5-31]

15 **Perçinel S**, Savaş B, Ensari A, Kuzu I, Kuzu MA, Bektaş M, Cetinkaya H, Kurşun N. Mucins in the colorectal neoplastic spectrum with reference to conventional and serrated adenomas. *Turk J Gastroenterol* 2007; **18**: 230-238 [PMID: 18080919]

16 **Perez RO**, Bresciani BH, Bresciani C, Proscurshim I, Kiss D, Gama-Rodrigues J, Pereira DD, Rawet V, Cecconnello I, Habr-Gama A. Mucinous colorectal adenocarcinoma: influence of mucin expression (Muc1, 2 and 5) on clinico-pathological features and prognosis. *Int J Colorectal Dis* 2008; **23**: 757-765 [PMID: 18458918 DOI: 10.1007/s00384-008-0486-0]

17 **Saeland E**, Belo AI, Mongera S, van Die I, Meijer GA, van Kooyk Y. Differential glycosylation of MUC1 and CEACAM5 between normal mucosa and tumour tissue of colon cancer patients. *Int J Cancer* 2012; **131**: 117-128 [PMID: 21823122 DOI: 10.1002/ijc.26354]

18 **Terada T**. An immunohistochemical study of primary signet-ring cell carcinoma of the stomach and colorectum: II. Expression of MUC1, MUC2, MUC5AC, and MUC6 in normal mucosa and in 42 cases. *Int J Clin Exp Pathol* 2013; **6**: 613-621 [PMID: 23573307]

19 **Kesari MV**, Gaopande VL, Joshi AR, Babanagare SV, Gogate BP, Khadilkar AV. Immunohistochemical study of MUC1, MUC2 and MUC5AC in colorectal carcinoma and review of literature. *Indian J Gastroenterol* 2015; **34**: 63-67 [PMID: 25731647 DOI: 10.1007/s12664-015-0534-y]

20 **Wang HS**, Wang LH. The expression and significance of Gal-3 and MUC1 in colorectal cancer and colon cancer. *Onco Targets Ther* 2015; **8**: 1893-1898 [PMID: 26251612 DOI: 10.2147/OTT.S83502]

21 **Debunne H**, Ceelen W. Mucinous differentiation in colorectal cancer: molecular, histological and clinical aspects. *Acta Chir Belg* 2013; **113**: 385-390 [PMID: 24494463]

22 **Xu F**, Liu F, Zhao H, An G, Feng G. Prognostic Significance of Mucin Antigen MUC1 in Various Human Epithelial Cancers: A Meta-Analysis. *Medicine* (Baltimore) 2015; **94**: e2286 [PMID: 26683959 DOI: 10.1097/MD.0000000000002286]

23 **Zeng Y**, Zhang Q, Zhang Y, Lu M, Liu Y, Zheng T, Feng S, Hao M, Shi H. MUC1 Predicts Colorectal Cancer Metastasis: A Systematic Review and Meta-Analysis of Case Controlled Studies. *PLoS One* 2015; **10**: e0138049 [PMID: 26367866 DOI: 10.1371/journal.pone.0138049]

24 **Chiang JM**, Yeh CY, Changchien CR, Chen JS, Tang R, Chen JR. Mucinous adenocarcinoma showing different clinicopathological and molecular characteristics in relation to different colorectal cancer subgroups. *Int J Colorectal Dis*2010; **25**: 941-947 [PMID: 20532535 DOI: 10.1007/s00384-010-0958-x]

25 **Winder T**, Lenz HJ. Mucinous adenocarcinomas with intra-abdominal dissemination: a review of current therapy. *Oncologist* 2010; **15**: 836-844 [PMID: 20656916 DOI: 10.1634/theoncologist.2010-0052]

26 **You JF**, Hsieh LL, Changchien CR, Chen JS, Chen JR, Chiang JM, Yeh CY, Hsieh PS, Fan CW, Liu CT, Tang R. Inverse effects of mucin on survival of matched hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer patients. *Clin Cancer Res* 2006; **12**: 4244-4250 [PMID: 16857798 DOI: 10.1158/1078-0432.CCR-06-0202]

27 **Verhulst J**, Ferdinande L, Demetter P, Ceelen W. Mucinous subtype as prognostic factor in colorectal cancer: a systematic review and meta-analysis. *J Clin Pathol* 2012; **65**: 381-388 [PMID: 22259177 DOI: 10.1136/jclinpath-2011-200340]

28 **Benedix F**, Kuester D, Meyer F, Lippert H. [Influence of mucinous and signet-ring cell differentiation on epidemiological, histological, molecular biological features, and outcome in patients with colorectal carcinoma]. *Zentralbl Chir*2013; **138**: 427-433 [PMID: 22274919 DOI: 10.1055/s-0031-1283870]

29 **Manne U**, Weiss HL, Grizzle WE. Racial differences in the prognostic usefulness of MUC1 and MUC2 in colorectal adenocarcinomas. *Clin Cancer Res* 2000; **6**: 4017-4025 [PMID: 11051251]

30 **Betge J**, Schneider NI, Harbaum L, Pollheimer MJ, Lindtner RA, Kornprat P, Ebert MP, Langner C. MUC1, MUC2, MUC5AC, and MUC6 in colorectal cancer: expression profiles and clinical significance. *Virchows Arch* 2016; **469**: 255-265 [PMID: 27298226 DOI: 10.1007/s00428-016-1970-5]

31 **ASTLER VB**, COLLER FA. The prognostic significance of direct extension of carcinoma of the colon and rectum. *Ann Surg* 1954; **139**: 846-852 [PMID: 13159135 DOI: 10.1097/00000658-195406000-00015]

32 **Puppa G**, Sonzogni A, Colombari R, Pelosi G. TNM staging system of colorectal carcinoma: a critical appraisal of challenging issues. *Arch Pathol Lab Med* 2010; **134**: 837-852 [PMID: 20524862]

33 **Kasprzak A**, Szaflarski W, Szmeja J, Andrzejewska M, Przybyszewska W, Kaczmarek E, Koczorowska M, Kościński T, Zabel M, Drews M. Differential expression of IGF-1 mRNA isoforms in colorectal carcinoma and normal colon tissue. *Int J Oncol* 2013; **42**: 305-316 [PMID: 23165777 DOI: 10.3892/ijo.2012.1706]

34 **Gurgul E**, Kasprzak A, Blaszczyk A, Biczysko M, Surdyk-Zasada J, Seraszek-Jaros A, Ruchala M. Ghrelin and obestatin in thyroid gland - immunohistochemical expression in nodular goiter, papillary and medullary cancer. *Folia Histochem Cytobiol* 2015; **53**: 19-25 [PMID: 25765090 DOI: 10.5603/FHC.a2015.0004]

35 **Gatter KC**, Dunnill MS, Gerdes J, Stein H, Mason DY. New approach to assessing lung tumours in man. *J Clin Pathol* 1986; **39**: 590-593 [PMID: 3013945 DOI: 10.1136/jcp.39.6.590]

36 **Lugli A**, Zlobec I, Baker K, Minoo P, Tornillo L, Terracciano L, Jass JR. Prognostic significance of mucins in colorectal cancer with different DNA mismatch-repair status. *J Clin Pathol* 2007; **60**: 534-539 [PMID: 16816167 DOI: 10.1136/jcp.2006.039552]

37 **Biemer-Hüttmann AE**, Walsh MD, McGuckin MA, Ajioka Y, Watanabe H, Leggett BA, Jass JR. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. *J Histochem Cytochem* 1999; **47**: 1039-1048 [PMID: 10424888 DOI: 10.1177/002215549904700808]

38 **Winterford CM**, Walsh MD, Leggett BA, Jass JR. Ultrastructural localization of epithelial mucin core proteins in colorectal tissues. *J Histochem Cytochem* 1999; **47**: 1063-1074 [PMID: 10424891 DOI:

10.1177/002215549904700811]
39 MUC1. Accessed March 20, 2018 Available from: URL: http://www.humanproteinatlas.org
40 **Ishizu H**, Kumagai J, Eishi Y, Takizawa T, Koike M. Mucin core protein expression by colorectal mucinous carcinomas with or without mucus hyperplasia. *J Gastroenterol* 2004; **39**: 125-132 [PMID: 15069618 DOI: 10.1007/s00535-003-1263-z]
41 **Matsuda K**, Masaki T, Watanabe T, Kitayama J, Nagawa H, Muto T, Ajioka Y. Clinical significance of MUC1 and MUC2 mucin and p53 protein expression in colorectal carcinoma. *Jpn J Clin Oncol* 2000; **30**: 89-94 [PMID: 10768872 DOI: 10.1093/jjco/hyd023]
42 **Shao B**, Wahrenbrock MG, Yao L, David T, Coughlin SR, Xia L, Varki A, McEver RP. Carcinoma mucins trigger reciprocal activation of platelets and neutrophils in a murine model of Trousseau syndrome. *Blood* 2011; **118**: 4015-4023 [PMID: 21860019 DOI: 10.1182/blood-2011-07-368514]
43 **Imai Y**, Yamagishi H, Fukuda K, Ono Y, Inoue T, Ueda Y. Differential mucin phenotypes and their significance in a variation of colorectal carcinoma. *World J Gastroenterol* 2013; **19**: 3957-3968 [PMID: 23840140 DOI: 10.3748/wjg.v19.i25.3957]
44 **El-Sayed IH**, Lotfy M, Moawad M. Immunodiagnostic potential of mucin (MUC2) and Thomsen-Friedenreich (TF) antigens in Egyptian patients with colorectal cancer. *Eur Rev Med Pharmacol Sci* 2011; **15**: 91-97 [PMID: 21381504]
45 **Ogata S**, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. *Cancer Res* 1992; **52**: 5971-5978 [PMID: 1394223]
46 **Sylvester PA**, Myerscough N, Warren BF, Carlstedt I, Corfield AP, Durdey P, Thomas MG. Differential expression of the chromosome 11 mucin genes in colorectal cancer. *J Pathol* 2001; **195**: 327-335 [PMID: 11673830 DOI: 10.1002/path.951]
47 **Hanski C**, Hofmeier M, Schmitt-Gräff A, Riede E, Hanski ML, Borchard F, Sieber E, Niedobitek F, Foss HD, Stein H, Riecken EO. Overexpression or ectopic expression of MUC2 is the common property of mucinous carcinomas of the colon, pancreas, breast, and ovary. *J Pathol* 1997; **182**: 385-391 [PMID: 9306958 DOI: 10.1002/(SICI)1096-9896(199708)182:4<385::AID-PATH861>3.0.CO;2-Q]
48 **Al-Khayal K**, Abdulla M, Al-Obaid O, Zubaidi A, Vaali-Mohammed MA, Alsheikh A, Ahmad R. Differential expression of mucins in Middle Eastern patients with colorectal cancer. *Oncol Lett* 2016; **12**: 393-400 [PMID: 27347157 DOI: 10.3892/ol.2016.4672]
49 **Blank M**, Klussmann E, Krüger-Krasagakes S, Schmitt-Gräff A, Stolte M, Bornhoeft G, Stein H, Xing PX, McKenzie IF, Verstijnen CP. Expression of MUC2-mucin in colorectal adenomas and carcinomas of different histological types. *Int J Cancer* 1994; **59**: 301-306 [PMID: 7927933 DOI: 10.1002/ijc.2910590302]
50 **Li A**, Goto M, Horinouchi M, Tanaka S, Imai K, Kim YS, Sato E, Yonezawa S. Expression of MUC1 and MUC2 mucins and relationship with cell proliferative activity in human colorectal neoplasia. *Pathol Int* 2001; **51**: 853-860 [PMID: 11844051 DOI: 10.1046/j.1440-1827.2001.01291.x]
51 **Walsh MD**, Clendenning M, Williamson E, Pearson SA, Walters RJ, Nagler B, Packenas D, Win AK, Hopper JL, Jenkins MA, Haydon AM, Rosty C, English DR, Giles GG, McGuckin MA, Young JP, Buchanan DD. Expression of MUC2, MUC5AC, MUC5B, and MUC6 mucins in colorectal cancers and their association with the CpG island methylator phenotype. *Mod Pathol* 2013; **26**: 1642-1656 [PMID: 23807779 DOI: 10.1038/modpathol.2013.101]
52 **Elzagheid A**, Emaetig F, Buhmeida A, Laato M, El-Faitori O, Syrjänen K, Collan Y, Pyrhönen S. Loss of MUC2 expression predicts disease recurrence and poor outcome in colorectal carcinoma. *Tumour Biol* 2013; **34**: 621-628 [PMID: 23179399 DOI: 10.1007/s13277-012-0588-8]
53 **Kang H**, Min BS, Lee KY, Kim NK, Kim SN, Choi J, Kim H. Loss of E-cadherin and MUC2 expressions correlated with poor survival in patients with stages Ⅱ and Ⅲ colorectal carcinoma. *Ann Surg Oncol* 2011; **18**: 711-719 [PMID: 20865330 DOI: 10.1245/s10434-010-1338-z]

P- Reviewer: Boscá L, Lim SC, Wang SK **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4178-4185

DOI: 10.3748/wjg.v24.i36.4178

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

ORIGINAL ARTICLE

Basic Study

Mechanism of combined use of vitamin D and puerarin in anti-hepatic fibrosis by regulating the Wnt/β-catenin signalling pathway

Gan-Rong Huang, Si-Jun Wei, Yan-Qiang Huang, Wei Xing, Lu-Yao Wang, Ling-Ling Liang

Gan-Rong Huang, Yan-Qiang Huang, Wei Xing, Lu-Yao Wang, Ling-Ling Liang, Youjiang Medical University for Nationalities, School of Basic Medical Sciences, Baise 533000, Guangxi Zhuang Autonomous Region, China

Si-Jun Wei, Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

ORCID number: Gan-Rong Huang (0000-0001-5580-8689); Si-Jun Wei (0000-0001-6948-2747); Yan-Qiang Huang (0000-0002-2121-8258); Wei Xing (0000-0001-5640-1151); Lu-Yao Wang (0000-0001-5545-0170); Ling-Ling Liang (0000-0002-1498-2370).

Author contributions: Huang GR and Wei SJ contributed equally to this work; Huang YQ, Wang LY and Liang LL performed the majority of the experiments; Xing W performed the experimental design, writing and analysis.

Supported by Key Laboratory Cultivation Base of Universities in Guangxi (Guangxi Education Research), No. [2014] 6.

Institutional animal care and use committee statement: This study was reviewed and approved by the Youjiang Medical University for Nationalities.

Conflict-of-interest statement: All authors have no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The manuscript has been revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Wei Xing, MD, Professor, Youjiang Medical University for Nationalities, School of Basic Medical Sciences, 98 Urban-Rural Road, Baise 533000, Guangxi Zhuang Autonomous Region, China. 116895009@qq.com
Telephone: 18907767511

Received: June 5, 2018
Peer-review started: June 5, 2018
First decision: June 21, 2018
Revised: July 30, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: September 28, 2018

Abstract

AIM

To reveal the protective mechanism of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis induced by carbon tetrachloride (CCl_4).

METHODS

Eight-week-old male Wistar rats were randomly divided into a normal control group (C group), a CCl_4 group (CCl_4 group), a vitamin D group (V group), a puerarin group (P group), and a combined group of vitamin D and puerarin (V + P group), each of which contained ten rats. In this way, we built a rat model of CCl_4-induced hepatic fibrosis with intervention by vitamin D, puerarin, or a combination of the two. After eight weeks, the mice were sacrificed to collect serum and liver specimens. Blood was collected to detect the hyaluronic acid (HA). We also measured hydroxyproline (Hyp) and prepared paraffin sections of liver. After Sirius red staining, the liver specimens were observed under a microscope. RT-PCR and western blot analysis were adopted to detect the mRNA and the protein

levels of Collagen I, Collagen III, Wnt1, and β-catenin in the liver tissues, respectively.

RESULTS

Hepatic fibrosis was observed in the CCl_4 group. In comparison, hepatic fibrosis was attenuated in the V, P, and V + P groups: the HA level in blood and the Hyp level in liver were reduced, and the mRNA levels of Collagen I, Collagen III, Wnt, and β-catenin in liver were also decreased, as well as the protein levels of Wnt1 and β-catenin. Among these groups, the V + P group demonstrated the greatest amelioration of hepatic fibrosis.

CONCLUSION

The combined application of vitamin D and puerarin is capable of alleviating CCl_4-induced hepatic fibrosis of rats. As to the mechanism, it is probably because the combined use is able to silence the Wnt1/β-catenin pathway, suppress the activation of hepatic stellate cells, and reduce the secretion of collagen fibers, therefore improving the anti-hepatic fibrosis effect.

Key words: Carbon tetrachloride; Hepatic fibrosis; Vitamin D; Puerarin; Wnt/β-catenin

Core tip: The proliferation of hepatic stellate cells (HSCs) is associated with hepatic fibrosis. The activated HSCs, as well as Wnt1 and β-catenin, have become important targets in anti-hepatic fibrosis therapy. This research investigated the protective effect of the combined use of vitamin D and puerarin against CCl_4-induced hepatic fibrosis in rats. The protective effect of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis is closely associated with the function of silencing the Wnt1/β-catenin pathway, suppressing the activation of HSCs, and decreasing the secretion of collagen fibers, which provided a useful reference for those in clinical practice.

Huang GR, Wei SJ, Huang YQ, Xing W, Wang LY, Liang LL. Mechanism of combined use of vitamin D and puerarin in anti-hepatic fibrosis by regulating the Wnt/β-catenin signalling pathway. *World J Gastroenterol* 2018; 24(36): 4178-4185 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4178.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4178

INTRODUCTION

Hepatic fibrosis is the change of pathological structures due to all kinds of chronic liver diseases. Essentially, it occurs because extracellular matrix (ECM) synthesized by hepatic stellate cells (HSCs) under the actions of various pathogenic factors substantially increases, exceeding the degradation ability of the liver itself. The long-term accumulation of ECM will lead to hepatic fibrosis and thus cause liver cirrhosis[1,2]. In recent years, it has been proven that the β-catenin protein plays a critical role in the occurrence and development of hepatic fibrosis[3,4]. Previous studies revealed that the more serious the hepatic fibrosis, the higher the expression of β-catenin in liver tissues compared with normal liver tissues, and silencing β-catenin is able to suppress the secretion of collagen and the proliferation of HSCs and mediate cell apoptosis. β-catenin participates in many signalling pathways, among which the Wnt/β-catenin pathway is the most common along which β-catenin plays its role. Apart from this, β-catenin also participates in alternative pathways including E-cadherin, NF-κB, and TGF-β[5-7]. Therefore, β-catenin is key to the intersection of multiple signalling pathways. Existing experiments revealed that HSC-T6 cell membranes and cytoplasm with activated phenotypes show β-catenin expression, and β-catenin expression is also observed in nuclei. This indicates that the Wnt/β-catenin signalling pathway is activated in activated HSCs. The expression of α-SMA in HSC-T6 cells was down-regulated by blocking the transduction of the Wnt/β-catenin signalling pathway, and the expression of types I and III collagen (Collagen I and Collagen III) was also significantly down-regulated. The analysis based on the String database finds that DVL1, DVL2, and DVL3 play their functions in signal transduction pathways mediated by multiple Wnt genes. They are regulatory factors for the Wnt signalling pathway and ER-to-Golgi transport and participate in the ER-to-Golgi transport, thus KLHL12 plays a critical role in the export of collagen, as shown in Figure 1. This implies that the Wnt/β-catenin signalling pathway is closely associated with the activation of HSCs.

Carbon tetrachloride (CCl_4) is one of the classical poisons used in establishing hepatic fibrosis models and is therefore widely used in fundamental research[8]. Previous research has proven *in vivo* that vitamin D is able to alleviate hepatic fibrosis, and *in vitro* experiments confirmed that vitamin D can reduce the secretion of collagen fibers of HSCs[9,10]. There are also evidences from existing research that puerarin is capable of attenuating hepatic fibrosis, which is probably associated with the inhibition of the activation of HSCs by blocking the TNF-α signalling pathway[11,12]. A preliminary study by the present research team has confirmed that the combined use of vitamin D and puerarin is able to enhance the anti-hepatic fibrosis effect; however, the related mechanism has not been revealed. Therefore, the current research investigated the effects of vitamin D combined with puerarin on the expression of key factors, including Wnt1, β-catenin, Collagen I, and Collagen III, in the Wnt/β-catenin signalling pathway based on the aforementioned preliminary study. By doing so, we attempted to further clarify the mechanism of the combined use of vitamin D

Table 1 Designations and sequences of primers

Designation (mice)	Sequence (5′-3′)
GAPDH	F: GGCATCCTGACCCTCAAGTA
	R: GGGGTGTGAACCTCTCAAA
Collagen Ⅰ	F: GGACACTACTGGATCGACCTAAC
	R: CTCACCTGTCTCCATGTTGCA
Collagen Ⅲ	F: CTACCTTGCTCAGTCCTATGAGTCTAGA
	R: TCCCGAGTCGCAGACACATAT
Wnt1	F: GAAACCGCCGCTGGAACT
	R: CCCTGCCTCGTTATTGTGAAG
β-*catenin*	F: ACC TCC CAAGTC CTG TAT
	R: CCT GGT CCT CGT CAT TTA

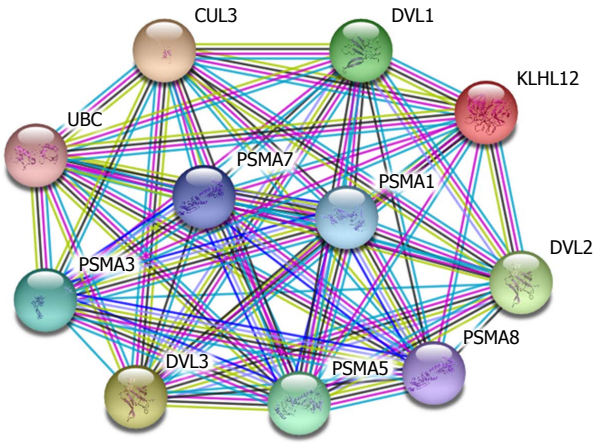


Figure 1 Interaction between WNT-related genes and collagen-related genes.

and puerarin in anti-hepatic fibrosis.

MATERIALS AND METHODS

Experimental materials

Fifty clean-grade healthy male Wistar rats (with a body mass of about 200 g) of similar age were provided by the Animal Center of Youjiang Medical College for Nationalities. Analytically pure CCl_4 was purchased from Sinopharm Chemical Reagent Co., Ltd, and vitamin D and puerarin were purchased from Sigma. In addition, corn oil, Sirius red staining solution, and real-time fluorescence quantitative PCR kits were purchased from Wako Pure Chemical Industries, Ltd, Beijing Leagene Biotech Co., Ltd, and Roche (Switzerland), respectively. Wnt1 and β-catenin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Table 1 shows the designations and sequences of primers.

Experimental methods

Fifty Wistar rats were randomly divided into five groups. Rats in the CCl_4 group were administered 3 mL/kg of corn oil solution containing 45% CCl_4 through intraperitoneal injection twice a week. The normal control group (C group) was administered the same dose of normal saline through intraperitoneal injection twice a week. The V, P, and V + P groups were the drug intervention groups. On the basis of the models established using corn oil solution containing CCl_4, mice in the three groups received 2 μg/kg of vitamin D, 0.4 g/kg of puerarin, and a combination of D vitamin (2 μg/kg) and puerarin (0.4 g/kg) through intragastric administration twice a week. The mice were subjected to fasting for one hour before each administration of treatment. The C group received the same quantity of normal saline through intragastric administration. After eight weeks, the mice were fasted overnight the day before the end of the experiments and sacrificed the next day. Whole blood was collected and stood for 20 min at room temperature. Then, the blood was centrifuged at 5000 rpm for 15 min. Thereafter, the supernatant and then the serum were obtained, followed by the detection of hyaluronic acid (HA) in blood using the aforementioned kit. After collecting the liver of the rats, the kit was used to detect hydroxyproline (Hyp) in the liver. Part of the liver was fixed in a paraformaldehyde solution, and paraffin sections were prepared. After Sirius red staining, liver tissue slices were placed under the microscope to observe their pathological changes and degree of fibrosis. Moreover, real-time fluorescence quantitative PCR was applied to detect the mRNA levels of Collagen I, Collagen III, Wnt1, and β-catenin, and a western blot assay was used to measure the protein levels of Wnt1 and β-catenin.

Statistical analysis

The experiments were repeated three times for each group. SPSS 17.0 statistical software was used for subsequent analysis. The analytical results were expressed using mean ± SD, and a *t*-test was used when comparing paired groups. The difference was deemed statistically significant when $P < 0.05$.

RESULTS

Determination of biochemical indices of blood and liver

HA is a type of proteoglycan distributed around hepatic cells, and its content significantly increases in blood when hepatic fibrosis occurs[13]. Hyp in liver is an important part of the collagen fibers in liver, and the amount thereof also increases when hepatic fibrosis occurs[14]. Therefore, these two factors are important diagnostic indices of hepatic fibrosis. As shown in Table 2, the HA and Hyp of the CCl_4 group were significantly higher than those of the C group, which indicated, to some extent, that the hepatic fibrosis model had been successfully established. The HA and Hyp levels of the V, P, and V + P groups were much lower than those of the CCl_4 group. Among these groups, the V + P group showed the most significant reduction in HA and Hyp levels.

Observation of the pathological sections of liver tissues

The Sirius-red-stained sections of each group were observed under the microscope. As illustrated in Figure 2A-F, mice in the CCl_4 group and the medication groups

Table 2 Biochemical indices of blood and liver

Groups	HA (μg/L)	Hyp (μg/g)
C	61 ± 20.6^{bcde}	195.6 ± 10.5^{bcde}
CCl_4	157.3 ± 44.3^{acde}	503 ± 31.7^{acde}
V	70.2 ± 12.9^{b}	375.2 ± 26.9^{ab}
P	79.5 ± 11.6^{b}	361.3 ± 24.1^{ab}
V + P	65 ± 12.1^{b}	353.7 ± 21.6^{ab}

In the comparison of the mean ± standard deviation (SD) of various groups, differences are statistically significant when $P < 0.05$, with $n = 10$. In addition, a, b, c, d, and e represent statistical differences *vs* C, CCl_4, V, P, and V + P groups, respectively. C: Control; V: Vitamin; P: Puerarin.

showed larger areas of fibrosis in their liver tissues than in the C group. In addition, the areas of fibrosis of liver tissues in rats in each of the medicated groups were smaller than that of the CCl_4 group ($P < 0.05$). Among the three groups, the V + P group presented the smallest area of fibrosis.

mRNA levels of collagen I, collagen III, Wnt1, and β-catenin

RT-PCR detection was conducted to test the mRNA levels of Collagen I, Collagen III, Wnt1, and β-catenin of the rats in each group. It can be seen from Table 3 that the mRNA levels of Collagen I, Collagen III, Wnt1, and β-catenin of liver tissues of rats in the CCl_4 group and the medicated groups are all higher than those of the C group. The mRNA levels of these indices of rats in the three medicated groups were all lower than those of the CCl_4 group ($P < 0.05$). Among the medicated groups, the V + P group showed the lowest mRNA levels.

Protein levels of Wnt1 and β-catenin of liver

As the Wnt/β-catenin signalling pathway plays a significant role in the progression of hepatic fibrosis, a western blot assay was applied to detect the protein levels of Wnt1 and β-catenin in the liver. Figure 3 shows that the protein levels of Wnt1 and β-catenin in the liver tissues of rats in the CCl_4 group and the medicated groups were higher than the C group. The medicated groups showed lower protein levels than the CCl_4 group ($P < 0.05$), among which the V + P group exhibited the lowest protein levels ($P < 0.01$).

DISCUSSION

Hepatic fibrosis is the inevitable pathological change during the development of chronic liver diseases. If it is not blocked, its further development would cause liver cirrhosis and even liver cancer. Hepatic fibrosis can be induced by many pathogenic factors, including viruses, parasites, alcohol, and some poisons such as CCl_4. It is essentially a disturbance of the balance between the production and degradation of ECM outside hepatocytes. The ECM accumulates in the liver and its main component is collagen fibers[15,16]. HSCs, as the principal participants in the production and degradation of ECM, when activated, are the key link necessary for the occurrence of hepatic fibrosis. Existing in the Disse space, HSCs mainly play their roles in metabolizing and storing vitamin A in normal conditions. They can synthesize and secrete small amounts of ECM and produce collagenase. When hepatic fibrosis occurs, HSCs in their resting state are activated. Active oxygen, lipid peroxide, and molecules secreted by nearby cells, such as activated Kupffer cells, and liver sinusoidal endothelial cells, as well as damaged hepatic cells, all can facilitate the activation of resting HSCs. After activation, HSCs have different morphologies, and the changes include the secretion of alpha smooth muscle actin (α-SMA), loss of vitamin A stored in cells, and increase in the rough endoplasmic reticulum. The activation of HSCs is also accompanied by a series of changes in genetic expression, including the appearance of receptors that can respond to paracrine stimulation on the cytomembrane of activated HSCs and a series of signalling cascade reactions in cells. The presence of the signalling cascade reactions in cells is beneficial to maintaining the phenotype of activated cells and controlling the occurrence of fibrosis, the proliferation of HSCs, and the increases in the transcription and translation of Collagen I and Collagen III[17-19].

The canonical Wnt signal transduction pathway is also known as the Wnt/β-catenin signal transduction pathway. It is the pathway that has been the subject of most of the research to date. As a highly conserved signalling pathway activated during the evolution of some species, it is a basic pathway that is able to regulate cell proliferation and cell polarity, control cell fate, and maintain homeostasis in the embryo, and influence tissue development. Therefore, the pathway plays significant roles in various physiological processes including early development of animal embryos, organogenesis, and tissue regeneration, as shown in Figure 4[20]. When Wnt is deficient, β-catenin in the cytoplasm is generally degraded via Axin complexes; in addition, glycogen and casein kinase 1 (CK1) synthesize glycogen synthase kinase 3 (GSK3), which sequentially phosphorylates the amino terminus of β-catenin. As a result, β-catenin is recognized by β-Trcp and the sub-unit of E3 ubiquitin ligases and is then degraded by the ubiquitination pathway. The continuous removal of β-catenin inhibits the nuclear import of β-catenin and the combination of the protein family of T cell factor/lymphoid enhancer factor (TCF/LEF) with DNA, thus suppressing the transcription of Wnt target genes[21,22]. When Wnt ligands are bound with transmembrane Frizzled (Fz) receptors and their co-receptor–low-density lipoprotein receptor related protein 6 (LRP6) or similar LRP5, the Wnt/β-catenin signalling pathway and dishevelled Dsh/Dvl proteins in cells are activated. Consequently, the GSK-3β activity is suppressed, Axin falls off, and the formation of biodegradable composite film (mainly composed of Axin, APC, and GSK-3β) of β-catenin is inhibited. Therefore, β-catenin

Table 3 The mRNA levels of collagen I, collagen III, Wnt1, and β-catenin of liver

Groups	Collagen Ⅰ	Collagen Ⅲ	Wnt1	β-catenin
C	1.17 ± 0.16^{bcde}	0.62 ± 0.15^{bcde}	0.56 ± 0.13^{bcde}	0.48 ± 0.21^{bcde}
CCl_4	4.73 ± 0.76^{acde}	2.47 ± 1.12^{acde}	1.77 ± 0.32^{acde}	2.16 ± 0.42^{acde}
V	1.97 ± 0.31^{b}	1.29 ± 0.19^{ab}	1.06 ± 0.35^{ab}	1.25 ± 0.33^{ab}
P	1.75 ± 0.46^{b}	1.35 ± 0.17^{ab}	0.83 ± 0.19^{ab}	0.71 ± 0.46^{ab}
V + P	1.53 ± 0.12^{abcd}	1.03 ± 0.08^{abcd}	0.61 ± 0.24^{abcd}	0.59 ± 0.14^{abcd}

Differences are considered to exhibit statistical significance when $P < 0.05$ ($n = 10$) in the comparison of the mean ± standard deviation (SD) between various groups. a, b, c, d, and e indicate that there are statistical differences *vs* C, CCl_4, V, P, and V + P groups, respectively. C: Control; V: Vitamin; P: Puerarin.

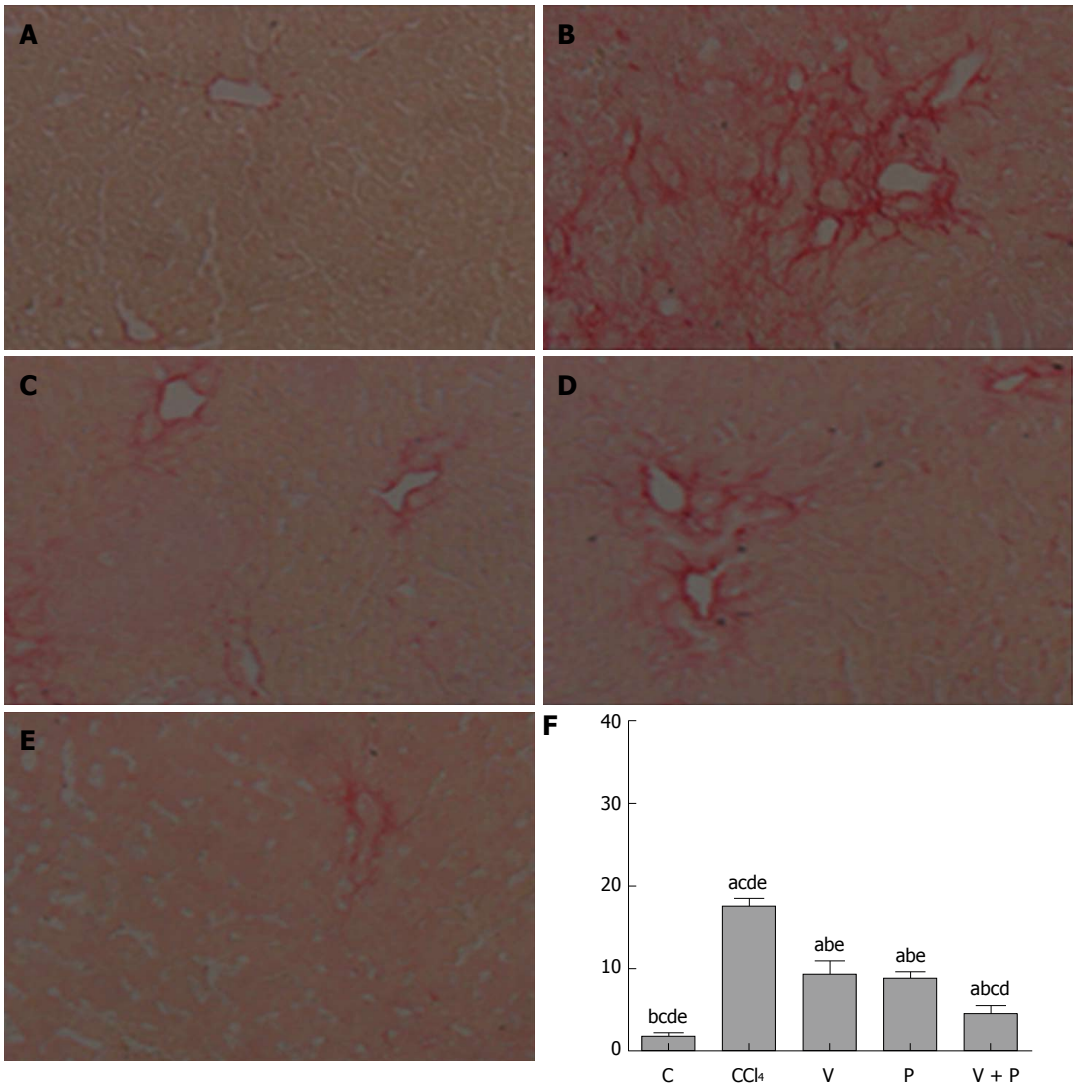


Figure 2 Sirius red staining. A: Control (C) group; B: CCl_4 group; C: Vitamin D (V) group; D: Puerarin (P) group; E: V + P group (200 × magnification); F: Statistical graph of positive Sirius-red-stained areas (in the comparison of the mean ± SD of various groups, differences are statistically significant when $P < 0.05$, $n = 10$. a, b, c, d, and e represent statistical differences *vs* C, CCl_4, V, P, and V + P groups, respectively).

will aggregate, not be recognized, and degraded *via* the ubiquitination pathway[23,24]. As β-catenin accumulates to a certain level, it dissociates. Under these conditions, β-catenin is likely to undergo nuclear translocation, thus combining with the transcription factor TCF/LEF, forming transcriptional activation complex, and finally upregulating or downregulating the expressions of some downstream genes. At present, the known target downstream genes include D1 (cyclin D1), c-myc, MMP7, survivin, CD44, and growth factor, and new target genes are constantly being discovered[25,26]. In the Wnt/β-catenin pathway, Wnt1 is the key gene participating in the aggregation and disappearance of β-catenin and is closely related to the occurrence and development of hepatic fibrosis and tumors.

The Wnt/β-catenin pathway and the activation and

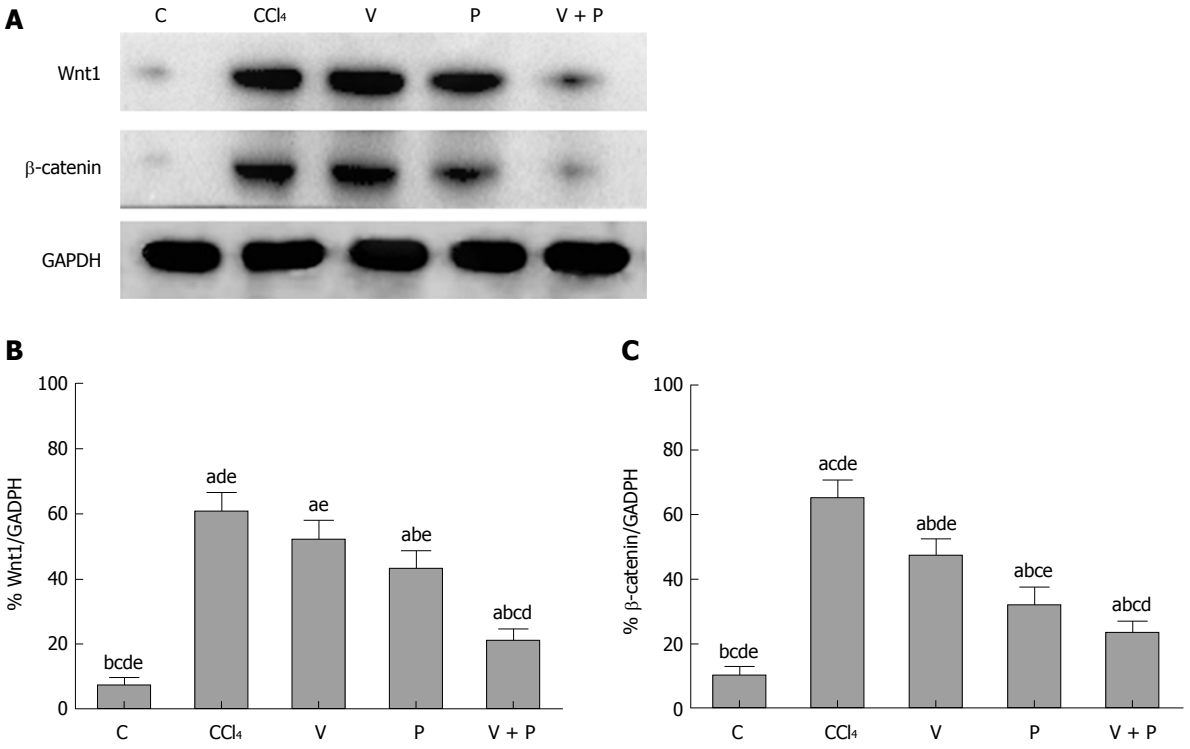


Figure 3 The protein levels of Wnt1 and β-catenin of liver tissues in rats in the CCl_4 group and the medicated groups were higher than the Control (C) group. A: Detection of protein levels of Wnt1, and β-catenin using western blot assay; B: Grey analysis of Wnt1; C: Grey analysis of β-catenin [in the comparison of mean ± standard deviation (SD) of various groups, differences are statistically significant when $P < 0.05$, $n = 10$. In addition, a, b, c, d, and e represent statistical differences *vs* C, CCl_4, V, P, and V + P groups, respectively]. C: Control; V: Vitamin; P: Puerarin.

proliferation of HSCs are therefore associated with hepatic fibrosis. The Wnt/β-catenin pathway, dominated as it is by Wnt and β-catenin, plays a significant role in regulating the activation of HSCs and the secretion of Collagen I and III. Although the mechanism *via* which the Wnt/β-catenin signalling pathway takes part in hepatic fibrosis by activating HSCs is not completely understood, activated HSCs, as well as Wnt1 and β-catenin, have become important targets in anti-hepatic fibrosis therapy.

Most of the vitamin D in the body is produced by the liver, which is also one of the target organs of vitamin D. Previous studies have revealed that vitamin D is capable of ameliorating CCl_4-induced hepatic fibrosis in mice. *In vitro* experiments have shown that this is probably because it reduces the activation of HSCs and the secretion of collagen fibers. Puerarin, as a type of flavonoid drug that is widely applied in clinical practice, is also able to alleviate CCl_4-induced hepatic fibrosis (according to the evidence of existing research): however, there is no report on the issue as to whether the Wnt/β-catenin signalling pathway can be significantly suppressed after the combined use of vitamin D and puerarin. Whether using vitamin D or puerarin alone, their protective effects on the liver during the progression of hepatic fibrosis have been found to be closely associated with their functions of suppressing HSCs. Our preliminary research revealed that the combined use of the two drugs is able to impart greater protective function, so we speculatd that the mechanism of the combined use is probably related to HSCs, Wnt, and β-catenin.

The research demonstrated that CCl_4 greatly increased the HA level in blood and Hyp level in rat liver. By observing the Sirius-red-stained specimens under a microscope, it was found that CCl_4 caused the deposition of collagen fibers in the liver of the rats. All these results indicated that the rat model of hepatic fibrosis was successfully established. Vitamin D and puerarin can both reduce the HA level in blood and Hyp level in liver of the rats, thus greatly decreasing the amount of collagen fibers in the liver. The combined use of vitamin D and puerarin most significantly reduced various damage indices and alleviated the deposition of collagen fibers, which proved that the two drugs have synergistic effects. Meanwhile, we detected the mRNA and protein levels of Collagen I, Collagen III, Wnt1, and β-catenin molecules in the liver of the rats. The results indicated that both vitamin D and puerarin are capable of decreasing the mRNA and protein levels of these molecules. Likewise, the most significant reduction effect was also observed in the V + P group. This suggested that the protective effect of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis is closely associated with the functions of silencing the Wnt1/β-catenin pathway, suppressing the activation of HSCs, and decreasing the secretion of collagen fibers.

The research investigated the protective effect of the

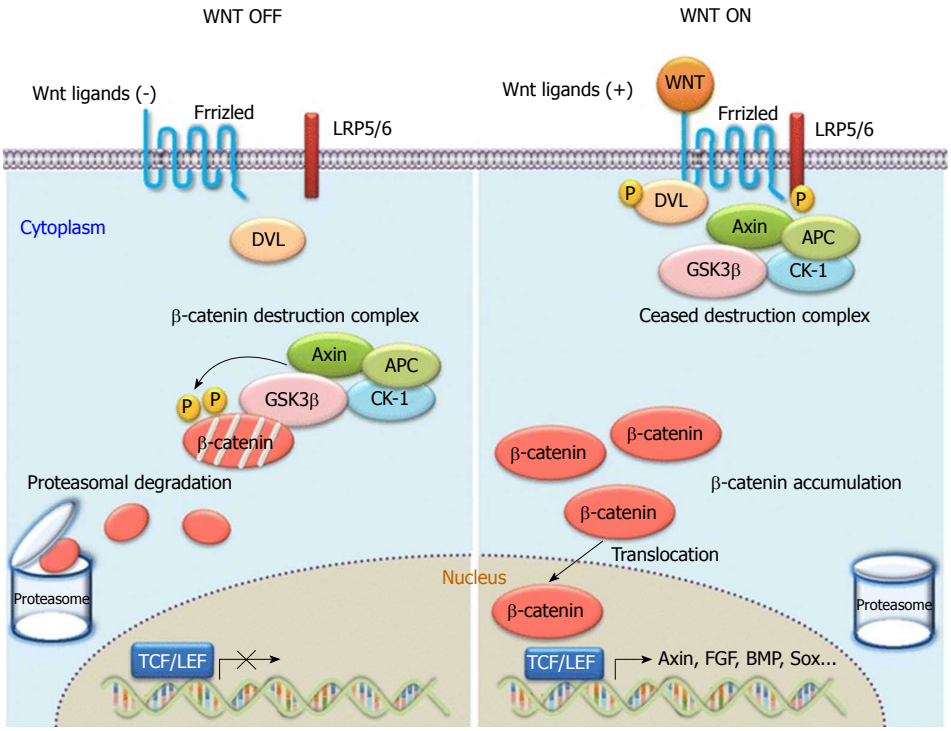


Figure 4 Overview of WNT/β-catenin signalling. Without WNT signalling ("WNT OFF"), the destruction complex phosphorylates cytosolic β-catenin, and phosphorylated β-catenin is recognized and degraded by the proteasomes. With active WNT signalling ("WNT ON"), the function of the "destruction complex" is inhibited to phosphorylate cytosolic β-catenin. Then unphosphorylated β-catenin accumulates in the cytosol, translocates into the nucleus, and activates WNT target gene expression, such as the targets of the T-cell factor and lymphoid enhancer factor-1 (TCF/LEF1) family of transcription factors.

combined use of vitamin D and puerarin against CCl_4-induced hepatic fibrosis in rats. The results revealed that the combined use of the two drugs exhibited a superior anti-hepatic fibrosis effect compared to that of each of the agents used as a single medication. It was preliminarily found that the protective mechanism of the combined use was related to the Wnt/β-catenin pathway, which provided a useful reference for those in clinical practice. However, the regulatory mechanism is complex and involves multiple factors and pathways. Other mechanisms of action warrant further investigation.

ARTICLE HIGHLIGHTS

Research background

Hepatic fibrosis is seriously endangering the safety of life. At present, there is no ideal way to treat hepatic fibrosis. The combination of vitamin D and puerarin can improve the effects of anti-hepatic fibrosis, but the mechanism is not clear. Therefore, the aim of this study is to explore the mechanism of the combined use of vitamin D and puerarin in the treatment of hepatic fibrosis, so as to improve the theoretical basis for the treatment of hepatic fibrosis.

Research motivation

The combination of vitamin D and puerarin can improve the effect of anti-hepatic fibrosis, but its mechanism is not clear. The Wnt/β-catenin pathway is closely related to liver fibrosis. In this study, we found that vitamin D and puerarin are closely related to the regulation of Wnt/β-catenin pathway in hepatic fibrosis, which provided a useful reference for those in clinical practice.

Research objectives

The research aimed to reveal the protective mechanism of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis induced by carbon tetrachloride (CCl_4).

Research methods

In this study, a Wistar rat model of hepatic fibrosis was constructed by CCl_4. Vitamin D combined with puerarin was used in the treatment of hepatic fibrosis rats, and the liver pathology and the genes related to the Wnt/β-catenin pathway were detected. The protective mechanism of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis was explored at the molecular level.

Research results

The research demonstrated that CCl_4 greatly increased the HA level in blood and Hyp level in the rat liver. CCl_4 caused the deposition of collagen fibers in the liver of the rats. Vitamin D and puerarin can both reduce the HA level in blood and Hyp level in liver of the rats, thus greatly decreasing the amount of collagen fibers in the liver. The combined use of vitamin D and puerarin most significantly reduced the various damage indices, and alleviated the deposition of collagen fibers, which proved that the two drugs had synergistic effects.

Research conclusions

The combined application of vitamin D and puerarin is capable to alleviating the CCl_4-induced hepatic fibrosis of rats.

Research perspectives

We have learned to induce an animal model of hepatic fibrosis and study the drug effects by the animal model, but also because we were not skilled enough in the experimental technique and spent more time doing repetitive work. The future research direction is to explore the prevention and treatment measures of hepatic fibrosis. The drug targets will be studied by transcriptome, proteomics and other methods.

REFERENCES

1 **Ding BS**, Cao Z, Lis R, Nolan DJ, Guo P, Simons M, Penfold ME,

Shido K, Rabbany SY, Rafii S. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature* 2014; **505**: 97-102 [PMID: 24256728 DOI: 10.1038/nature12681]
2 **Friedman SL**. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250 [PMID: 10644669 DOI: 10.1074/jbc.275.4.2247]
3 **Ge WS**, Wang YJ, Wu JX, Fan JG, Chen YW, Zhu L. β-catenin is overexpressed in hepatic fibrosis and blockage of Wnt/β-catenin signaling inhibits hepatic stellate cell activation. *Mol Med Rep* 2014; **9**: 2145-2151 [PMID: 24691643 DOI: 10.3892/mmr.2014.2099]
4 **Li W**, Zhu C, Li Y, Wu Q, Gao R. Mest attenuates CCl4-induced liver fibrosis in rats by inhibiting the Wnt/β-catenin signaling pathway. *Gut Liver* 2014; **8**: 282-291 [PMID: 24827625 DOI: 10.5009/gnl.2014.8.3.282]
5 **Barrott JJ**, Cash GM, Smith AP, Barrow JR, Murtaugh LC. Deletion of mouse Porcn blocks Wnt ligand secretion and reveals an ectodermal etiology of human focal dermal hypoplasia/Goltz syndrome. *Proc Natl Acad Sci USA* 2011; **108**: 12752-12757 [PMID: 21768372 DOI: 10.1073/pnas.1006437108]
6 **Qian J**, Niu M, Zhai X, Zhou Q, Zhou Y. β-catenin pathway is required for TGF-β1 inhibition of PPARγ expression in cultured hepatic stellate cells. *Pharmacol Res* 2012; **66**: 219-225 [PMID: 22706027 DOI: 10.1016/j.phrs.2012.06.003]
7 **Nejak-Bowen K**, Kikuchi A, Monga SP. Beta-catenin-NF-κB interactions in murine hepatocytes: a complex to die for. *Hepatology* 2013; **57**: 763-774 [PMID: 22941935 DOI: 10.1002/hep.26042]
8 **Ma ZG**, Lv XD, Zhan LL, Chen L, Zou QY, Xiang JQ, Qin JL, Zhang WW, Zeng ZJ, Jin H, Jiang HX, Lv XP. Human urokinase-type plasminogen activator gene-modified bone marrow-derived mesenchymal stem cells attenuate liver fibrosis in rats by down-regulating the Wnt signaling pathway. *World J Gastroenterol* 2016; **22**: 2092-2103 [PMID: 26877613 DOI: 10.3748/wjg.v22.i6.2092]
9 **Abramovitch S**, Dahan-Bachar L, Sharvit E, Weisman Y, Ben Tov A, Brazowski E, Reif S. Vitamin D inhibits proliferation and profibrotic marker expression in hepatic stellate cells and decreases thioacetamide-induced liver fibrosis in rats. *Gut* 2011; **60**: 1728-1737 [PMID: 21816960 DOI: 10.1136/gut.2010.234666]
10 **Ding N**, Yu RT, Subramaniam N, Sherman MH, Wilson C, Rao R, Leblanc M, Coulter S, He M, Scott C, Lau SL, Atkins AR, Barish GD, Gunton JE, Liddle C, Downes M, Evans RM. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 2013; **153**: 601-613 [PMID: 23622244 DOI: 10.1016/j.cell.2013.03.028]
11 **Li R**, Xu L, Liang T, Li Y, Zhang S, Duan X. Puerarin mediates hepatoprotection against CCl4-induced hepatic fibrosis rats through attenuation of inflammation response and amelioration of metabolic function. *Food Chem Toxicol* 2013; **52**: 69-75 [PMID: 23146695 DOI: 10.1016/j.fct.2012.10.059]
12 **Kaji K**, Yoshiji H, Ikenaka Y, Noguchi R, Aihara Y, Douhara A, Moriya K, Kawaratani H, Shirai Y, Yoshii J, Yanase K, Kitade M, Namisaki T, Fukui H. Dipeptidyl peptidase-4 inhibitor attenuates hepatic fibrosis via suppression of activated hepatic stellate cell in rats. *J Gastroenterol* 2014; **49**: 481-491 [PMID: 23475323 DOI: 10.1007/s00535-013-0783-4]
13 **Lei X**, Wang YL, Tu-er-xun AL, Zhang H, Zhang RL, Yang Y, Wu G, Cheng W. Correlation between radiation-induced hepatic fibrosis and serum levels of hyaluronic acid, type IV collagen and laminin in rats. *Zhonghua Quanke Yixue* 2015, **13**: 18-20
14 **Wang ZL**, Liu ZJ, Song T, Sun XH, Luo WM, Liu S. Effects of FuzhengHuoxue recipes on liver fibrosis associated serum indicators and hepatic hydroxyproline in liver fibrosis rats. *Zhongyao Xinyao Yu Linchuang Yaoli* 2010, **21**: 611-613
15 **Lu J**, Chen B, Li S, Sun Q. Tryptase inhibitor APC 366 prevents hepatic fibrosis by inhibiting collagen synthesis induced by tryptase/protease-activated receptor 2 interactions in hepatic stellate cells. *Int Immunopharmacol* 2014; **20**: 352-357 [PMID: 24735816 DOI: 10.1016/j.intimp.2014.04.001]
16 **Wang Y**, Zhao Z, Yan Y, Qiang X, Zhou C, Li R, Chen H, Zhang Y. Demethyleneberberine Protects against Hepatic Fibrosis in Mice by Modulating NF-κB Signaling. *Int J Mol Sci* 2016; **17**: [PMID: 27376272 DOI: 10.3390/ijms17071036]
17 **Schuppan D**, Kim YO. Evolving therapies for liver fibrosis. *J Clin Invest* 2013; **123**: 1887-1901 [PMID: 23635787 DOI: 10.1172/jci66028]
18 **Friedman SL**. Hepatic fibrosis -- overview. *Toxicology* 2008; **254**: 120-129 [PMID: 18662740]
19 **Atzori L**, Poli G, Perra A. Hepatic stellate cell: a star cell in the liver. *Int J Biochem Cell Biol* 2009; **41**: 1639-1642 [PMID: 19433304 DOI: 10.1016/j.biocel.2009.03.001]
20 **Ota C**, Baarsma HA, Wagner DE, Hilgendorff A, Königshoff M. Linking bronchopulmonary dysplasia to adult chronic lung diseases: role of WNT signaling. *Mol Cell Pediatr* 2016; **3**: 34 [PMID: 27718180 DOI: 10.1186/s40348-016-0062-6]
21 **Qi B**, Wang Y, Chen ZJ, Li XN, Qi Y, Yang Y, Cui GH, Guo HZ, Li WH, Zhao S. Down-regulation of miR-30a-3p/5p promotes esophageal squamous cell carcinoma cell proliferation by activating the Wnt signaling pathway. *World J Gastroenterol* 2017; **23**: 7965-7977 [PMID: 29259372 DOI: 10.3748/wjg.v23.i45.7965]
22 **Wang D**, Lu G, Shao Y, Xu D. MiR-182 promotes prostate cancer progression through activating Wnt/β-catenin signal pathway. *Biomed Pharmacother* 2018; **99**: 334-339 [PMID: 29353209 DOI: 10.1016/j.biopha.2018.01.082]
23 **Wang C**, Zhu H, Sun Z, Xiang Z, Ge Y, Ni C, Luo Z, Qian W, Han X. Inhibition of Wnt/β-catenin signaling promotes epithelial differentiation of mesenchymal stem cells and repairs bleomycin-induced lung injury. *Am J Physiol Cell Physiol* 2014; **307**: C234-C244 [PMID: 24898581 DOI: 10.1152/ajpcell.00366.2013]
24 **Dijksterhuis JP**, Petersen J, Schulte G. WNT/Frizzled signalling: receptor-ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3. *Br J Pharmacol* 2014; **171**: 1195-1209 [PMID: 24032637 DOI: 10.1111/bph.12364]
25 **Kim JT**, Liu C, Zaytseva YY, Weiss HL, Townsend CM Jr, Evers BM. Neurotensin, a novel target of Wnt/β-catenin pathway, promotes growth of neuroendocrine tumor cells. *Int J Cancer* 2015; **136**: 1475-1481 [PMID: 25098665 DOI: 10.1002/ijc.29123]
26 **Gibbons GS**, Owens SR, Fearon ER, Nikolovska-Coleska Z. Regulation of Wnt signaling target gene expression by the histone methyltransferase DOT1L. *ACS Chem Biol* 2015; **10**: 109-114 [PMID: 25361163 DOI: 10.1021/cb500668u]

P- Reviewer: Facciorusso A, Musquer N, Shin T **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4186-4196

DOI: 10.3748/wjg.v24.i36.4186

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

ORIGINAL ARTICLE

Retrospective Study

Frequency, types, and treatment of anemia in Turkish patients with inflammatory bowel disease

Göksel Bengi, Hatice Keyvan, Seda Bayrak Durmaz, Hale Akpınar

Göksel Bengi, Hale Akpınar, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Dokuz Eylül University, İzmir 35360, Turkey

Hatice Keyvan, Seda Bayrak Durmaz, Department of Internal Medicine, Faculty of Medicine, Dokuz Eylül University, İzmir 35360, Turkey

ORCID number: Göksel Bengi (0000-0002-7859-4094); Hatice Keyvan (0000-0002-7032-6124); Seda Bayrak Durmaz (0000-0001-8272-5686); Hale Akpınar (0000-0002-8734-2518).

Author contributions: Bengi G, Keyvan H, Durmaz SB, and Akpınar H contributed equally to this work, designed the research, drafted the manuscript, and provided administrative and technical support.

Institutional review board statement: This study was approved by the Dokuz Eylul University Ethics Committee in June 2017.

Informed consent statement: Informed consent was provided by all participants.

Conflict-of-interest statement: The authors have declared no conflicts of interest.

Manuscript source: Unsolicited manuscript

Correspondence to: Göksel Bengi, MD, PhD, Assistant Professor, Department of Gastroenterology, Dokuz Eylul University Hospital, İnciraltı, İzmir 35000, Turkey. goksel.bengi@deu.edu.tr
Telephone: +90-532-4626972
Fax: +90-232-4123799

Received: May 2, 2018
Peer-review started: May 2, 2018
First decision: June 6, 2018
Revised: July 30, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 28, 2018

Abstract

AIM

To specify the type and prevalence of anemia along with a treatment approach for inflammatory bowel disease (IBD).

METHODS

We conducted a retrospective study on 465 patients who were diagnosed with IBD and followed up at our hospital from June 2015 to June 2016 [male: 254, female: 211; average age: 47 ± 14.4; Crohn's disease (CD): 257, Ulcerative Colitis (UC): 208]. Epidemiological and clinical data, such as sex, age, age of diagnosis, type of IBD, disease extension, disease behavior and duration, treatments for IBD and anemia, and surgical history were obtained for each patient. Per World Health Organization guidelines, anemia was diagnosed for males if hemoglobin values were less than 13 g/dL and for females if hemoglobin values were less than 12 g/dL.

RESULTS

We determined that 51.6% of the patients had anemia, which was more frequent in women then men (64% *vs* 41.3%, $P < 0.001$). Anemia frequency was higher in CD cases (57.6%) than in UC cases (44.2%) ($P = 0.004$). CD involvements were as follows: 48.2% in ileal involvement, 19% in colonic involvement, and 32.8% in ileocolonic involvement. Furthermore, 27.5% of UC patients had proctitis (E1) involvement, 41% of

them had involvement in left colitis (E2), and 31.5% had pancolitis involvement. There was no significant relationship between anemia frequency and duration of disease (P = 0.55). Iron deficiency anemia (IDA) was the most common type of anemia in this cohort. Moreover, because anemia parameters have not been evaluated during follow-up of 15.3% of patients, the etiology of anemia has not been clarified. Fifty percent of patients with anemia received treatment. Twenty-three percent of IDA patients had oral iron intake and forty-one percent of IDA patients had parenteral iron treatment. Fifty-three percent of patients who were suffering from megaloblastic anemia received B_{12}/folic acid treatment.

CONCLUSION

We found out that almost half of all IBD patients (51.6%) had anemia, the most frequent of which was IDA. Almost half of these patients received treatment. We should increase the treatment rate in our IBD patients that have anemia.

Key words: Anemia; Inflammatory bowel disease; Anemia of iron deficiency

Core tip: We conducted a retrospective study on 465 patients, who were diagnosed with inflammatory bowel disease (IBD). We determined that 51.6% of patients had anemia, which was more frequent in women then men. Anemia frequency was higher in Crohn's disease cases than in ulcerative colitis cases. No relation has been found between the presence of anemia and disease duration. Iron deficiency anemia was the most common type of anemia. The factors that are related to anemia among IBD patients are being female, drug therapy (corticosteroids, AZA/MTX, Anti-TNF), and high C-reactive protein levels. Fifty percent of patients with anemia received treatment.

Bengi G, Keyvan H, Durmaz SB, Akpınar H. Frequency, types and treatment of anemia in Turkish patients with inflammatory bowel disease. *World J Gastroenterol* 2018; 24(36): 4186-4196 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4186.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4186

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic idiopathic disease with a relapsing and remitting course. There are two major types of IBD, including Crohn's disease (CD) and ulcerative colitis (UC). The most common extraintestinal finding seen in IBD patients is anemia, which decreases both the quality of life and the ability to work. Fatigue and weakness are the most common complaints reported in IBD-related anemia. Moreover, IBD-related anemia has been associated with frequent hospitalizations, late hospital discharge, increased health expenditures, co-morbidity for other diseases [*e.g.*, transfusion-related Hepatitis C virus (HCV), *etc.*], and most importantly, a significant increase in the risk of mortality[1].

The prevalence of anemia is higher in IBD patients than in the general population, and ranges between 6% and 74%[2,3]. According to a review of 22 articles[2], the mean prevalence of anemia among IBD patients is 17% (95%CI: 16-18). In a meta-analysis[4], the prevalence of anemia in 2192 IBD patients was reported as 24% (27% in CD patients and 21% in UC patients). A recent study by Koutroubakis *et al*[5] including 1821 patients (1077 CD, 744 UC) reported the prevalence of anemia as 50.1% (CD: 53.3%, UC: 44.7%). The first study to report on the incidence of anemia in Turkish patients with IBD reported that 58.2% of 941 patients (62.1% of 375 CD patients and 55.7% of 566 UC patients) had anemia at least once during an 18-year follow-up period[6].

The most common causes of anemia in IBD are iron deficiency anemia (IDA) and chronic disease anemia (CDA)[6-10]. Other causes of anemia in IBD include macrocytic anemias (such as vitamin B_{12} deficiency and/or folate deficiency), hemolytic anemia, and drug-related bone marrow suppression.

Although IBD-related anemia has a relatively high prevalence, its diagnosis and treatment is generally overlooked. Iron therapy is recommended for all patients with IDA-related IBD, and the treatment should aim to return the patient to normal hemoglobin levels and provide adequate iron storage[11]. Recently, intravenous iron therapy has been recommended for the treatment of IDA-related IBD[12]. This is because intravenous iron treatment exerts its effects quickly, particularly among those who have active disease, have been intolerant to previous oral iron therapy, have severe anemia (Hb < 10 g/dL), and require erythropoiesis-stimulating agents[13].

The current study aimed to determine the frequency and types of anemia in IBD patients, to determine the relationship between anemia and disease characteristics, and to determine the most effective treatment approach.

MATERIALS AND METHODS

Study design and data collection

Patients: This study retrospectively evaluated 465 patients who were diagnosed with IBD and followed-up between June 2015 and June 2016 in the Gastroenterology/IBD outpatient clinic or ward of Dokuz Eylül University, Medical Faculty Hospital.

The IBD diagnoses were made in accordance with the new European Crohn's and Colitis Organization (ECCO) guidelines, and were confirmed according to standard clinical, endoscopic, histologic, and radiological criteria[14,15]. In order to obtain epidemiological and

Table 1 Types of anemia according to iron parameters

	Ferritin (ng/mL)	Transferrin saturation (%)	CRP (5 mg/L)
IDA	< 30	< 20	-
CDA	> 100	< 20	> 5
Mixed anemia (IDA + CDA)	> 30 and < 100	< 20	

IDA: Iron deficiency anemia; CDA: Chronic disease anemia; CRP: C reactive protein.

clinical data, the following data were recorded for each patient: sex, age, age at diagnosis, type of IBD, disease extension, disease behavior and duration, treatments for IBD and anemia, and surgical history. Patients were excluded from this study if they had indeterminate colitis, were pregnant, were monitored for less than one year, or had diseases such as chronic renal insufficiency, gastrectomy, hematological diseases, *etc*. Demographic and clinical data as well as endoscopic activities were obtained from hospital records.

Definition of anemia: We used the World Health Organization guidelines to diagnose anemia in our IBD patients. Males were diagnosed with anemia if they had hemoglobin values less than 13 g/dL, and females were diagnosed if they had hemoglobin values less than 12 g/dL[16]. Severe anemia was defined as having Hb values below 10 g/dL for both sexes. We evaluated the lowest hemoglobin levels of each patient during follow-up, as well as iron levels and other anemia parameters. The following were obtained from each patient's laboratory records: hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), serum ferritin, serum iron level, transferrin saturation (TS), serum iron binding capacity (SIBC), folic acid, vitamin B_{12}, CRP, and albumin.

Three main classifications of anemia were selected in accordance with the European consensus on anemia in IBD, including IDA, CDA, and mixed anemia[17] (Table 1). Aside from these, other causes of anemia were determined by examining the peripheral smear, and in cases with suspected macrocytic anemia, vitamin B_{12} and folic acid levels were evaluated. Additionally, medications that may cause macrocytic anemia (*i.e.*, thiopurines and sulfasalazine) were also taken into account. In cases with suspected hemolytic anemia, reticulocyte ratio and haptoglobulin levels were evaluated.

Statistical analysis

In our current study, variables indicated by census are presented as percentage distributions, and variables indicated by measurements are presented as means and standard deviations. In univariate analyses, Chi Square and Fisher's exact test were used to compare the variables indicated by census. The variables indicated by measurement were compared by Student's *t*-test. The logistic regression model was used in multivariate analysis. Values of $P < 0.05$ were considered significant. All analyses were made using the Statistical package for the Social Sciences (SPSS) (version 22.0; SPSS Inc., Chicago, IL, United States).

Ethical considerations

This study was approved by Dokuz Eylül University School of Medicine's non-invasive clinical research ethics committee (15.06.2017 3387-GOA). Patient information was kept confidential, and the study was conducted according to the Helsinki declaration.

RESULTS

Patient characteristics

This study included the data from 465 IBD patients [254 male (54.6%) and 211 female (45.4%)] who were newly diagnosed or were being followed-up with in our hospital. Of these patients, 55.3% were diagnosed with CD and 44.7% with UC. The mean age at IBD diagnosis was 40.2 ± 13.9 years (39.8 ± 13.7 years in CD patients, 40.7 ± 14.6 years in UC patients, $P = 0.46$). The patient characteristics are presented in Table 2. There was no significant difference between mean disease duration of CD (6.45 years) and UC (7.36 years) ($P = 0.07$).

Among patients with CD, 48.2% had ileal involvement (L1), 19% had colonic involvement (L2), and 32.8% had ileocolonic involvement (L3). Isolated upper GI involvement (L4) was not observed in any of the patients. In terms of CD behavior, 60.9% of patients had inflammatory type CD (B1), 16.5% had structuring type CD (B2), and 22.6% had penetrating type CD (B3).

In patients with UC, 27.5% had proctitis involvement (E1), 41% had left colitis involvement (E2), and 31.5% had pancolitis involvement (Table 2).

Frequency and type of anemia among IBD patients

In our current study, 51.6% ($n = 240$) of the patients had anemia. Anemia frequency was higher in CD (57.6%) than in UC (44.2%) ($P = 0.004$). The mean hemoglobin concentration was 12.3 g/dL in IBD patients, and was significantly lower in those with CD (12.1 g/dL) than in those with UC (12.5 g/dL) ($P = 0.03$). The frequency of anemia and hematological profiles at the time of IBD diagnosis is shown in Table 3.

Anemia was more common among women than men (64% *vs* 41.3%, $P < 0.001$) (Table 4). Severe anemia (Hb < 10 g/dL) was observed in 21.6% of patients with CD and 9.8% of patients with UC (*P*

Table 2 Demographic data of inflammatory bowel disease patients *n* (%)

	IBD overall	Crohn's disease	Ulcerative colitis	*P* value
n	465	257 (55.3)	208 (44.7)	
Age (yr) mean (SD; range)	47.1 (14.3; 18-83)	46.2 (13.4; 17-78)	48 (15.3; 12-83)	0.183
Sex (male/female)	254 (54.6)/211 (45.4)	153 (59.5)/104 (40.5)	101 (48.6)/107 (51.4)	0.019
Disease characteristics				
Age at diagnosis (yr)	40.2	39.7	40.7	0.46
Location of the disease				
L1		122 (48.2)		
L2		48 (19)		
L3		83 (32.8)		
L4		-		
Disease behavior				
B1		148 (60.9)		
B2		40 (16.5)		
B3		55 (22.6)		
Disease extension				
E1			49 (27.5)	
E2			73 (41)	
E3			56 (31.5)	
Drugs				
SZP/5-ASA	360 (77.4)	171 (66.5)	189 (90.6)	< 0.001
Corticosteroids	55 (11.8)	30 (11.7)	25 (12)	0.90
Budenoside	17 (3.7)	15 (5.8)	2 (1)	0.005
AZA / MTX	200 (43)	155 (60.3)	45 (21.6)	< 0.001
IFX/ADA	81 (17.4)	72 (28)	9 (4.3)	< 0.001
Antibiotic	92 (19.8)	68 (26.5)	24 (11.5)	< 0.001
Surgery	70 (15.1)	60 (23.3)	10 (4.8)	< 0.001

IBD: Inflammatory bowel disease; L: Disease location (for Crohn's disease); L1: Ileal disease; L2: Colonic disease; L3: Ileocolonic disease; L4: Upper gastrointestinal tract disease; B: Disease behavior (for Crohn's disease); B1: Inflammatory disease; B2: Stricturing disease; B3: Penetrating disease; E: Disease extension (for ulcerative colitis); E1: Ulcerative proctitis; E2: Left-sided ulcerative colitis; E3: Extensive disease; SZP: Sulphasalazinep; 5-ASA: 5-aminosalicyclate; AZA: Azathiopurine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab.

Table 3 Prevalence of anemia and hematological profile at the time of diagnosis in patients with inflammatory bowel disease *n* (%)

	IBD overall	Crohn's disease	Ulcerative colitis	*P* value
Anemia	240 (51.6)	148 (57.6)	92 (44.2)	0.005
Severe anemia	64 (16.4)	47 (21.6)	17 (9.8)	0.002
Hemoglobin (g/dL) mean (SD; range)	12.3 (2.1; 5.3-17.9)	12.1 (2.2; 5.3-16.8)	12.5 (1.9; 7.1-17.9)	0.03
Hematocrit (%) mean (SD; range)	37.41 (6.1; 10-53)	36.8 (6.6; 10-52)	38.16 (5.3; 22-53)	0.02
MCV (fL) mean (SD; range)	84.54 (7.7; 57-112)	84.75 (8.3; 58-112)	84.28 (7; 57-100)	0.46
Iron (μg/dL) mean (SD; range)	43.76 (30.3; 4-160)	43.26 (30.4; 4-126)	44.5 (30; 4-160)	0.73
Ferritin (μg/dL) mean (SD; range)	40.3 (74; 2-754)	48.96 (91; 2-754)	27.26 (37; 2-167)	0.01
TS (%) mean (SD; range)	15.35 (13; 2-100)	15.14 (12.7; 2-100)	15.67 (12; 2-92)	0.9
CRP (mg/L) mean (SD; range)	13 (29; 1-289)	17.1 (32.8; 0-289)	7.9 (7.93; 1-242)	0.001
Albumin mean (SD; range)	4.2 (0.4; 1.9-5.0)	4.1 (0.4; 1.9-4.9)	4.1 (0.4; 2.0-5.0)	0.17

MCV: Mean corpuscular volume; TS: Transferrin saturation; CRP: C reactive protein.

= 0.002). No relationship was found between the presence of anemia and disease duration (P = 0.55).

IDA was the most common type of anemia (29.9%). The frequencies of CDA and mixed anemia (IDA + CDA) were 8% and 3.4%, respectively. In addition, vitamin B_{12}/folic acid deficiency anemia was observed in 14% of the patients (Figure 1). Moreover, since anemia parameters were not evaluated during the follow-up of 15.3% of our IBD patients, the etiology of anemia was not clarified in these patients.

Factors related to anemia among IBD patients

Results of the current study indicate that anemia in IBD patients is more common among women than men, regardless of disease type or age. While elevated CRP levels (> 5 mg/L) are indicative of active disease, CD and UC patients with CRP levels > 5 mg/L had significantly higher rates of anemia compared to those with lower levels of CRP (70.4% *vs* 45.8% for CD patients, P < 0.001, and 54.7% *vs* 36.8% for UC patients, P = 0.006). The factors related to anemia in IBD are summarized in Table 5. Data from this study indicate that disease type and duration do not have a significant effect on the frequency of anemia in CD.

Corticosteroid users had a higher frequency of anemia than non-users (80% *vs* 47.8%, P < 0.001).

Table 4 Evaluation of disease characteristics according to types of anemia in inflammatory bowel disease *n* (%)

	Anemia	IDA	CDA	Mixed anemia	B12/Folic acid anemia	Other anemia
Sex						
M	105 (41.3)	48 (18.9)	18 (7.1)	8 (3.8)	34 (13.4)	35 (13.8)
F	135 (64)	91 (43.1)	19 (9)	8 (3.1)	31 (14.6)	36 (17.1)
P value	< 0.001	< 0.001	0.44	0.7	0.6	0.32
Disease duration (yr)						
A+	6.6	6.7	6.1	5.9	5.9	6.8
A -	7.1	6.9	6.9	6.9	7	6.9
P value	0.55	0.45	0.31		0.14	0.36
Disease type						
CD	148 (57.6)	84 (32.7)	31 (12.1)	13 (5.1)	50 (19.4)	39 (15.2)
UC	92 (44.2)	55 (26.4)	6 (2.9)	3 (1.4)	15 (7.2)	32 (15.4)
P value	0.004	0.14	< 0.001	0.03	0.001	0.95
Disease localization						
L1	66 (54.1)	34 (27.9)	13 (10.7)	5 (4.1)	25 (20.5)	19 (15.6)
L2	29 (60.4)	17 (35.4)	8 (16.7)	1 (2.1)	3 (6.3)	9 (18.8)
L3	50 (60.2)	31 (37.3)	9 (13.8)	6 (7.2)	22 (26.5)	10 (12)
P value	0.6	0.32	0.51		0.02	0.56
Disease behavior						
B1	90 (60.8)	53 (35.8)	18 (12.2)	8 (5.4)	23 (15.6)	25 (16.9)
B2	27 (67.5)	12 (30)	5 (12.5)	1 (2.5)	14 (35)	6 (15)
B3	26 (47.3)	16 (29.1)	8 (14.5)	4 (7.3)	12 (21.8)	6 (10.9)
P value	0.1	0.59	0.9		0.02	0.57
Disease extension						
E1	16 (32.7)	6 (12.2)	1 (2)	0	2 (4.1)	9 (18.4)
E2	32 (48.8)	19 (26)	2 (2.7)	2 (2.7)	6 (8.2)	12 (16.4)
E3	35 (62.5)	26 (46.4)	3 (5.4)	1 (1.8)	6 (10.7)	6 (10.7)
P value	0.008	< 0.001				0.51
Corticosteroid use						
+	44 (80)	28 (50.9)	10 (18.2)	4 (7.3)	15 (27.3)	10 (18.2)
None	196 (47.8)	111 (27.1)	27 (6.6)	12 (2.9)	50 (12.2)	61 (14.9)
P value	< 0.001	< 0.001	0.003	0.097	0.003	0.52
AZA/MTX use						
+	129 (64.5)	73 (36.5)	25 (12.5)	11 (5.5)	45 (22.5)	36 (18)
None	111 (41.9)	66 (24.5)	12 (4.5)	5 (1.9)	20 (7.5)	35 (13.2)
P value	< 0.001	0.007	0.002	0.03	< 0.001	0.15
Anti-TNF use						
+	56 (69.1)	38 (46.9)	13 (16)	7 (8.6)	21 (26)	11 (13.6)
None	184 (47.9)	101 (26.3)	24 (6.3)	9 (2.3)	44 (11.5)	60 (15.6)
P value	0.001	< 0.001	0.003	0.01	0.002	0.64
Surgery						
+	44 (62.9)	23 (32.9)	11 (15.7)	5 (7.19)	15 (21.4)	14 (20)
None	196 (49.6)	116 (29.4)	26 (6.6)	11 (2.8)	50 (12.6)	57 (14.4)
P value	0.041	0.55	0.009	0.07	0.55	0.23
CRP (mg/L)						
< 5	109 (41.3)	70 (26.5)	7 (2.7)	3 (1.1)	31 (11.8)	29 (11)
> 5	120 (65.6)	63 (34.4)	29 (15.8)	12 (6.6)	30 (16.4)	39 (21.3)
P value	0.001	0.07	0.001	0.002	0.37	0.003

M: Male; F: Female; A+: Anemia present; A-: Anemia absent; IBD: Inflammatory bowel disease; L: Disease location (for Crohn's disease); L1: Ileal disease; L2: Colonic disease; L3: Ileocolonic disease; L4: Upper gastrointestinal tract disease; B: Disease behavior (for Crohn's disease); B1: Inflammatory disease; B2: Stricturing disease; B3: Penetrating disease; E: Disease extension (for ulcerative colitis); E1: Ulcerative proctitis; E2: Left-sided ulcerative colitis; E3: Extensive disease; SZP: Sulphasalazine; 5-ASA: 5-aminosalicyclate; AZA: Azathiopurine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab.

Further, those using immunomodulator therapy (such as AZA and MTX) also had higher rates of anemia than non-users (64.5% *vs* 41.9%, $P < 0.001$). Vitamin B12/folic acid deficiency anemia was the most common type of anemia in this immunomodulator users group. On the other hand, anemia rates were significantly higher among patients who did not use anti-TNF, compared to those who did use anti-TNF (69.1% *vs* 30.9%, $P = 0.001$). Further, those who had undergone previous surgeries had higher rates of anemia than those who did not (62.9 % *vs* 37.1%, $P = 0.04$).

In UC patients, left colon involvement and pancolitis involvement were associated with a higher incidence of anemia than proctitis involvement. Also in the UC patients, as disease duration increased, the incidence of anemia significantly increased ($P = 0.008$) (Table 4). IDA is significantly more prevalent in UC with pancolitis involvement compared to other types of disease involvement ($P < 0.001$).

Anemia management in patients with IBD

Approximately 50.4% of all of the IBD patients who

Table 5 Factors associated with anemia by logistic regression in patients with Crohn's disease and ulcerative colitis at the diagnosis

	Odds ratio	95%CI	*P* value
All IBD patients			
Sex (female)	3.19	2.07-4.91	< 0.001
Corticosteroids	3.21	1.52-6.80	0.002
AZA/MTX	2.28	1.48-3.49	< 0.001
Anti-TNF (INF/ADA)	2.32	1.30-4.11	0.004
Elevated CRP	2.75	1.78-4.24	< 0.001
Crohn's disease			
Sex (female)	4.10	2.19-7.68	< 0.001
Corticosteroids	4.06	1.27-12.96	0.018
AZA/MTX	2.30	1.26-4.20	0.006
Anti-TNF (INF/ADA)	2.34	1.20-4.53	0.012
Elevated CRP	3.05	1.67-5.58	< 0.001
Ulcerative colitis			
Sex (female)	3.12	1.58-6.18	0.001
Corticosteroids	3.62	1.22-10.67	0.02
Elevated CRP	2.03	0.96-4.26	0.062
Disease extension	3.79	1.51-9.55	0.005

IBD: Inflammatory bowel disease; E: Disease extension (for ulcerative colitis); E1: Ulcerative proctitis; E2: Left-sided ulcerative colitis; E3: Extensive disease; AZA: Azathiopurine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab.

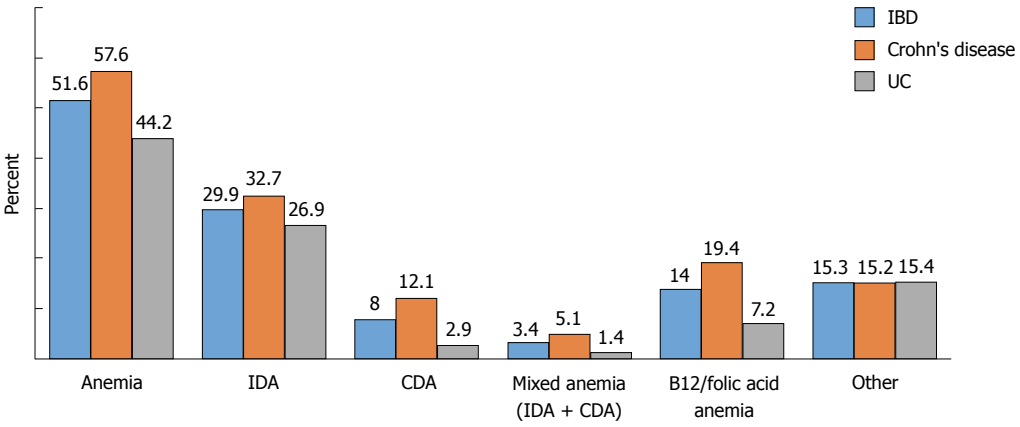


Figure 1 Types of anemia seen in inflammatory bowel disease. IBD: Inflammatory bowel disease; IDA: Iron deficiency anemia; CDA: Chronic disease anemia; UC: Ulcerative colitis.

were diagnosed with anemia received treatment. Of the patients with IDA, 23% received oral iron therapy and 40.3% received parenteral iron preparations. Of those with B_{12}/folic acid anemia, 53.3% received B_{12}/folic acid treatment. None of the patients in the current study received blood transfusions or were given erythropoiesis stimulant agents.

DISCUSSION

Anemia is the most common extraintestinal finding in IBD. While anemia significantly impairs quality of life, the majority of IBD patients with anemia are not aware that some of their symptoms and/or complaints may be related to their anemia. In our current study, 52% of the IBD patients had anemia, and the rate of anemia was higher in CD patients compared to UC patients. These results are similar to those of previous studies[4,5,18,19]. In their population-based study including 756 patients (235 CD and 519 UC), Hoivik *et al*[18] found that 48.8% of CD patients and 20.2% of UC patients were diagnosed with anemia. Another population-based study[6] reported that among 749 IBD patients, 30% had anemia, and the rate was higher in CD patients (42%) compared to UC patients (24%). Another study conducted in Spain[19] revealed a similar incidence of anemia in IBD (41.2%). Half of the CD patients had anemia, and only one third of the UC patients were anemic. In a study conducted in Turkey[20], the anemia rate was found to be 22% in UC and 24% in CD among 398 patients with IBD. The higher rate of anemia observed repeatedly in CD may be explained not only by increased bleeding, but also by additional mechanisms such as systemic inflammation (which can be more severe in CD) and decreased iron absorption (due to involvement of the proximal gastrointestinal tract). However, it is important to note that some studies have reported no difference in anemia rates between IBD sub-types[21].

In our patient population, the severe anemia (Hb < 10 g/dL) rate was significantly higher in CD patients

compared to UC patients. However, Lucendo *et al*[19] reported a lower rate of severe anemia, and that the rate of severe anemia between CD and UC was not significantly different. Another study conducted in Korea reported that the anemia rate in UC was 36.3% (similar to that of Western countries), while the rate in CD patients was 41.6%; however, the severe anemia rates between CD and UC were not significantly different[22].

While the underlying cause of anemia in IBD patients is most likely multi-factorial, the most common causes are IDA and CDA. Multivariate analyses from published studies have repeatedly shown that being female[5,23,24] and disease activity[18,25] are the most important determining factors that increase the frequency of anemia in both UC and CD. Our results are consistent with these previous studies. The fact that anemia is more common among young women could be related to blood loss during menstrual cycles, gestation, and lactation.

In our current study, anemia was detected at a higher rate in those with active disease (CRP levels higher than 5 mg/L) than in those without. Moreover, CRP level is suggested to be the predictive factor for unresponsiveness to oral iron therapy. Therefore, intravenous iron therapy is proposed to be the first line therapy in active disease[26]. Similar to our current study, previous reports have shown that the frequency of anemia increases with increased clinical activity in IBD. These data are supported by the results of the current study indicating a high incidence of anemia in patients taking corticosteroids or immunomodulator treatment due to active disease. Immune system activation and disease-related lesions in the gastrointestinal tract have been shown to contribute to the association between disease activity and anemia. It should be kept in mind that anemia is sometimes seen in IBD patients who are in remission (rate of 18%), and therefore, these patients should be evaluated for anemia as well[24].

Results of the current study revealed no difference in the frequency of anemia in CD patients with regards to disease behavior. However, in UC the rate of anemia was significantly increased in parallel with disease duration. The increased rate of anemia in the more extensive disease may be a result of both increased blood loss and increased burden of inflammation.

Smoking has been shown to have a low risk for causing anemia in IBD patients. This is due to these patients often developing compensatory polycythemia due to increased carbon monoxide consumption[18,24]. Previous studies have reported that while smoking is a risk factor for anemia in CD, it is a protective factor in UC[19]. Our current study did not elucidate the role of smoking in the development of anemia in IBD patients.

Although it is known that the incidence of anemia is higher in hospitalized patients than in outpatients, we could not perform a robust statistical comparison in our current study due to the low the number of hospitalized patients. In addition, the current study did not establish a significant relationship between surgery and frequency of anemia. It has been reported that the incidence of anemia decreases as IBD disease duration progresses. However, similar to the study of Koutroubakis *et al*[5], results of our current study indicate no relationship between disease duration and anemia.

In our current study, the most common cause of anemia was IDA. Similar to a previously published review[7], results of our current study reveal that IDA was more frequent in CD (32%) than in UC (26%). IDA can develop due to intestinal bleeding, dietary restrictions, or malabsorption[27]. Pro-inflammatory cytokines, such as IL-6 and bone morphogenetic protein, are increased in the circulation in active IBD. This causes increased secretion of hepcidin, which is produced in the liver and responsible for the absorption of iron. Increased hepcidin levels may cause the degradation of ferroportin channels, which allow iron to be transferred through the basolateral membrane of enterocytes, thus causing malabsorption of iron. In addition, it is known that iron accumulates in macrophages and monocytes. Basseri *et al*[28] revealed that hepcidin expression increases in parallel with increased levels of IL-6 in CD. Concordant with this finding, Semrin *et al*[29] showed that intestinal iron absorption is decreased in active CD compared to patients in remission.

While the diagnosis of IDA is routinely made via low serum ferritin levels (< 30 ng/mL) as well as a decrease in the TS and MCV indices, these criteria may not be valid in IBD patients, since ferritin is an acute phase reactant. When evaluating IBD patients, TS and disease activity status should always be considered along with ferritin levels[25]. In our current study, no difference was observed between CD and UC in terms of serum iron levels and mean TS. However, serum ferritin and CRP were significantly higher in CD (P = 0.001) (Table 3).

CDA is the second most common type of anemia in IBD patients. In our current study, CDA was seen a rate of 8%, while IDA was seen at a rate of 3.4%. In IBD patients, anemia often develops due to increased levels of cytokines [*e.g.*, TNF-α, IL-1, interferon (IFN)-γ] and hepcidin, and decreased levels of erythropoietin[30,31]. IFN-γ is known to inhibit the development of erythroid progenitor cells and enhance erythrocyte destruction in the spleen[32], while TNF-α causes increased apoptosis in the erythroid progenitor leading to anemia[33].

In CD, vitamin B_{12} and folic acid deficiency due to malabsorption is reported to be 29%-33%[34]. However, in our current study, this rate was estimated to be 19%. Anemia in CD patients can be caused by dietary restrictions, malabsorption due to ileal inflammation, bacterial overgrowth, fistula development, and/or surgical resection. In UC, vitamin B_{12} deficiency has been reported at a rate of 16%, which is lower than that reported for CD[34]. According to a study conducted in Turkey, there is a higher rate of vitamin B_{12} deficiency in CD than UC[35]. In our current study, the rate of anemia due to vitamin B_{12} or folic acid deficiency in UC patients was 7%. The responsible mechanisms in this situation may include ileal dysfunction following proctocolectomy and ileal pouch-anal anastomosis, bacterial overgrowth,

and reduced intestinal transit time[36].

Anemia may sometimes occur as a side effect of drugs used for the treatment of IBD. Anemia has been reported to occur at a rate of 3% per year due to the toxic effects of thiopurines on bone marrow[37]. Other reports indicate that sulfazalazine is rarely associated with reduced folate absorption, and does not often cause anemia due to aplasia or hemolysis[38]. Results of the current study indicate a 64% anemia rate among AZA users; this is often due to vitamin B_{12} and folic acid deficiency. The combination of IDA and megaloblastic anemia caused by thiopurines may present as normocytic normochromic anemia. Testa *et al*[23] observed only two UC patients had autoimmune hemolytic anemia due to antibody development caused by cross-reaction against erythrocytes as a result of AZA and INF administration. In the current study, we found no evidence of drug-related hemolytic anemia in our patients.

It is recommended that IBD patients with active inflammation (high inflammatory bio-markers or endoscopic evidence due to disease activity) be evaluated for signs of anemia at least once every three months, while those in remission should be checked every six months. If the patient has anemia, further tests should be performed to determine etiology. Regular follow-up is recommended, as there is a risk of B_{12} or folic acid deficiency in patients with small bowel disease or history of resection[12]. In our current study, further examinations were not performed in 15% of patients during follow-up, and therefore the etiology of anemia in these patients remains unknown.

Unfortunately, many clinicians still believe in the concept of asymptomatic anemia, in which anemia in IBD slowly progresses and may resolve once patients adapt to low Hb levels. In these cases, anemia is often not treated until it is severe. Only half of the patients in our follow-up were treated for anemia, which is better than the rates of previous studies.

In their 2013 study including gastroenterologists from nine European countries, Stein *et al*[8] reported that patients with IBD were not adequately monitored and treated for anemia. The diagnosis of anemia was made based on Hb levels in 88% of patients, serum ferritin levels in 75%, and on TS in 25%. Iron deficiency (ferritin < 30 ng/mL) was detected in 76% of the patients in that study, and only 28% of them were prescribed IV iron therapy.

Danese *et al*[1] reported that only 33% of IBD patients with anemia were being treated despite having been diagnosed. A recent study including 55 German gastroenterology centers reported that only 43.5% of IBD patients with anemia were treated, and 56% of those had received oral iron therapy[39]. In our current study, IV iron therapy was prescribed more often than oral iron therapy.

A sufficient response in the treatment of IDA after four weeks of treatment is indicated by an increase of 2 g/dL Hb or a > 30% increase in TS[40]. The target ferritin level in IV iron therapy is 400 μg/L. Oral iron replacement therapy alone is typically only successful in cases with mild disease activity and mild anemia[41]. Due to its low cost and safety, oral iron replacement therapy is usually used by clinicians as a first line treatment. However, oral iron therapy has been associated with some side effects and mucosal injury events, and therefore the efficacy and tolerability of this therapy must be monitored during treatment[42]. In animal studies, oral and rectal iron administration has been shown to increase disease activity because they increase pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, and IFN-α[43]. Therefore, IV iron therapy is the preferred treatment for IBD patients, especially those with severe anemia who have had an inadequate response to oral iron therapy or cannot tolerate oral iron therapy[44]. The benefits of IV iron therapy include quicker improvement of iron deficiency, quicker alleviation of patients' complaints, and higher satisfaction rate from patients. Moreover, it has been shown that IV-administered iron has no effect on disease activity[45]. Since IDA has a tendency to recur in IBD, maintenance treatment should be continued for at least three months[46]. In cases of unresponsiveness to all types of anemia therapies, the patient should be referred to a hematologist[40].

In IBD patients with anemia, it is of utmost importance to treat the underlying cause(s) and to control inflammation. Over time, attacks of inflammation in IBD lead to decreased iron absorption. In addition, TNF-α is known to increase bone marrow suppression. In our current study, there was a lower rate of anemia among patients undergoing anti-TNF therapy compared to other treatment groups. Anti-TNF therapy is becoming an increasingly preferred treatment. These patients had a lower rate of anemia, which may be related to the fact that anti-TNF agents suppress adverse effects on bone marrow, decrease inflammation, and provide effective mucosal improvement. Similarly, another study reported that anti-TNF therapy improved anemia by controlling inflammation and disease activity in IBD patients[47]. It has been suggested that anti-TNF therapy regulates erythropoiesis at various levels. However, some studies have shown that the prevalence of anemia was higher in patients treated with anti-TNF agents than those treated with other drugs (*e.g.*, immunomodulators, corticosteroids, aminosalicylates)[48]. The higher prevalence reported in those studies is associated with the fact that these drugs (anti-TNF agents) are often used in patients with increased disease severity.

One of the limitations of our current study is that it was retrospective. Therefore, we did not have any information about disease activity or smoking status. However, since we could not obtain or confirm the accuracy of the patients' clinical activity indices, we utilized CRP levels to interpret disease activity. Moreover, we could not evaluate the etiology of anemia in 15.3% of our patients because their anemia parameters were not evaluated during follow-up. In addition, we did not separately evaluate the anemia rates of pediatric-

onset IBD and adult-onset IBD, and therefore we could not compare the frequency of anemia between these groups. Further, we did not evaluate anemia-associated symptom rates or quality of life in this study. Lastly, the current study may not represent the general Turkish patient population as it was conducted with IBD patients with more severe and problematic conditions who were being followed at a tertiary referral university hospital.

In conclusion, because almost half of IBD patients have anemia and anemia causes a multitude of negative effects on patients, its presence should be further examined, and if necessary treated with regards to disease activity. It should be kept in mind that the most common cause of anemia is iron deficiency. Being female and disease activity are important risk factors in the development of anemia, and disease involvement is an additional factor in UC. Treatment rates should be increased in IBD patients with anemia. To conclude, anemia should be recognized, investigated, and treated in IBD patients.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is a chronic idiopathic disease with a relapsing and remitting course. The most common extraintestinal finding seen in IBD patients is anemia, which decreases both the quality of life and the ability to work. The first study to report the incidence of anemia in Turkish patients with IBD reported that 58.2% had anemia at least once during an 18-year follow-up period.

Research motivation

The prevalence of anemia is higher in IBD patients than in the general population. The most common causes of anemia in IBD are iron deficiency anemia (IDA) and chronic disease anemia (CDA). Although IBD-related anemia has a relatively high prevalence, its diagnosis and treatment is generally overlooked.

Research objectives

The current study aimed to determine the frequency and types of anemia in IBD patients, to determine the relationship between anemia and disease characteristics, and to determine the most effective treatment approach.

Research methods

This study retrospectively evaluated 465 patients who were diagnosed with IBD and followed-up between June 2015 and June 2016 in the Gastroenterology/IBD outpatient clinic or ward of Dokuz Eylül University, Medical Faculty Hospital. The IBD diagnoses were made in accordance with the new European Crohn's and Colitis Organization (ECCO) guidelines, and were confirmed according to standard clinical, endoscopic, histologic, and radiological criteria. Demographic and clinical data as well as endoscopic activities were obtained from hospital records. We used the World Health Organization guidelines to diagnose anemia in our IBD patients. Males were diagnosed with anemia if they had hemoglobin values less than 13 g/dL, and females were diagnosed if they had hemoglobin values less than 12 g/dL. Severe anemia was defined as having Hb values below 10 g/dL for both sexes. We evaluated the lowest hemoglobin levels of each patient during follow-up, as well as iron levels and other anemia parameters. Three main classifications of anemia were selected in accordance with the European consensus on anemia in IBD, including IDA, CDA, and mixed anemia.

Research results

This study included the data from 465 IBD patients (54.6% male and 45.4% female) who were newly diagnosed or were being followed-up with in our hospital. Of these patients, 55.3% were diagnosed with CD and 44.7% with UC. Approximately fifty-two percent of the IBD patients had anemia. Anemia frequency was higher in CD than in UC. Anemia was more common among women than men. Severe anemia was observed in 21.6% of patients with CD and 9.8% of patients with UC. IDA was the most common type of anemia (29.9%).

Approximately 50.4% of all of the IBD patients who were diagnosed with anemia in this study received treatment. Of the patients with IDA, 23% received oral iron therapy and 40.3% received parenteral iron preparations. Of those with B_{12}/folic acid anemia, 53.3% received B_{12}/folic acid treatment. None of the patients in the current study received blood transfusions or were given erythropoiesis stimulant agents.

Research conclusions

Since almost half of IBD patients have anemia, and because anemia causes a multitude of negative effects on patients, its presence should be further examined, and if necessary, treated with regards to disease activity. It should be kept in mind that the most common cause of anemia is iron deficiency. Treatment rates should be increased in IBD patients with anemia.

Research perspectives

Anemia in IBD patients must be monitored throughout active and remissive disease and treated accordingly.

REFERENCES

1 **Danese S**, Hoffman C, Vel S, Greco M, Szabo H, Wilson B, Avedano L. Anaemia from a patient perspective in inflammatory bowel disease: results from the European Federation of Crohn's and Ulcerative Colitis Association's online survey. *Eur J Gastroenterol Hepatol* 2014; **26**: 1385-1391 [PMID: 25264983 DOI: 10.1097/MEG.0000000000000200]

2 **Gisbert JP**, Gomollón F. Common misconceptions in the diagnosis and management of anemia in inflammatory bowel disease. *Am J Gastroenterol* 2008; **103**: 1299-1307 [PMID: 18477354 DOI: 10.1111/j.1572-0241.2008.01846.x]

3 **Kulnigg S**, Gasche C. Systematic review: managing anaemia in Crohn's disease. *Aliment Pharmacol Ther* 2006; **24**: 1507-1523 [PMID: 17206940 DOI: 10.1111/j.1365-2036.2006.03146.x]

4 **Filmann N**, Rey J, Schneeweiss S, Ardizzone S, Bager P, Bergamaschi G, Koutroubakis I, Lindgren S, Morena Fde L, Moum B, Vavricka SR, Schröder O, Herrmann E, Blumenstein I. Prevalence of anemia in inflammatory bowel diseases in european countries: a systematic review and individual patient data meta-analysis. *Inflamm Bowel Dis* 2014; **20**: 936-945 [PMID: 24572205 DOI: 10.1097/01.MIB.0000442728.74340.fd]

5 **Koutroubakis IE**, Ramos-Rivers C, Regueiro M, Koutroumpakis E, Click B, Schwartz M, Swoger J, Baidoo L, Hashash JG, Barrie A, Dunn MA, Binion DG. Five-Year Period Prevalence and Characteristics of Anemia in a Large US Inflammatory Bowel Disease Cohort. *J Clin Gastroenterol* 2016; **50**: 638-643 [PMID: 26485103 DOI: 10.1097/MCG.0000000000000417]

6 **Atuğ Ö**, Kani HT, Banzragch M, İmeryüz N, Akın H. Incidence rate of anemia in inflammatory bowel diseases. *Turk J Gastroenterol* 2016; **27**: 143-148 [PMID: 27015619 DOI: 10.5152/tjg.2016.16011]

7 **Gomollón F**, Gisbert JP. Anemia and inflammatory bowel diseases. *World J Gastroenterol* 2009; **15**: 4659-4665 [PMID: 19787829]

8 **Stein J**, Hartmann F, Dignass AU. Diagnosis and management of iron deficiency anemia in patients with IBD. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 599-610 [PMID: 20924367 DOI: 10.1038/nrgastro.2010.151]

9 **Ott C**, Schölmerich J. Extraintestinal manifestations and complications in IBD. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 585-595 [PMID: 23835489 DOI: 10.1038/nrgastro.2013.117]

10 **Reinisch W**, Staun M, Bhandari S, Muñoz M. State of the iron: how to diagnose and efficiently treat iron deficiency anemia in

inflammatory bowel disease. *J Crohns Colitis* 2013; **7**: 429-440 [PMID: 22917870 DOI: 10.1016/j.crohns.2012.07.031]
11 **de Silva AD**, Mylonaki M, Rampton DS. Oral iron therapy in inflammatory bowel disease: usage, tolerance, and efficacy. *Inflamm Bowel Dis* 2003; **9**: 316-320 [PMID: 14555915]
12 **Gasche C**, Berstad A, Befrits R, Beglinger C, Dignass A, Erichsen K, Gomollon F, Hjortswang H, Koutroubakis I, Kulnigg S, Oldenburg B, Rampton D, Schroeder O, Stein J, Travis S, Van Assche G. Guidelines on the diagnosis and management of iron deficiency and anemia in inflammatory bowel diseases. *Inflamm Bowel Dis* 2007; **13**: 1545-1553 [PMID: 17985376 DOI: 10.1002/ibd.20285]
13 **Dignass AU**, Gasche C, Bettenworth D, Birgegård G, Danese S, Gisbert JP, Gomollon F, Iqbal T, Katsanos K, Koutroubakis I, Magro F, Savoye G, Stein J, Vavricka S; European Crohn's and Colitis Organisation [ECCO]. European consensus on the diagnosis and management of iron deficiency and anaemia in inflammatory bowel diseases. *J Crohns Colitis* 2015; **9**: 211-222 [PMID: 25518052 DOI: 10.1093/ecco-jcc/jju009]
14 **Van Assche G**, Dignass A, Panes J, Beaugerie L, Karagiannis J, Allez M, Ochsenkühn T, Orchard T, Rogler G, Louis E, Kupcinskas L, Mantzaris G, Travis S, Stange E; European Crohn's and Colitis Organisation (ECCO). The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis. *J Crohns Colitis* 2010; **4**: 7-27 [PMID: 21122488 DOI: 10.1016/j.crohns.2009.12.003]
15 **Dignass A**, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, Mantzaris G, Reinisch W, Colombel JF, Vermeire S, Travis S, Lindsay JO, Van Assche G. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohns Colitis* 2012; **6**: 965-990 [PMID: 23040452 DOI: 10.1016/j.crohns.2012.09.003]
16 **WHO,** UNICEF, UNU. Iron deficiency anemia: assessment, prevention and control. Report of a joint WHO/UNICEF/UNU consultation. Geneva: World Health Organization; 1998
17 **Weiss G**. Anemia of Chronic Disorders: New Diagnostic Tools and New Treatment Strategies. *Semin Hematol* 2015; **52**: 313-320 [PMID: 26404443 DOI: 10.1053/j.seminhematol.2015.07.004]
18 **Høivik ML**, Reinisch W, Cvancarova M, Moum B; IBSEN study group. Anaemia in inflammatory bowel disease: a population-based 10-year follow-up. *Aliment Pharmacol Ther* 2014; **39**: 69-76 [PMID: 24172277 DOI: 10.1111/apt.12541]
19 **Lucendo AJ**, Arias Á, Roncero Ó, Hervías D, Verdejo C, Naveas-Polo C, Bouhmidi A, Lorente R, Alcázar LM, Salueña I, García-Quiñones JA, Carrillo-Ramos MJ. Anemia at the time of diagnosis of inflammatory bowel disease: Prevalence and associated factors in adolescent and adult patients. *Dig Liver Dis* 2017; **49**: 405-411 [PMID: 28096058 DOI: 10.1016/j.dld.2016.12.005]
20 **Toruner M,** Kalkan C, Keskin O, Cetinkaya H, Soykan I. P599. Prevalence of iron deficiency anemia and iron deficiency in a single center Turkish IBD cohort. J Crohns Colitis 2014; **8**: S318-S319
21 **Portela F**, Lago P, Cotter J, Gonçalves R, Vasconcelos H, Ministro P, Lopes S, Eusébio M, Morna H, Cravo M, Peixe P, Cremmers I, Sousa H, Deus J, Duarte M, Magro F; CAPOR Investigators and GEDII. Anaemia in Patients with Inflammatory Bowel Disease - A Nationwide Cross-Sectional Study. *Digestion* 2016; **93**: 214-220 [PMID: 27022722 DOI: 10.1159/000443927]
22 **Lee DS**, Bang KB, Kim JY, Jung YS, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI, Choi KY, Park DI. The prevalence and clinical characteristics of anemia in Korean patients with inflammatory bowel disease. *Intest Res* 2016; **14**: 43-49 [PMID: 26884734 DOI: 10.5217/ir.2016.14.1.43]
23 **Testa A**, Rispo A, Romano M, Riegler G, Selvaggi F, Bottiglieri E, Martorano M, Rea M, Gravina A, Nardone OM, Patturelli M, Pellino G, Miranda A, Caporaso N, Castiglione F. The burden of anaemia in patients with inflammatory bowel diseases. *Dig Liver Dis* 2016; **48**: 267-270 [PMID: 26698411 DOI: 10.1016/j.dld.2015.10.012]
24 **Antunes CV**, Hallack Neto AE, Nascimento CR, Chebli LA, Moutinho IL, Pinheiro Bdo V, Reboredo MM, Malaguti C, Castro AC, Chebli JM. Anemia in inflammatory bowel disease outpatients: prevalence, risk factors, and etiology. *Biomed Res Int* 2015; **2015**: 728925 [PMID: 25705682 DOI: 10.1155/2015/728925]
25 **Gasche C**, Lomer MC, Cavill I, Weiss G. Iron, anaemia, and inflammatory bowel diseases. *Gut* 2004; **53**: 1190-1197 [PMID: 15247190 DOI: 10.1136/gut.2003.035758]
26 **Herrera-deGuise C**, Casellas F, Robles V, Navarro E, Borruel N. Iron Deficiency in the Absence of Anemia Impairs the Perception of Health-Related Quality of Life of Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis*2016; **22**: 1450-1455 [PMID: 27057682 DOI: 10.1097/MIB.0000000000000768]
27 **Murawska N**, Fabisiak A, Fichna J. Anemia of Chronic Disease and Iron Deficiency Anemia in Inflammatory Bowel Diseases: Pathophysiology, Diagnosis, and Treatment. *Inflamm Bowel Dis* 2016; **22**: 1198-1208 [PMID: 26818422 DOI: 10.1097/MIB.0000000000000648]
28 **Basseri RJ**, Nemeth E, Vassilaki ME, Basseri B, Enayati P, Shaye O, Bourikas LA, Ganz T, Papadakis KA. Hepcidin is a key mediator of anemia of inflammation in Crohn's disease. *J Crohns Colitis* 2013; **7**: e286-e291 [PMID: 23219355 DOI: 10.1016/j.crohns.2012.10.013]
29 **Semrin G**, Fishman DS, Bousvaros A, Zholudev A, Saunders AC, Correia CE, Nemeth E, Grand RJ, Weinstein DA. Impaired intestinal iron absorption in Crohn's disease correlates with disease activity and markers of inflammation. *Inflamm Bowel Dis* 2006; **12**: 1101-1106 [PMID: 17119383 DOI: 10.1097/01.mib.0000235097.86360.04]
30 **Jelkmann W**. Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res* 1998; **18**: 555-559 [PMID: 9726435 DOI: 10.1089/jir.1998.18.555]
31 **Dallalio G**, Law E, Means RT Jr. Hepcidin inhibits in vitro erythroid colony formation at reduced erythropoietin concentrations. *Blood* 2006; **107**: 2702-2704 [PMID: 16332970 DOI: 10.1182/blood-2005-07-2854]
32 **Libregts SF**, Gutiérrez L, de Bruin AM, Wensveen FM, Papadopoulos P, van Ijcken W, Ozgür Z, Philipsen S, Nolte MA. Chronic IFN-γ production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU.1 axis. *Blood* 2011; **118**: 2578-2588 [PMID: 21725055 DOI: 10.1182/blood-2010-10-315218]
33 **Felli N**, Pedini F, Zeuner A, Petrucci E, Testa U, Conticello C, Biffoni M, Di Cataldo A, Winkles JA, Peschle C, De Maria R. Multiple members of the TNF superfamily contribute to IFN-gamma-mediated inhibition of erythropoiesis. *J Immunol* 2005; **175**: 1464-1472 [PMID: 16034083]
34 **Ward MG**, Kariyawasam VC, Mogan SB, Patel KV, Pantelidou M, Sobczyńska-Malefora A, Porté F, Griffin N, Anderson SH, Sanderson JD, Harrington DJ, Irving PM. Prevalence and Risk Factors for Functional Vitamin B12 Deficiency in Patients with Crohn's Disease. *Inflamm Bowel Dis* 2015; **21**: 2839-2847 [PMID: 26296064 DOI: 10.1097/MIB.0000000000000559]
35 **Yakut M**, Ustün Y, Kabaçam G, Soykan I. Serum vitamin B12 and folate status in patients with inflammatory bowel diseases. *Eur J Intern Med* 2010; **21**: 320-323 [PMID: 20603044 DOI: 10.1016/j.ejim.2010.05.007]
36 **M'Koma AE**. Follow-up results of hematology data before and after restorative proctocolectomy. Clinical outcome. *Dis Colon Rectum* 1994; **37**: 932-937 [PMID: 8076494]
37 **Gisbert JP**, Gomollón F. Thiopurine-induced myelotoxicity in patients with inflammatory bowel disease: a review. *Am J Gastroenterol* 2008; **103**: 1783-1800 [PMID: 18557712 DOI: 10.1111/j.1572-0241.2008.01848.x]
38 **Ransford RA**, Langman MJ. Sulphasalazine and mesalazine: serious adverse reactions re-evaluated on the basis of suspected adverse reaction reports to the Committee on Safety of Medicines. *Gut* 2002; **51**: 536-539 [PMID: 12235076]
39 **Blumenstein I**, Dignass A, Vollmer S, Klemm W, Weber-Mangal S, Stein J. Current practice in the diagnosis and management of IBD-associated anaemia and iron deficiency in Germany: the German AnaemIBD Study. *J Crohns Colitis* 2014; **8**: 1308-1314 [PMID:

24721157 DOI: 10.1016/j.crohns.2014.03.010]
40 **Akpınar H**, Çetiner M, Keshav S, Örmeci N, Törüner M. Diagnosis and treatment of iron deficiency anemia in patients with inflammatory bowel disease and gastrointestinal bleeding: iron deficiency anemia working group consensus report. *Turk J Gastroenterol* 2017; **28**: 81-87 [PMID: 28119272 DOI: 10.5152/tjg.2017.17593]
41 **Kulnigg S**, Stoinov S, Simanenkov V, Dudar LV, Karnafel W, Garcia LC, Sambuelli AM, D'Haens G, Gasche C. A novel intravenous iron formulation for treatment of anemia in inflammatory bowel disease: the ferric carboxymaltose (FERINJECT) randomized controlled trial. *Am J Gastroenterol* 2008; **103**: 1182-1192 [PMID: 18371137 DOI: 10.1111/j.1572-0241.2007.01744.x]
42 **Lugg S**, Beal F, Nightingale P, Bhala N, Iqbal T. Iron treatment and inflammatory bowel disease: what happens in real practice? *J Crohns Colitis* 2014; **8**: 876-880 [PMID: 24486177 DOI: 10.1016/j.crohns.2014.01.011]
43 **Oldenburg B**, van Berge Henegouwen GP, Rennick D, Van Asbeck BS, Koningsberger JC. Iron supplementation affects the production of pro-inflammatory cytokines in IL-10 deficient mice. *Eur J Clin Invest* 2000; **30**: 505-510 [PMID: 10849019]
44 **Lee TW**, Kolber MR, Fedorak RN, van Zanten SV. Iron replacement therapy in inflammatory bowel disease patients with iron deficiency anemia: a systematic review and meta-analysis. *J Crohns Colitis* 2012; **6**: 267-275 [PMID: 22405161 DOI: 10.1016/j.crohns.2011.09.010]
45 **Schröder O**, Mickisch O, Seidler U, de Weerth A, Dignass AU, Herfarth H, Reinshagen M, Schreiber S, Junge U, Schrott M, Stein J. Intravenous iron sucrose versus oral iron supplementation for the treatment of iron deficiency anemia in patients with inflammatory bowel disease--a randomized, controlled, open-label, multicenter study. *Am J Gastroenterol* 2005; **100**: 2503-2509 [PMID: 16279906 DOI: 10.1111/j.1572-0241.2005.00250.x]
46 **Schmidt C**, Ahmad T, Tulassay Z, Baumgart DC, Bokemeyer B, Howaldt S, Stallmach A, Büning C; AEGIS Study Group. Ferric maltol therapy for iron deficiency anaemia in patients with inflammatory bowel disease: long-term extension data from a Phase 3 study. *Aliment Pharmacol Ther* 2016; **44**: 259-270 [PMID: 27237709 DOI: 10.1111/apt.13665]
47 **Bergamaschi G**, Di Sabatino A, Albertini R, Ardizzone S, Biancheri P, Bonetti E, Cassinotti A, Cazzola P, Markopoulos K, Massari A, Rosti V, Porro GB, Corazza GR. Prevalence and pathogenesis of anemia in inflammatory bowel disease. Influence of anti-tumor necrosis factor-alpha treatment. *Haematologica* 2010; **95**: 199-205 [PMID: 19815838 DOI: 10.3324/haematol.2009.009985]
48 **Magro F**, Ramos J, Correia L, Lago P, Peixe P, Gonçalves AR, Rodrigues Ã, Vieira C, Ferreira D, Pereira Silva J, Túlio MA, Salgueiro P, Fernandes S. [Portuguese Consensus on the Diagnosis, Prevention and Treatment of Anaemia in Inflammatory Bowel Disease]. *Acta Med Port* 2016; **29**: 144-156 [PMID: 27234956]

P- Reviewer: Chiba T, Lin J, Serban ED, Sergi CM, Yücel O
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4197-4207

DOI: 10.3748/wjg.v24.i36.4197

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

ORIGINAL ARTICLE

Prospective Study

Low-dose spectral insufflation computed tomography protocol preoperatively optimized for T stage esophageal cancer - preliminary research experience

Yue Zhou, Dan Liu, Ping Hou, Kai-Ji Zha, Feng Wang, Kun Zhou, Wei He, Jian-Bo Gao

Yue Zhou, Ping Hou, Kai-Ji Zha, Jian-Bo Gao, Department of Radiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Dan Liu, Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Feng Wang, Wei He, Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Kun Zhou, Department of Thoracic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

ORCID number: Yue Zhou (0000-0001-7781-7228); Dan Liu (0000-0003-3081-792X); Ping Hou (0000-0002-3932-8789); Kai-Ji Zha (0000-0002-8774-0698); Feng Wang (0000-0002-1607-6478); Kun Zhou (0000-0002-2234-3069); Wei He (0000-0001-5214-5162); Jian-Bo Gao (0000-0003-1252-7144).

Author contributions: Gao JB and Zhou Y designed the research; Zhou Y, Hou P, Zha KJ, Liu D, Zhou K and He W performed the research and data analysis; Zhou Y, Hou P and Zha KJ contributed to the statistical analysis; Liu D, Zhou K and He W collected the data and assigned the forms; Zhou Y and Gao JB wrote the paper.

Supported by Scientific and Technological Support Plan Projects of China, No. 2007BAI05B05; and Medical Science and Technology Project of Henan Province, No. 201602012.

Institutional review board statement: The study was reviewed and approved by the institutional review boards of the First Affiliated Hospital of Zhengzhou University.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Jian-Bo Gao, MD, PhD, Professor, Department of Radiology, The First Affiliated Hospital of Zhengzhou University, No. 1 East Jianshe Road, Zhengzhou 450052, Henan Province, China. cjr_gaojianbo@163.com
Telephone: +86-371-67966890
Fax: +86-371-66970906

Received: June 25, 2018
Peer-review started: July 2, 2018
First decision: July 17, 2018
Revised: July 28, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 28, 2018

Abstract

AIM

To evaluate the T stage of esophageal squamous cell carcinoma (ESCC) using preoperative low-dose esophageal insufflation computed tomography (EICT).

METHODS

One hundred and twenty ESCC patients confirmed by surgery or esophagoscopy were divided into three groups. Groups B and C were injected with 300 mgI/kg contrast medium for automatic spectral imaging assist

(GSI assist), while group A underwent a conventional 120 kVp computed tomography (CT) scan with a 450 mgI/kg contrast medium injection. EICT was performed in group C. Group A was reconstructed with filtered back projection, and groups B and C were reconstructed with 50% adaptive statistical iterative reconstruction. The contrast-to-noise ratio of lesion-to-mediastinal adipose tissue and the radiation dose were measured. Specific imaging features were observed, and T stage ESCCs were evaluated.

RESULTS

The sensitivity and accuracy of the T1/2 stage were higher in group C than in groups A and B (sensitivity: 43.75% *vs* 31.82% and 33.33%; accuracy: 54.29% *vs* 46.67% and 52.50%, respectively). With regard to the T3 stage, the sensitivity and specificity in group C were higher than those in groups A and B (sensitivity: 56.25% *vs* 41.17% and 44.44%; specificity: 73.68% *vs* 67.86% and 63.64%, respectively). The diagnostic sensitivity, specificity and accuracy of the T4 stage were similar among all groups. There were no significant differences in volume CT dose index [(5.91 ± 2.57) mGy *vs* (3.24 ± 1.20) *vs* (3.65 ± 1.77) mGy], dose-length product [(167.10 ± 99.08) mGy•cm *vs* (113.24 ± 54.46) mGy•cm *vs* (117.98 ± 32.32) mGy•cm] and effective dose [(2.52 ± 1.39) *vs* (1.63 ± 0.76) *vs* (1.73 ± 0.44) mSv] among the groups ($P > 0.05$). However, groups B and C received similar effective doses but lower iodine loads than group A [(300 *vs* 450) mgI/kg].

CONCLUSION

EICT combined with GSI assist allows differential diagnosis between the T1/2 and T3 stages. The ability to differentially diagnose the T3 and T4 stages of medullary ESCC can be improved by quantitatively and qualitatively analyzing the adipose tissue in front of the vertebral body.

Key words: Esophageal neoplasms; tomography; tumor staging

Core tip: Esophageal insufflation computed tomography (EICT) is a method of insufflating air into the stomach before computed tomography examination, which fully expands the esophageal lumen. The optimal monochromatic energy level clearly displays esophageal lesions and surrounding adipose infiltration by means of effectively improving the image quality and resolution. Our study demonstrates that EICT combined with GSI assist technology contributes to better performance in the differential diagnosis between the T1/2 *vs* T3 stages and the T3 *vs* T4 stages in medullary esophageal cancer.

Zhou Y, Liu D, Hou P, Zha KJ, Wang F, Zhou K, He W, Gao JB. Low-dose spectral insufflation computed tomography protocol preoperatively optimized for T stage esophageal cancer - preliminary research experience. *World J Gastroenterol* 2018; 24(36): 4197-4207 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4197.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4197

INTRODUCTION

Esophageal carcinoma (EC) is a common malignant tumor of the digestive system. Mortality due to EC is approximately 300000 people per year worldwide[1]. The most common pathology subtype in Asia is esophageal squamous cell carcinoma (ESCC). As it is rare for patients to exhibit early symptoms of ESCC, patients typically receive treatment in the mid- and late stages (T3/4, N+ or M1)[2]. The overall five-year survival rate of progressive ESCC (T3/4 or N+) is only 42% after surgical resection or preoperative neoadjuvant chemotherapy[3]. Identification of the correct T stage of ESCC by preoperative imaging plays a critical role in the development, treatment and prognosis of patients.

Endoscopic ultrasonography (EUS) can be used clinically to determine the infiltration of ESCC and the possibility of surgical resection. However, the detection range is limited to centimeters from the center of the ultrasonic probe without interference or severe stenosis. Griffin *et al*[4] reported that inflammation or fibrous tissue surrounding ESCC tissue leads to over-staging of the local T stage. Currently, computed tomography (CT) and positron emission tomography/computed tomography (PET/CT) are common methods used to evaluate the T stage before ESCC treatment[5-7]. Variations in the sensitivity and specificity of these common methods is 27%-67% and 33%-93%, respectively[8,9]. Some studies continue to use traditional CT enhancement with low spatial and density resolutions. Konieczny *et al*[10] asserted that the accuracy of traditional 64-slice CT enhancement was 34% for EC. The sensitivity and specificity of CT or PET/CT are approximately 31% and 59% for diagnosis of the T1/2 stage, 60% and 64% for diagnosis of the T3 stage, and 100% and 4% for diagnosis of the T4 stage, respectively, which are not satisfactory.

Conventional CT has limitations for ESCC staging or restaging after treatment. Esophageal insufflation CT (EICT) is a method of insufflating air into the stomach before CT examination, which fully expands the esophageal lumen[11]. Diagnosis of the T1 or T2 stage has low accuracy because of the difficulty in visualizing the esophageal mucosa[12,13]. The optimal monochromatic energy level clearly displays esophageal lesions and the surrounding adipose infiltration by effectively improving the image quality and resolution. The optimal monochromatic energy level can be used for diagnosis, treatment selection, and therapeutic monitoring. Hence, we aimed to evaluate the T stage of ESCC using low-dose spectral insufflation CT, and we

discuss the accuracy of this technique for diagnosing the T stage preoperatively.

MATERIALS AND METHODS

Subject enrollment

This study was approved by the Institutional Review Board. All patients enrolled in this study provided informed consent.

In this single-institution study, 120 patients with a biopsy-proven esophageal malignancy who were being considered for radical treatment and who had already undergone EUS with a median age of 58 years (range, 48-83 years) were recruited from November 2015 to August 2017. The patients included 66 males and 54 females, with a median age of 55.4 years (range, 48-83 years). The typical clinical symptoms included vomiting, progressive dysphagia, intermittent sternal sensation, hematemesis and a sense of frustration. All patients considered for radical treatment were staged according to spectral CT and EUS within 6 wk.

The exclusion criteria included the following: (1) patients with esophageal cancer undergoing spectral CT to detect recurrence; (2) patients with a poor physical condition or a combination of severe heart, liver or kidney dysfunction; (3) patients with a history of iodine allergy, making them unsuitable for enhanced examination; and (4) patients with a history of other cancers.

Spectral CT protocol and data acquisition

Patients were divided into three groups that included 45 patients (group A), 40 patients (group B) and 35 patients (group C). Patients were required to fast for 6 h prior to the investigation and were administered an intramuscular injection of amidoamine (20 mg) 10-15 min before experimental procedures. EUS was performed using a PHILIP IU22 Color Doppler Diagnostic Apparatus (Philip, Eindhoven, The Netherlands) with a 5-10 Hz radical or linear endoscope. Then, a dual-phase contrast enhancement spectral spiral CT was performed with a spectral CT scanner (Discovery CT, GE Healthcare, Waukesha, WI, United States) from the thoracic inlet to the bottom of the lungs. The imaging parameters for group B were as follows: tube voltage: 80 kV and 140 kV with a fast kV-switching technique; tube current: auto mA with a slice thickness of 5 mm. Iobitrido (Guerbet, Paris, French), containing 350 mg/mL of iodine, was injected at a dose of 300 mgI/kg. The injection rate was calculated as the weight in kilograms divided by 30 s. A triggering scan was performed when the CT attenuation of the aortic arch reached the level of 100 HU. The starting time was 90 s after triggering. The saline tracer injection rate was similar to that of the contrast medium. For group A, conventional 120 kVp chest-enhanced CT scanning was performed with an injection dose of 450 mgI/kg. The remaining parameters were similar to those of group B.

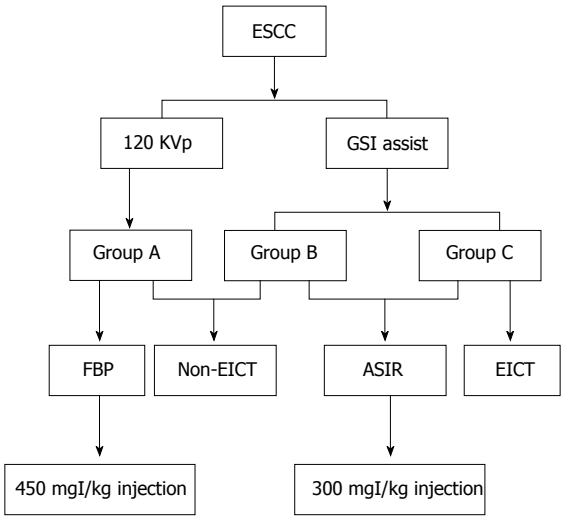


Figure 1 Study flow chart. ESCC: Esophageal squamous cell carcinoma; FBP: Filtered back projection; EICT: Esophageal insufflation computed tomography; ASIR: Adaptive statistical iterative reconstruction.

For group C, a gastric tube was inserted into the stomach *via* the nasal cavity 10-15 min before the CT examination. The depth of insertion was referenced to the location of the esophageal lesion, and the end of the tube was fixed near the nostrils. Patients were asked to press a balloon to fill the stomach with air. Pressure was maintained between 4-4.67 kPa. Patients were required to keep their lips tightly closed and to fill the esophagus as much as possible during the process of filling. The rest of the parameters were similar to those of group B.

Qualitative and quantitative analyses

CT images (40-140 keV, monochromatic) were reconstructed using spectral imaging analysis software (GE Healthcare, Waukesha, WI, United States). The 50% adaptive statistical iterative reconstruction (ASIR) algorithm and standard filtered back projection reconstruction were applied to the decomposition images of group A and groups B and C, respectively. A flowchart of the study procedures is shown in Figure 1.

Special CT features observation

Two radiologists (Zha KJ and Zhou Y) with ten years of experience in CT diagnosis independently observed and recorded the special features of the CT images in a blinded and randomized manner using a dedicated workstation (Advantage Workstation 4.6, GE Healthcare, Waukesha, WI, United States). In the case of a discrepancy between interpretations, a consensus was reached by discussion. The main observations included enhanced features for ESCC (layered/unlayered enhancement) and morphological changes near ESCC tissue (a triangle area in front of the vertebral body, trachea, bronchus and aorta). A layered enhanced feature for ESCC was used to clearly identify the layers of the esophageal wall to effectively determine infiltration.

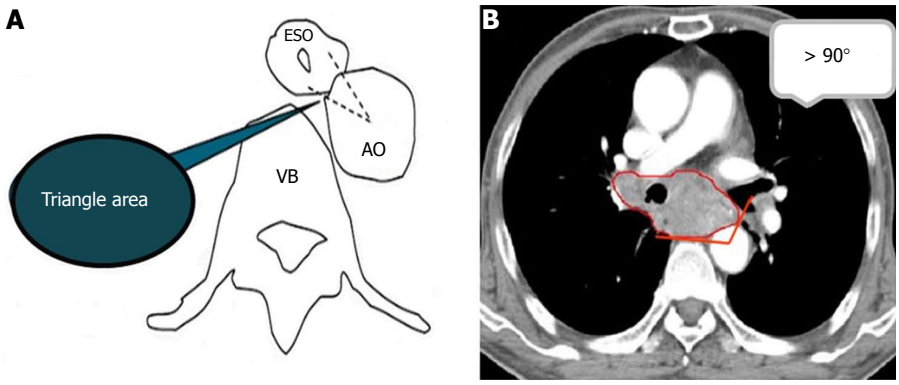


Figure 2 The morphological features of the triangular area in front of the vertebral body. A: Sketch of the triangular area. The triangular space is surrounded by the outer esophageal membrane, thoracic aorta and vertebral body. B: The contact arc between the tumor and thoracic aorta is less than 45°; a contact arc of 45-90° corresponds to suspected invasion, and a contact arc larger than 90° indicates thoracic aorta invasion. VB: Vertebral body.

As described in Figure 2A, the triangular area in front of the vertebral body is surrounded by the outer esophageal membrane, the thoracic aorta and the vertebral body. Under normal circumstances, this area is filled with adipose tissue; however, when invaded by ESCC, this area is blurred or disappears. The narrow space between the anterior wall of the esophagus and the trachea is connected by loose connective tissue. When the tracheal bronchus is invaded, the space is depicted as having an ill-defined boundary or tracheal bronchus deformation and displacement. In general, the contact arc between the tumor and the thoracic aorta is less than 45°, while an arc of 45°-90° indicates invasion, and an arc larger than 90° indicates thoracic aorta invasion (Figure 2B).

Subjective imaging evaluation

From the monochromatic images, an analysis was performed to obtain the optimal energy level to provide the best contrast-to-noise ratio (CNR) between ESCC tissue and surrounding adipose tissue. Selected circular or oval-shaped areas from 70-80 mm^2 were used for the regions of interest (ROI) measurement, which contained ESCC tissue and surrounding adipose tissue. The GSI Viewer software package automatically calculated the best CNR values from 101 sets of monochromatic images. The standard deviation (SD) of adipose tissue inside the mediastinal space at the same level represents image noise. The ROI was placed in the region as homogeneously as possible (an average of three ROIs). CNR was calculated using the following formula: $CNR=(CT_{ESCC}-CT_{adipose})/SD_{adipose}$. The normalized iodine concentration was obtained by dividing the iodine concentration (IC) for ESCC tissue (IC_{ESCC}) by that for the aorta (IC_{aorta}). The normalized iodine concentration (NIC) was calculated as $NIC = IC_{ESCC}/IC_{aorta}$.

Radiation dose

The volume CT dose index (CTDIvol, mGy) and dose-length product (DLP, mGy•cm) in the dose report were also recorded. The estimated effective dose (ED, mSv) was calculated by multiplying the DLP by 0.014 (as recommended by the International Commission on Radiological Protection (ICRP) for chest CT examinations).

Pathological subtype and T stage

The spectral CT results were compared with the results of other combined staging investigations, such as EUS and PET. For unresectable disease, sections were obtained for histological assessment, additional imaging [PET/CT, magnetic resonance imaging (MRI)] or clinical course determination, such as rapidly progressive disease or response to treatment. For potentially resectable disease, lesion sections were taken and frozen at the time of resection when appropriate, and information was obtained upon subsequent relapse and survival.

The T stage was reported according to the maximum wall thickness of ESCC tissue using the criteria of the classification system by Konieczny *et al*[10] and Jones *et al*[14], and consistent with the 7th TNM edition[4,15]. The T status for CT diagnosis was defined as follows: the T1 and T2 stages were combined because it was impossible to differentiate between the esophageal wall layers on MDCT images. The T1/2 stage was defined as a tumor wall thickness of at least 5-10 mm without evidence of mediastinal involvement. The T3 stage was defined as a tumor wall thickness greater than 10 mm with mediastinal involvement but no invasion of adjacent structures. The T4a (invasion of pleura, pericardium and diaphragm) and T4b (invasion of other structures, *e.g.*, aorta, vertebral body and trachea) stages were defined as a tumor wall thickness greater than 10 mm and invaded adjacent structures.

The pathological subtype was classified based on the advanced esophagus cancer pathology classification criteria of the NCCN guidelines (2017. V3). The pathological subtypes included medullary type (wall thickness with symmetry or partial lumen stenosis), mushroom type (wall thickness similar to a flat mushroom mass), ulcer type (a larger and deeper ulcer on the surface of the wall) and narrowing type (narrow and obstructed lumen with a dilated upper segment).

Table 1 Basic clinical characteristics of patients *n* (%)

Variable	Group A (*n* = 45)	Group B (*n* = 40)	Group C (*n* = 35)
Gender			
Male	25 (56)	19 (48)	22 (64)
Female	50 (44)	21 (52)	13 (36)
Age (yr)			
Medium	67	61	63
Range	55-82	52-79	50-81
Location			
Upper esophagus	11 (24)	8 (20)	6 (16)
Middle esophagus	14 (32)	11 (28)	11 (32)
Lower esophagus	20 (44)	22 (56)	18 (52)
Differentiation degree			
High	17 (38)	9 (23)	10 (28)
Medium	23 (50)	25 (62)	14 (40)
Low	5 (12)	6 (15)	11 (32)
Symptom			
Progressive dysphagia	18 (40)	19 (48)	21 (60)
Vomiting	13 (28)	8 (20)	4 (12)
Intermittent sternal sensation	9 (20)	8 (20)	6 (16)
Hematemesis and sense of frustration	5 (12)	5 (12)	4 (12)

Statistical analysis

The Statistical Package for the Social Sciences version 19.0 software program (SPSS, Inc., Chicago, IL, United States) was used for statistical analyses. Quantitative variables are expressed as the mean ± SD. Paired *t*-tests were used to compare age, BMI and radiation dose among the image reconstruction protocols. One-way analysis of variance was used to compare objective image noise. The least significant difference correction was used for multiple comparisons. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for determining the T stage of the three groups were calculated, and the positive predictive value and negative predictive value were calculated. Inter-observer agreements between the two radiologists were based on the percentage agreement and simple Cohen's kappa statistic. The significance level for all tests was 5% (two-sided).

RESULTS

The basic characteristics of the patients are summarized in Table 1. No significant differences were found in gender, tumor location, differentiation, and clinical symptoms among groups ($P < 0.05$ for all). The medullary type comprised the largest proportion in each group, followed by the mushroom and ulcer types; the narrowing type corresponded to the smallest proportion.

Special CT features for T1/2 and T3 stage differentiation

The proportions of layered enhancement in medullary ESCC tissue in groups A, B and C were 33%, 56% and 75% (for the T1/2 stage) and 20%, 20% and 11% (for the T3 stage), respectively; those for ulcer type were 33%, 0% and 33% (for the T1/2 stage) and 0%, 0% and 20% (for the T3 stage); and those for mushroom type were 20%, 0% and 60% (for the T1/2 stage) and 0% for all groups (for the T3 stage). The presentation of layered enhancement significantly differed between T1/2 and T3 stage medullary ESCC in group C ($P < 0.05$) but not between those stages in groups A and B ($P > 0.05$), and there was no significant difference between the T1/2 and T3 stages in the ulcer and mushroom types ($P > 0.05$ for all) (Table 2, Figures 3 and 4).

Special CT features for T3 and T4 stage differentiation

The optimal monochromatic image with the best CNR in groups B and C was mainly located at (50.18 ± 2.64) KeV. The $CNR_{lesion\text{-}to\text{-}adipose}$ at 50 keV in groups B and C was higher than that of group A ($P < 0.05$); however, there was no significant difference in the $CNR_{lesion\text{-}to\text{-}adipose}$ between groups B and C ($P < 0.05$). In terms of the morphological change of the triangular area in front of the vertebral body, the proportion of adipose blur or disappearance in groups A, B and C was 40%, 30% and 22% (for the T3 stage) and 50%, 57% and 54% (for the T4 stage) for medullary ESCC, respectively; 25%, 0% and 0% (for the T3 stage) and 0% in all groups (for the T4 stage) for the ulcer type; and 0% in all groups (for the T3 and T4 stage) for the mushroom type. There were no significant differences in morphological changes between the T3 and T4 stages for medullary type, ulcerative type and mushroom type ESCC (Table 3).

The quantitative parameters IC and NIC of adipose tissue in the triangular area in front of the vertebral body during arterial phase (AP) and venous phase showed significant differences in their ability to discriminate the T3 and T4 stages ($P < 0.05$). The receiver operating characteristic curve demonstrated that the area under the curve for NIC was higher than that for IC. When the threshold of NIC during AP was -0.03, the sensitivity and specificity for identifying the T3 stage were 83.30% and 83.33%, respectively (Figure 5).

Combined analyses of the morphological features

Table 2 Comparison between esophageal squamous cell carcinoma-enhanced features and pathological T stage

Group	Enhancement feature	Medullary type		Ulcerative type		Mushroom type		Total
		T1/2	T3	T1/2	T3	T1/2	T3	
A	Layered	3	2	2	0	1	0	8
	Unlayered	6	8	6	4	4	3	31
Total		9	10	8	4	5	3	39
B	Layered	5	2	0	0	0	0	7
	Unlayered	4	8	2	5	4	3	26
Total		9	10	2	5	4	3	33
C	Layered	6	1	1	1	3	0	12
	Unlayered	2	8	2	4	2	2	20
Total		8	9	3	5	5	2	22

Table 3 Comparisons between morphological changes of the triangular area in front of the vertebral body and pathological T stage

Group	Morphological change of triangle area in front of vertebral body	Medullary type		Ulcerative type		Mushroom type		Total
		T3	T4	T3	T4	T3	T4	
A	Clear	6	3	3	0	3	0	15
	Blurred or disappeared	4	3	1	0	0	0	8
Total		10	6	4	0	3	0	23
B	Clear	7	3	5	0	3	0	18
	Blurred or disappeared	3	4	0	0	0	0	7
Total		10	7	5	0	3	0	25
C	Clear	7	6	5	0	2	0	20
	Blurred or disappeared	2	7	0	0	0	0	9
Total		9	13	5	0	2	0	29

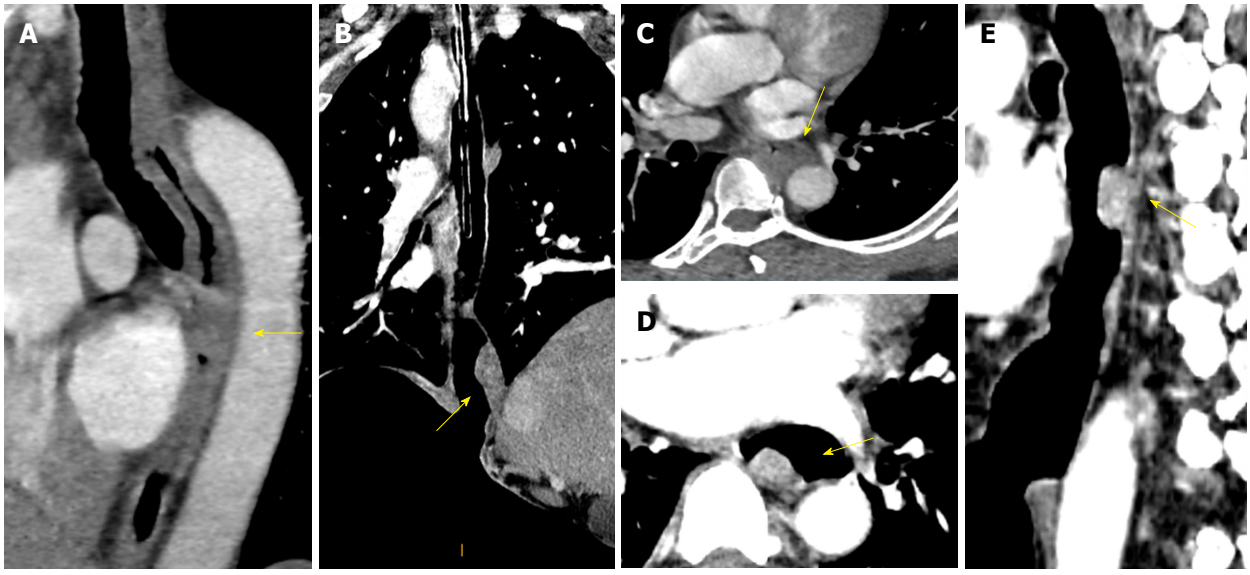


Figure 3 Typical cases showing the esophageal wall of group A, B and C. A: A sagittal reformatted image obtained *via* 120 kVp combined with FBP reconstruction shows unlayered enhanced esophageal cancer in a 53-year old patient (yellow arrow). The esophageal wall appears to be generally thickened over a long distance but without an enhanced layer. B: Moderately enhanced esophageal cancer in the lower esophagus of a 61-year old patient. The coronal reformatted image obtained *via* EICT combined with GSI assist at 50 KeV shows that the lesion appears to protrude into the lumen (yellow arrow). The lumen is filled with air without esophageal wall shrinkage. C: A 63-year old patient with histopathological T2N0M0. The axial image in the venous phase shows esophageal wall thickening, but it is difficult to identify layers (yellow arrow). Due to nose or mouth leakage, non-EICT is displayed in the venous phase for this patient. D-F: The same patient as in (C); axial and sagittal images obtained *via* EICT combined with GSI assist at 50 KeV show the wall thickness as if a partial mass was present with a moderate enhancement in the arterial phase (yellow arrow). EICT: Esophageal insufflation computed tomography.

and NIC during AP in the triangular area in front of the vertebral body highlighted a significant difference in discriminating T3 and T4 stage medullary ESCC in groups B and C ($P < 0.05$), and there were no significant differences between T3 and T4 stage ulcer and mushroom type ESCC ($P > 0.05$ for all) (Table 4, Figure 6).

T stage comparisons

The sensitivity and accuracy in group C in terms of diagnosing the T1/2 stage were higher than those in the

Table 4 Comparison of pathological T stage and combined diagnosis of the triangular area in front of the vertebral body

Group	T stage	Medullary type		Ulcerative type		Mushroom type		Total
		T3	T4	T3	T4	T3	T4	
A	T3	6	1	3	0	3	0	13
	T4	4	5	1	0	0	0	10
Total		10	6	4	0	3	0	23
B	T3	8	1	5	0	3	0	17
	T4	2	6	0	0	0	0	8
Total		10	7	5	0	3	0	25
C	T3	6	2	5	0	2	0	15
	T4	3	11	0	0	0	0	14
Total		9	13	5	0	2	0	29

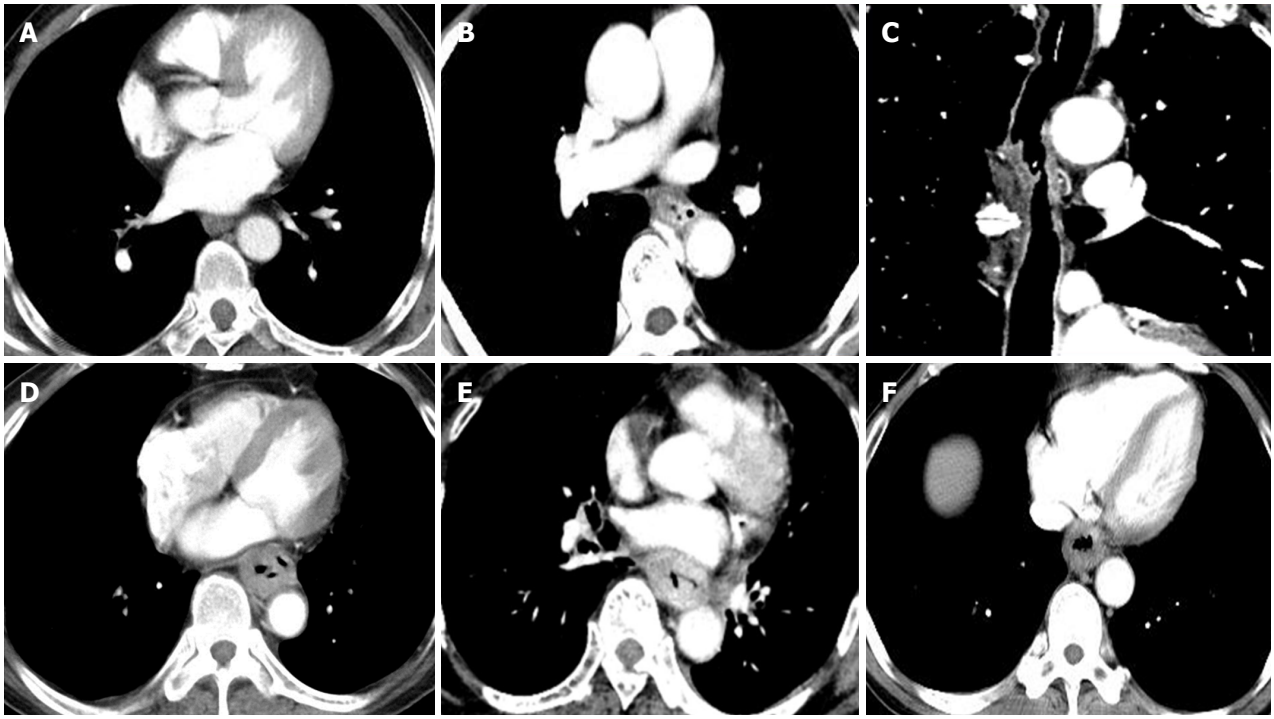


Figure 4 Typical cases between T1/2 and T3 stages of esophageal squamous cell carcinoma in group A, B and C. A: An axial image obtained *via* 120 kVp combined with FBP reconstruction of a 56-year old patient shows unlayered enhanced wall thickening at the lower esophagus with histopathological T2N0M0. B: A 62-year old, correctly-staged patient with histopathological T2N0M0ESCC of the middle esophagus. The axial image at 50 KeV obtained by GSI assist depicts significantly layered enhanced wall thickening, which was regarded as invasion within the submucosal or muscle layer. C: A 58-year old patient with histopathological T2N0M0; sagittal reformatted image obtained *via* EICT combined with GSI assist at 50 KeV shows slightly mucosal enhanced wall thickening with an ulcer on the surface. D: An axial image obtained *via* 120 kVp combined with FBP reconstruction of a 59-year old patient shows unlayered enhanced wall thickening at the lower esophagus with histopathological T3N1M0. E: A 55-year old patient with T3N0M0; axial image obtained using GSI assist at 50 KeV shows more obvious enhanced wall thickening than the image obtained by conventional 120 kVp. F: A 61-year old patient with T3N0M0; EICT combined with GSI assist exhibits unlayered enhanced esophageal wall thickening surrounding the air-filled lumen. EICT: Esophageal insufflation computed tomography; ESCC: Esophageal squamous cell carcinoma.

other groups. With regard to diagnosing the T3 stage, the sensitivity and specificity were higher in group C than in the other groups. The accuracy of diagnosing the T4 stage between groups was similar (Table 5).

Compared with the pathological results, mucosa enhancement was identified in 31.82% (7/22), 33.33% (5/15) and 43.75% (7/16) of cases for T1/2 stage ESCC in groups A, B and C, respectively; 68.18% (15/22), 66.67% (10/15) and 56.25% (9/16) of these cases were upstaged to T3, respectively.

There were 41.17% (7/17), 44.44% (8/18) and 56.25% (9/16) cases with T3 stage ESCC in groups A, B and C, respectively; 35.29% (6/17), 27.78% (5/18) and 25% (4/16) of these cases were upstaged to the T4 stage. Four cases were characterized by blurring of adipose tissue between tumor and adjacent structures, seven cases showed significant enhancement of the esophageal layer, three cases had an unclear boundary mass or ulcer, and 23.53% (4/17), 27.78% (5/18) and 18.75% (3/16) cases were down-staged to T1/2.

Radiation dose

There were no significant differences in CTDIvol [(5.91 ± 2.57) mGy *vs* (3.24 ± 1.20) mGy *vs* (3.65 ± 1.77) mGy], DLP [(167.10 ± 99.08) mGy•cm *vs* (113.24 ± 54.46) mGy•cm *vs* (117.98 ± 32.32) mGy•cm] and ED

Table 5 Accuracy of T stage comparisons between groups

Stage	Group	*n*	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
T1/2	A	45	7	9	15	14	31.82	60.87	46.67	43.75	48.28
	B	40	5	9	10	16	33.33	64.00	52.50	35.71	61.54
	C	35	7	7	9	12	43.75	63.16	54.29	50.00	57.14
T3	A	45	7	9	10	19	41.17	67.86	57.78	43.75	65.52
	B	40	8	8	10	14	44.44	63.64	55.00	50.00	58.33
	C	35	9	5	7	14	56.25	73.68	65.71	60.00	66.67
T4	A	45	5	12	1	27	83.33	69.23	71.11	29.41	96.43
	B	40	6	10	1	23	85.71	69.70	72.50	37.50	95.83
	C	35	11	7	2	15	84.62	68.18	74.29	61.11	94.12

TP: True positive; TN: True negative; FP: False positive; FN: False negative; PPV: Positive predictive value; NPV: Negative predictive value.

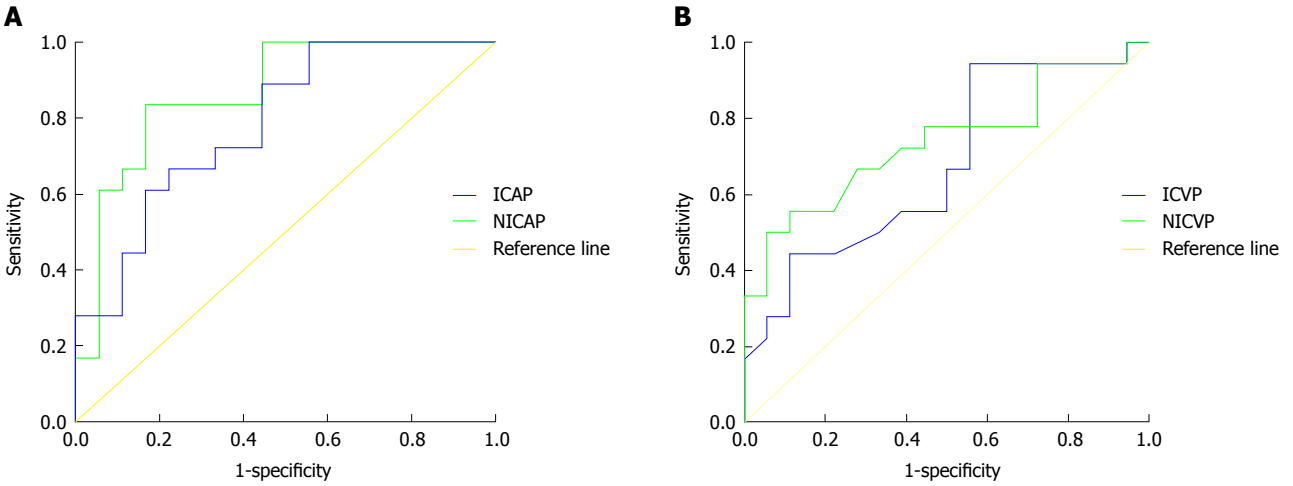


Figure 5 Receiver operating characteristic curves. Graphs showing the sensitivity and specificity of both iodine concentration and normalized iodine concentration of adipose tissue in the triangular area in front of the vertebral body during the arterial phase and the venous phase for differential diagnosis of T3 and T4 stages. AP: Arterial phase; VP: Venous phase; IC: Iodine concentration; NIC: Normalized iodine concentration.

[(2.52 ± 1.39) mSv *vs* (1.63 ± 0.76) mSv *vs* (1.73 ± 0.44) mSv] among the groups ($P > 0.05$). However, groups B and C received similar effective doses but lower iodine loads than group A [(300 *vs* 450) mgI/kg].

DISCUSSION

Reducing the radiation dose while maintaining image quality has become a key issue in CT research[16]. Reasonable adjustments of the ASIR-weighted value with the appropriate reduction in the scanning conditions are the main factors for low-dose scanning[17]. As a type of automatic dynamic real-time radiation dose control technology, GSI appropriately changes the tube to compensate for the loss of image contrast by adjusting the field of view, rotation speed and detector width[18,19]. Our study shows that the CNRs of groups B and C were superior to the CNR of group A. Thus, GSI assist combined with ASIR achieved equal or higher image quality than conventional scanning. Low-dose scanning brings benefits to patients with esophageal cancer[20]. Radiation from multiple follow-ups can be potentially harmful to patients who receive multiple radiation or chemotherapy treatments.

The esophageal wall is composed of a mucosal layer, a submucosal layer, a muscle layer and an outer membrane layer. The infiltration depth of ESCC determines the T stage. In fact, esophageal wall thickening observed on CT enhancement is largely dependent on the pathological classification. We found that T staging not only depends on the advantages of imaging techniques, but is also closely connected to the degree of esophageal lumen filling. In addition, esophageal wall shrinkage leads to incomplete lumen filling and makes it difficult to identify layers. In our study, the proportion of layered enhancement for the medullary T1/2 and T3 stages increased when we performed EICT in group C. However, no significant differences between the T1/2 and T3 stages in the ulcer and mushroom types were found. We argue that insufflation CT promotes the ability to identify lesions located on one side or around the lumen, but limitations for flat masses or local ulcers remain.

We reported that there were no significant differences between the T3 and T4 stages in all types of ESCC when only observing the morphological changes of the triangular area in front of the vertebral body. We inferred that the changes in the triangular area in medullary

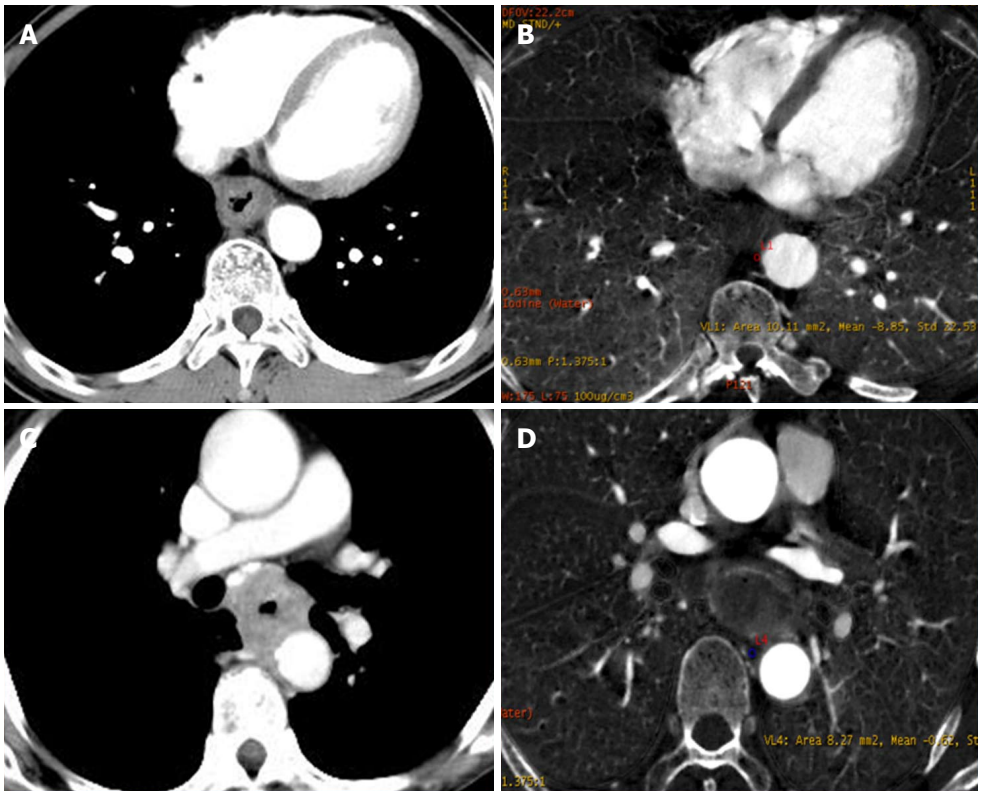


Figure 6 Typical cases showing differences between the T3 and T4 stages of esophageal squamous cell carcinoma. A: Image from a 62-year old patient with T3N0M0 obtained using EICT combined GSI assist shows a clear triangular area in front of the vertebral body. B: IC in the triangle area during the AP was quantified to be -8.85 μg/cm^3, and NIC during the AP was -0.05 μg/cm^3. C: An axial image of a 57-year old patient obtained by EICT combined with GSI assist during the AP shows that the adipose tissue of the triangular area disappeared with suspected tumor invasion. D: IC in the triangle area during the AP was quantified to be -0.62 μg/cm^3, and NIC during the AP was -0.01 μg/cm^3. EICT: Esophageal insufflation computed tomography; IC: Iodine concentration; NIC: Normalized iodine concentration; AP: Arterial phase.

ESCC were affected by the size of the adipose tissue and connections with adjacent structures. Less adipose tissue and a close correlation with the triangular area are sometimes observed as blurring. Combined analysis of subjective observations and quantitative measurements revealed significant differences that could be used to discriminate T3 and T4 stage medullary ESCC in groups B and C. Hence, the better performance of the combined analysis in the triangular area was mainly attributed to the ability of NIC to discern invasion during the AP.

Based on the above analysis, the ability to diagnose T1-T3 stages is consistent with previous research[9,13]. The accuracy and sensitivity in distinguishing the T4 stage were similar to those reported by Cerfolio *et al*[13] and Konieczny *et al*[10], while the specificity was significantly higher than that reported by Konieczny *et al*[10]. Our study included more T4 stage patients than that of Konieczny *et al*[10], and, thus, our results may be more reliable than previous reports. Therefore, our findings demonstrate the relatively stable ability of our proposed technique to discriminate peripheral invasion, such as invasion of the trachea, aorta, muscle and pericardium.

There are several limitations of our study. First, the small sample size of T4 stage patients likely impacted the comparison of the diagnostic value in this preliminary study. Second, incomplete lumen filling associated with uncertain lesions restricts tumor localization. Third, nose or mouth leaks were unavoidable when patients held their breath for a long period of time. Fourth, there is still a certain false-positive rate when analyzing the triangular area in front of the vertebral body. Furthermore, the EICT process is influenced by the patient's age and tolerance coordination. Lastly, other modalities, especially functional MRI, perform better in displaying the esophagus layer and distant metastases. Comparisons of EICT with EUS, MRI and PET-CT will be performed in the future. As mentioned above, fewer adipose tissue and close adjacent structural connections are easily misdiagnosed. The future direction of our research will focus on local expansion by lumen filling.

In conclusion, GSI optimizes the image contrast, maintains the radiation dose and reduces the contrast medium injection dose. EICT combined with GSI assist promotes differential diagnosis between T1/2 and T3 stage ESCC. The ability to differentially diagnose the T3 and T4 stages in medullary ESCC can be improved by quantitatively and qualitatively analyzing adipose tissue in front of the vertebral body.

ARTICLE HIGHLIGHTS

Research background

Conventional computed tomography (CT) has limitations for esophageal cancer staging or restaging after treatment. Diagnoses of the T1 and T2 stages exhibit

low accuracy due to difficulty visualizing the esophageal mucosa. The optimal monochromatic energy level clearly displays esophageal lesions and the surrounding adipose infiltration by effectively improving the image quality and resolution.

Research motivation

Radiation from multiple follow-ups can be potentially harmful to patients who receive multiple radiation or chemotherapy treatments. Low-dose scanning brings benefits to patients with esophageal cancer. GSI combined with ASIR achieved image quality equal to or greater than that of conventional scanning.

Research objectives

We aimed to evaluate the T stage of esophageal cancer using low-dose spectral insufflation CT, and we discuss the accuracy of this technique for preoperatively diagnosing the T stage.

Research methods

One hundred and twenty patients with esophageal cancer were divided into three groups that included 45 patients (group A underwent conventional 120 kVp CT with 450 mgI/kg contrast medium injection), 40 patients (group B underwent GSI assist and 300 mgI/kg contrast medium injection) and 35 patients (group C underwent insufflation CT combined GSI assist and 300 mgI/kg contrast medium injection). Specific imaging features were observed, and the contrast-to-noise ratio of lesion-to-mediastinal adipose tissue was calculated for qualitative and quantitative T stage evaluation. The radiation dose was measured in each group.

Research results

When performed with insufflation CT combined with GSI assist technology, the ability to present layered enhancement was significantly different for the identification of T1/2 and T3 stage medullary esophageal cancer. Combined analyses of the morphological features and normalized iodine concentration during the arterial phase in the triangular area in front of the vertebral body highlighted a significant difference in discriminating T3 and T4 stage medullary esophageal cancer.

Research conclusions

EUS can be clinically used to determine the infiltration of esophageal cancer and the possibility of surgical resection. However, the detection range is limited to centimeters from the center of the ultrasonic probe without interference or severe stenosis. Currently, CT and PET/CT are common methods used to evaluate the T stage before esophageal cancer treatment. Hence, we aimed to evaluate the T stage of esophageal squamous cell carcinoma using low-dose spectral insufflation CT, and we discuss the accuracy of this technique for preoperatively diagnosing the T stage. We propose the new idea that the T stage for esophageal cancer can be assessed quantitatively and qualitatively methods using low-dose spectral CT scanning. We found that insufflation CT combined GSI assist technology allows a differential diagnosis between the T1/2 and T3 stages. The ability to differentially diagnose the T3 and T4 stages in medullary esophageal cancer can be improved by analyzing the adipose tissue in front of the vertebral body.

Research perspectives

Nose or mouth leaks are unavoidable when patients hold their breath for a long period of time. Furthermore, the process of insufflation CT is influenced by the patient's age and ability to tolerate the procedures. The future direction of our research will focus on local expansion by lumen filling.

REFERENCES

1 **Roth MJ**, Liu SF, Dawsey SM, Zhou B, Copeland C, Wang GQ, Solomon D, Baker SG, Giffen CA, Taylor PR. Cytologic detection of esophageal squamous cell carcinoma and precursor lesions using balloon and sponge samplers in asymptomatic adults in Linxian, China. *Cancer* 1997; **80**: 2047-2059 [PMID: 9392326]

2 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10-29 [PMID: 22237781 DOI: 10.3322/caac.20138]

3 **Sjoquist KM**, Burmeister BH, Smithers BM, Zalcberg JR, Simes RJ, Barbour A, Gebski V; Australasian Gastro-Intestinal Trials Group. Survival after neoadjuvant chemotherapy or chemoradiotherapy for resectable oesophageal carcinoma: an updated meta-analysis. *Lancet Oncol* 2011; **12**: 681-692 [PMID: 21684205 DOI: 10.1016/S1470-2045(11)70142-5]

4 **Griffin JM**, Reed CE, Denlinger CE. Utility of restaging endoscopic ultrasound after neoadjuvant therapy for esophageal cancer. *Ann Thorac Surg* 2012; **93**: 1855-1859; discussion 1860 [PMID: 22516835 DOI: 10.1016/j.athoracsur.2011.12.095]

5 **Wolf MC**, Stahl M, Krause BJ, Bonavina L, Bruns C, Belka C, Zehentmayr F. Curative treatment of oesophageal carcinoma: current options and future developments. *Radiat Oncol* 2011; **6**: 55 [PMID: 21615894 DOI: 10.1186/1748-717X-6-55]

6 **Lorenzen S**, von Gall C, Stange A, Haag GM, Weitz J, Haberkorn U, Lordick F, Weichert W, Abel U, Debus J, Jäger D, Münter MW. Sequential FDG-PET and induction chemotherapy in locally advanced adenocarcinoma of the Oesophago-gastric junction (AEG): the Heidelberg Imaging program in Cancer of the oesophago-gastric junction during Neoadjuvant treatment: HICON trial. *BMC Cancer* 2011; **11**: 266 [PMID: 21702914 DOI: 10.1186/1471-2407-11-266]

7 **Yen TJ**, Chung CS, Wu YW, Yen RF, Cheng MF, Lee JM, Hsu CH, Chang YL, Wang HP. Comparative study between endoscopic ultrasonography and positron emission tomography-computed tomography in staging patients with esophageal squamous cell carcinoma. *Dis Esophagus* 2012; **25**: 40-47 [PMID: 21595776 DOI: 10.1111/j.1442-2050.2011.01204.x]

8 **Westerterp M**, van Westreenen HL, Reitsma JB, Hoekstra OS, Stoker J, Fockens P, Jager PL, Van Eck-Smit BL, Plukker JT, van Lanschot JJ, Sloof GW. Esophageal cancer: CT, endoscopic US, and FDG PET for assessment of response to neoadjuvant therapy--systematic review. *Radiology* 2005; **236**: 841-851 [PMID: 16118165 DOI: 10.1148/radiol.2363041042]

9 **Sloof GW**. Response monitoring of neoadjuvant therapy using CT, EUS, and FDG-PET. *Best Pract Res Clin Gastroenterol* 2006; **20**: 941-957 [PMID: 16997171 DOI: 10.1016/j.bpg.2006.04.004]

10 **Konieczny A**, Meyer P, Schnider A, Komminoth P, Schmid M, Lombriser N, Weishaupt D. Accuracy of multidetector-row CT for restaging after neoadjuvant treatment in patients with oesophageal cancer. *Eur Radiol* 2013; **23**: 2492-2502 [PMID: 23645329 DOI: 10.1007/s00330-013-2844-8]

11 **Liu BR**, Liu BL, Wang XH, Zhan L, Liu LL, Song JT, Du B, Cui L, Liu SQ. Esophageal insufflation computed tomography for the diagnosis and management of esophageal submucosal tumors. *Surg Endosc* 2017; **31**: 2350-2355 [PMID: 27631315 DOI: 10.1007/s00464-016-5220-3]

12 **Shi Q**, Zhong YS, Yao LQ, Zhou PH, Xu MD, Wang P. Endoscopic submucosal dissection for treatment of esophageal submucosal tumors originating from the muscularis propria layer. *Gastrointest Endosc* 2011; **74**: 1194-1200 [PMID: 21963065 DOI: 10.1016/j.gie.2011.07.039]

13 **Cerfolio RJ**, Bryant AS, Ohja B, Bartolucci AA, Eloubeidi MA. The accuracy of endoscopic ultrasonography with fine-needle aspiration, integrated positron emission tomography with computed tomography, and computed tomography in restaging patients with esophageal cancer after neoadjuvant chemoradiotherapy. *J Thorac Cardiovasc Surg* 2005; **129**: 1232-1241 [PMID: 15942562 DOI: 10.1016/j.jtcvs.2004.12.042]

14 **Jones DR**, Parker LA Jr, Detterbeck FC, Egan TM. Inadequacy of computed tomography in assessing patients with esophageal carcinoma after induction chemoradiotherapy. *Cancer* 1999; **85**: 1026-1032 [PMID: 10091784]

15 **Misra S**, Choi M, Livingstone AS, Franceschi D. The role of endoscopic ultrasound in assessing tumor response and staging after neoadjuvant chemotherapy for esophageal cancer. *Surg Endosc* 2012; **26**: 518-522 [PMID: 21938577 DOI: 10.1007/s00464-011-1911-y]

16 **Yasaka K**, Katsura M, Hanaoka S, Sato J, Ohtomo K. High-

resolution CT with new model-based iterative reconstruction with resolution preference algorithm in evaluations of lung nodules: Comparison with conventional model-based iterative reconstruction and adaptive statistical iterative reconstruction. *Eur J Radiol* 2016; **85**: 599-606 [PMID: 26860673 DOI: 10.1016/j.ejrad.2016.01.001]

17 **Uhrig M**, Simons D, Kachelrieß M, Pisana F, Kuchenbecker S, Schlemmer HP. Advanced abdominal imaging with dual energy CT is feasible without increasing radiation dose. *Cancer Imaging* 2016; **16**: 15 [PMID: 27329159 DOI: 10.1186/s40644-016-0073-5]

18 **Zhu Z**, Zhao XM, Zhao YF, Wang XY, Zhou CW. Feasibility Study of Using Gemstone Spectral Imaging (GSI) and Adaptive Statistical Iterative Reconstruction (ASIR) for Reducing Radiation and Iodine Contrast Dose in Abdominal CT Patients with High BMI Values. *PLoS One* 2015; **10**: e0129201 [PMID: 26079259 DOI: 10.1371/journal.pone.0129201]

19 **Kulkarni NM**, Uppot RN, Eisner BH, Sahani DV. Radiation dose reduction at multidetector CT with adaptive statistical iterative reconstruction for evaluation of urolithiasis: how low can we go? *Radiology* 2012; **265**: 158-166 [PMID: 22891359 DOI: 10.1148/radiol.12112470]

20 **Zhao E**, Ling T, Xu J, Zhao G, Cao H, Giacopuzzi S, Bencivenga M, de Manzoni G. Turning left or right? A comparative analysis in adenocarcinomas of the esophagogastric junction according to the seventh AJCC TNM classification for cancers of the esophagus and stomach: experience in a Chinese single institution. *Int J Clin Exp Med* 2015; **8**: 10668-10677 [PMID: 26379858]

P- Reviewer: Eleftheriadis NP, Kim SM, Surucu E
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 28; 24(36): 4208-4216

DOI: 10.3748/wjg.v24.i36.4208

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

CASE REPORT

Novel methionyl-tRNA synthetase gene variants/ phenotypes in interstitial lung and liver disease: A case report and review of literature

Kuerbanjiang Abuduxikuer, Jia-Yan Feng, Yi Lu, Xin-Bao Xie, Lian Chen, Jian-She Wang

Kuerbanjiang Abuduxikuer, Yi Lu, Xin-Bao Xie, Jian-She Wang, Department of Hepatology, Children's Hospital of Fudan University, Shanghai 201102, China

Jia-Yan Feng, Lian Chen, Department of Pathology, Children's Hospital of Fudan University, Shanghai 201102, China

Jian-She Wang, Department of Pediatrics, Jinshan Hospital of Fudan University, Shanghai 201508, China

ORCID number: Kuerbanjiang Abuduxikuer (0000-0003-0298-3269); Jia-Yan Feng (0000-0002-6651-4675); Yi Lu (0000-0002-3311-4501); Xin-Bao Xie (0000-0002-3692-7356); Lian Chen (0000-0002-0545-2108); Jian-She Wang (0000-0003-0823-586X).

Author contributions: Wang JS designed the report and approved the final submission; Abuduxikuer K collected data, analyzed relevant information, and wrote the manuscript; Wang JS, Lu Y, Xie XB, and Abuduxikuer K clinically managed the patient; Feng JY, Chen L analyzed liver biopsy samples.

Supported by the National Natural Science Foundation of China, No. 81570468.

Informed consent statement: Informed consent was obtained from the parents.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

CARE Checklist (2013) statement: The authors have read the CARE Checklist (2013), and the manuscript was prepared and revised according to the CARE Checklist (2013).

Manuscript source: Unsolicited manuscript

Correspondence to: Jian-She Wang, MD, PhD, Professor, Department of Hepatology, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China. jshwang@shmu.edu.cn
Telephone: +86-21-64931171
Fax: +86-21-64931171

Received: June 21, 2018
Peer-review started: June 22, 2018
First decision: July 31, 2018
Revised: August 2, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: September 28, 2018

Abstract

Interstitial lung and liver disease (ILLD) is caused by biallelic mutations in the methionyl-tRNA synthetase (*MARS*) gene. To date, no genetic changes other than missense variants were reported in the literature. Here, we report a five-month old female infant with typical ILLD (failure to thrive, developmental delay, jaundice, diffuse interstitial lung disease, hepatomegaly with severe steatosis, anemia, and thrombocytosis) showing novel phenotypes such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant (c.2158C>T/p.Gln720Stop) together with a novel tri-nucleotide insertion (c.893_894insTCG that caused the insertion of an arginine at amino acid position 299) in the *MARS* gene.

Key words: Methionyl-tRNA synthetase; Infant; Kidney stone; Hip dysplasia; Leukocytosis; Interstitial lung and

liver disease; Methionyl-tRNA synthetase gene

Core tip: Previously reported cases of interstitial lung and liver disease (ILLD) were associated with biallelic missense mutations in the methionyl-tRNA synthetase (*MARS*) gene. Here, we report a Chinese infant with typical ILLD (failure to thrive, developmental delay, interstitial lung disease, cholestasis, hepatomegaly, steatosis, anemia, and thrombocytosis) with novel phenotypes, such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant (c.2158C>T/p.Gln720Stop), and a novel tri-nucleotide insertion (c.893_894insTCG) in the *MARS* gene. Despite the resolution of cholestasis, this patient died of respiratory failure at the age of 11 mo.

Abuduxikuer K, Feng JY, Lu Y, Xie XB, Chen L, Wang JS. Novel methionyl-tRNA synthetase gene variants/phenotypes in interstitial lung and liver disease: A case report and review of literature. *World J Gastroenterol* 2018; 24(36): 4208-4216 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i36/4208.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i36.4208

INTRODUCTION

The methionyl-tRNA synthetase (*MARS*) gene encodes cytoplasmic methionyl-tRNA synthetase (MetRS) responsible for catalyzing the ligation of methionine to tRNA[1]. MetRS belongs to a family of aminoacyl-tRNA synthetases that play critical roles in protein biosynthesis by charging tRNAs with their cognate amino acids[2]. Interstitial lung and liver disease (ILLD) (OMIM#615486) is caused by homozygous or compound heterozygous mutations in the *MARS* gene (156560) on chromosome 12q13[3-5]. Heterozygous *MARS* mutations have been reported to be associated with autosomal dominant Charcot-Marie-Tooth disease (CMT)[6-9]. The same *MARS* mutation may cause both ILLD and CMT[10]. *MARS* is also a candidate gene for hereditary spastic paraplegias (HSPs), a neuro-degenerative motor neuron disorder[11]. To date, no genetic changes other than missense variants have been reported in the literature. Here, we report a Chinese infant with lethal ILLD showing novel phenotypes such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant together with a novel tri-nucleotide insertion in the *MARS* gene.

CASE REPORT

A five-month old female infant was presented with a failure to thrive, developmental delay, jaundice, and dark urine. She was born full-term with a normal birth weight (3100 g) after an uncomplicated first pregnancy and vaginal delivery. Weight gain and developmental milestones were normal until three months of age (weighted 6000 g), when she failed to thrive with a body weight of 5700 g at the age of 5 mo without the ability of rolling over.

At in-patient admission, this patient was 5.2 mo old with a body weight of 5500 g (2nd percentile by WHO standards), length of 55 cm (lower than the 1st percentile), and head circumference of 39 cm (2nd percentile). This infant had prolonged low-grade fever, pulmonary effusion, diffuse interstitial lung disease, significant leukocytosis, high procalcitonin (PCT)/CRP levels, and required nasal oxygen therapy. Serial chest X-rays showed some improvement in pulmonary effusion, but no improvement in interstitial lung involvement (Figure 1A). After serial antibiotic treatments (ceftriaxone, cefoperazone + slubactam, meropenem, norvancomycin, and fluconazole), body temperature was normalized, oxygen therapy was no longer needed, and leukocytosis improved, however the interstitial lung disease stayed the same. After treatment with ursodeoxycholic acid and fat-soluble vitamins, cholestasis improved significantly (Table 1).

The patient was discharged with normal oxygen saturation on room air without apparent respiratory distress or cough. A liver function test and complete blood count were normal at a 9.5 mo follow-up. However, the infant was admitted to a provincial level pediatric intensive care unit for acute respiratory distress at 11 mo of age and received mechanical ventilation. Despite treatment, she died of respiratory failure and hypoxic encephalopathy.

A genetic cause was suspected due to multiple system involvement, although a liver panel consisting of 41 genes (Table 2) related to liver diseases came back negative. Lysosomal storage disease was considered, but an enzyme panel for the screening of common lysosomal storage diseases was normal, as was the urine acidoglycoprotein level. This patient was enrolled for the undiagnosed disease patient program in our hospital, and whole exome sequencing was ordered. Compound heterozygous *MARS* gene variants, c.2158C>T/p.Gln720Stop and c.893_894insTCG/p.Arg299dup, were detected. Presence of these mutations was confirmed with Sanger sequencing, and parental origins were ascertained. Both variants were not reported in the dbSNP137 (http://www. ncbi.nlm.nih.gov/snp/), 1000 Genome Database (http://www.1000genomes.org/), and Exome Variant Server (http://evs.gs.washington.edu/EVS/). The c.2158C>T mutation was inherited from the healthy mother, which caused the change of a glutamine amino acid at position 720 to a stop codon, which was predicted to be disease-causing by MutationTaster (http://www.mutationtaster.org). The tri-nucleotide insertion (c.893_894insTCG)

Table 1 Changes in complete blood count, procalcitonin, serum biochemistry, and blood coagulation profiles

Age (mo) ([1]in-patient admission; [2]discharge to out-patient follow-up)		5.1	5.6[1]	6	6.2	6.5	6.8	7	7.2[2]	9.5
Complete blood count (reference range)	White blood cell (4-10 × 10^9/L)	16.9	21.1	71.7	26.4	33.7	45.8	30.3	24.3	14.3
	Neutrophil (20%-50%)	58.1	39.8	58.0	63.1	62.0	63.0	62.7	64.4	38.9
	Lymphocyte (45%-75%)	36.2	53.1	31.1	28.7	28.9	15.0	29.5	27.8	51.4
	Abnormal lymphocytes (0%)	NA	0.0	0.0	NA	0.0	17.0	0.0	0.0	NA
	Platelet count (100-300 × 10^9/L)	764.0	513.0	993.0	464	387.0	494.0	279.0	397	386.0
	Hemoglobin (110-160 g/L)	78.0	85.2	78.2	60.1	64.0	65.2	90.0	88.0	122.0
	Red blood cell count (4.0-5.5 × 10^{12} /L)	3.5	3.1	2.8	2.0	2.0	2.2	2.9	2.9	4.3
	Reticulocyte (0.5%-1.5%)	NA	2.9	6.7	NA	6.3	7.8	3.3	6.8	1.0
	C-reactive protein (< 8 mg/L)	1.0	8.0	90.0	32.0	43.0	37.0	45.0	8.0	8.0
Procalcitonin (< 0.05 ng/mL)		NA	4.6	17.4	7.7	13.4	NA	NA	NA	NA
Serum biochemistry (reference range)	Albumin (35-55 g/L)	29.0	34.6	27.3	30.8	32.3	28.7	38.5	39.1	43.0
	Alanine aminotransferase (0-40 IU/L)	41.0	45.0	17.0	13.0	4.0	50.0	49.0	38.0	29.0
	Aspartate aminotransferase (0-40 IU/L)	100.0	104.0	46.0	37.0	66.0	98.0	70.0	62.0	41.0
	Total bilirubin (5.1-17.1 μmol/L)	68.0	120.4	133.0	132.9	126.8	110.6	90.1	42.9	8.1
	Direct bilirubin (0-6 μmol/L)	53.0	76.9	93.7	96.1	86.6	70.4	61.8	29.8	4.4
	γ-glutamyl transferase (7-50 IU/L)	73.0	61.0	76.0	58.0	54.0	57.0	107.0	230.0	122.0
	Total bile acid (0-10 μmol/L)	NA	182.8	123.3	152.4	137.2	157.4	311.7	282.3	34.6
	Alkaline phosphatase (42-383 IU/L)	307.0	137.0	149.0	119.0	122.0	148.0	178.0	214.0	378.0
	Blood glucose (3.9-5.8 mmol/L)	NA	1.2	1.6	8.4	1.1	NA	NA	3.6	NA
	Lactic acid (0-2 mmol/L)	NA	3.9	NA	3.6	3.6	NA	NA	NA	NA
	Ammonia (10-47 μmol/L)	NA	88.0	NA	NA	NA	NA	NA	55.0	NA
	Total cholesterol (3.1-5.2 mmol/L)	3.1	2.0	NA	2.3	2.5	NA	2.8	4.4	3.1
	LDL-cholesterol (1.30-3.90 mmol/L)	NA	NA	NA	1.0	NA	NA	NA	NA	NA
	HDL-cholesterol (0.91-2.05 mmol/L)	NA	NA	NA	0.3	NA	NA	NA	NA	NA
	Triglyceride (0.56-1.70 mmol/L)	NA	2.0	NA	2.7	2.1	NA	2.1	1.8	1.5
Blood coagulation profiles (reference range)	Activated partial thromboplastin time (28.0-44.5 s)	NA	48.1	NA	57.5	56.4	53.9	47.7	42.3	43.8
	D-dimer (0-0.3 mg/L)	NA	0.94	NA	2.06	1.15	0.97	0.7	0.51	NA
	Fibrinogen (2-4 g/L)	NA	1.45	NA	1.82	2.29	2.54	3.03	3.46	3.44
	Fibrinogen degradation products (0-5 μg/ML)	NA	1.31	NA	5.22	2.35	2.78	1.47	1.16	NA
	Thrombin time (14-21 s)	NA	20.4	NA	19.1	19.9	19.9	15.8	18.4	15.2
	International normalized ratio (0.8-1.2)	NA	NA	NA	1.29	1.26	1.35	1.3	1.03	0.99
	Prothrombin time (12.0-14.8 s)	NA	NA	NA	16	15.7	16.5	16.1	13.5	13.1
	Prothrombin time activity (80%-100%)	NA	NA	NA	67	69	63	66	95	103

NA: Not available.

inherited from her healthy father caused the insertion of a single amino acid (arginine) at position 299, which was predicted to be disease-causing by MutationTaster (Figure 2A). The detailed genetic testing results and secondary findings are provided in Table 2.

Liver biopsy results showed severe steatosis of hepatic cells with ballooning, lobular disarray, and cholestasis. Mild changes, such as fibrosis, lymphocyte infiltration, and bile duct proliferation, were seen within the portal region. Hepatic iron deposition was seen after iron staining, but copper staining was negative (Figure 1C). Sinosoids and Kupffer cells seemed normal. Immunohistochemical staining for hepatitis B surface antigen, core antigen, Epstein-Barr virus, and langerin cells were negative. Immunohistochemical staining for cholestasis-related proteins, such as BSEP, MDR3, MRP2, TJP2, and MYO5B, were all normal. After genetic diagnosis, we used a rabbit anti-MARS monoclonal antibody (purchased from http://www.abcam.cn, product code: ab180497) to perform immunohistochemical staining on paraffin-embedded liver biopsy samples. When compared to a normal liver sample (donated for liver transplantation), coarsely granular pigments within the cytoplasm were seen in the index patient sample.

Ultrasound examination revealed marked hepatomegaly (liver 4 cm below the right costal margin, and 5 cm below the xiphoid process) and reduced hepatic echogenicity. Hyper-echoic lesions consistent with stone formation were seen on both kidneys. Abdominal computed tomography scans showed hepatic steatosis and hyper-echoic lesions suggestive of kidney stones in the left kidney but not in the right kidney (Figure 1B). X-ray imaging of the skull was normal, as were the long bones of both arms and legs. X-ray imaging also picked up abnormally shallow hip sockets on both sides, which is suggestive of acetabular dysplasia or congenital hip dysplasia (Figure 1B). Other diagnostic evaluations are provided in Table 3.

DISCUSSION

MetRS is one of 20 ubiquitously expressed enzymes essential for protein biosynthesis, and covalently links methionine with its cognate tRNA. Since initial reports of *MARS* gene mutations causing ILLD[3] and CMT[6] in 2013, a total of 34 cases of ILLD[4,5,10] and eight cases of CMT[7-10] have been reported.

Table 2 Genetic testing results

Genetic Tests	Gene	Transcript ID	Associated conditions (Inheritance patterns) in OMIM	Variant	Amino-acid change	Hom/Het	Parental origin	Prediction of pathogenicity			
								Mutation taster	SIFT	Provean	Polyphen2
Liver Panel[1]	*ATP8B1*	NM _005603	Cholestasis, benign recurrent, intrahepatic (AR); cholestasis, intrahepatic, of pregnancy, 1 (AD); cholestasis, progressive familial intrahepatic 1 (AR)	c.234C> G	p.His78Gln	Het	NA	Polymorphism	Tolerated	Neutral	Benign
				c.1729A>G	p.Ile577Val	Het	NA	Polymorphism	Tolerated	Neutral	Possibly damaging
				c.2021T>C	p. Met674Thr	Het	NA	Polymorphism	Tolerated	Neutral	Benign
				c.3477C>T	Synonymous	Het	NA	Polymorphism	Tolerated	Neutral	NA
				c.3744C>A	Synonymous	Het	NA	Polymorphism	Tolerated	Neutral	NA
Whole exome sequencing	*MARS*	NM_004990	Charcot-Marie-Tooth disease, axonal, type 2U (AD); Interstitial lung and liver disease (AR)	c.2158C>T	p.Gln720Stop	Het	Maternal	Disease causing	NA	NA	NA
				c.893_894insTCG	p.Arg299dup	Het	Paternal	Disease causing	NA	Deleterious	NA
	ATP8B1	NM_005603	Cholestasis, benign recurrent, intrahepatic (AR); cholestasis, intrahepatic, of pregnancy, 1 (AD); cholestasis, progressive familial intrahepatic 1 (AR)	c.2021T>C	p. Met674Thr	Het	Paternal	polymorphism	Tolerated	Neutral	Benign
	CPT1A	NM_001876	CPT deficiency, hepatic, type IA (AR)	c.1163+5G>A	-	Het	Maternal	Disease causing	NA	NA	NA
	LRPPRC	NM_133259	Leigh syndrome, French-Canadian type (AR)	c.2965C>T	p.Arg989Cys	Het	Maternal	Disease causing	Damaging	Deleterious	Probably damaging
	FLG	NM_002106	Ichthyosis vulgaris (AD); (Dermatitis, atopic, susceptibility to, 2)	c.5841G>A	p.Trp1947Stop	Het	Maternal	Disease causing	NA	NA	NA
	G6PD	NM_00104251	Hemolytic anemia, G6PD deficient (favism) (XLD); (Resistance to malaria due to G6PD deficiency)	c.241C>T	p.Arg81Cys	Het	Maternal	Disease causing	Damaging	Deleterious	Benign
	POMGNT1	NM_017739	Muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), type A, 3 (AR); Muscular dystrophy-dystroglycanopathy (congenital with mental retardation), type B, 3 (AR); Muscular dystrophy-dystroglycanopathy (limb-girdle), type C, 3 (AR); Retinitis pigmentosa 76 (AR)	c.794G>A	p.Arg265His	Het	Maternal	Disease causing	Damaging	Deleterious	Probably damaging
	SERPINC1	NM_000488	Thrombophilia due to antithrombin III deficiency (AD/AR)	c.719A>G	p.Asn240Ser	Het	Maternal	Polymorphism	Tolerated	Neutral	Benign
	TG	NM_003235	Thyroid dyshormonogenesis 3 (AR); (autoimmune thyroid disease, susceptibility to, 3)	c.5791A>G	p.Ile1931Val	Het	Paternal	Polymorphism	Tolerated	Neutral	Benign
	USH2A	NM_206933	Retinitis pigmentosa 39; Usher syndrome type 2A (AR)	c.8559-2A>G	-	Het	Paternal	Disease causing	NA	NA	NA

[1]Genes included in liver panel: *ATP8B1, ABCB11, ABCB4, TJP2, BAAT, CLDN1, HSD3B7, AKR1D1, CYP7B1, AMACR, CYP27A1, DHCR7, JAG1, NOTCH2, SLC25A13, DGUOK, MPV17, FAH, ABCC2, UGT1A1, NPC1, NPC2, GALT, GALE, ALDOA, ALDOB, KRT18, KRT8, CIRH1A, CFTR, GFDM1, EARS2, HSD17B4, LIPA, PEX1, PEX5, POU1F1, HESX1, SERPINA1, VIPAS39*, and *VPS33B*. NA: Not available.

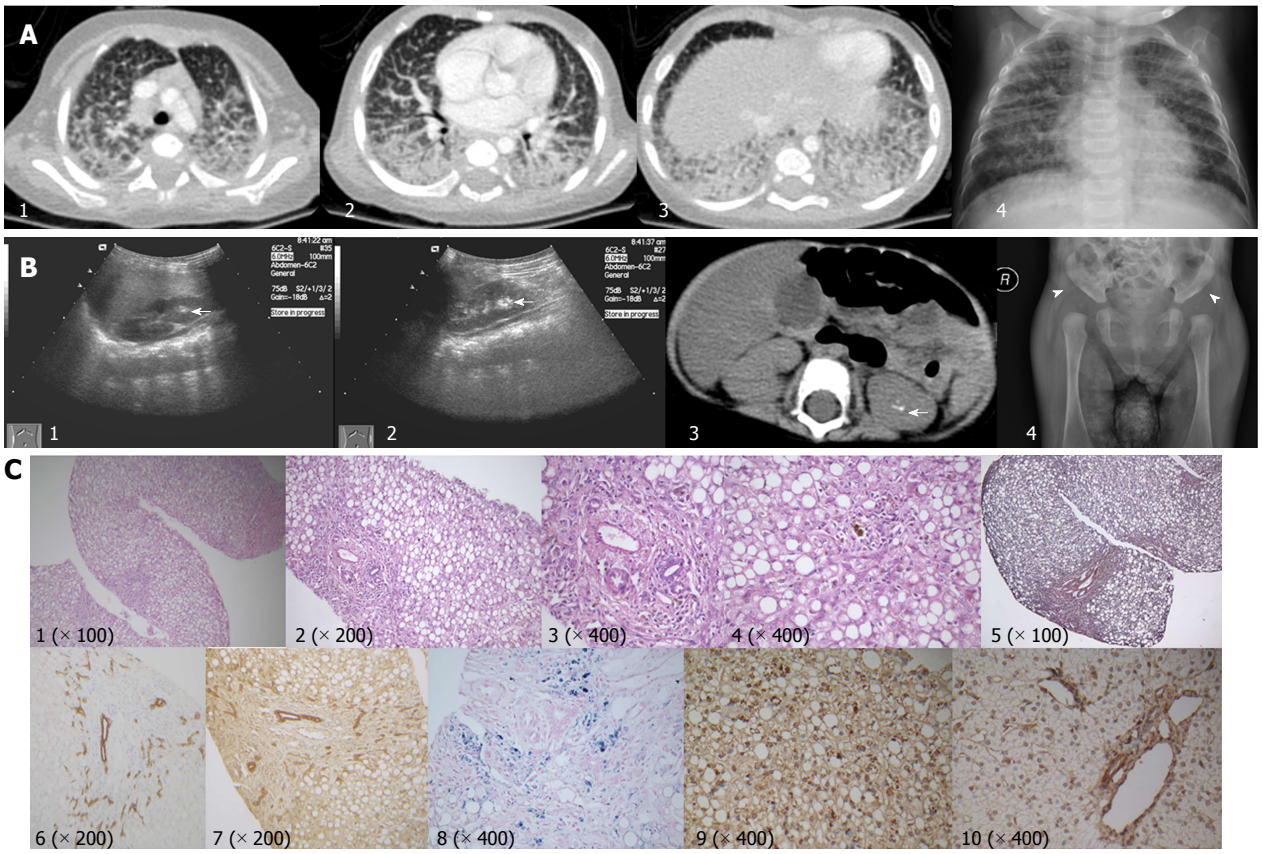


Figure 1 Imaging and histopathological features. A: Contrast enhanced pulmonary CT scan (1-3), and chest X-ray (4) showing pulmonary effusion with marked interstitial lung involvement; B: Hyper-echoic lesions consistent with stone formation on ultrasonography (arrows; 1, right kidney; 2, left kidney) and non-contrast abdominal computed tomography scan (arrow, 3). Acetabular dysplasia (4, arrowhead showing abnormally shallow hip socket); C: Liver biopsy (all originally magnified principal images): severe steatosis of hepatic cells with ballooning, lobular disarray, and cholestasis (1-4), mild fibrosis (5), mild lymphocyte infiltration (4), bile duct proliferation (6 CK-7, 7 CK-19), and hepatic iron deposition (8). *MARS* immunohistochemistry staining, coarsely granular pigments within the cytoplasm in the index patient (9), but not in samples of a healthy control (10). *MARS*: Methionyl-tRNA synthetase gene.

Similar to previous reports, the patient in our case showed a failure to thrive, developmental delay, interstitial lung disease, liver involvement (hepatomegaly, cholestasis, hepatic steatosis, fibrosis, and iron deposition), anemia, and thrombocytosis. An active proliferation of bone marrow cells has been reported by Sun *et al*[5]. Our patient had marked leukocytosis (white blood cell count up to 71.7 × 10^9/L), and a bone marrow biopsy showed extreme proliferation of bone marrow cells with few hemophagocytic cells. MetRS is also a component of a cytoplasmic multiaminoacyl-tRNA synthetase complex with multiple roles in immune response, inflammation, and tumorigenesis[12,13]. Prolonged low-grade fever, leukocytosis, thrombocytosis, and elevated c-reactive protein in this patient responded to intensive antibiotic treatment, and could be viewed as an exaggerated inflammatory or immune response to infection. Unlike previous reports of an arrest in red blood cell maturity[3,5], a bone marrow biopsy from this patient showed marked proliferation of normal erythrocyte precursors.

While aminoaciduria has been reported[3], kidney stones have never been reported to be associated with a *MARS* mutation. No evidence of urinary tract infection, proteinuria, or organic aciduria was found in our case, and serum electrolytes with urea and creatinine were essentially normal. An evaluation of urinary citrate, calcium, and 24 h urine output in future ILLD cases might be necessary in order to rule out factors that promote renal stone formation[14]. Mutations in genes encoding mitochondrial seryl-tRNA synthetases have been reported to cause renal damage[15,16], but no association of cytoplasmic aminoacyl-tRNA synthetases, including *MARS,* have been reported. Since previously reported mutations were all non-synonymous in nature, severe mutations (such as a truncation or single amino acid insertion as in our case) may have caused some renal impairment leading to stone formation.

No skeletal abnormality has been reported, with the exception of two ILLD cases with delayed bone age[5]. Our case had marked acetabular dysplasia consistent with developmental hip dysplasia. Other than being female, this infant did not have other risk factors[17], such as breach presentation upon delivery, local infection, or trauma. Whole exome sequencing did not reveal abnormalities in previously reported susceptible genes such as *GDF5*, *TBX4*, *ASPN*, *IL-6*, *TGF-b1*, and *PAPPA2*[18]. Hip dysplasia is associated with CMT[19],

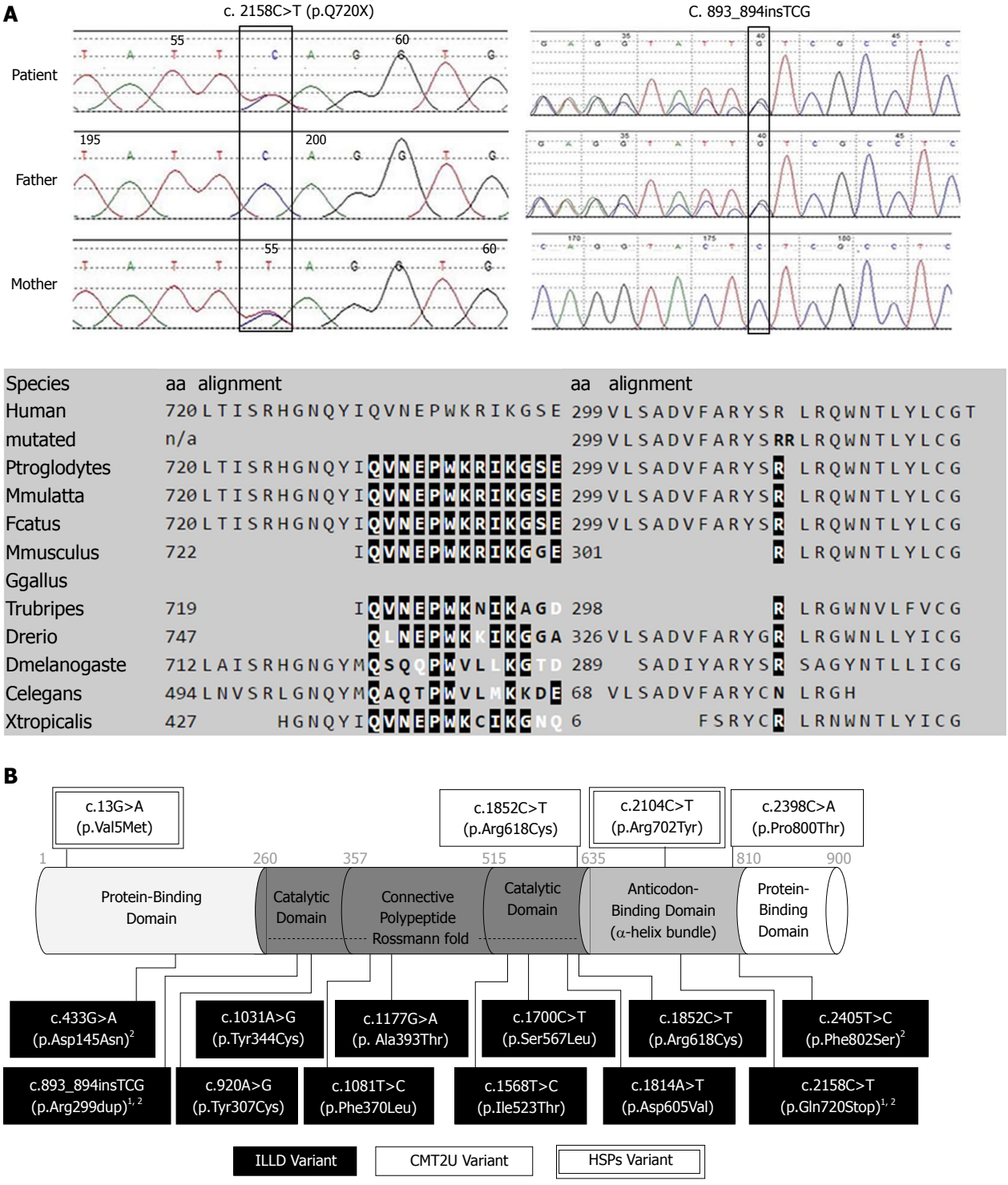


Figure 2 Genetic testing results, protein features, and distribution of reported variants within the methionyl-tRNA synthetase protein. A: Sanger sequencing confirmation of the index case and parents, both variants affect highly conserved amino acid residues of the MetRS protein; B: Illustration of MetRS protein domains, location of amino acid changes of the reported variants so far. [1]Variants from our report; [2]Variants from Chinese ILLD cases. MetRS: Methionyl-tRNA synthetase; ILLD: Interstitial lung and liver disease.

and the rate of hip dysplasia among children with CMT ranges from 6% to 8.1%[20]. Novarino *et al*[11] reported four cases of HSPs with compound heterozygous variants of the *MARS* gene in a family with infantile onset delayed motor milestones and disabilities upon crawling/walking. Two cases had bilateral Achilles contracture, one had scoliosis, but none had hip-joint abnormalities. A recent report of an ILLD case[10] with a p.Arg618Cys variant was also associated with CMT in a previous report[6], indicating ILLD and CMT may share a similar disease-causing mechanism. All reported cases of CMT, ILLD, and HSPs associated with the *MARS* gene

Table 3 Diagnostic evaluation of the patient with a methionyl-tRNA synthetase mutation

Etiological assessment	Investigations performed (normal unless otherwise indicated)
Infections	Serum procalcitonin levels (significantly elevated, Table 1); Serology for Hepatitis B, C, HIV, syphilis, EBV, CMV, HSV, toxoplasmin, and rubella virus; PCR for CMV; beta-d-glucan assay; galactomannan assay; T-Spot.TB test; Cerebrospinal fluid analysis for white blood cell count, protein, and glucose level; Complete blood count: anemia, elevated WBC and C-reactive protein (Table 1); Culture for blood, urine, sputum, alveolar lavage fluid, and cerebrospinal fluid; Sputum and alveolar lavage fluid for mycoplasma/chlamydia DNA detection; Sputum and alveolar lavage fluid for detection of respiratory syncytial virus, adenovirus, influenza virus, and para-influenza virus antigens; Alveolar lavage fluid smear for fungus detection
Radiology, endoscopy, and histopathology	Multiple chest X-rays and a contrast-enhanced computed tomography scan of the lung (alveolar effusions with severe interstitial lung disease) (Figure 1); Abdominal ultrasonography and CT scan (hepatomegaly, liver steatosis, kidney stones) (Figure 1); Bronchoscopy (chronic inflammatory changes in bronchiolar mucosa); X-ray imaging of the skull; CT scan of adrenal gland; X-ray imaging of long bones: (abnormally shallow hip socket that is suggestive of acetabular dysplasia or congenital hip dysplasia) (Figure 1); Liver biopsy (severe steatosis of hepatic cells with ballooning, lobular disarrays; mild changes, such as cholestasis, fibrosis, lymphocyte infiltration, Iron deposition, and bile duct proliferation); Bone marrow aspirate (extreme proliferation of bone marrow cells with few hemophagocytic cells); peripheral blood smear
Immunology	Immunoglobulin levels (after IVIG therapy at local hospital): elevated IgG (20.2 g/L, normal range 3.7-8.3 g/L), IgM (1.47 g/L, normal range 0.33-1.25 g/L), and IgA (0.63 g/L, normal range 0.14-0.5) levels; normal IgE, complement 4, and complement 3 levels; Neutrophil oxidative burst activity, and lymphocyte subpopulations; Autoimmune antibodies
Biochemical, metabolic and endocrine profiling	Glucose profiling (hypoglycemia); slightly elevated serum lactate (Table 1); Liver function test: cholestasis, hypoalbuminemia, abnormal blood coagulation profiles (Table 1); Creatine kinase, lactate dehydrogenase; Serum amino acids (proline 1803 μmol/L, normal range: 165-700 μmol/L; threonine 171 μmol/L, normal range: 17-90 μmol/L) and acyl-carnitine profile; urine organic acids (including succinylacetone); Urine acidoglycoprotein (51.98 mg/mmol creatinine, normal range: 59.70-78.52 mg/mmol creatinine). Low levels of total serum cholesterol, HDL and LDL cholesterol (Table 1). Serum cortisol level; thyroid function test (total triiodothyronine 52.6 ng/dL, normal range: 70-220 ng/dL) Ophthalmology, electrocardiology, and echocardiogram (patent foramen ovale, 2.6 mm)
Genetic disorders	White blood cell lysosomal enzyme screening for GM1 gangliosidosis, GM2 gangliosidosis, Sandhoff disease, Krabbe leukodystrophy, Gaucher disease, Fabry disease, Pompe disease, metachromatic leukodystrophy, Nieman-Pick disease, neuronal ceroid lipofuscinoses (1 and 2), mucopolysaccharidosis (type I-VII, IX), muculipidosis (type II and III). Liver panel including 41 genes known to cause liver diseases, and trio whole exome sequencing (Table 2).

had missense mutations. Our case had a truncating mutation and an insertion of a single amino acid. Severe mutations may have been responsible for the hip dysplasia, which could be an early manifestation of CMT in this patient.

The c.2158C>T/p.Gln720Stop, which was inherited from the mother, caused the glutamine amino acid change at position 720, leading to a stop codon at a well-conserved α-helix bundle domain (anti-codon binding domain) of the methionyl-tRNA synthetase protein.

The tri-nucleotide insertion (c.893_894insTCG) with paternal origin caused the insertion of a single amino acid (arginine) at position 299 in the Rossmann fold domain (catalysis center). Nine out of 12 ILLD variants reported so far affected an amino acid in the Rossmann fold domain (Figure 2B). Arg299 is adjacent to the active methionine-binding site of human MetRS, which is surrounded by the amino acid residues Arg12, Leu13, Pro14, Thr257, Gly259, Tyr260, Asn297, and His301[21].

All eight mutations from European ILLD cases were located in the Rossmann fold of the MARS protein. However, only one out of four mutations from Chinese cases carried mutations in the Rossmann fold domain, and the location of mutations among Chinese ILLD cases was significantly different from that of European ILLD cases (Fisher's exact = 0.018) (Figure 2B). Our case also suggested that severe mutations may lead to more organ/system involvement and severe outcomes.

In vivo yeast complementation assays were used to predict the effects of *MARS* variants, including 1852C>T/p.Arg618Cys[6], c.920A>G/p.Tyr307Cys[10] and 1852C>T/p.Arg618Cys[10]. The *in vitro* aminoacylation assay with HEK293 cells was used to confirm the effects of c.1108T>C/p.Phe370Leu, and c.1568T>C/p.Ile523Thr *MARS* variants[3]. The effects of c.1031A>G/p.Tyr344Cys, c.1177G>A/p.Ala393Thr, c.1700C>T/p.Ser567Leu and c.1814A>T/p.Asp605Val were studied using the *in vitro* yeast aminoacylation assay[4], and later by Comisso *et al*[22] using the *E. Coli*-based aminoacylation assay. Further functional

studies are needed to confirm the effects of variants in our case, as well as variants reported by others (c.2398C>A/p.Pro800Thr[7], c.433G>A/p.Asp145Asn and c.2405T>C/p.Phe802Ser[5]). Besides previously used methods, one may consider the use of animal models such as *Drosophila* and *C. elegans* to predict the pathogenicity of other aminoacyl-tRNA synthetase mutations[23].

There is currently no cure for ILLD, and thus treatment is only supportive. Provided that *in vitro* enzyme activity may partly be restored by increasing methionine[22], methionine supplementation could be considered in studies of animal models, or possibly even in humans. However, plasma levels of methionine and its toxic product homocysteine should be closely monitored.

In conclusion, truncation and insertion variants in the *MARS* gene may cause ILLD, and phenotypes of ILLD may also include kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis.

ARTICLE HIGHLIGHTS

Case characteristics

A five-month old female infant presented with failure to thrive, developmental delay, jaundice, and dark urine.

Clinical diagnosis

Typical clinical findings and whole exome sequencing results led to a diagnosis of interstitial lung and liver disease (ILLD).

Differential diagnosis

Genetic cause was suspected due to multiple system involvement, but a liver panel consisting of 41 genes related to liver diseases came back negative. Lysosomal storage disease was considered, but an enzyme panel for screening common lysosomal storage diseases was normal, as was the urine acidoglycoprotein level.

Laboratory diagnosis

Laboratory findings were Cholestasis, anemia, abnormal blood coagulation profiled, thrombocytosis, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant (c.2158C>T/p.Gln720Stop) and a novel tri-nucleotide insertion (c.893_894insTCG) in the methionyl-tRNA synthetase (*MARS*) gene.

Imaging diagnosis

X-ray, computed tomography scan, and ultrasound imaging revealed interstitial lung disease, hepatomegaly, kidney stones, and acetabular dysplasia.

Pathological diagnosis

Liver biopsy results showed severe hepatic steatosis, hepatic cells ballooning, lobular disarray, cholestasis, iron deposition, and mild fibrosis/lymphocyte infiltration/bile duct proliferation within the portal region.

Treatment

Ursodeoxycholic acid, fat-soluble vitamins, antibiotics, oxygen therapy, and supportive treatment.

Related reports

Previous reports of ILLD were associated with biallelic missense mutations in the *MARS* gene. Phenotypes, such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis have never been reported to be associated with ILLD.

Term explanation

ILLD is interstitial lung and liver disease caused by homozygous or compound heterozygous mutations in the *MARS* gene. Typical findings in ILLD include failure to thrive, developmental delay, interstitial lung disease, liver involvement (hepatomegaly, cholestasis, hepatic steatosis, fibrosis, and iron deposition), anemia, and thrombocytosis.

Experiences and lessons

Regardless of race or ethnicity, ILLD should be considered in all patients with chronic liver diseases showing progressive interstitial lung involvement. Severe mutations may lead to more organ/system involvement and severe outcomes.

REFERENCES

1 **Deniziak MA**, Barciszewski J. Methionyl-tRNA synthetase. *Acta Biochim Pol* 2001; **48**: 337-350 [PMID: 11732605]

2 **Ibba M**, Soll D. Aminoacyl-tRNA synthesis. *Annu Rev Biochem* 2000; **69**: 617-650 [PMID: 10966471 DOI: 10.1146/annurev.biochem.69.1.617]

3 **van Meel E**, Wegner DJ, Cliften P, Willing MC, White FV, Kornfeld S, Cole FS. Rare recessive loss-of-function methionyl-tRNA synthetase mutations presenting as a multi-organ phenotype. *BMC Med Genet* 2013; **14**: 106 [PMID: 24103465 DOI: 10.1186/1471-2350-14-106]

4 **Hadchouel A**, Wieland T, Griese M, Baruffini E, Lorenz-Depiereux B, Enaud L, Graf E, Dubus JC, Halioui-Louhaichi S, Coulomb A, Delacourt C, Eckstein G, Zarbock R, Schwarzmayr T, Cartault F, Meitinger T, Lodi T, de Blic J, Strom TM. Biallelic Mutations of Methionyl-tRNA Synthetase Cause a Specific Type of Pulmonary Alveolar Proteinosis Prevalent on Réunion Island. *Am J Hum Genet* 2015; **96**: 826-831 [PMID: 25913036 DOI: 10.1016/j.ajhg.2015.03.010]

5 **Sun Y**, Hu G, Luo J, Fang D, Yu Y, Wang X, Chen J, Qiu W. Mutations in methionyl-tRNA synthetase gene in a Chinese family with interstitial lung and liver disease, postnatal growth failure and anemia. *J Hum Genet* 2017; **62**: 647-651 [PMID: 28148924 DOI: 10.1038/jhg.2017.10]

6 **Gonzalez M**, McLaughlin H, Houlden H, Guo M, Yo-Tsen L, Hadjivassilious M, Speziani F, Yang XL, Antonellis A, Reilly MM, Züchner S; Inherited Neuropathy Consortium. Exome sequencing identifies a significant variant in methionyl-tRNA synthetase (MARS) in a family with late-onset CMT2. *J Neurol Neurosurg Psychiatry* 2013; **84**: 1247-1249 [PMID: 23729695 DOI: 10.1136/jnnp-2013-305049]

7 **Hyun YS**, Park HJ, Heo SH, Yoon BR, Nam SH, Kim SB, Park CI, Choi BO, Chung KW. Rare variants in methionyl- and tyrosyl-tRNA synthetase genes in late-onset autosomal dominant Charcot-Marie-Tooth neuropathy. *Clin Genet* 2014; **86**: 592-594 [PMID: 24354524 DOI: 10.1111/cge.12327]

8 **Nam SH**, Hong YB, Hyun YS, Nam da E, Kwak G, Hwang SH, Choi BO, Chung KW. Identification of Genetic Causes of Inherited Peripheral Neuropathies by Targeted Gene Panel Sequencing. *Mol Cells* 2016; **39**: 382-388 [PMID: 27025386 DOI: 10.14348/molcells.2016.2288]

9 **Hirano M**, Oka N, Hashiguchi A, Ueno S, Sakamoto H, Takashima H, Higuchi Y, Kusunoki S, Nakamura Y. Histopathological features of a patient with Charcot-Marie-Tooth disease type 2U/AD-CMTax-MARS. *J Peripher Nerv Syst* 2016; **21**: 370-374 [PMID: 27717217 DOI: 10.1111/jns.12193]

10 **Rips J**, Meyer-Schuman R, Breuer O, Tsabari R, Shaag A, Revel-Vilk S, Reif S, Elpeleg O, Antonellis A, Harel T. MARS variant associated with both recessive interstitial lung and liver disease and dominant Charcot-Marie-Tooth disease. *Eur J Med Genet* 2018; **61**: 616-620 [PMID: 29655802 DOI: 10.1016/j.ejmg.2018.04.005]

11 **Novarino G**, Fenstermaker AG, Zaki MS, Hofree M, Silhavy JL, Heiberg AD, Abdellateef M, Rosti B, Scott E, Mansour L, Masri A,

Kayserili H, Al-Aama JY, Abdel-Salam GMH, Karminejad A, Kara M, Kara B, Bozorgmehri B, Ben-Omran T, Mojahedi F, El Din Mahmoud IG, Bouslam N, Bouhouche A, Benomar A, Hanein S, Raymond L, Forlani S, Mascaro M, Selim L, Shehata N, Al-Allawi N, Bindu PS, Azam M, Gunel M, Caglayan A, Bilguvar K, Tolun A, Issa MY, Schroth J, Spencer EG, Rosti RO, Akizu N, Vaux KK, Johansen A, Koh AA, Megahed H, Durr A, Brice A, Stevanin G, Gabriel SB, Ideker T, Gleeson JG. Exome sequencing links corticospinal motor neuron disease to common neurodegenerative disorders. *Science* 2014; **343**: 506-511 [PMID: 24482476 DOI: 10.1126/science.1247363]

12 **Guo M**, Schimmel P. Essential nontranslational functions of tRNA synthetases. *Nat Chem Biol* 2013; **9**: 145-153 [PMID: 23416400 DOI: 10.1038/nchembio.1158]

13 **Yao P**, Fox PL. Aminoacyl-tRNA synthetases in medicine and disease. *EMBO Mol Med* 2013; **5**: 332-343 [PMID: 23427196 DOI: 10.1002/emmm.201100626]

14 **Bevill M**, Kattula A, Cooper CS, Storm DW. The Modern Metabolic Stone Evaluation in Children. *Urology* 2017; **101**: 15-20 [PMID: 27838366 DOI: 10.1016/j.urology.2016.09.058]

15 **Rivera H**, Martín-Hernández E, Delmiro A, García-Silva MT, Quijada-Fraile P, Muley R, Arenas J, Martín MA, Martínez-Azorín F. A new mutation in the gene encoding mitochondrial seryl-tRNA synthetase as a cause of HUPRA syndrome. *BMC Nephrol* 2013; **14**: 195 [PMID: 24034276 DOI: 10.1186/1471-2369-14-195]

16 **Belostotsky R**, Ben-Shalom E, Rinat C, Becker-Cohen R, Feinstein S, Zeligson S, Segel R, Elpeleg O, Nassar S, Frishberg Y. Mutations in the mitochondrial seryl-tRNA synthetase cause hyperuricemia, pulmonary hypertension, renal failure in infancy and alkalosis, HUPRA syndrome. *Am J Hum Genet* 2011; **88**: 193-200 [PMID: 21255763 DOI: 10.1016/j.ajhg.2010.12.010]

17 **Shaw BA**, Segal LS; Section on orthopaedics. Evaluation and Referral for Developmental Dysplasia of the Hip in Infants. *Pediatrics* 2016; **138**: [PMID: 27940740 DOI: 10.1542/peds.2016-3107]

18 **Shi D**, Dai J, Ikegawa S, Jiang Q. Genetic study on developmental dysplasia of the hip. *Eur J Clin Invest* 2012; **42**: 1121-1125 [PMID: 22594447 DOI: 10.1111/j.1365-2362.2012.02682.x]

19 **Novais EN**, Bixby SD, Rennick J, Carry PM, Kim YJ, Millis MB. Hip dysplasia is more severe in Charcot-Marie-Tooth disease than in developmental dysplasia of the hip. *Clin Orthop Relat Res* 2014; **472**: 665-673 [PMID: 23943527 DOI: 10.1007/s11999-013-3127-z]

20 **Walker JL**, Nelson KR, Heavilon JA, Stevens DB, Lubicky JP, Ogden JA, VandenBrink KA. Hip abnormalities in children with Charcot-Marie-Tooth disease. *J Pediatr Orthop* 1994; **14**: 54-59 [PMID: 8113373]

21 **Nadarajan SP**, Mathew S, Deepankumar K, Yun H. An in silico approach to evaluate the polyspecificity of methionyl-tRNA synthetases. *J Mol Graph Model* 2013; **39**: 79-86 [PMID: 23228618 DOI: 10.1016/j.jmgm.2012.11.005]

22 **Comisso M**, Hadchouel A, de Blic J, Mirande M. Mutations in MARS identified in a specific type of pulmonary alveolar proteinosis alter methionyl-tRNA synthetase activity. *FEBS J* 2018 [PMID: 29775242 DOI: 10.1111/febs.14510]

23 **Oprescu SN**, Griffin LB, Beg AA, Antonellis A. Predicting the pathogenicity of aminoacyl-tRNA synthetase mutations. *Methods* 2017; **113**: 139-151 [PMID: 27876679 DOI: 10.1016/j.ymeth.2016.11.013]

P- Reviewer: Al-Haggar M, Arslan N, Hamaguchi M
S- Editor: Wang XJ **L- Editor**: Filipodia **E- Editor**: Huang Y

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 October 7; 24(37): 4217-4296

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 37 October 7, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Cesare Tosetti, MD, Doctor, Professor, Department of Primary Care, Health Care Agency of Bologna, Porretta Terme 40046, Bologna, Italy

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
October 7, 2018

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4217-4223

DOI: 10.3748/wjg.v24.i37.4217

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

EDITORIAL

Focus on the gut-brain axis: Multiple sclerosis, the intestinal barrier and the microbiome

Carlos R Camara-Lemarroy, Luanne M Metz, V Wee Yong

Carlos R Camara-Lemarroy, Luanne M Metz, V Wee Yong, Department of Clinical Neurosciences, Cumming School of Medicine, University of Calgary, Calgary T2N 2T9, Canada

Carlos R Camara-Lemarroy, Luanne M Metz, V Wee Yong, Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, Calgary T2N 2T9, Canada

ORCID number: Carlos R Camara-Lemarroy (0000-0003-0676-6675); Luanne M Metz (0000-0002-9868-6842); V Wee Yong (0000-0002-2600-3563).

Author contributions: Camara-Lemarroy CR conceived the study and drafted the manuscript; Metz LM and Yong VW revised and approved the final version of the article.

Supported by the Lejoie-Lake Fellowship (to Camara-Lemarroy CR) awarded by the Hotchkiss Brain Institute.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Carlos R Camara-Lemarroy, MD, Academic Fellow, Postdoctoral Fellow, Department of Clinical Neurosciences, Cumming School of Medicine, University of Calgary, 1403 29 Street NW, Calgary T2N 2T9, Canada. carlos.camara-lemarroy@albertahealthservices.ca
Telephone: +1-403-9444253
Fax: +1-403-2707162

Received: July 5, 2018
Peer-review started: July 5, 2018
First decision: July 25, 2018
Revised: July 30, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: October 7, 2018

Abstract

The brain-gut axis serves as the bidirectional connection between the gut microbiome, the intestinal barrier and the immune system that might be relevant for the pathophysiology of inflammatory demyelinating diseases. People with multiple sclerosis have been shown to have an altered microbiome, increased intestinal permeability and changes in bile acid metabolism. Experimental evidence suggests that these changes can lead to profound alterations of peripheral and central nervous system immune regulation. Besides being of pathophysiological interest, the brain-gut axis could also open new avenues of therapeutic targets. Modification of the microbiome, the use of probiotics, fecal microbiota transplantation, supplementation with bile acids and intestinal barrier enhancers are all promising candidates. Hopefully, pre-clinical studies and clinical trials will soon yield significant results.

Key words: Multiple sclerosis; Microbiome; Intestinal barrier; Bile acids; Gut-brain axis

Core tip: Many studies suggest that the brain-gut connection can contribute to our knowledge of the pathophysiology of neurological conditions. Recent evidence suggests that people with multiple sclerosis have changes in their gut microbiome, their intestinal barrier and even in the metabolism of bile acids. All of these represent relevant therapeutic targets that could feasibly be addressed by pre-clinical and clinical studies. This knowledge acquired in the bench might soon be translated to the bedside.

Camara-Lemarroy CR, Metz LM, Yong VW. Focus on the gut-brain axis: Multiple sclerosis, the intestinal barrier and the microbiome. *World J Gastroenterol* 2018; 24(37): 4217-4223 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4217.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4217

INTRODUCTION

Clinical and preclinical studies have shown bidirectional interactions within the brain-gut axis and the gut microbiome, the intestinal barrier and the immune system, both in health and disease. These complex interactions might be relevant for the pathophysiology of inflammatory demyelinating diseases, and in particular, multiple sclerosis, where much interest has been placed in the recent literature.

THE GUT MICROBIOME

Much interest has been placed recently on the possible role of the gut microbiome in multiple sclerosis (MS) pathophysiology. Many review articles on this subject have recently been published[1-3], perhaps more than original research articles that actually characterize the gut microbiome in patients with MS. This research is in keeping with the essential role that the gut microbiome has in regulating the development of the immune system[4]. This area of research has also been the subject of recent symposia in international MS conferences[5,6].

Much of the experimental evidence is derived from studies using the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. Modifying the gut microbiota with either antibiotic cocktails or probiotics leads to EAE attenuation as well as a multitude of regulatory immune responses[7-9]. Animals bred in germ-free conditions are resistant to EAE induction and show an attenuated immunological response[10,11], an effect lost when mice are repopulated with gut commensals[11]. In recent intriguing experiments, transgenic mice prone to spontaneous brain autoimmunity developed severe disease when transplanted with fecal microbiota from MS patients, as opposed to mice that received fecal microbiota from healthy matching twins[12]. Germ-free mice receiving similar transplants also developed severe EAE, while showing altered peripheral immune responses[13].

From studies attempting to characterize the composition of the microbiome, it is clear there are some differences in people with MS compared to controls. People with relapsing-remitting MS (RRMS) have an abundance of *Anaerostipes*, *Faecalibacterium*, *Pseudomonas*, *Mycoplasma*, *Haemophilus*, *Blautia*, and *Dorea* and a relative decrease of *Bacteroides*, *Prevotella*, *Parabacteroides* and *Adlercreutzia*[14-16]. In pediatric MS, patients have higher levels of members of *Desulfovibrionaceae* and depletion in *Lachnospiraceae* and *Ruminococcaceae*[17,18]. Issues are further complicated by complex analyses at the taxa, phylum and species levels, and a myriad of microbes have been implicated. For example, studies have found a significant depletion in clostridial species[15,19], *Butyricimonas*[20], *Roseburia*[21] and an increase in *Streptococcus*[22], *Methanobrevibacter*, *Akkermansia* and *Coprococcus*[14,20].

However, there are some limitations to these studies. The methods used to analyze the microbiome have been heterogeneous, with most (but not all) studies using a variation of 16S sequencing. There are differences in sample processing, DNA extraction, choice of primers, databases and hyper-variable regions analyzed across studies. Furthermore, close to two thirds of patients with MS have gastrointestinal symptoms such as constipation, dyspepsia and other functional gastrointestinal disorders[23], and many of these have been also associated with an altered gut microbiota[24]. Studies so far have not properly accounted for these symptoms or other relevant variables such as diet. An ongoing International MS Microbiome study aims to define a "core microbiome"[3]. It might shine some light into this complicated field.

Nonetheless, there is mounting experimental evidence that the gut microbiome may play a role in MS pathophysiology and human studies suggest that patients have a different microbiome compared to controls. Of course, the true significance of the results obtained so far is unclear, considering that there has often been a failure to replicate microbiome animal studies in humans. But the next question that comes to mind is whether this can also constitute a relevant therapeutic target. Although this appears to be the case in experimental models, translation to clinical practice may prove challenging.

Modifying the microbiome through medications, possibly antibiotics, could be the simplest method, but several issues arise that question the feasibility of this approach. Targeting specific commensals might prove difficult and requires appropriate identification of these targets. The case of minocycline is an interesting example. Recently shown to delay the occurrence of a second demyelinating event in patients with a clinically isolated syndrome[25], minocycline is an antibiotic known to alter the gut microbiome[26]. Whether this is an additional mechanism of action remains unknown; it is noteworthy that the initial rationale for testing minocycline in early MS is based on its various immune-modifying properties[27]. On the other hand, there is also evidence that MS disease modifying therapies (DMTs) may alter the microbiome directly[26], and indeed, it also appears that a multitude of other medications such as antidepressants, antipsychotics and immune modulators may also do so[28]. Issues such as the generation of resistant strains are also worthy of consideration.

Probiotics are a popular option but there are various issues with their practical implementation. Probiotics do not modify the host microbiome in a robust and persistent manner, although they are purported to be able to influence gut immunity and homeostasis. Despite success

in showing a benefit for probiotics in animal models[29,30], there are only a handful of clinical trials in MS. Results have been preliminary, with some modest beneficial trends in clinical variables and some biochemical markers of changes in peripheral immune function[31-33]. However, they have included very small numbers of patients and the duration of these trials have been too short to shed any light onto clinically meaningful outcomes. There are many barriers to be overcome, such as selecting the appropriate formulation, dose and study design. There is also a lesson to be learned from the multiple clinical trials in inflammatory bowel disease (IBD), where despite a wealth of available studies (although heterogeneous in design and quality), the evidence supporting their clinical use is limited to carefully selected subpopulations[34,35].

Fecal microbiota transplantation (FMT) would constitute the optimal strategy to modify the gut microbiome. It has proven to be remarkably effective in managing *C. difficile* colitis, and isolated case reports describe beneficial effects over MS disease course, through mechanisms that remain unclear[36,37]. A clinical trial of FMT is underway[38], but even before its completion, many questions arise. It is unclear which population should be studied and what characterizes an ideal donor, not to mention the dose, route of administration and dose scheduling (single dose *vs* multiple doses). Patient with *C. diff* colitis who undergo FMT have been previously treated with antibiotics such as vancomycin and metronidazole, and presumably, have had some of their microbiota depleted. Would patients with MS require "ablation" of their microbiome before FMT? DMTs have immune modulating properties and they may also directly alter the microbiome[26], so their possible effects on the "engraftment" cannot be underestimated.

THE INTESTINAL BARRIER

The intestinal barrier is the physical and functional zone of interaction between the gut microbiome and the organism. It is a complex multi-layered structure that includes major portions of the gut immunological system and is essential for homeostasis[26]. However, it has been comparatively ignored regarding its possible role in MS pathophysiology.

In experimental models, mice undergoing EAE show an altered intestinal barrier, with increased permeability and various gross morphological changes, as well as alterations in the expression of tight junction proteins in the intestinal mucosa[39]. The peak of intestinal barrier dysfunction mirrors the peak of EAE clinical severity and preventing intestinal barrier breakdown leads to attenuation of EAE[40]. These alterations have also been associated with several abnormal immunological responses.

Patients with MS also have an altered intestinal barrier. Almost 2 decades ago, investigators found that patients with MS had increased intestinal permeability when compared to controls, using an *in vivo* mannitol/lactulose ratio test[41]. Increased intestinal permeability was also found to be associated with the number of peripheral CD45RO+ B cells[41]. A more recent study confirmed this finding; up to 70% of MS patients had increased intestinal permeability[42]. It has been hypothesized that an altered intestinal barrier might lead to bacterial translocation thus allowing the passage of noxious molecules such as microbial associated molecular patterns. This could then alter peripheral immune responses or allow these molecules to enter the CNS and alter neuroimmunity[26,43].

Although the evidence linking the intestinal barrier with MS is much more limited than evidence linking MS with alterations of the gut microbiome, the question of whether it constitutes a viable therapeutic target is the same. Of course, the issue is complicated by the fact that the microbiome is essential in the regulation of intestinal barrier function, so it could be arbitrary to think of them as separate entities. An altered intestinal barrier is also a crucial aspect of the pathophysiology of IBD and celiac disease, so research from these fields has shed light on possible strategies to maintain intestinal barrier integrity.

One of the first components of the intestinal barrier is a thick mucus layer forming a protective film, enriched by secretory IgA and antimicrobial peptides and proteins. Oral supplementation with lecithin and phosphatidylcholine can adhere to the intestinal mucosa, strengthening the mucus layer and improving barrier function[44-46]. Regulators of tight junctions, such as larazotide, are under development. Larazotide is a peptide able to re-arrange tight junctions and prevent intestinal barrier dysfunction. It has been studied in patients with celiac disease with promising results[47-49]. Designing pre-clinical studies using these methods to enhance barrier function in the setting of autoimmune neurological disease should be straightforward.

Although probiotics may not be the ideal method to modify the microbiome, they have been suggested to play a significant role in modulating barrier function. *E. coli* strain *nissle* has been marketed in Europe for many years as a probiotic with beneficial effects on the intestinal barrier[50]. It has moderate evidence from randomized trials showing it may lead to remission in ulcerative colitis[51] and in the EAE mouse model of MS it reduced disease severity by maintaining intestinal barrier function[40]. VSL#3 is another probiotic mixture with putative barrier-protecting properties[52]. There is evidence of clinical effectiveness in the management of chronic pouchitis in patients with ulcerative colitis[35,53]. VSL#3 administered to a small number of MS patients leads to an anti-inflammatory peripheral immune response[33]. These two probiotic agents would be good candidates for a large, well-designed clinical trial.

Finally, we go full circle and return to FMT. It is believed that after successful modification of the microbiome, this strategy might lead to improved intestinal barrier function[54]. The gut microbiome is essential for the regulation of intestinal barrier homeostasis[55], partly through the production of short chain fatty acids (SCFA) such as butyrate, propionate and acetate. SCFAs can modulate tight junctions in the gut and modulate inflammatory responses in the intestinal mucosa[44,55]. Other interesting alternatives have also recently been described

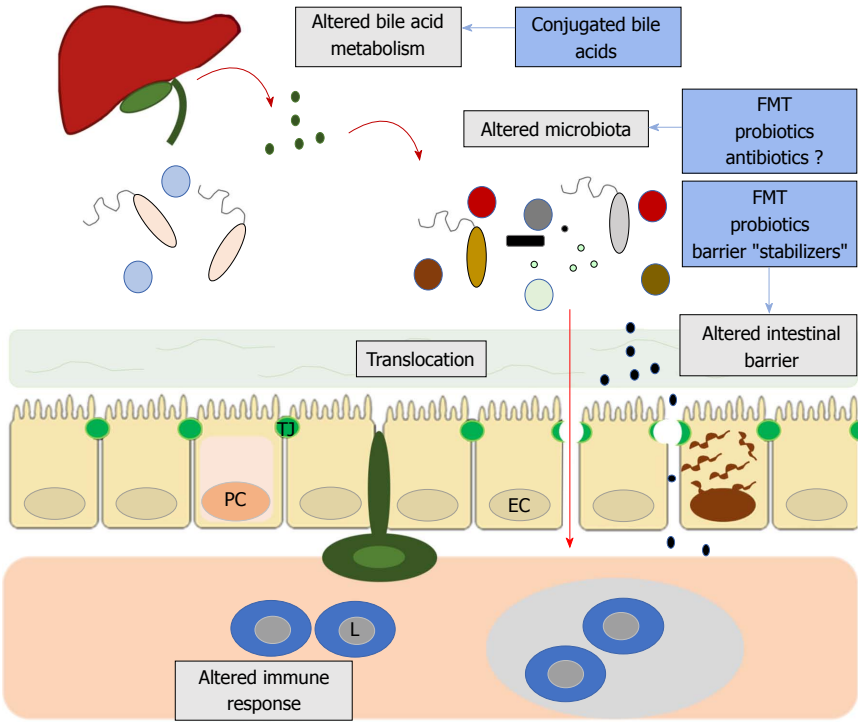


Figure 1 Alterations in intestinal homeostasis described in multiple sclerosis as therapeutic targets. Altered bile acid metabolism, altered microbiota and alterations in intestinal barrier function all lead to local and systemic alterations in immune responses that could negatively impact MS pathophysiology (grey squares). Bile acid supplementation, fecal microbiota transplantation, probiotics, antibiotics and barrier protectors are all possible therapeutic interventions (blue squares). MS: Multiple sclerosis; FMT: Fecal microbiota transplantation; PC: Paneth cells; EC: Epithelial cells; TJ: Tight junctions; L: Lymphocytes.

including the use of stool substitute preparations made from purified intestinal bacterial cultures derived from a single healthy donor[56]. Of course, many questions would need to be settled before clinical trials as discussed above.

BILE ACIDS

Bile acids are the main regulators of fat and fat-soluble vitamins digestion. They also significantly affect gut physiology and homeostasis. Bile acids can modulate the intestinal barrier function through complex mechanisms[57,58], and can shape the gut microbiota community. In turn, the microbiome can change bile acid metabolism[59]. Remarkably, bile acids may also modulate inflammatory signaling in the central nervous system. Ursodeoxycholic acid can inhibit the inflammatory activity of microglia *in vitro*[60], and tauroursodeoxycholic acid can shift microglia phenotypes towards an anti-inflammatory state through activation of the G protein-coupled bile acid receptor 1/Takeda G protein-coupled receptor 5[61]. Bile acids are also agonists of the nuclear hormone receptor farnesoid X receptor. Bile acid farnesoid X agonism led to attenuation of EAE and modulation of neuroinflammatory responses[62]. Mice fed a high-fat diet show dysregulated bile acid synthesis, gut dysbiosis and increased microglial activation[63]. Furthermore, metabolomics studies have found alterations in bile acids in patients with MS compared to healthy controls[64]. Conjugated bile acids such as ursodeoxycholic acid have been used in the management of some gastrointestinal diseases for decades. A clinical trial of bile acid supplementation in MS is underway[65].

CONCLUSION

Exciting research suggests that the brain-gut axis, once an almost esoteric concept, might yield novel therapeutic targets in neuroimmunological diseases such as MS (Figure 1). The often-symbiotic roles of the gut microbiome, intestinal barrier and even bile acids in the regulation of neuroimmune responses is beginning to be elucidated. If future pre-clinical and clinical studies confirm the relevance of intestinal barrier dysfunction, bile acid metabolism and the gut microbiome in the pathophysiology of MS, the next step will be to translate these findings into therapeutics. Only well designed clinical trials will answer whether interventions such as FMT, probiotics or barrier protectors yield clinically meaningful results. The time is right to assess whether the gut-brain axis can be transferred from the bench to the bedside.

REFERENCES

1 **Freedman SN**, Shahi SK, Mangalam AK. The "Gut Feeling": Breaking Down the Role of Gut Microbiome in Multiple Sclerosis. *Neurotherapeutics* 2018; **15**: 109-125 [PMID: 29204955 DOI: 10.1007/s13311-017-0588-x]

2 **Calvo-Barreiro L**, Eixarch H, Montalban X, Espejo C. Combined therapies to treat complex diseases: The role of the gut microbiota in multiple sclerosis. *Autoimmun Rev* 2018; **17**: 165-174 [PMID:

29191793 DOI: 10.1016/j.autrev.2017.11.019]
3 **Pröbstel AK**, Baranzini SE. The Role of the Gut Microbiome in Multiple Sclerosis Risk and Progression: Towards Characterization of the "MS Microbiome". *Neurotherapeutics* 2018; **15**: 126-134 [PMID: 29147991 DOI: 10.1007/s13311-017-0587-y]
4 **Thaiss CA**, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature* 2016; **535**: 65-74 [PMID: 27383981 DOI: 10.1038/nature18847]
5 Hot Topic 6: Gut microbiota in multiple sclerosis. Symposium held at the 7th Joint ECTRIMS-ACTRIMS meeting, 25-28 October, Paris, France. Available from: URL: https://www.ectrims-congress.eu/2017/scientific-programme/scientific-programme.html
6 Brain-Gut Axis: a focus on finding a cure for Multiple Sclerosis. University of Alberta MS Centre Research Symposium, held May 4, 2018, Edmonton, Alberta, Canada. Available from: URL: https://cloudfront.ualberta.ca/-/media/medicine/ms-centre/2018-ualberta-ms-centre-program-25apr18-pdfa.pdf
7 **Ochoa-Repáraz J**, Mielcarz DW, Haque-Begum S, Kasper LH. Induction of a regulatory B cell population in experimental allergic encephalomyelitis by alteration of the gut commensal microflora. *Gut Microbes* 2010; **1**: 103-108 [PMID: 21326918 DOI: 10.4161/gmic.1.2.11515]
8 **Colpitts SL**, Kasper EJ, Keever A, Liljenberg C, Kirby T, Magori K, Kasper LH, Ochoa-Repáraz J. A bidirectional association between the gut microbiota and CNS disease in a biphasic murine model of multiple sclerosis. *Gut Microbes* 2017; **8**: 561-573 [PMID: 28708466 DOI: 10.1080/19490976.2017.1353843]
9 **Wang Y**, Begum-Haque S, Telesford KM, Ochoa-Repáraz J, Christy M, Kasper EJ, Kasper DL, Robson SC, Kasper LH. A commensal bacterial product elicits and modulates migratory capacity of CD39(+) CD4 T regulatory subsets in the suppression of neuroinflammation. *Gut Microbes* 2014; **5**: 552-561 [PMID: 25006655 DOI: 10.4161/gmic.29797]
10 **Berer K**, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, Wekerle H, Krishnamoorthy G. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 2011; **479**: 538-541 [PMID: 22031325 DOI: 10.1038/nature10554]
11 **Lee YK**, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4615-4622 [PMID: 20660719 DOI: 10.1073/pnas.1000082107]
12 **Berer K**, Gerdes LA, Cekanaviciute E, Jia X, Xiao L, Xia Z, Liu C, Klotz L, Stauffer U, Baranzini SE, Kümpfel T, Hohlfeld R, Krishnamoorthy G, Wekerle H. Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci USA* 2017; **114**: 10719-10724 [PMID: 28893994 DOI: 10.1073/pnas.1711233114]
13 **Cekanaviciute E**, Yoo BB, Runia TF, Debelius JW, Singh S, Nelson CA, Kanner R, Bencosme Y, Lee YK, Hauser SL, Crabtree-Hartman E, Sand IK, Gacias M, Zhu Y, Casaccia P, Cree BAC, Knight R, Mazmanian SK, Baranzini SE. Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci USA* 2017; **114**: 10713-10718 [PMID: 28893978 DOI: 10.1073/pnas.1711235114]
14 **Cantarel BL**, Waubant E, Chehoud C, Kuczynski J, DeSantis TZ, Warrington J, Venkatesan A, Fraser CM, Mowry EM. Gut microbiota in multiple sclerosis: possible influence of immunomodulators. *J Investig Med* 2015; **63**: 729-734 [PMID: 25775034 DOI: 10.1097/JIM.0000000000000192]
15 **Miyake S**, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, Chihara N, Tomita A, Sato W, Kim SW, Morita H, Hattori M, Yamamura T. Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters. *PLoS One* 2015; **10**: e0137429 [PMID: 26367776 DOI: 10.1371/journal.pone.0137429]
16 **Chen J**, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, Luckey DH, Marietta EV, Jeraldo PR, Chen X, Weinshenker BG, Rodriguez M, Kantarci OH, Nelson H, Murray JA, Mangalam AK. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* 2016; **6**: 28484 [PMID: 27346372 DOI: 10.1038/srep28484]
17 **Tremlett H**, Fadrosh DW, Faruqi AA, Zhu F, Hart J, Roalstad S, Graves J, Lynch S, Waubant E; US Network of Pediatric MS Centers. Gut microbiota in early pediatric multiple sclerosis: a case-control study. *Eur J Neurol* 2016; **23**: 1308-1321 [PMID: 27176462 DOI: 10.1111/ene.13026]
18 **Tremlett H**, Fadrosh DW, Faruqi AA, Hart J, Roalstad S, Graves J, Lynch S, Waubant E; US Network of Pediatric MS Centers. Gut microbiota composition and relapse risk in pediatric MS: A pilot study. *J Neurol Sci* 2016; **363**: 153-157 [PMID: 27000242 DOI: 10.1016/j.jns.2016.02.042]
19 **Rumah KR**, Linden J, Fischetti VA, Vartanian T. Isolation of Clostridium perfringens type B in an individual at first clinical presentation of multiple sclerosis provides clues for environmental triggers of the disease. *PLoS One* 2013; **8**: e76359 [PMID: 24146858 DOI: 10.1371/journal.pone.0076359]
20 **Jangi S**, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, Patel B, Mazzola MA, Liu S, Glanz BL, Cook S, Tankou S, Stuart F, Melo K, Nejad P, Smith K, Topçuolu BD, Holden J, Kivisäkk P, Chitnis T, De Jager PL, Quintana FJ, Gerber GK, Bry L, Weiner HL. Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* 2016; **7**: 12015 [PMID: 27352007 DOI: 10.1038/ncomms12015]
21 **Swidsinski A**, Dörffel Y, Loening-Baucke V, Gille C, Göktas Ö, Reißhauer A, Neuhaus J, Weylandt KH, Guschin A, Bock M. Reduced Mass and Diversity of the Colonic Microbiome in Patients with Multiple Sclerosis and Their Improvement with Ketogenic Diet. *Front Microbiol* 2017; **8**: 1141 [PMID: 28702003 DOI: 10.3389/fmicb.2017.01141]
22 **Cosorich I**, Dalla-Costa G, Sorini C, Ferrarese R, Messina MJ, Dolpady J, Radice E, Mariani A, Testoni PA, Canducci F, Comi G, Martinelli V, Falcone M. High frequency of intestinal TH17 cells correlates with microbiota alterations and disease activity in multiple sclerosis. *Sci Adv* 2017; **3**: e1700492 [PMID: 28706993 DOI: 10.1126/sciadv.1700492]
23 **Levinthal DJ**, Rahman A, Nusrat S, O'Leary M, Heyman R, Bielefeldt K. Adding to the burden: gastrointestinal symptoms and syndromes in multiple sclerosis. *Mult Scler Int* 2013; **2013**: 319201 [PMID: 24163768 DOI: 10.1155/2013/319201]
24 **De Palma G**, Collins SM, Bercik P. The microbiota-gut-brain axis in functional gastrointestinal disorders. *Gut Microbes* 2014; **5**: 419-429 [PMID: 24921926 DOI: 10.4161/gmic.29417]
25 **Metz LM**, Li DKB, Traboulsee AL, Duquette P, Eliasziw M, Cerchiaro G, Greenfield J, Riddehough A, Yeung M, Kremenchutzky M, Vorobeychik G, Freedman MS, Bhan V, Blevins G, Marriott JJ, Grand'Maison F, Lee L, Thibault M, Hill MD, Yong VW; Minocycline in MS Study Team. Trial of Minocycline in a Clinically Isolated Syndrome of Multiple Sclerosis. *N Engl J Med* 2017; **376**: 2122-2133 [PMID: 28564557 DOI: 10.1056/NEJMoa1608889]
26 **Camara-Lemarroy CR**, Metz L, Meddings JB, Sharkey KA, Wee Yong V. The intestinal barrier in multiple sclerosis: implications for pathophysiology and therapeutics. *Brain* 2018; **141**: 1900-1916 [PMID: 29860380 DOI: 10.1093/brain/awy131]
27 **Yong VW**, Wells J, Giuliani F, Casha S, Power C, Metz LM. The promise of minocycline in neurology. *Lancet Neurol* 2004; **3**: 744-751 [PMID: 15556807 DOI: 10.1016/S1474-4422(04)00937-8]
28 **Maier L**, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, Brochado AR, Fernandez KC, Dose H, Mori H, Patil KR, Bork P, Typas A. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* 2018; **555**: 623-628 [PMID: 29555994 DOI: 10.1038/nature25979]
29 **Kwon HK**, Kim GC, Kim Y, Hwang W, Jash A, Sahoo A, Kim JE, Nam JH, Im SH. Amelioration of experimental autoimmune encephalomyelitis by probiotic mixture is mediated by a shift in T helper cell immune response. *Clin Immunol* 2013; **146**: 217-227 [PMID: 23416238 DOI: 10.1016/j.clim.2013.01.001]
30 **Lavasani S**, Dzhambazov B, Nouri M, Fåk F, Buske S, Molin G, Thorlacius H, Alenfall J, Jeppsson B, Weström B. A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. *PLoS One* 2010; **5**: e9009 [PMID: 20126401 DOI: 10.1371/

journal.pone.0009009]
31 **Kouchaki E**, Tamtaji OR, Salami M, Bahmani F, Daneshvar Kakhaki R, Akbari E, Tajabadi-Ebrahimi M, Jafari P, Asemi Z. Clinical and metabolic response to probiotic supplementation in patients with multiple sclerosis: A randomized, double-blind, placebo-controlled trial. *Clin Nutr* 2017; **36**: 1245-1249 [PMID: 27669638 DOI: 10.1016/j.clnu.2016.08.015]
32 **Tamtaji OR**, Kouchaki E, Salami M, Aghadavod E, Akbari E, Tajabadi-Ebrahimi M, Asemi Z. The Effects of Probiotic Supplementation on Gene Expression Related to Inflammation, Insulin, and Lipids in Patients With Multiple Sclerosis: A Randomized, Double-Blind, Placebo-Controlled Trial. *J Am Coll Nutr* 2017; **36**: 660-665 [PMID: 28922099 DOI: 10.1080/07315724.2017.1347074]
33 **Tankou SK**, Regev K, Healy BC, Cox LM, Tjon E, Kivisakk P, Vanande IP, Cook S, Gandhi R, Glanz B, Stankiewicz J, Weiner HL. Investigation of probiotics in multiple sclerosis. *Mult Scler* 2018; **24**: 58-63 [PMID: 29307299 DOI: 10.1177/1352458517737390]
34 **Abraham BP**, Quigley EMM. Probiotics in Inflammatory Bowel Disease. *Gastroenterol Clin North Am* 2017; **46**: 769-782 [PMID: 29173520 DOI: 10.1016/j.gtc.2017.08.003]
35 **Derwa Y**, Gracie DJ, Hamlin PJ, Ford AC. Systematic review with meta-analysis: the efficacy of probiotics in inflammatory bowel disease. *Aliment Pharmacol Ther* 2017; **46**: 389-400 [PMID: 28653751 DOI: 10.1111/apt.14203]
36 **Borody TJ**, Leis SM, Campbell J, Torres M, Nowak A. Fecal microbiota transplantation (FMT) in multiple sclerosis (MS). *Am J Gastroenterol* 2011; **106**: S352
37 **Makkawi S**, Camara-Lemarroy C, Metz L. Fecal microbiota transplantation associated with 10 years of stability in a patient with SPMS. *Neurol Neuroimmunol Neuroinflamm* 2018; **5**: e459 [PMID: 29619403 DOI: 10.1212/NXI.0000000000000459]
38 **Kremenchutzky M**. Fecal Microbial Transplantation in Relapsing Multiple Sclerosis Patients. cited 2018-05-22; In ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: URL: https://clinicaltrials.gov/ct2/show/NCT03183869
39 **Nouri M**, Bredberg A, Weström B, Lavasani S. Intestinal barrier dysfunction develops at the onset of experimental autoimmune encephalomyelitis, and can be induced by adoptive transfer of auto-reactive T cells. *PLoS One* 2014; **9**: e106335 [PMID: 25184418 DOI: 10.1371/journal.pone.0106335]
40 **Secher T**, Kassem S, Benamar M, Bernard I, Boury M, Barreau F, Oswald E, Saoudi A. Oral Administration of the Probiotic Strain *Escherichia coli* Nissle 1917 Reduces Susceptibility to Neuroinflammation and Repairs Experimental Autoimmune Encephalomyelitis-Induced Intestinal Barrier Dysfunction. *Front Immunol* 2017; **8**: 1096 [PMID: 28959254 DOI: 10.3389/fimmu.2017.01096]
41 **Yacyshyn B**, Meddings J, Sadowski D, Bowen-Yacyshyn MB. Multiple sclerosis patients have peripheral blood CD45RO+ B cells and increased intestinal permeability. *Dig Dis Sci* 1996; **41**: 2493-2498 [PMID: 9011463 DOI: 10.1007/BF02100148]
42 **Buscarinu MC**, Cerasoli B, Annibali V, Policano C, Lionetto L, Capi M, Mechelli R, Romano S, Fornasiero A, Mattei G, Piras E, Angelini DF, Battistini L, Simmaco M, Umeton R, Salvetti M, Ristori G. Altered intestinal permeability in patients with relapsing-remitting multiple sclerosis: A pilot study. *Mult Scler* 2017; **23**: 442-446 [PMID: 27270497 DOI: 10.1177/1352458516652498]
43 **Buscarinu MC**, Romano S, Mechelli R, Pizzolato Umeton R, Ferraldeschi M, Fornasiero A, Reniè R, Cerasoli B, Morena E, Romano C, Loizzo ND, Umeton R, Salvetti M, Ristori G. Intestinal Permeability in Relapsing-Remitting Multiple Sclerosis. *Neurotherapeutics* 2018; **15**: 68-74 [PMID: 29119385 DOI: 10.1007/s13311-017-0582-3]
44 **Sun J**, Shen X, Li Y, Guo Z, Zhu W, Zuo L, Zhao J, Gu L, Gong J, Li J. Therapeutic Potential to Modify the Mucus Barrier in Inflammatory Bowel Disease. *Nutrients* 2016; **8**: [PMID: 26784223 DOI: 10.3390/nu8010044]
45 **Merga Y**, Campbell BJ, Rhodes JM. Mucosal barrier, bacteria and inflammatory bowel disease: possibilities for therapy. *Dig Dis* 2014; **32**: 475-483 [PMID: 24969297 DOI: 10.1159/000358156]
46 **Stremmel W**, Gauss A. Lecithin as a therapeutic agent in ulcerative colitis. *Dig Dis* 2013; **31**: 388-390 [PMID: 24246994 DOI: 10.1159/000354707]
47 **Pearce SC**, Al-Jawadi A, Kishida K, Yu S, Hu M, Fritzky LF, Edelblum KL, Gao N, Ferraris RP. Marked differences in tight junction composition and macromolecular permeability among different intestinal cell types. *BMC Biol* 2018; **16**: 19 [PMID: 29391007 DOI: 10.1186/s12915-018-0481-z]
48 **Gopalakrishnan S**, Tripathi A, Tamiz AP, Alkan SS, Pandey NB. Larazotide acetate promotes tight junction assembly in epithelial cells. *Peptides* 2012; **35**: 95-101 [PMID: 22401910 DOI: 10.1016/j.peptides.2012.02.016]
49 **Khaleghi S**, Ju JM, Lamba A, Murray JA. The potential utility of tight junction regulation in celiac disease: focus on larazotide acetate. *Therap Adv Gastroenterol* 2016; **9**: 37-49 [PMID: 26770266 DOI: 10.1177/1756283X15616576]
50 **Sonnenborn U**. Escherichia coli strain Nissle 1917-from bench to bedside and back: history of a special Escherichia coli strain with probiotic properties. *FEMS Microbiol Lett* 2016; **363**: [PMID: 27619890 DOI: 10.1093/femsle/fnw212]
51 **Scaldaferri F**, Gerardi V, Mangiola F, Lopetuso LR, Pizzoferrato M, Petito V, Papa A, Stojanovic J, Poscia A, Cammarota G, Gasbarrini A. Role and mechanisms of action of Escherichia coli Nissle 1917 in the maintenance of remission in ulcerative colitis patients: An update. *World J Gastroenterol* 2016; **22**: 5505-5511 [PMID: 27350728 DOI: 10.3748/wjg.v22.i24.5505]
52 **Dai C**, Zhao DH, Jiang M. VSL#3 probiotics regulate the intestinal epithelial barrier in vivo and in vitro via the p38 and ERK signaling pathways. *Int J Mol Med* 2012; **29**: 202-208 [PMID: 22089663 DOI: 10.3892/ijmm.2011.839]
53 **Singh S**, Stroud AM, Holubar SD, Sandborn WJ, Pardi DS. Treatment and prevention of pouchitis after ileal pouch-anal anastomosis for chronic ulcerative colitis. *Cochrane Database Syst Rev* 2015; CD001176 [PMID: 26593456 DOI: 10.1002/14651858.CD001176.pub3]
54 **Reinisch W**. Fecal Microbiota Transplantation in Inflammatory Bowel Disease. *Dig Dis* 2017; **35**: 123-126 [PMID: 28147375 DOI: 10.1159/000449092]
55 **Wells JM**, Brummer RJ, Derrien M, MacDonald TT, Troost F, Cani PD, Theodorou V, Dekker J, Méheust A, de Vos WM, Mercenier A, Nauta A, Garcia-Rodenas CL. Homeostasis of the gut barrier and potential biomarkers. *Am J Physiol Gastrointest Liver Physiol* 2017; **312**: G171-G193 [PMID: 27908847 DOI: 10.1152/ajpgi.00048.2015]
56 **Petrof EO**, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, Brown EM, Schroeter K, Allen-Vercoe E. Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut. *Microbiome* 2013; **1**: 3 [PMID: 24467987 DOI: 10.1186/2049-2618-1-3]
57 **Pavlidis P**, Powell N, Vincent RP, Ehrlich D, Bjarnason I, Hayee B. Systematic review: bile acids and intestinal inflammation-luminal aggressors or regulators of mucosal defence? *Aliment Pharmacol Ther* 2015; **42**: 802-817 [PMID: 26223936 DOI: 10.1111/apt.13333]
58 **Keating N**, Keely SJ. Bile acids in regulation of intestinal physiology. *Curr Gastroenterol Rep* 2009; **11**: 375-382 [PMID: 19765365 DOI: 10.1007/s11894-009-0057-8]
59 **Li Y**, Tang R, Leung PSC, Gershwin ME, Ma X. Bile acids and intestinal microbiota in autoimmune cholestatic liver diseases. *Autoimmun Rev* 2017; **16**: 885-896 [PMID: 28698093 DOI: 10.1016/j.autrev.2017.07.002]
60 **Joo SS**, Won TJ, Lee DI. Potential role of ursodeoxycholic acid in suppression of nuclear factor kappa B in microglial cell line (BV-2). *Arch Pharm Res* 2004; **27**: 954-960 [PMID: 15473667 DOI: 10.1007/BF02975850]
61 **Yanguas-Casás N**, Barreda-Manso MA, Nieto-Sampedro M, Romero-Ramírez L. TUDCA: An Agonist of the Bile Acid Receptor GPBAR1/TGR5 With Anti-Inflammatory Effects in Microglial Cells. *J Cell Physiol* 2017; **232**: 2231-2245 [PMID: 27987324 DOI: 10.1002/jcp.25742]

62 **Ho PP**, Steinman L. Obeticholic acid, a synthetic bile acid agonist of the farnesoid X receptor, attenuates experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 2016; **113**: 1600-1605 [PMID: 26811456 DOI: 10.1073/pnas.1524890113]
63 **Jena PK**, Sheng L, Di Lucente J, Jin LW, Maezawa I, Wan YY. Dysregulated bile acid synthesis and dysbiosis are implicated in Western diet-induced systemic inflammation, microglial activation, and reduced neuroplasticity. *FASEB J* 2018; **32**: 2866-2877 [PMID: 29401580 DOI: 10.1096/fj.201700984RR]
64 **Bhargava P**, Mowry E, Calabresi P. Global metabolomics identifies perturbation of multiple metabolic pathways in Multiple Sclerosis (Abstract). *Neurology* 2015; **84**: P5.242
65 **Bhargave P.** A Trial of Bile Acid Supplementation in Patients With Multiple Sclerosis. Cited 2018-05-22; In ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: URL: https://clinicaltrials.gov/ct2/show/NCT03423121

P- Reviewer: Abalo R, Quigley EM **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4224-4229

DOI: 10.3748/wjg.v24.i37.4224

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

EDITORIAL

Hepatocellular carcinoma in Latin America: Diagnosis and treatment challenges

Federico Piñero, Jaime Poniachik, Ezequiel Ridruejo, Marcelo Silva

Federico Piñero, Ezequiel Ridruejo, Marcelo Silva, Liver Unit, Hospital Universitario Austral, Facultad de Ciencias Biomédicas, Universidad Austral, Pilar, Buenos Aires B1629HJ, Argentina

Federico Piñero, Ezequiel Ridruejo, Marcelo Silva, Latin American Liver Research Educational and Awareness Network, Buenos Aires B1629HJ, Argentina

Jaime Poniachik, Department of Gastroenterology and Hepatology, Hospital Clínico Universidad de Chile, Santiago de Chile, Región Metropolitana Santiago 46010, Chile

Jaime Poniachik, Gastroenterology and Hepatology, Clínica Santa María, Santiago de Chile, Región Metropolitana Santiago 834-0518, Chile

Ezequiel Ridruejo, Hepatology Section, Centro de Educación Médica e Investigaciones Clínicas Norberto Quirno, Ciudad de Buenos Aires C1425ASG, Argentina

ORCID number: Federico Piñero (0000-0002-9528-2279); Jaime Poniachik (0000-0001-7958-3357); Ezequiel Ridruejo (0000-0002-3321-0683); Marcelo Silva (0000-0002-2287-7351).

Author contributions: Piñero F and Poniachik J contributed to concept and writing of the article; Ridruejo E and Silva M contributed to critical review and final approval of the article.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Federico Piñero, MD, MSCE, Academic Research, Doctor, Hepatology and Liver Transplant Unit, Hospital Universitario Austral, Av. Presidente Perón 1500, Pilar, Buenos Aires B1629HJ, Argentina. fpinerof@cas.austral.edu.ar
Telephone: +54-230-4482000
Fax: +54-230-4482236

Received: July 5, 2018
Peer-review started: July 5, 2018
First decision: August 1, 2018
Revised: August 2, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

Latin America, a region with a population greater than 600000000 individuals, is well known due to its wide geographic, socio-cultural and economic heterogeneity. Access to health care remains as the main barrier that challenges routine screening, early diagnosis and proper treatment of hepatocellular carcinoma (HCC). Therefore, identification of population at risk, implementation of surveillance programs and access to curative treatments has been poorly obtained in the region. Different retrospective cohort studies from the region have shown flaws in the implementation process of routine surveillance and early HCC diagnosis. Furthermore, adherence to clinical practice guidelines recommendations assessed in two studies from Brazil and Argentina demonstrated that there is also room for improvement in this field, similarly than the one observed in Europe and the United States. In summary, Latin America shares difficulties in HCC decision-making processes similar to those from developed countries. However, a transversal limitation in the region is the poor access to health care with the consequent limitation to standard treatments for overall population. Specifically, universal health care access to the different World Health Organization levels is crucial, including improvement in research, education and continuous

medical training in order to expand knowledge and generation of data promoting a continuous improvement in the care of HCC patients.

Key words: Latin America; Liver cancer; Limitations; Challenge

Core tip: Which are the implications in regard to clinical decision making processes related to hepatocellular carcinoma (HCC) in daily practice in Latin America? Should we consider making these decisions taking into account both, local experiences and their feasibility together with the best available evidence in parallel with patient preferences? These decision-making processes must be individualized according to local barriers to health care systems. Primary prevention programs of liver diseases, surveillance for HCC and intervention programs following the best evidence will be possible only if we are aware of local barriers and develop efficient strategies to overcome them.

Piñero F, Poniachik J, Ridruejo E, Silva M. Hepatocellular carcinoma in Latin America: Diagnosis and treatment challenges. *World J Gastroenterol* 2018; 24(37): 4224-4229 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4224.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4224

INTRODUCTION

Latin American comprises a region of the Americas of Latin origin, in which the most common speaking languages are Spanish and Portuguese. The region accounts for more than twenty million square kilometers of surface area, with more than six hundred million population. Due to its geographic extension, Latin America has a great socio-cultural heterogeneity and an important socio-economic difference among countries. While there are high earners like Chile and Uruguay with a gross domestic product (GDP) per capita over $20000, others like Haiti and Honduras have GDPs per capita lower than $ 5000[1]. At the same time, each country in itself is highly unequal, presenting some of the highest Globalization of Inequality (GINI) scores in the world. Brazil, Chile, Ecuador and Colombia all present GINIs above 0.45 for the year 2016; Argentina and Uruguay having slightly better scores[1]. In comparison, Sweden, Norway, Netherlands and Denmark all have GINI scores less than 0.30[1].

It is in this socio-cultural and economic scenario, where settles a large variety in access to health care systems in the region. These systems are mainly made up of a common payer and provider that is the state. However, in several countries, there are other type of health providers through social security and private insurances and providers. Furthermore, expenditure on access care in many Latin American countries comes from out-of-pocket money among high to middle income people. On the other hand, among low socio-economic classes, expenditure comes purely and exclusively from public services, which in most of the cases provide with low to regular quality of medical care services and shortage of appropriate medical supplies and devices.

WHERE DO WE STAND IN LATIN AMERICA REGARDING HEPATOCELLULAR CARCINOMA?

Hepatocellular carcinoma (HCC) is the second leading cause of cancer related death worldwide and the main cause of cancer in patients with cirrhosis. Incidence of HCC varies according to geographic location, depending on the prevalence of viral hepatitis among the world. The predominant reported causes of HCC in different geographic areas around the world have been related with chronic hepatitis C virus (HCV) or hepatitis B virus (HBV) infection and alcoholic liver disease[2-5]. Heterogeneous data regarding epidemiology of HCC in Latin America has been reported[6-12]. While HCV and alcoholic liver disease are the most frequent etiologies of HCC in the region, HBV is a leading cause in some countries, mainly in Brazil. More recently, we have observed a changing epidemiological trend of HCC towards an increasing non-alcoholic fatty liver disease, becoming an important public health burden in the region[6,7].

As previously proposed by the World Health Organization the structural challenge in the region is the uneven access to health care. To our knowledge there is not even one country with an integrated program to assist on the prevention of chronic liver diseases and early identification of the population at risk for developing HCC. Consequently, the common challenge for scientific societies is to induce regional policy makers to develop interventions and strategies able to identify the population at risk, implement surveillance programs, and improve access to curative and palliative treatments. Once we have assured access to adequate care we should move into next step which is the correct adherence to recommendations from clinical practice guidelines[2-5].

A clinical case scenario as an example of where do we fail in Latin America

The following clinical case demonstrates the regional shortcomings related to HCC diagnosis at late stages and its therapeutic consequences. A 60-year-old male patient with compensated cirrhosis and clinically significant portal hypertension due to chronic HCV infection, who started antiviral treatment with direct-acting antiviral agents, began an erratic path of ultrasound (US) screening for HCC. Surveillance was performed by non-liver expert

Table 1 Surveillance for hepatocellular carcinoma in Latin America

Study	Population	Design	Results
Fassio *et al*[8]	*n* = 240 HCC Brazil, Arg, Colombia, Chile, Uruguay, Venezuela	Prospective cohort (Surveillance retrospectively analyzed)	54% under surveillance; BCLC A 70% *vs* 39% not under surveillance; No survival analysis
Paranaguá-Vezozzo *et al*[9]	*n* = 884 Cirrhosis Child A-B Brazil, Sao Paulo	Retrospective cohort US ± AFP annual	HCC annual incidence 2.9%; 75% under annual surveillance; 80% within Milan, better survival
Piñero *et al*[10]	*n* = 643 Cirrhosis, waiting list for liver transplantation. Argentina	Retrospective cohort Surveillance Failure = incidental HCC in the explant	US accuracy: S 33% and E 99%
Campos Appel-da-Silva *et al*[11]	*n* = 453 Child A-C Cirrhosis Brazil, Porto Alegre	Retrospective cohort US ± AFP every 6 mo	50.7% under surveillance; More BCLC 0-A *vs* no screening; Better survival within Milan criteria
Debes *et al*[12]	*n* = 1336 HCC Brazil, Argentina, Colombia, Peru, Uruguay, Ecuador	Retrospective cohort	47% under surveillance; Better survival *vs* symptomatic diagnosis (adjusted for lead-time bias)

BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; US: Ultrasound.

sonographers due to insurance's related lack of access to academic sites. Initially a 24-mm nodule was visualized and he was recommended to stay on a follow-up visit with no further imaging evaluation. Twelve months later, another US was performed; this time the nodule grew to 38 mm. He performed an abdominal computed tomography (CT) scan with oral contrast only, and the finding of an "uncharacteristic" nodule leaded to a CT-guided biopsy. The pathologic report was "nodules of hepatocellular regeneration separated by broad fibrous septa, cirrhosis". Result: No cancer. His physician suggested him to continue life normally and the patient happily went home.

A year later, a liver specialist suggested him to perform an abdominal CT scan with intravenous contrast. A heterogeneous 80-mm diameter lesion in the right hepatic lobe with "non-characteristic findings" was observed. Not satisfied, the patient looked for a second opinion. A second hepatologist performed a three-phase dynamic abdominal magnetic resonance imaging (MRI). Result: One lesion with arterial enhancement and wash out during portal and late phases: HCC of 83 mm, without vascular invasion. Serum alpha-fetoprotein value was 1200 ng/mL.

In the end, the patient consulted at least 4 medical doctors during a 2-year period, with extended and inadmissible delay in HCC diagnosis that at this point will probably exclude him from potentially curative treatments. Where did we fail?

Early diagnosis of HCC: Challenges and areas of improvement

This case, clearly illustrates some of the reasons for failure in routine surveillance and HCC diagnosis at early stages in Latin America, and as a consequence, failure in the appropriate staging and selection of therapies.

Screening failure entails three important points to be considered. First, absence of early identification of the population at risk, such as chronic HBV or HCV. Second, ineffective application of routine surveillance (semi-annual ultrasound performed by expert operators) and third, errors in interpretation of a positive or negative screening tests, misinterpreting its sensitivity and specificity.

Surveillance for HCC in Latin America demands a continuous improvement. Different retrospective cohort studies have shown flaws in the implementation process of routine surveillance, the consequent failure in the diagnosis in early stages and finally a notorious negative impact upon patient survival[8-12] (Table 1).

Overall, surveillance programs reported to be applied in less than 50% of the patients in Latin America. This number perhaps does not show the "real" regional situation, since most of this data came from academic rather than general hospitals. Consequently, screening failure for HCC in this region might be even greater, demanding strategies to improve its implementation such as application of US done by experts, correct interpretation of imaging tests and finally, adequacy of therapeutic decisions according to the best evidence-based-medicine. Consequently, early HCC diagnosis should be the aim of these strategies.

As exemplified in the clinical case, the misuse of diagnostic tools delays the correct diagnosis. HCC diagnosis implies an appropriate oncologic imaging paradigm, not requiring histological confirmation for diagnosis in most of the cases. However, discordance between images and histology may occur. This situation has been reported up to 10% in Argentina when comparing imaging reports and explanted liver data from liver transplanted patients[13,14]. In a multicenter Latin American cohort study, false positives cases were less than 3%[15]. Two different situations need to be further clarified when discussing imaging accuracy against histological confirmation of HCC. On one hand, when false positives are considered, it should be important to address if complete necrotic nodules were included as false positive cases resulting in a biased report. On the other hand, discrepancy between images

Table 2 Adherence to clinical practice guidelines around the world and in Latin America

Study	Population	Design	Results
Leoni *et al*[20]	*n* = 227 HCV 58% Child A 54%	Retrospective cohort (2005-2010) One center	At HCC diagnosis: BCLC 0-A 55%; Adherence to BCLC 60%; Higher adherence among BCLC A 86%
Gashin *et al*[21]	*n* = 137 HCV 62%	Retrospective cohort (2009-2010) One center	Adherence to BCLC 62%; Better overall survival; Heterogeneous causes of non-adherence
Kim *et al*[22]	*n* = 3515 HBV 77% Child A 82%	Retrospective cohort (2005-2009) One center	At HCC diagnosis: BCLC A 59%; Adherence to BCLC 49%; Better survival for adherence, except BCLC-D (BCLC D who were transplanted were considered "non-adherence")
Wallace *et al*[23]	*n* = 292 OH-HCV 65%	Retrospective cohort (2006-2014) One center	At HCC diagnosis: BCLC 0-A 64%; Adherence to BCLC 48% *vs* HKLC 56% (P.001); No better survival among BCLC adherence *vs* no-adherence but better survival among HKLC (TACE before transplant was considered "no-adherence")
Guarino *et al*[24]	*n* = 1008 HCV Child A 73%	Retrospective cohort (2013-2015) Multicenter study	At HCC diagnosis: BCLC 0-A 59%; Adherence BCLC 71%, lower in BCLC B 36% and C 46%; No better survival (TACE before transplant was considered "no-adherence")
Kikuchi *et al*[25]	*n* = 364 HBV 53% Child A 53%	Retrospective cohort (2010-2012) One center	At HCC diagnosis: BCLC A 36%; Adherence BCLC 52%; Lower adherence in BCLC C-D; No better survival, except in BCLC A (BCLC D who were transplanted were considered "non-adherence")
Piñero *et al*[26]	*n* = 708 HCV 58% Child A 54%	Dual cohort (2009-2016) Multicenter study	At HCC diagnosis: BCLC 0-A 47%; Adherence BCLC 53% initial, 63% subsequently; Adherence to BCLC: better survival HR 0.67 (CI: 0.52-0.87)

BCLC: Barcelona Clinic Liver Cancer; HKLC: Hong Kong Liver Cancer algorithm; HCV: Hepatitis C virus; HBV: Hepatitis B virus; TACE: Transarterial chemoembolization.

and explanted liver should be considered taking into account potential tumor progression, and locoregional response to treatments during the waiting list period.

Nevertheless this led to changes in diagnostic criteria for HCC in patients enrolled for liver transplantation in Argentina aimed to improve imaging diagnostic accuracy. Although the idea was novel, LIRADS criteria implementation leaded even to a greater uncertainty for those cases where HCC diagnosis is probable or possible (LIRADS 3 or 4). Moreover, imaging expert's agreement on LIRADS in the daily practice has been not assessed at all. Thus, LIRADS system seemed to make the clinical decision making process even more complex in daily practice in that country[16,17].

Challenges regarding staging and adherence to recommended treatment options from clinical practice guidelines

HCC staging considering the Barcelona Clinic Liver Cancer (BCLC) algorithm has been recommended in different clinical practice guidelines[3,4], including that from the Latin American Association for the Study of the Liver (ALEH)[3]. However, strict adherence to these therapeutic recommendations is often not feasible in daily practice. This does not contradict the BCLC algorithm, since its explicitly recommends that the therapeutic choice must be individualized considering feasibility, access and preferences of the patients[18]. In addition, there are different guidelines and recommendations, including those from Asia (APASL)[5], Japan and South Korea. Consequently, there is a wide range of treatment algorithms when considering HCC.

The BRIDGE study demonstrated the great heterogeneity in terms of the treatments performed worldwide at each stage and far from that recommended in the ideal situation[19]. Global and individual context makes therapeutic decisions in HCC heterogeneous in real life. Adherence to clinical practice guidelines recommendations varies between 40%-70% in different retrospective cohort studies[20-26]. Two Latin American studies evaluated adherence to BCLC and its impact on survival. In a study from Brazil, adherence to BCLC did not have a favorable impact on survival[25]. However, there was a selection bias when "non-adherence" was categorized in those patients within BCLC-D stage who were candidates for liver transplantation. Precisely, the BCLC clarifies in its footnote that these patients must be transplanted. In a dual cohort study in Argentina, adherence to BCLC was greater than 50%, being associated with better overall survival[26] (Table 2).

In summary, although Latin America shares some difficulties in HCC decision-making processes similar to those reported in some developed countries, we still have big gaps when compared to them. These gaps are seen in medical education, on early and accurate HCC diagnosis, and in universal access to good diagnostic technology and to curative treatments. Until they are corrected, discrepancy on HCC related survival would remain present.

PERSPECTIVE

Consequently, we shall make decisions considering local education, expertise and feasibility together with

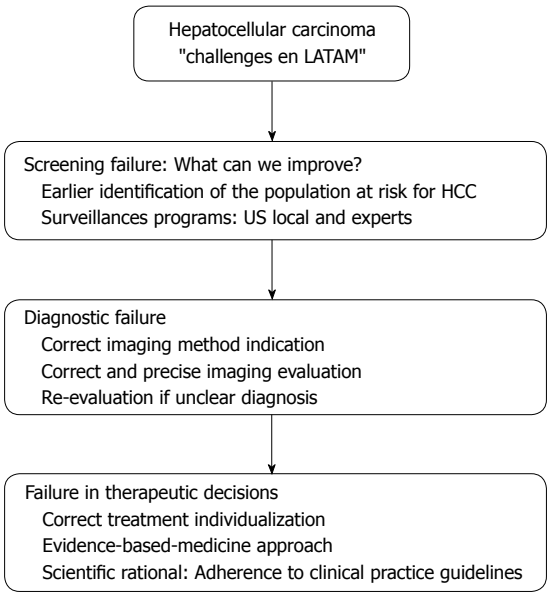


Figure 1 Areas of improvement regarding hepatocellular carcinoma in Latin America. HCC: Hepatocellular carcinoma.

the best available evidence. Ultimately, this decision-making-process must be individualized[27].

Which are the areas for improvement in Latin America? Specifically, universal health care access as per World Health Organization recommendation is crucial. This includes improvement in transmission of information and medical education from academic to primary health care centers, focusing on prevention of development of liver diseases, identification of population at risk for HCC, systematic implementation of routine surveillance programs, improvement in the diagnostic work-up process and finally, promoting overall access to all treatments strategies which have shown improvement in patient's survival (Figure 1). Finally, an important field to promote in the region is the development of research consortia such as the Latin American Liver Research Educational and Awareness Network, through which we can multiply medical education and generation of regional data necessary to develop efficient health interventions for improvement the care of patients with HCC[28].

REFERENCES

1 **World Bank.** International Comparison Program database. Available from: URL: https://data.worldbank.org

2 **Heimbach JK**, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, Zhu AX, Murad MH, Marrero JA. AASLD guidelines for the treatment of hepatocellular carcinoma. *Hepatology* 2018; **67**: 358-380 [PMID: 28130846 DOI: 10.1002/hep.29086]

3 **Méndez-Sánchez N,** Ridruejo E, Alves de Mattos A, Chávez-Tapia NC, Zapata R, Paraná R, Mastai R, Strauss E, Guevara-Casallas LG, Daruich J, Gadano A, Parise ER, Uribe M, Aguilar-Olivos NE, Dagher L, Ferraz-Neto BH, Valdés-Sánchez M, Sánchez-Avila JF. Latin American Association for the Study of the Liver clinical practice guidelines: management of hepatocellular carcinoma. *Ann Hepatol* 2014;**13** Suppl 1: S4–S40 [PMID 24998696]

4 **European Association for the Study of the Liver.** European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

5 **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]

6 **Piñero F**, Pages J, Marciano S, Fernández N, Silva J, Anders M, Zerega A, Ridruejo E, Ameigeiras B, D'Amico C, Gaite L, Bermúdez C, Cobos M, Rosales C, Romero G, McCormack L, Reggiardo V, Colombato L, Gadano A, Silva M. Fatty liver disease, an emerging etiology of hepatocellular carcinoma in Argentina. *World J Hepatol* 2018; **10**: 41-50 [PMID: 29399277 DOI: 10.4254/wjh.v10.i1.41]

7 **Piñero F**, Costa P, Boteon YL, Duque SH, Marciano S, Anders M, Varón A, Zerega A, Poniachik J, Soza A, Padilla Machaca M, Menéndez J, Zapata R, Vilatoba M, Muñoz L, Maraschio M, Podestá LG, McCormack L, Gadano A, Boin ISFF, García P, Silva M; Latin American Liver Research, Education, Awareness Network (LALREAN). A changing etiologic scenario in liver transplantation for hepatocellular carcinoma in a multicenter cohort study from Latin America. *Clin Res Hepatol Gastroenterol* 2018; Epub ahead of print [PMID: 29773419 DOI: 10.1016/j.clinre.2018.03.014]

8 **Fassio E**, Díaz S, Santa C, Reig ME, Martínez Artola Y, Alves de Mattos A, Míguez C, Galizzi J, Zapata R, Ridruejo E, de Souza FC, Hernández N, Pinchuk L; Multicenter Group for Study of Hepatocarcinoma in Latin America; Asociación Latinoamericana para el Estudio del Hígado (ALEH). Etiology of hepatocellular carcinoma in Latin America: a prospective, multicenter, international study. *Ann Hepatol* 2010; **9**: 63-69 [PMID: 20332549]

9 **Paranaguá-Vezozzo DC**, Ono SK, Alvarado-Mora MV, Farias AQ, Cunha-Silva M, França JI, Alves VA, Sherman M, Carrilho FJ. Epidemiology of HCC in Brazil: incidence and risk factors in a ten-year cohort. *Ann Hepatol* 2014; **13**: 386-393 [PMID: 24927609]

10 **Piñero F**, Marciano S, Anders M, Orozco F, Zerega A, Cabrera CR, Baña MT, Gil O, Andriani O, de Santibañes E, McCormack L, Gadano A, Silva M. Screening for liver cancer during transplant waiting list: a multicenter study from South America. *Eur J Gastroenterol Hepatol* 2015; **27**: 355-360 [PMID: 25563142 DOI: 10.1097/MEG.0000000000000272]

11 **Appel-da-Silva MC**, Miozzo SA, Dossin IA, Tovo CV, Branco F, de Mattos AA. Incidence of hepatocellular carcinoma in outpatients with cirrhosis in Brazil: A 10-year retrospective cohort study. *World J Gastroenterol* 2016; **22**: 10219-10225 [PMID: 28028370 DOI: 10.3748/wjg.v22.i46.10219]

12 **Debes JD**, Chan AJ, Balderramo D, Kikuchi L, Gonzalez Ballerga E, Prieto JE, Tapias M, Idrovo V, Davalos MB, Cairo F, Barreyro FJ, Paredes S, Hernandez N, Avendaño K, Diaz Ferrer J, Yang JD, Carrera E, Garcia JA, Mattos AZ, Hirsch BS, Gonçalves PT, Carrilho FJ, Roberts LR. Hepatocellular carcinoma in South America: Evaluation of risk factors, demographics and therapy. *Liver Int* 2018; **38**: 136-143 [PMID: 28640517 DOI: 10.1111/liv.13502]

13 **McCormack L**, Gadano A, Lendoire J, Imventarza O, Andriani O, Gil O, Toselli L, Bisigniano L, de Santibañes E. Model for end-stage liver disease-based allocation system for liver transplantation in Argentina: does it work outside the United States? *HPB* (Oxford) 2010; **12**: 456-464 [PMID: 20815854 DOI: 10.1111/j.1477-2574.2010.00199.x]

14 **Cejas NG**, Villamil FG, Lendoire JC, Tagliafichi V, Lopez A, Krogh DH, Soratti CA, Bisigniano L. Improved waiting-list outcomes in Argentina after the adoption of a model for end-stage liver disease-based liver allocation policy. *Liver Transpl* 2013; **19**: 711-720 [PMID: 23775946 DOI: 10.1002/lt.23665]

15 **Piñero F**, Tisi Baña M, de Ataide EC, Hoyos Duque S, Marciano S, Varón A, Anders M, Zerega A, Menéndez J, Zapata R, Muñoz L, Padilla Machaca M, Soza A, McCormack L, Poniachik J, Podestá LG, Gadano A, Boin IS, Duvoux C, Silva M; Latin American Liver

Research, Education and Awareness Network (LALREAN). Liver transplantation for hepatocellular carcinoma: evaluation of the alpha-fetoprotein model in a multicenter cohort from Latin America. *Liver Int* 2016; **36**: 1657-1667 [PMID: 27169841 DOI: 10.1111/liv.13159]

16 **Ayuso C**, Rimola J, Vilana R, Burrel M, Darnell A, García-Criado Á, Bianchi L, Belmonte E, Caparroz C, Barrufet M, Bruix J, Brú C. Diagnosis and staging of hepatocellular carcinoma (HCC): current guidelines. *Eur J Radiol* 2018; **101**: 72-81 [PMID: 29571804 DOI: 10.1016/j.ejrad.2018.01.025]

17 **Mitchell DG**, Bruix J, Sherman M, Sirlin CB. LI-RADS (Liver Imaging Reporting and Data System): summary, discussion, and consensus of the LI-RADS Management Working Group and future directions. *Hepatology* 2015; **61**: 1056-1065 [PMID: 25041904 DOI: 10.1002/hep.27304]

18 **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

19 **Park JW**, Chen M, Colombo M, Roberts LR, Schwartz M, Chen PJ, Kudo M, Johnson P, Wagner S, Orsini LS, Sherman M. Global patterns of hepatocellular carcinoma management from diagnosis to death: the BRIDGE Study. *Liver Int* 2015; **35**: 2155-2166 [PMID: 25752327 DOI: 10.1111/liv.12818]

20 **Leoni S**, Piscaglia F, Serio I, Terzi E, Pettinari I, Croci L, Marinelli S, Benevento F, Golfieri R, Bolondi L. Adherence to AASLD guidelines for the treatment of hepatocellular carcinoma in clinical practice: experience of the Bologna Liver Oncology Group. *Dig Liver Dis* 2014; **46**: 549-555 [PMID: 24630947 DOI: 10.1016/j.dld.2014.02.012]

21 **Gashin L**, Tapper E, Babalola A, Lai KC, Miksad R, Malik R, Cohen E. Determinants and outcomes of adherence to recommendations from a multidisciplinary tumour conference for hepatocellular carcinoma. *HPB* (Oxford) 2014; **16**: 1009-1015 [PMID: 24888730 DOI: 10.1111/hpb.12280]

22 **Kim KM**, Sinn DH, Jung SH, Gwak GY, Paik YH, Choi MS, Lee JH, Koh KC, Paik SW. The recommended treatment algorithms of the BCLC and HKLC staging systems: does following these always improve survival rates for HCC patients? *Liver Int* 2016; **36**: 1490-1497 [PMID: 26936471 DOI: 10.1111/liv.13107]

23 **Wallace MC**, Huang Y, Preen DB, Garas G, Adams LA, MacQuillan G, Tibballs J, Ferguson J, Samuelson S, Jeffrey GP. HKLC Triages More Hepatocellular Carcinoma Patients to Curative Therapies Compared to BCLC and Is Associated with Better Survival. *Dig Dis Sci* 2017; **62**: 2182-2192 [PMID: 28547649 DOI: 10.1007/s10620-017-4622-y]

24 **Guarino M**, Tortora R, de Stefano G, Coppola C, Morisco F, Salomone Megna A, Izzo F, Nardone G, Piai G, Adinolfi LE, D' Adamo G, Gaeta GB, Messina V, Francica G, De Girolamo V, Coppola N, Persico M, Di Costanzo GG; Progetto Epatocarcinoma Campania Group. Adherence to Barcelona Clinic Liver Cancer guidelines in field practice: Results of Progetto Epatocarcinoma Campania. *J Gastroenterol Hepatol* 2018; **33**: 1123-1130 [PMID: 28994145 DOI: 10.1111/jgh.14013]

25 **Kikuchi L**, Chagas AL, Alencar RSSM, Tani C, Diniz MA, D'Albuquerque LAC, Carrilho FJ. Adherence to BCLC recommendations for the treatment of hepatocellular carcinoma: impact on survival according to stage. *Clinics* (Sao Paulo) 2017; **72**: 454-460 [PMID: 28954003 DOI: 10.6061/clinics/2017(08)01]

26 **Piñero F**, Marciano S, Fernández N, Silva J, Zambelo Y, Cobos M, Zerega A, Ridruejo E, Miguez C, Ameigeiras B, D'Amico C, Gaite L, Coronel M, Bermúdez C, Rosales C, Romero G, McCormack L, Reggiardo V, Colombato L, Gadano A, Rubinstein F, Silva M; Argentinean Association for the Study of Liver Diseases (A.A.E.E.H). Adherence to Barcelona Clinic Liver Cancer therapeutic algorithm for hepatocellular carcinoma in the daily practice: a multicenter cohort study from Argentina. *Eur J Gastroenterol Hepatol* 2018; **30**: 376-383 [PMID: 29509603 DOI: 10.1097/MEG.0000000000001049]

27 **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

28 **Mendizabal M**, Silva MO. Developing multicenter consortia in liver disease in Latin America: Challenges and opportunities. *Liver Transpl* 2017; **23**: 1210-1215 [PMID: 28590520 DOI: 10.1002/lt.24793]

P- Reviewer: Jang BK, Marcos M **S- Editor**: Ma RY **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4230-4242

DOI: 10.3748/wjg.v24.i37.4230

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

REVIEW

New prognostic biomarkers of mortality in patients undergoing liver transplantation for hepatocellular carcinoma

Leonardo Lorente

Leonardo Lorente, Intensive Care Unit, Hospital Universitario de Canarias, Santa Cruz de Tenerife 38320, Spain

ORCID number: Leonardo Lorente (0000-0003-4902-4065).

Author contributions: Lorente L drafted the manuscript.

Conflict-of-interest statement: The author has no financial or other conflicts of interest to declare related to the submitted manuscript.

Manuscript source: Invited manuscript

Correspondence to: Leonardo Lorente, MD, PhD, Attending Doctor, Medical Assistant, Intensive Care Unit, Hospital Universitario de Canarias, Ofra, s/n, Santa Cruz de Tenerife 38320, Spain. lorentemartin@msn.com
Telephone: +34-922-678000
Fax: +34-922-647112

Received: May 25, 2018
Peer-review started: May 25, 2018
First decision: July 4, 2018
Revised: August 18, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

The outcome prediction of hepatocellular carcinoma (HCC) patients undergoing liver transplantation (LT) was classically established using various macromorphological factors and serum alpha-fetoprotein levels prior to LT. However, other biomarkers have recently been reported to be associated with the prognosis of HCC patients undergoing to LT. This review summarizes clinical data on these new biomarkers. High blood levels of malondialdehyde, total antioxidant capacity, caspase-cleaved cytokeratin-18, soluble CD40 ligand, substance P, C-reactive protein, and vascular endothelial growth factor, increased neutrophil to lymphocyte ratio and platelet to lymphocyte ratio in blood, high peripheral blood expression of human telomerase reverse transcriptase messenger ribonucleic acid, and high HCC expression of dickkopf-1 have recently been associated with decreased survival rates. In addition, high blood levels of des-gamma-carboxy prothrombin, and high HCC expression of glypican-3, E-cadherin and beta-catenin have been associated with increased HCC recurrence. Additional research is necessary to establish the prognostic role of these biomarkers in HCC prior to LT. Furthermore, some of these biomarkers are also interesting because their potential modulation could help to create new research lines for improving the outcomes of those patients.

Key words: Liver transplantation; Hepatocellular carcinoma; Biomarkers; Outcome; Survival; Recurrence; Genomic

Core tip: The outcome of liver transplantation (LT) for hepatocellular carcinoma (HCC) patients are generally predicted using various macromorphological factors and serum alpha-fetoprotein levels prior to LT. However, other biomarkers have recently been reported to be associated with the prognosis of HCC patients undergoing LT. Furthermore, some of these biomarkers are also interesting because their potential modulation could help to create new research lines for improving

the outcomes of those patients. This review summarizes clinical data on those new biomarkers.

Lorente L. New prognostic biomarkers of mortality in patients undergoing liver transplantation for hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(37): 4230-4242 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4230.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4230

INTRODUCTION

Hepatocellular carcinoma (HCC), the most frequent primary liver malignancy, is one of the most common malignancies and causes of cancer-related deaths[1-3]. In liver transplantation (LT), the primary tumor is removed, and liver failure is treated. Therefore, LT is considered the treatment of choice for some HCC patients[1-11].

Various macromorphological factors assessed prior to LT have been classically used to predict the outcome of HCC patients undergoing LT. These factors include the tumor size, tumor number, degree of differentiation, hepatic microvascular invasion, hepatic macrovascular invasion, being outside the Milan criteria and infiltration[1-11].

However, establishing biomarkers to be assessed prior to LT could strengthen the predictions of prognoses for HCC patients undergoing LT. Currently, the most commonly studied biomarker are the serum alphafetoprotein levels[1-11]. However, other biomarkers have recently been reported to be associated with the prognosis of HCC patients undergoing to LT. This review summarizes clinical data on these new biomarkers.

BIOMARKERS

Malondialdehyde

Oxidative stress can lead to membrane lipid peroxidation, which creates many end products, including malondialdehyde, which is a low molecular weight aldehyde that is produced during the degradation of cellular membrane phospholipids. It is formed when free radicals attack polyunsaturated fatty acids. Malondialdehyde can be released into the extracellular space, and it can ultimately reach the bloodstream. Therefore, it has been used as a circulating biomarker of lipid oxidation[12,13].

Some studies have reported higher levels of serum malondialdehyde in HCC patients[14-16] and patients with chronic liver disease than in healthy controls[17,18]. Additionally, studies have reported that the tumoral tissue of HCC patients has higher malondialdehyde concentrations than non-tumoral tissue[19]. Studies have also found higher free radical intensity in the erythrocytes of HCC patients than in healthy controls[20], higher serum concentrations of reactive oxygen metabolites in HCC patients who exhibit recurrence after curative treatment by radiofrequency ablation or surgical resection[21], and higher circulating lipid peroxide levels prior to LT in patients who do not survive LT than in survivors[22].

A study by our team reported, for the first time, that serum malondialdehyde levels prior to LT were higher in non-surviving patients than in patients who survived for one year after LT. We also found an association between serum malondialdehyde levels in HCC patients prior to LT and their survival at one year after LT[23]. These findings are consistent with those from other studies that have reported an association between circulating malondialdehyde levels and mortality in patients with sepsis[24], traumatic brain injuries[25], brain infarctions[26] and spontaneous intracerebral hemorrhaging[27].

Total antioxidant capacity

The production of reactive oxygen species (ROS) is balanced by the production of antioxidant defenses, and the analysis of total antioxidant capacity (TAC) could provide a global information in respect to the antioxidant status[28].

Some studies have found lower circulating TAC levels in LT patients than in healthy controls[18], and lower circulating TAC levels in HCC patients than in healthy controls[14,15].

A study by our team was the first to find that serum TAC levels prior to LT were lower in non-surviving than in surviving patients during the first one year after LT[29]. Besides, we found that there is an association between low serum TAC levels in HCC patients prior to LT and their survival at one year after LT. In addition, we found a negative association between serum levels of TAC and malondialdehyde; thus, patients with lower serum TAC levels showed higher lipid peroxidation.

I think that those findings could suggest that it is possible that non-survivor LT patients remains during the first one year after LT with low serum TAC levels and high serum malondialdehyde levels (due to a higher lipid peroxidation because the high ROS production is not balanced by an insufficient antioxidant capacity) compared to survivor patients.

Antioxidant agents have been shown to reduce malondialdehyde concentrations in animal models of sepsis[30] and brain trauma[31] as well as in clinical trials involving septic newborns[32], acute ischemic stroke[33] and traumatic brain injuries[34]. Additionally, in a clinical trial of traumatic brain injuries[34], the administration of antioxidant agents reduced mortality rate. Thus, since non-surviving HCC patients showed higher serum malondialdehyde levels prior to LT than surviving patients, it could be interesting to explore the benefit of the administration of antioxidant agents to HCC patients undergoing LT. Antioxidant treatment could potentially improve their prognoses, especially for patients with a higher oxidative state.

Caspase-cleaved cytokeratin-18

Apoptosis, which leads to active and programmed cell

elimination, is increased in liver diseases[35-37]. Two main pathways exist (extrinsic and intrinsic) for cell death by apoptosis. The apoptotic extrinsic pathway is initiated when the tumor necrosis factor receptor superfamily (TNFRSF) is activated by its ligand (TNFSF). This leads to the formation of a death signal that activates caspase-8 and ultimately activates caspase-3. The intrinsic apoptotic pathway is activated *via* oxygen free radicals, interleukin (IL)-1, IL-6 and nitric oxide. These factors release cytochromes from the mitochondria to the cytosol, which activates caspase-3. Thus, both apoptotic pathways ultimately activate caspase 3, which leads to cell death.

Cytokeratin-18 is the main protein found in the intermediate filaments of the liver and is present in most parenchymal and epithelial cells. During hepatocyte apoptosis, cytokeratin-18 is cleaved by caspases and can be released into the bloodstream as caspase-cleaved cytokeratin (CCCK)-18[35-39], which can be detected using M30 monoclonal antibodies[40,41].

Some studies have reported higher circulating CCCK-18 levels in patients with tumoral diseases than in healthy controls[42,43] and in patients with tumoral diseases that had a poor evolution[44-48]. Additionally, HCC patients have higher circulating CCCK-18 levels than healthy controls[49,50] or cirrhotic patients[51,52]. Studies have reported an association between serum CCCK-18 levels and mortality in HCC patients[53].

A study by our team found, for the first time, that serum CCCK-18 levels prior to LT were higher in non-surviving patients than in patients who survived for one year after LT. Additionally, an association was found between serum CCCK-18 levels in HCC patients prior to LT and their survival for one year after LT[54]. These findings are consistent with the results of other studies that have shown that circulating CCCK-18 levels are associated with the prognosis of patients with various tumoral diseases[44-48], HCC[53], sepsis[55], traumatic brain injury[56] and cerebral artery infarction[57]. Additionally, circulating CCCK-18 levels have been associated with metastasis[45], serum AFP levels[46,54] and tumor size[47,48].

Soluble CD40 ligand

Soluble CD40 ligand (sCD40L) is a member in the TNFSF of proteins. It has proinflammatory and prothrombotic effects when bound to its receptor, CD40, which is also a member of the TNFRSF[58-65]. CD40L is mainly found in platelets and activated T-lymphocytes, although it is also present in smooth muscle cells, endothelial cells, microglia, monocytes, and B cells. When CD40L is cleaved, it is released into circulation and is present in its soluble form, sCD40L[58-65].

Some studies have reported higher circulating sCD40L levels in patients with ischemic stroke[66-69], acute coronary syndrome[70,71], and sepsis[72,73] than in healthy subjects. Additionally, high circulating sCD40L levels are associated with a poor prognosis in patients with ischemic stroke[69], acute coronary syndrome[74], sepsis[72,73] and traumatic brain injuries[75]. Patients with chronic hepatitis C virus infection[76], cirrhosis[77], and non-alcoholic fatty liver disease have been shown to exhibit higher circulating sCD40L levels than control subjects[78]. Furthermore, high circulating sCD40L levels are associated with a poor prognosis in HCC patients[79].

A study by our team was the first to report that serum sCD40L levels prior to LT were higher in patients who did not survive for one year after transplantation than in the surviving patients, and an association was also found between serum sCD4L levels in HCC patients prior to LT and survival for one year after LT[80]. These findings are consistent with the results of other studies reporting an association between circulating sCD40L levels and mortality in patients with cerebral infarction[69], acute coronary syndrome[74], sepsis[72,73] and traumatic brain injuries[75].

Circulating sCD40L levels could play a role in patients receiving LT for HCC by their proinflammatory[81,82] and procoagulant[83-88] effects. The proinflammatory effects of sCD40L may be due to an increase in the expression of proinflammatory mediators such as IL-1, IL-6, IL-12, TNF-alpha and interferon-gamma[81,82]. The procoagulant effects of sCD40L may be due to the induction of tissue factor expression[83-86], reduced expression of thrombomodulin[85,86] and its binding to glycoprotein Ⅱb/Ⅲa platelet receptor[87,88]. These proinflammatory and procoagulant effects could potentially favor the development of vascular thrombosis and organ dysfunction, ultimately resulting in patient death.

The statin administration has been associated with a reduction of circulating sCD40L levels in patients with coronary artery disease[89-91] and an improvement in the prognosis of patients with ischemic stroke[92] and infections[93-96]. Therefore, as non-surviving HCC patients showed higher serum sCD40L levels prior to LT than patients who survived for one year after LT, it could be interesting to explore the benefit of administering sCD40L modulators to HCC patients who are undergoing to LT to improve their prognosis, especially for patients with higher sCD40L levels.

Substance P

Substance P is a member of the tachykinin family, which is distributed by the central and peripheral nervous, respiratory and urinary systems and by the gut. Tachykinins may play a role in nociceptive responses, inflammation, vasodilation and plasma protein extravasation[97-99].

Circulating substance P levels are elevated in patients with liver diseases compared to control subjects[100-106] and in patients with severe liver diseases[104-106].

A study by our team was the first to report that serum levels of substance P prior to LT were higher in patients who did not survive for one year after LT than in surviving patients. The study also found an association between serum levels of substance P in HCC patients prior to LT and mortality within one year after LT[107].

These findings are consistent with the results of other studies that have reported an association between circulating serum P levels and mortality in patients with traumatic brain injuries[108] or ischemic stroke[109].

Substance P plays a role in the inflammatory response by producing inflammatory cytokines such as IL-1, IL-6 and TNF-alpha[110-114]. Various agents that reduce substance P activity have been identified in animal models of ischemic stroke[115-117] and traumatic brain injury[118,119]. These agents have been associated with a reduction in the inflammation process and edema. HCC patients who did not survive for one year after LT showed higher serum substance P levels prior to LT than surviving patients. Therefore, it could be interesting to explore the benefit of administering agents to control substance P activity to HCC patients undergoing LT, especially in patients with high circulating substance P levels.

Neutrophil to lymphocyte ratio and platelet to lymphocyte ratio

The blood neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) have both been used as biomarkers to evaluate systemic inflammatory responses. A meta-analysis published in 2017 by Zheng *et al*[120] analyzed the association between the NLR and PLR in the blood of HCC patients prior to receiving different treatments and its overall survival and HCC recurrence. The treatments included curative resection, transarterial chemoembolization (TACE), radiofrequency ablation (RFA), LT and chemotherapy. The authors examined the associations between the NLR and all treatments combined and individually. They found an association between a high NLR and both of the outcomes (poor overall survival and HCC recurrence) for all types of treatment. They also reported an association between a high NLR and survival when specifically analyzing LT. However, no association was found between the NLR and HCC recurrence when specifically analyzing LT. The authors also found an association between a high PLR and poor outcomes for all treatments combined and when analyzing LT specifically.

C-reactive protein

C-reactive protein (CRP) is synthesized in the liver by hepatocytes in response to factors released by macrophages and adipocytes during inflammation and afterwards is released to blood; thus, blood levels of CRP increase in response to inflammation. Elevated blood CRP levels are associated in multivariate analyses with an increase in the risk of HCC recurrence and decreased survival in patients undergoing LT for HCC[121,122], overall in patients with HCC beyond the Milan criteria[121].

Des-gamma-carboxy prothrombin or protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ)

Des-gamma-carboxy prothrombin (DCP) is a nonfunctional prothrombin form produced by the liver. The normal liver converts the glutamic acid residues in the N-terminal portion of prothrombin by carboxylation in gamma-carboxyglutamic acid residues before its release into the peripheral blood. In many of HCC cells, the vitamin K dependent carboxylase that produces this carboxylation is absent; thus, an abnormal prothrombin is secreted. Several studies have found in multivariate analyses that high blood DCP levels are associated with a higher risk of HCC recurrence in HCC patients who undergo LT[123-127].

Glypican-3

Glypican (GPC)-3 is a member of the glypican protein family, which plays a role in regulating cell division and growth. One study reported that the protein expression of GPC-3 in HCC tissue samples prior to LT was associated with a higher rate of HCC recurrence after LT[128]; in addition, there was found that GPC-3 was expressed in 68% of HCC tissues, but not in adjacent non-tumoral tissues and in tissues of liver controls. Another study found that serum GPC-3 levels were higher in HCC patients than in cirrhosis patients[129]; however, the study did not examine the prognostic role of serum GPC-3 in HCC patients prior to LT.

Human telomerase reverse transcriptase messenger ribonucleic acid

Human telomerase reverse transcriptase messenger ribonucleic acid (h-TERT mRNA) is a ribonucleoprotein polymerase that maintains telomere ends in chromosomes. High h-TERT mRNA expression in the peripheral blood of HCC patients who undergo LT has been associated with decreased survival and increased HCC recurrence[130,131]; however, in another study, h-TERT mRNA concentrations in the peripheral blood were not associated with HCC recurrence after LT[132]. Therefore, additional research is necessary to determine the prognostic role of h-TERT mRNA expression in the peripheral blood of HCC patients prior to LT.

Matrix metalloproteinase-9

Matrix metalloproteinase (MMP)-9 is a member of the matrix metalloproteinases (MMPs), which are involved in degradation and remodeling of the extracellular matrix. MMPs play a role in physiological functions such as morphogenesis, tissue remodeling and the menstrual cycle. They are also involved in various diseases such as arthritis, tumors, atherosclerosis and sepsis. The activity of MMPs is regulated by several tissue inhibitor of matrix metalloproteinases (TIMPs).

Contradictory results have been found with regard to MMP-9. Patients who undergo LT for HCC and have high MMP-9 expression in the tumor have exhibited an unfavorable rates of overall survival and HCC recurrence[133,134]. Another study in patients undergoing LT due to HCC or for cirrhosis without HCC found that high serum MMP-9 levels at one week after LT were associated

with a higher rate of LT rejection[135]. However, one study also reported that high serum MMP-9 levels and low serum TIMP-1 levels in HCC patients receiving different treatments (curative resection, TACE, thermoablation, and LT) were associated with a higher survival rate, although the study did not specifically examine patients receiving LT because the sample size for that group was small[136]. Our group has previously reported a lower survival rate in patients with cerebral artery infarction[137], traumatic brain injury[138] and sepsis[139,140] who have high serum TIMP-1 levels than in patients who have low TIMP-1 levels. Therefore, additional research is necessary to establish the prognostic role of MMP-9 expression in the peripheral blood of HCC patients prior to LT.

E-cadherin

E-cadherin is a member of the cadherin family of proteins, which are cell adhesion molecules that participate in the formation of junctions between cells. One study found that high serum levels of soluble E-cadherin were associated with increased recurrence of HCC after a curative resection[141]. Another study of HCC patients who underwent LT found that E-cadherin expression in the liver was associated with HCC recurrence after LT[142].

Beta-catenin

Beta-catenin is a member of the catenin family of proteins, which also constitute a group of cell adhesion molecules that are involved in the formation of bonds between cells. A study of HCC patients who underwent LT found that beta-catenin expression in the liver was associated with HCC recurrence after LT[142]. However, in other recently published study, no association was found between beta-catenin expression in the liver prior to LT and the survival of HCC patients[143]. Therefore, additional research is necessary to establish the prognostic role of beta-catenin expression in the liver in HCC patients prior to LT.

AFP

AFP is a glycoprotein that is produced by the yolk sac and the fetal liver during fetal development. It is the most abundant plasma protein in the human fetus. Increased values are found in newborns (values gradually decrease to normal over the first year of life), pregnant women (values return to normal after delivery), and patients with various AFP-producing tumors such as HCC and tumors of the ovary and testis.

The blood AFP level is the most extensively studied biomarker in HCC patients undergoing LT. Elevated blood AFP levels are associated with decreased survival[144] as well as an increase in HCC recurrence[145] in patients undergoing LT for HCC.

A review of 13 observational studies published in 2012 involving 12,159 patients who underwent LT for HCC examined the role of pre-LT circulating AFP levels in predicting survival and HCC recurrence[144]. Nine of the 13 studies reported data about pre-LT serum AFP levels and survival. Only four studies reported absolute serum AFP values for all included patients, and the other studies used varying cut-off points for serum AFP levels. This heterogeneity precluded pooling of the data for a valid meta-analysis. The majority of the studies concluded that a high pre-LT serum AFP level was an independent predictor of death following LT for HCC. These studies also suggested that serum AFP levels higher than 1000 ng/mL may predict poorer survival. Ten of the 13 studies reported data on HCC recurrence and pre-LT serum AFP values. All of these studies concluded that high AFP levels were associated with increased HCC recurrence following LT for HCC. The authors were unable to perform a meta-analysis on this research question due to the heterogeneity in the data reported by the studies. Additionally, some of the studies included in the review found that pre-LT serum AFP levels were correlated with vascular invasion and poor differentiation of HCC.

A review and meta-analysis published in 2016 examined the prognostic role of biomarkers in HCC recurrence in patients who underwent LT for HCC[145]. The review included 49 studies with a total of 13693 patients that reported data on pre-LT serum AFP levels and HCC recurrence. However, the studies had 88% heterogeneity due to their use of varying definitions and cut-off values for AFP. Therefore, it was not possible to conduct a valid meta-analysis for this topic. However, a meta-analysis was performed using 17 of the studies with different cut-off values for pre-LT serum AFP levels, but the meta-analysis required a cut-off value higher than 400 ng/mL. In this analysis, an association was found between elevated pre-LT serum AFP levels and the risk of HCC recurrence (HR = 2.69; 95%CI: 2.08-3.47), with a heterogeneity of 46%.

Dickkopf-1

In several studies have been found higher circulating Dickkopf-1 (DKK1) levels in HCC patients than in healthy subjects[146-149] or than in patients with liver cirrosis without HCC[150,151]. In addition, in a meta-analysis published in 2014 including 4 studies[152] and in other recently published study[153] was found that higher DKK1 expression levels in HCC patients were associated with lower survival. Besides, in one study was found that higher DKK1 expression is associated with lower survival and higher recurrence in HCC patients after LT[154].

Vascular endothelial growth factor

In a meta-analysis of 11 studies was found that high serum Vascular endothelial growth factor (VEGF) leves in HCC patients were associated with lower survival[155]. In addition, in one study was found that high plasma VEGF levels in HCC patients previously to LT were associated with HCC recurrence and survival[156].

Caspase-1

Pyroptosis is a form of programmed cell death, which is dependent of caspase-1. In some studies has been

Table 1 New prognostic biomarkers in patients undergoing liver transplantation for hepatocellular carcinoma

Biomarker	Alteration	Outcome	Ref.
Malondialdehyde	High circulating levels	Lower survival	[23]
Total antioxidant capacity	High circulating levels	Lower survival	[29]
Caspase-cleaved cytokeratin-18	High circulating levels	Lower survival	[54]
Soluble CD40 ligand	High circulating levels	Lower survival	[80]
Substance P	High circulating levels	Lower survival	[107]
Neutrophil to lymphocyte ratio	High circulating ratio	Lower survival	[120]
Platelet to lymphocyte ratio	High circulating ratio	Lower survival	[120]
C-reactive protein	High circulating levels	Lower survival	[121,122]
Des-gamma-carboxy prothrombin	High circulating levels	Higher recurrence	[123-127]
Glypican-3	High HCC expression	Higher recurrence	[128]
H-TERT mRNA	High peripheral blood expression	Lower survival	[130,131]
E-cadherin	High HCC expression	Higher recurrence	[142]
Beta-catenin	High HCC expression	Higher recurrence	[142]
Dickkopf-1	High HCC expression	Lower survival	[154]
Vascular endothelial growth factor	High circulating levels	Lower survival	[156]

HCC: Hepatocellular carcinoma; H-TERT mRNA: Human telomerase reverse transcriptase messenger ribonucleic acid.

found lower caspase-1 expression in HCC tissues[157,158]. In a study was determined caspase-1 expression in HCC patients (from HCC tissues and adjacent normal tissues) and in hepatocyte cell lines[157]. There was found a significant decrease in caspase-1 expression in HCC tissues compared to adjacent normal tissues and hepatocyte cell lines. Besides, the use of berberine increased the expression of caspase-1, decreased cell number, and increased cell swelling in hepatocyte cell lines; and the use of the caspase-1 inhibitor Ac-YVAD-CMK attenuated the effects of berberine.

However, in one study has been found that liver tissue of patients infected with hepatitis C virus (HCV) showed caspase-1-mediated pyroptosis[159]. Besides, in other study of patients with resection of HCC was found lower survival in patients with high of caspase-1 expression in normal tissues[160].

Angiopoietin-2

Angiopoietin-2 is a protein that is involved in angiogenesis and inflammation[161]. In a recently published study of chronic HCV patients treated with direct acting antivirals (DAA) was found that angiopoietin-2 in liver tissue was related with the risk of HCC recurrence or de novo ocurrence.

Another interesting finding of that study was that patients with HCC recurrence or de novo ocurrence had significantly higher portal pressure than patients never developing HCC[162]; and in previous studies was found that portal hypertension was associated with poor prognosis in patients undergoing to LT[163] or with HCC[164,165].

The risk of HCC occurrence or recurrence following DAA remains unclear due to that the results of different studies are contradictories. In a review published in 2017 including 10 studies was found in meta-analyses a higher incidence of HCC occurrence and HCC recurrence with the administration of DAA[166]. However, in meta-regression analyses after adjusting for study follow-up and age, DAA therapy was not associated with higher HCC de novo ocurrence and neither with HCC recurrence. In the study by Faillaci *et al*[162] was found that the use of DAA was associated with de novo HCC, and that this risk is higher in patients with higher angiopoietin-2 expression.

Genomic

The Cancer Genome Atlas (TCGA) Research Network published in 2017 the genomic characterization of HCC[167]. There were analyzed 363 HCC cases by whole-genome sequencing and DNA copy number, and 196 HCC cases by DNA methylation, mRNA, miRNA, and proteomic expression. In total, 12136 genes had non-silent mutations, and 26 genes were determined to be significantly mutated genes. Of these 26 genes, 18 were reported in at least one previous HCC genome sequencing study and 8 were not previously associated with HCC. Whithin of know mutated genes, the most included TERTpromoter (51%), TP53 (31%), CTNNB1 (27%), ALB (13%), APOB (10%), ARID1A (7%), AXIN1 (8%), ARID2 (5%), BAP1(5%), KEAP1 (5%), RB1 (4%), and NFE2L2 (3%). There were identified 8 novel mutated gene with a low frequence (2%-3% of HCC patients), including LTZR1, EEF1A1, AZIN1, RP1L 1, GPATCH4, CREB3L3, AHCTF1, and HIST1H1C. In addition, other two mutated genes previously associated with other cancer types were associated with HCC in this study, F3B1 and SMARCA4. Besides, integrative clustering of datasets of DNA copy number, DNA methylation, mRNA expression and miRNA expression could define three HCC subtypes (iClust 1 to 3), and iClust1 subtype had a poor prognosis. In addition, the analysis of these mutations and pathways provide potential directions for future potential therapeutic in HCC patients by the use of inhibitors of WNT, MDM4, MET, VEGFA, MCL1, IDH1, TERT. Thus, this genome-wide characterization has been very important in improving our knowledge about mutated genes associated with HCC, prognostic gene signatures and potential treatments[168].

CONCLUSION

Various macromorphological factors measured prior to LT have been classically used to estimate the outcomes of HCC patients undergoing LT. Additionally, the determination of some valid biomarkers prior to LT could help predict the prognoses of HCC patients undergoing LT. The most frequently examined biomarker is the serum AFP level. Recently, an association was reported between decreased survival rates and high blood levels of malondialdehyde, TAC, CCCK-18, sCD40L, substance P, CRP, and VEGF, NLR and PLR in blood, high peripheral blood expression of h-TERT mRNA, and high HCC expression of DKK1. In addition, an association has been found between increased HCC recurrence and high blood levels of Des-gamma-carboxy prothrombin, and high HCC expression of GPC-3, E-cadherin and beta-catenin. Additional research is necessary to establish the prognostic role of these biomarkers for HCC prior to LT. Furthermore, some of these biomarkers are also interesting because their potential modulation could help to create new research lines for improving the outcomes of those patients. Those new biomarkers are summarized on Table 1.

REFERENCES

1 **Bodzin AS**, Busuttil RW. Hepatocellular carcinoma: Advances in diagnosis, management, and long term outcome. *World J Hepatol* 2015; **7**: 1157-1167 [PMID: 26019732 DOI: 10.4254/wjh.v7.i9.1157]

2 **Guerrero-Misas M**, Rodríguez-Perálvarez M, De la Mata M. Strategies to improve outcome of patients with hepatocellular carcinoma receiving a liver transplantation. *World J Hepatol* 2015; **7**: 649-661 [PMID: 25866602 DOI: 10.4254/wjh.v7.i4.649]

3 **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 DOI: 10.1016/j.jhep.2011.12.001]

4 **Slotta JE**, Kollmar O, Ellenrieder V, Ghadimi BM, Homayounfar K. Hepatocellular carcinoma: Surgeon's view on latest findings and future perspectives. *World J Hepatol* 2015; **7**: 1168-1183 [PMID: 26019733 DOI: 10.4254/wjh.v7.i9.1168]

5 **Gomaa AI**, Waked I. Recent advances in multidisciplinary management of hepatocellular carcinoma. *World J Hepatol* 2015; **7**: 673-687 [PMID: 25866604 DOI: 10.4254/wjh.v7.i4.673]

6 **Clark T**, Maximin S, Meier J, Pokharel S, Bhargava P. Hepatocellular Carcinoma: Review of Epidemiology, Screening, Imaging Diagnosis, Response Assessment, and Treatment. *Curr Probl Diagn Radiol* 2015; **44**: 479-486 [PMID: 25979220 DOI: 10.1067/j.cpradiol.2015.04.004]

7 **Toyoda H**, Kumada T, Tada T, Sone Y, Kaneoka Y, Maeda A. Tumor Markers for Hepatocellular Carcinoma: Simple and Significant Predictors of Outcome in Patients with HCC. *Liver Cancer* 2015; **4**: 126-136 [PMID: 26020034 DOI: 10.1159/000367735]

8 **Cescon M**, Bertuzzo VR, Ercolani G, Ravaioli M, Odaldi F, Pinna AD. Liver transplantation for hepatocellular carcinoma: role of inflammatory and immunological state on recurrence and prognosis. *World J Gastroenterol* 2013; **19**: 9174-9182 [PMID: 24409045 DOI: 10.3748/wjg.v19.i48.9174]

9 **Lee HW**, Suh KS. Expansion of the criteria for living donor liver transplantation for hepatocellular carcinoma. *Curr Opin Organ Transplant* 2016; **21**: 231-237 [PMID: 26918880 DOI: 10.1097/MOT.0000000000000294]

10 **Cillo U**, Giuliani T, Polacco M, Herrero Manley LM, Crivellari G, Vitale A. Prediction of hepatocellular carcinoma biological behavior in patient selection for liver transplantation. *World J Gastroenterol* 2016; **22**: 232-252 [PMID: 26755873 DOI: 10.3748/wjg.v22.i1.232]

11 **Romano M**, De Francesco F, Pirozzi G, Gringeri E, Boetto R, Di Domenico M, Zavan B, Ferraro GA, Cillo U. Expression of cancer stem cell biomarkers as a tool for a correct therapeutic approach to hepatocellular carcinoma. *Oncoscience* 2015; **2**: 443-456 [PMID: 26097877]

12 **Draper HH**, Hadley M. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* 1990; **186**: 421-431 [PMID: 2233309]

13 **Dalle-Donne I**, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. *Clin Chem* 2006; **52**: 601-623 [PMID: 16484333 DOI: 10.1373/clinchem.2005.061408]

14 **Yahya RS**, Ghanem OH, Foyouh AA, Atwa M, Enany SA. Role of interleukin-8 and oxidative stress in patients with hepatocellular carcinoma. *Clin Lab* 2013; **59**: 969-976 [PMID: 24273918]

15 **Zhao J**, Zhao Y, Wang H, Gu X, Ji J, Gao C. Association between metabolic abnormalities and HBV related hepatocelluar carcinoma in Chinese: a cross-sectional study. *Nutr J* 2011; **10**: 49 [PMID: 21569630 DOI: 10.1186/1475-2891-10-49]

16 **Tsai SM**, Lin SK, Lee KT, Hsiao JK, Huang JC, Wu SH, Ma H, Wu SH, Tsai LY. Evaluation of redox statuses in patients with hepatitis B virus-associated hepatocellular carcinoma. *Ann Clin Biochem* 2009; **46**: 394-400 [PMID: 19641006 DOI: 10.1258/acb.2009.009029]

17 **Trevisani F**, Caraceni P, Simoncini M, Micati M, Domenicali M, Dazzani F, Zambruni A, Stefanelli C, Grazi G, Nardo B, Guarnieri C, Bernardi M. Evidence of oxidative imbalance in long-term liver transplant patients. *Dig Liver Dis* 2002; **34**: 279-284 [PMID: 12038812]

18 **Thorat VN**, Suryakar AN, Naik P, Tiwale BM. Total antioxidant capacity and lipid peroxidation in liver transplantation. *Indian J Clin Biochem* 2009; **24**: 102-104 [PMID: 23105816 DOI: 10.1007/s12291-009-0018-x]

19 **Czeczot H**, Scibior D, Skrzycki M, Podsiad M. Glutathione and GSH-dependent enzymes in patients with liver cirrhosis and hepatocellular carcinoma. *Acta Biochim Pol* 2006; **53**: 237-242 [PMID: 16404476]

20 **Váli L**, Hahn O, Kupcsulik P, Drahos A, Sárváry E, Szentmihályi K, Pallai Z, Kurucz T, Sípos P, Blázovics A. Oxidative stress with altered element content and decreased ATP level of erythrocytes in hepatocellular carcinoma and colorectal liver metastases. *Eur J Gastroenterol Hepatol* 2008; **20**: 393-398 [PMID: 18403940 DOI: 10.1097/MEG.0b013e3282f495c7]

21 **Suzuki Y**, Imai K, Takai K, Hanai T, Hayashi H, Naiki T, Nishigaki Y, Tomita E, Shimizu M, Moriwaki H. Hepatocellular carcinoma patients with increased oxidative stress levels are prone to recurrence after curative treatment: a prospective case series study using the d-ROM test. *J Cancer Res Clin Oncol* 2013; **139**: 845-852 [PMID: 23411687 DOI: 10.1007/s00432-013-1389-1]

22 **Liu C**, Zhou XS, Geng QM. [Evaluation oxygen free radicals related index before liver transplantation to forejudge prognosis]. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue* 2003; **15**: 560-562 [PMID: 12971857]

23 **Lorente L**, Rodriguez ST, Sanz P, Abreu-González P, Díaz D, Moreno AM, Borja E, Martín MM, Jiménez A, Barrera MA. Association between Pre-Transplant Serum Malondialdehyde Levels and Survival One Year after Liver Transplantation for Hepatocellular Carcinoma. *Int J Mol Sci* 2016; **17**: 500 [PMID: 27058525 DOI: 10.3390/ijms17040500]

24 **Lorente L**, Martín MM, Abreu-González P, Domínguez-Rodriguez A, Labarta L, Díaz C, Solé-Violán J, Ferreres J, Cabrera J, Igeño JC, Jiménez A. Sustained high serum malondialdehyde levels are associated with severity and mortality in septic patients. *Crit Care* 2013; **17**: R290 [PMID: 24326199 DOI: 10.1186/cc13155]

25 **Lorente L**, Martín MM, Abreu-González P, Ramos L, Argueso M, Cáceres JJ, Solé-Violán J, Lorenzo JM, Molina I, Jiménez A.

Association between serum malondialdehyde levels and mortality in patients with severe brain trauma injury. *J Neurotrauma* 2015; **32**: 1-6 [PMID: 25054973 DOI: 10.1089/neu.2014.3456]
26 **Lorente L**, Martín MM, Abreu-González P, Ramos L, Argueso M, Solé-Violán J, Riaño-Ruiz M, Jiménez A. Serum malondialdehyde levels in patients with malignant middle cerebral artery infarction are associated with mortality. *PLoS One* 2015; **10**: e0125893 [PMID: 25933254 DOI: 10.1371/journal.pone.0125893]
27 **Lorente L**, Martín MM, Abreu-González P, Sabatel R, Ramos L, Argueso M, Solé-Violán J, Riaño-Ruiz M, Jiménez A, García-Marín V. Serum Malondialdehyde Levels and Mortality in Patients with Spontaneous Intracerebral Hemorrhage. *World Neurosurg* 2018; **113**: e542-e547 [PMID: 29477698 DOI: 10.1016/j.wneu.2018.02.085]
28 **Ghiselli A**, Serafini M, Natella F, Scaccini C. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radic Biol Med* 2000; **29**: 1106-1114 [PMID: 11121717]
29 **Lorente L**, Rodriguez ST, Sanz P, Pérez-Cejas A, Abreu-González P, Padilla J, Díaz D, González A, Martín MM, Jiménez A, Cerro P, Barrera MA. Serum total antioxidant capacity prior to liver transplantation for hepatocellular carcinoma is associated with 1-year liver transplantation survival. *J Int Med Res* 2018; **46**: 2641-2649 [PMID: 29911482 DOI: 10.1177/0300060518768150]
30 **Lowes DA**, Webster NR, Murphy MP, Galley HF. Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. *Br J Anaesth* 2013; **110**: 472-480 [PMID: 23381720 DOI: 10.1093/bja/aes577]
31 **Horáková L**, Ondrejicková O, Bachratá K, Vajdová M. Preventive effect of several antioxidants after oxidative stress on rat brain homogenates. *Gen Physiol Biophys* 2000; **19**: 195-205 [PMID: 11156442]
32 **Gitto E**, Karbownik M, Reiter RJ, Tan DX, Cuzzocrea S, Chiurazzi P, Cordaro S, Corona G, Trimarchi G, Barberi I. Effects of melatonin treatment in septic newborns. *Pediatr Res* 2001; **50**: 756-760 [PMID: 11726736 DOI: 10.1203/00006450-200112000-00021]
33 **Ullegaddi R**, Powers HJ, Gariballa SE. Antioxidant supplementation enhances antioxidant capacity and mitigates oxidative damage following acute ischaemic stroke. *Eur J Clin Nutr* 2005; **59**: 1367-1373 [PMID: 16091766 DOI: 10.1038/sj.ejcn.1602248]
34 **Saniova B**, Drobny M, Lehotsky J, Sulaj M, Schudichova J. Biochemical and clinical improvement of cytotoxic state by amantadine sulphate. *Cell Mol Neurobiol* 2006; **26**: 1475-1482 [PMID: 16710757 DOI: 10.1007/s10571-006-9033-0]
35 **Luedde T**, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. *Gastroenterology* 2014; **147**: 765-783.e4 [PMID: 25046161 DOI: 10.1053/j.gastro.2014.07.018]
36 **Canbay A**, Feldstein A, Kronenberger B, Schulze-Osthoff K, Bantel H. [Cytokeratin 18 as marker for non-invasive diagnosis and prognosis of acute and chronic liver diseases]. *Z Gastroenterol* 2014; **52**: 290-295 [PMID: 24622872 DOI: 10.1055/s-0033-1356138]
37 **Yilmaz Y**. Systematic review: caspase-cleaved fragments of cytokeratin 18 - the promises and challenges of a biomarker for chronic liver disease. *Aliment Pharmacol Ther* 2009; **30**: 1103-1109 [PMID: 19769633 DOI: 10.1111/j.1365-2036.2009.04148.x]
38 **Chu PG**, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathology* 2002; **40**: 403-439 [PMID: 12010363]
39 **Caulín C**, Salvesen GS, Oshima RG. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol* 1997; **138**: 1379-1394 [PMID: 9298992]
40 **Leers MP**, Kölgen W, Björklund V, Bergman T, Tribbick G, Persson B, Björklund P, Ramaekers FC, Björklund B, Nap M, Jörnvall H, Schutte B. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 1999; **187**: 567-572 [PMID: 10398123 DOI: 10.1002/(SICI)1096-9896(199904)187:53.0.CO;2-J]
41 **Hägg M**, Bivén K, Ueno T, Rydlander L, Björklund P, Wiman KG, Shoshan M, Linder S. A novel high-through-put assay for screening of pro-apoptotic drugs. *Invest New Drugs* 2002; **20**: 253-259 [PMID: 12201488]
42 **Oyama K**, Fushida S, Kinoshita J, Okamoto K, Makino I, Nakamura K, Hayashi H, Inokuchi M, Nakagawara H, Tajima H, Fujita H, Takamura H, Ninomiya I, Kitagawa H, Fujimura T, Ohta T. Serum cytokeratin 18 as a biomarker for gastric cancer. *Clin Exp Med* 2013; **13**: 289-295 [PMID: 22825587 DOI: 10.1007/s10238-012-0202-9]
43 **Ueno T**, Toi M, Bivén K, Bando H, Ogawa T, Linder S. Measurement of an apoptotic product in the sera of breast cancer patients. *Eur J Cancer* 2003; **39**: 769-774 [PMID: 12651202]
44 **Ulukaya E**, Yilmaztepe A, Akgoz S, Linder S, Karadag M. The levels of caspase-cleaved cytokeratin 18 are elevated in serum from patients with lung cancer and helpful to predict the survival. *Lung Cancer* 2007; **56**: 399-404 [PMID: 17316892 DOI: 10.1016/j.lungcan.2007.01.015]
45 **Yaman E**, Coskun U, Sancak B, Buyukberber S, Ozturk B, Benekli M. Serum M30 levels are associated with survival in advanced gastric carcinoma patients. *Int Immunopharmacol* 2010; **10**: 719-722 [PMID: 20362077 DOI: 10.1016/j.intimp.2010.03.013]
46 **de Haas EC**, di Pietro A, Simpson KL, Meijer C, Suurmeijer AJ, Lancashire LJ, Cummings J, de Jong S, de Vries EG, Dive C, Gietema JA. Clinical evaluation of M30 and M65 ELISA cell death assays as circulating biomarkers in a drug-sensitive tumor, testicular cancer. *Neoplasia* 2008; **10**: 1041-1048 [PMID: 18813353]
47 **Koelink PJ**, Lamers CB, Hommes DW, Verspaget HW. Circulating cell death products predict clinical outcome of colorectal cancer patients. *BMC Cancer* 2009; **9**: 88 [PMID: 19302716 DOI: 10.1186/1471-2407-9-88]
48 **Wu YX**, Wang JH, Wang H, Yang XY. [Study on expression of Ki-67, early apoptotic protein M30 in endometrial carcinoma and their correlation with prognosis]. *Zhonghua Bing Li Xue Za Zhi* 2003; **32**: 314-318 [PMID: 14514374]
49 **Gonzalez-Quintela A**, Mallo N, Mella C, Campos J, Perez LF, Lopez-Rodriguez R, Tome S, Otero E. Serum levels of cytokeratin-18 (tissue polypeptide-specific antigen) in liver diseases. *Liver Int* 2006; **26**: 1217-1224 [PMID: 17105587 DOI: 10.1111/j.1478-3231.2006.01380.x]
50 **Yagmur E**, Trautwein C, Leers MP, Gressner AM, Tacke F. Elevated apoptosis-associated cytokeratin 18 fragments (CK18Asp386) in serum of patients with chronic liver diseases indicate hepatic and biliary inflammation. *Clin Biochem* 2007; **40**: 651-655 [PMID: 17306787 DOI: 10.1016/j.clinbiochem.2006.12.010]
51 **Godin C**, Louandre C, Bodeau S, Diouf M, Saidak Z, Conte MA, Chauffert B, Barbare JC, Barget N, Trinchet JC, Ganne N, Galmiche A. Biomarkers of apoptosis and necrosis in patients with hepatocellular carcinoma treated with sorafenib. *Anticancer Res* 2015; **35**: 1803-1808 [PMID: 25750346]
52 **Waidmann O**, Köberle V, Bettinger D, Trojan J, Zeuzem S, Schultheiß M, Kronenberger B, Piiper A. Diagnostic and prognostic significance of cell death and macrophage activation markers in patients with hepatocellular carcinoma. *J Hepatol* 2013; **59**: 769-779 [PMID: 23792028 DOI: 10.1016/j.jhep.2013.06.008]
53 **Morris KL**, Tugwood JD, Khoja L, Lancashire M, Sloane R, Burt D, Shenjere P, Zhou C, Hodgson C, Ohtomo T, Katoh A, Ishiguro T, Valle JW, Dive C. Circulating biomarkers in hepatocellular carcinoma. *Cancer Chemother Pharmacol* 2014; **74**: 323-332 [PMID: 24923562 DOI: 10.1007/s00280-014-2508-7]
54 **Lorente L**, Rodriguez ST, Sanz P, Pérez-Cejas A, Padilla J, Díaz D, González A, Martín MM, Jiménez A, Barrera MA. Prognostic Value of Serum Caspase-Cleaved Cytokeratin-18 Levels before Liver Transplantation for One-Year Survival of Patients with Hepatocellular Carcinoma. *Int J Mol Sci* 2016; **17** [PMID: 27618033 DOI: 10.3390/ijms17091524]
55 **Lorente L**, Martín MM, González-Rivero AF, Ferreres J, Solé-Violán J, Labarta L, Díaz C, Jiménez A, Borreguero-León JM. Serum levels of caspase-cleaved cytokeratin-18 and mortality are

associated in severe septic patients: pilot study. *PLoS One* 2014; **9**: e109618 [PMID: 25290885 DOI: 10.1371/journal.pone.0109618]
56 **Lorente L**, Martín MM, González-Rivero AF, Argueso M, Ramos L, Solé-Violán J, Cáceres JJ, Jiménez A, Borreguero-León JM. Serum levels of caspase-cleaved cytokeratin-18 in patients with severe traumatic brain injury are associated with mortality: a pilot study. *PLoS One* 2015; **10**: e0121739 [PMID: 25822281 DOI: 10.1371/journal.pone.0121739]
57 **Lorente L**, Martín MM, Pérez-Cejas A, Ramos L, Argueso M, Solé-Violán J, Cáceres JJ, Jiménez A, García-Marín V. High serum levels of caspase-cleaved cytokeratin-18 are associated with malignant middle cerebral artery infarction patient mortality. *BMC Neurol* 2018; **18**: 32 [PMID: 29573748 DOI: 10.1186/s12883-018-1038-z]
58 **Antoniades C**, Bakogiannis C, Tousoulis D, Antonopoulos AS, Stefanadis C. The CD40/CD40 ligand system: linking inflammation with atherothrombosis. *J Am Coll Cardiol* 2009; **54**: 669-677 [PMID: 19679244 DOI: 10.1016/j.jacc.2009.03.076]
59 **Ferroni P**, Santilli F, Guadagni F, Basili S, Davì G. Contribution of platelet-derived CD40 ligand to inflammation, thrombosis and neoangiogenesis. *Curr Med Chem* 2007; **14**: 2170-2180 [PMID: 17691955]
60 **Aukrust P**, Damas JK, Solum NO. Soluble CD40 ligand and platelets: self-perpetuating pathogenic loop in thrombosis and inflammation? *J Am Coll Cardiol* 2004; **43**: 2326-2328 [PMID: 15193701 DOI: 10.1016/j.jacc.2004.03.023]
61 **Anand SX**, Viles-Gonzalez JF, Badimon JJ, Cavusoglu E, Marmur JD. Membrane-associated CD40L and sCD40L in atherothrombotic disease. *Thromb Haemost* 2003; **90**: 377-384 [PMID: 12958605 DOI: 10.1160/TH03-05-0268]
62 **Zhang B**, Wu T, Chen M, Zhou Y, Yi D, Guo R. The CD40/CD40L system: a new therapeutic target for disease. *Immunol Lett* 2013; **153**: 58-61 [PMID: 23892087 DOI: 10.1016/j.imlet.2013.07.005]
63 **Zhang T**, Pierson RN 3rd, Azimzadeh AM. Update on CD40 and CD154 blockade in transplant models. *Immunotherapy* 2015; **7**: 899-911 [PMID: 26268734 DOI: 10.2217/IMT.15.54]
64 **Pinelli DF**, Ford ML. Novel insights into anti-CD40/CD154 immunotherapy in transplant tolerance. *Immunotherapy* 2015; **7**: 399-410 [PMID: 25917630 DOI: 10.2217/imt.15.1]
65 **Aloui C**, Prigent A, Sut C, Tariket S, Hamzeh-Cognasse H, Pozzetto B, Richard Y, Cognasse F, Laradi S, Garraud O. The signaling role of CD40 ligand in platelet biology and in platelet component transfusion. *Int J Mol Sci* 2014; **15**: 22342-22364 [PMID: 25479079 DOI: 10.3390/ijms151222342]
66 **Mao DJ**, Guo RY, Tang YC, Zang YH. [Expression of sCD40L in peripheral blood and NF-κBp65 in PBMC of patients with acute progressive cerebral infarction]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2011; **27**: 177-179 [PMID: 21315050]
67 **Tuttolomondo A**, Di Raimondo D, Di Sciacca R, Casuccio A, Bivona G, Bellia C, Barreca L, Serio A, D'Aguanno G, Ciaccio M, Licata G, Pinto A. Fetuin-A and CD40 L plasma levels in acute ischemic stroke: differences in relation to TOAST subtype and correlation with clinical and laboratory variables. *Atherosclerosis* 2010; **208**: 290-296 [PMID: 19709661 DOI: 10.1016/j.atherosclerosis.2009.07.032]
68 **Davì G**, Tuttolomondo A, Santilli F, Basili S, Ferrante E, Di Raimondo D, Pinto A, Licata G. CD40 ligand and MCP-1 as predictors of cardiovascular events in diabetic patients with stroke. *J Atheroscler Thromb* 2009; **16**: 707-713 [PMID: 19755790]
69 **Lorente L**, Martín MM, González-Rivero AF, Ramos L, Argueso M, Cáceres JJ, Solé-Violán J, Jiménez A, Borreguero-León JM. Association between Serum Soluble CD154 Levels and Mortality in Patients with Malignant Middle Cerebral Artery Infarction. *Int J Mol Sci* 2015; **16**: 12147-12158 [PMID: 26030673 DOI: 10.3390/ijms160612147]
70 **Aukrust P**, Müller F, Ueland T, Berget T, Aaser E, Brunsvig A, Solum NO, Forfang K, Frøland SS, Gullestad L. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation* 1999; **100**: 614-620 [PMID: 10441098]
71 **Varo N**, de Lemos JA, Libby P, Morrow DA, Murphy SA, Nuzzo R, Gibson CM, Cannon CP, Braunwald E, Schönbeck U. Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation* 2003; **108**: 1049-1052 [PMID: 12912804 DOI: 10.1161/01.CIR.0000088521.04017.13]
72 **Lorente L**, Martín MM, Varo N, Borreguero-León JM, Solé-Violán J, Blanquer J, Labarta L, Díaz C, Jiménez A, Pastor E, Belmonte F, Orbe J, Rodríguez JA, Gómez-Melini E, Ferrer-Agüero JM, Ferreres J, Llimiñana MC, Páramo JA. Association between serum soluble CD40 ligand levels and mortality in patients with severe sepsis. *Crit Care* 2011; **15**: R97 [PMID: 21406105 DOI: 10.1186/cc10104]
73 **Martínez de Lizarrondo S**, Roncal C, Calvayrac O, Rodríguez C, Varo N, Purroy A, Lorente L, Rodríguez JA, Doeuvre L, Hervás-Stubbs S, Angles-Cano E, Páramo JA, Martínez-González J, Orbe J. Synergistic effect of thrombin and CD40 ligand on endothelial matrix metalloproteinase-10 expression and microparticle generation in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2012; **32**: 1477-1487 [PMID: 22492089 DOI: 10.1161/ATVBAHA.112.248773]
74 **Dominguez-Rodriguez A**, Abreu-Gonzalez P, Garcia-Gonzalez MJ, Kaski JC. Soluble CD40 ligand:interleukin-10 ratio predicts in-hospital adverse events in patients with ST-segment elevation myocardial infarction. *Thromb Res* 2007; **121**: 293-299 [PMID: 17521712 DOI: 10.1016/j.thromres.2007.04.007]
75 **Lorente L**, Martín MM, González-Rivero AF, Ramos L, Argueso M, Cáceres JJ, Solé-Violán J, Serrano N, Rodríguez ST, Jiménez A, Borreguero-León JM. Serum soluble CD40 Ligand levels are associated with severity and mortality of brain trauma injury patients. *Thromb Res* 2014; **134**: 832-836 [PMID: 25123332 DOI: 10.1016/j.thromres.2014.07.034]
76 **Lapiński TW**, Pogorzelska J, Grzeszczuk A, Swiderska M, Kowalczuk O, Nikliński J, Flisiak R. The importance of sCD40 and sCD40L concentration in patients with chronic HCV infection and HIV co-infection. *Przegl Epidemiol* 2014; **68**: 11-15, 105-108 [PMID: 25004625]
77 **Basili S**, Raparelli V, Riggio O, Merli M, Carnevale R, Angelico F, Tellan G, Pignatelli P, Violi F; CALC Group. NADPH oxidase-mediated platelet isoprostane over-production in cirrhotic patients: implication for platelet activation. *Liver Int* 2011; **31**: 1533-1540 [PMID: 22093328 DOI: 10.1111/j.1478-3231.2011.02617.x]
78 **Sookoian S**, Castaño GO, Burgueño AL, Rosselli MS, Gianotti TF, Mallardi P, Martino JS, Pirola CJ. Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. *Atherosclerosis* 2010; **209**: 585-591 [PMID: 19896127 DOI: 10.1016/j.atherosclerosis.2009.10.011]
79 **Eltaher SM**, El-Gil R, Fouad N, Mitwali R, El-Kholy H. Evaluation of serum levels and significance of soluble CD40 ligand in screening patients with hepatitis C virus-related hepatocellular carcinoma. *East Mediterr Health J* 2016; **22**: 603-610 [PMID: 27834442]
80 **Lorente L**, Rodriguez ST, Sanz P, Pérez-Cejas A, Padilla J, Díaz D, González A, Martín MM, Jiménez A, Barrera MA. High serum soluble CD40L levels previously to liver transplantation in patients with hepatocellular carcinoma are associated with mortality at one year. *J Crit Care* 2018; **43**: 316-320 [PMID: 29020665 DOI: 10.1016/j.jcrc.2017.09.032]
81 **Noelle RJ**, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci USA* 1992; **89**: 6550-6554 [PMID: 1378631]
82 **Mach F**, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci USA* 1997; **94**: 1931-1936 [PMID: 9050882]
83 **Zhou L**, Stordeur P, de Lavareille A, Thielemans K, Capel P,

Goldman M, Pradier O. CD40 engagement on endothelial cells promotes tissue factor-dependent procoagulant activity. *Thromb Haemost* 1998; **79**: 1025-1028 [PMID: 9609241]
84 **Hezi-Yamit A**, Wong PW, Bien-Ly N, Komuves LG, Prasad KS, Phillips DR, Sinha U. Synergistic induction of tissue factor by coagulation factor Xa and TNF: evidence for involvement of negative regulatory signaling cascades. *Proc Natl Acad Sci USA* 2005; **102**: 12077-12082 [PMID: 16105945 DOI: 10.1073/pnas.0504526102]
85 **Miller DL**, Yaron R, Yellin MJ. CD40L-CD40 interactions regulate endothelial cell surface tissue factor and thrombomodulin expression. *J Leukoc Biol* 1998; **63**: 373-379 [PMID: 9500526]
86 **Slupsky JR**, Kalbas M, Willuweit A, Henn V, Kroczek RA, Müller-Berghaus G. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb Haemost* 1998; **80**: 1008-1014 [PMID: 9869175]
87 **Prasad KS**, Andre P, He M, Bao M, Manganello J, Phillips DR. Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. *Proc Natl Acad Sci USA* 2003; **100**: 12367-12371 [PMID: 14519852 DOI: 10.1073/pnas.2032886100]
88 **André P**, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. *Nat Med* 2002; **8**: 247-252 [PMID: 11875495 DOI: 10.1038/nm0302-247]
89 **Li J**, Zhao SP, Peng DQ, Xu ZM, Zhou HN. Early effect of pravastatin on serum soluble CD40L, matrix metalloproteinase-9, and C-reactive protein in patients with acute myocardial infarction. *Clin Chem* 2004; **50**: 1696-1699 [PMID: 15265816 DOI: 10.1373/clinchem.2003.030940]
90 **Hamdan R**, Hajj F, Kadry Z, Kassab R, Salame E, Aboujaoude S, Azar R, Badaoui G. Benefit and tolerability of the coadministration of ezetimibe and atorvastatin in acute coronary syndrome patients. *J Med Liban* 2011; **59**: 65-69 [PMID: 21834489]
91 **Han SH**, Koh KK, Quon MJ, Lee Y, Shin EK. The effects of simvastatin, losartan, and combined therapy on soluble CD40 ligand in hypercholesterolemic, hypertensive patients. *Atherosclerosis* 2007; **190**: 205-211 [PMID: 16500662 DOI: 10.1016/j.atherosclerosis.2006.01.021]
92 **Ní Chróinín D**, Asplund K, Åsberg S, Callaly E, Cuadrado-Godia E, Díez-Tejedor E, Di Napoli M, Engelter ST, Furie KL, Giannopoulos S, Gotto AM Jr, Hannon N, Jonsson F, Kapral MK, Martí-Fàbregas J, Martínez-Sánchez P, Milionis HJ, Montaner J, Muscari A, Pikija S, Probstfield J, Rost NS, Thrift AG, Vemmos K, Kelly PJ. Statin therapy and outcome after ischemic stroke: systematic review and meta-analysis of observational studies and randomized trials. *Stroke* 2013; **44**: 448-456 [PMID: 23287777 DOI: 10.1161/STROKEAHA.112.668277]
93 **Tleyjeh IM**, Kashour T, Hakim FA, Zimmerman VA, Erwin PJ, Sutton AJ, Ibrahim T. Statins for the prevention and treatment of infections: a systematic review and meta-analysis. *Arch Intern Med* 2009; **169**: 1658-1667 [PMID: 19822822 DOI: 10.1001/archinternmed.2009.286]
94 **Thomsen RW**, Riis A, Kornum JB, Christensen S, Johnsen SP, Sørensen HT. Preadmission use of statins and outcomes after hospitalization with pneumonia: population-based cohort study of 29,900 patients. *Arch Intern Med* 2008; **168**: 2081-2087 [PMID: 18955636 DOI: 10.1001/archinte.168.19.2081]
95 **Hsu J**, Andes DR, Knasinski V, Pirsch J, Safdar N. Statins are associated with improved outcomes of bloodstream infection in solid-organ transplant recipients. *Eur J Clin Microbiol Infect Dis* 2009; **28**: 1343-1351 [PMID: 19669657 DOI: 10.1007/s10096-009-0787-4]
96 **Dobesh PP**, Klepser DG, McGuire TR, Morgan CW, Olsen KM. Reduction in mortality associated with statin therapy in patients with severe sepsis. *Pharmacotherapy* 2009; **29**: 621-630 [PMID: 19476415 DOI: 10.1592/phco.29.6.621]
97 **Almeida TA**, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martín JD, Candenas ML. Tachykinins and tachykinin receptors: structure and activity relationships. *Curr Med Chem* 2004; **11**: 2045-2081 [PMID: 15279567]
98 **Pennefather JN**, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: a growing family. *Life Sci* 2004; **74**: 1445-1463 [PMID: 14729395]
99 **Satake H**, Kawada T. Overview of the primary structure, tissue-distribution, and functions of tachykinins and their receptors. *Curr Drug Targets* 2006; **7**: 963-974 [PMID: 16918325]
100 **Trivedi M**, Bergasa NV. Serum concentrations of substance P in cholestasis. *Ann Hepatol* 2010; **9**: 177-180 [PMID: 20526012]
101 **El-Raziky MS**, Gohar N, El-Raziky M. Study of substance P, renin and aldosterone in chronic liver disease in Egyptian children. *J Trop Pediatr* 2005; **51**: 320-323 [PMID: 15980023 DOI: 10.1093/tropej/fmi060]
102 **Li CP**, Lee FY, Hwang SJ, Chang FY, Lin HC, Lu RH, Hou MC, Chu CJ, Chan CC, Luo JC, Lee SD. Role of substance P in the pathogenesis of spider angiomas in patients with nonalcoholic liver cirrhosis. *Am J Gastroenterol* 1999; **94**: 502-507 [PMID: 10022654 DOI: 10.1111/j.1572-0241.1999.883_l.x]
103 **Uemura M**, Tsujii T, Kikuchi E, Fukui H, Tsukamoto N, Matsumura M, Fujimoto M, Koizumi M, Takaya A, Kojima H, Ishii Y, Okamoto S. Increased plasma levels of substance P and disturbed water excretion in patients with liver cirrhosis. *Scand J Gastroenterol* 1998; **33**: 860-866 [PMID: 9754735]
104 **Lee FY**, Lin HC, Tsai YT, Chang FY, Lu RH, Hou MC, Li CP, Chu CJ, Wang SS, Lee SD. Plasma substance P levels in patients with liver cirrhosis: relationship to systemic and portal hemodynamics. *Am J Gastroenterol* 1997; **92**: 2080-2084 [PMID: 9362197]
105 **Fernández-Rodriguez CM**, Prieto J, Quiroga J, Zozoya JM, Andrade A, Núñez M, Sangro B, Penas J. Plasma levels of substance P in liver cirrhosis: relationship to the activation of vasopressor systems and urinary sodium excretion. *Hepatology* 1995; **21**: 35-40 [PMID: 7528711]
106 **Hörtnagl H**, Singer EA, Lenz K, Kleinberger G, Lochs H. Substance P is markedly increased in plasma of patients with hepatic coma. *Lancet* 1984; **1**: 480-483 [PMID: 6199630]
107 **Lorente L**, Rodriguez ST, Sanz P, Pérez-Cejas A, Padilla J, Díaz D, González A, Martín MM, Jiménez A, Cerro P, Barrera MA. Patients with high serum substance P levels previously to liver transplantation for hepatocellular carcinoma have higher risk of one-year liver transplantation mortality. *Oncotarget* 2018; **9**: 21552-21559 [PMID: 29765559 DOI: 10.18632/oncotarget.25097]
108 **Lorente L**, Martín MM, Almeida T, Hernández M, Ramos L, Argueso M, Cáceres JJ, Solé-Violán J, Jiménez A. Serum substance P levels are associated with severity and mortality in patients with severe traumatic brain injury. *Crit Care* 2015; **19**: 192 [PMID: 25928056 DOI: 10.1186/s13054-015-0911-z]
109 **Lorente L**, Martín MM, Almeida T, Pérez-Cejas A, Ramos L, Argueso M, Riaño-Ruiz M, Solé-Violán J, Hernández M. Serum Levels of Substance P and Mortality in Patients with a Severe Acute Ischemic Stroke. *Int J Mol Sci* 2016; **17** [PMID: 27338372 DOI: 10.3390/ijms170609]
110 **Lotz M**, Vaughan JH, Carson DA. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* 1988; **241**: 1218-1221 [PMID: 2457950]
111 **Laurenzi MA**, Persson MA, Dalsgaard CJ, Haegerstrand A. The neuropeptide substance P stimulates production of interleukin 1 in human blood monocytes: activated cells are preferentially influenced by the neuropeptide. *Scand J Immunol* 1990; **31**: 529-533 [PMID: 1692157]
112 **Ansel JC**, Brown JR, Payan DG, Brown MA. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *J Immunol* 1993; **150**: 4478-4485 [PMID: 7683320]
113 **Rameshwar P**, Gascón P. Substance P (SP) mediates production of stem cell factor and interleukin-1 in bone marrow stroma: potential autoregulatory role for these cytokines in SP receptor expression and induction. *Blood* 1995; **86**: 482-490 [PMID: 7541664]
114 **Yamaguchi M**, Kojima T, Kanekawa M, Aihara N, Nogimura A, Kasai K. Neuropeptides stimulate production of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in human dental

pulp cells. *Inflamm Res* 2004; **53**: 199-204 [PMID: 15105969 DOI: 10.1007/s00011-003-1243-z]
115 **Turner RJ**, Helps SC, Thornton E, Vink R. A substance P antagonist improves outcome when administered 4 h after onset of ischaemic stroke. *Brain Res* 2011; **1393**: 84-90 [PMID: 21466790 DOI: 10.1016/j.brainres.2011.03.066]
116 **Turner RJ**, Vink R. NK1 tachykinin receptor treatment is superior to capsaicin pre-treatment in improving functional outcome following acute ischemic stroke. *Neuropeptides* 2014; **48**: 267-272 [PMID: 25151181 DOI: 10.1016/j.npep.2014.07.002]
117 **Yu Z,** Cheng G, Huang X, Li K, Cao X. Neurokinin-1 receptor antagonist SR140333: A novel type of drug to treat cerebral ischemia. *Neuroreport* 1997; **8**: 2117-2119
118 **Gabrielian L**, Helps SC, Thornton E, Turner RJ, Leonard AV, Vink R. Substance P antagonists as a novel intervention for brain edema and raised intracranial pressure. *Acta Neurochir Suppl* 2013; **118**: 201-204 [PMID: 23564132 DOI: 10.1007/978-3-7091-1434-6_37]
119 **Donkin JJ**, Cernak I, Blumbergs PC, Vink R. A substance P antagonist reduces axonal injury and improves neurologic outcome when administered up to 12 hours after traumatic brain injury. *J Neurotrauma* 2011; **28**: 217-224 [PMID: 21175297 DOI: 10.1089/neu.2010.1632]
120 **Zheng J**, Cai J, Li H, Zeng K, He L, Fu H, Zhang J, Chen L, Yao J, Zhang Y, Yang Y. Neutrophil to Lymphocyte Ratio and Platelet to Lymphocyte Ratio as Prognostic Predictors for Hepatocellular Carcinoma Patients with Various Treatments: a Meta-Analysis and Systematic Review. *Cell Physiol Biochem* 2017; **44**: 967-981 [PMID: 29179180 DOI: 10.1159/000485396]
121 **An HJ**, Jang JW, Bae SH, Choi JY, Yoon SK, Lee MA, You YK, Kim DG, Jung ES. Serum C-reactive protein is a useful biomarker for predicting outcomes after liver transplantation in patients with hepatocellular carcinoma. *Liver Transpl* 2012; **18**: 1406-1414 [PMID: 22821639 DOI: 10.1002/lt.23512]
122 **Mori S**, Choi Y, Park MS, Kim H, Hong G, Yi NJ, Lee KW, Suh KS. Usefulness of preoperative C-reactive protein and alpha-fetoprotein levels for prognostication of patients with hepatocellular carcinoma after living donor liver transplantation. *Hepatogastroenterology* 2014; **61**: 2353-2358 [PMID: 25699382]
123 **Harimoto N**, Yoshizumi T, Shimagaki T, Nagatsu A, Motomura T, Harada N, Okabe H, Itoh S, Ikegami T, Uchiyama H, Soejima Y, Maehara Y. Inflammation-based Prognostic Score in Patients with Living Donor Liver Transplantation for Hepatocellular Carcinoma. *Anticancer Res* 2016; **36**: 5537-5542 [PMID: 27798927 DOI: 10.21873/anticanres.11137]
124 **Fujiki M**, Takada Y, Ogura Y, Oike F, Kaido T, Teramukai S, Uemoto S. Significance of des-gamma-carboxy prothrombin in selection criteria for living donor liver transplantation for hepatocellular carcinoma. *Am J Transplant* 2009; **9**: 2362-2371 [PMID: 19656125 DOI: 10.1111/j.1600-6143.2009.02783.x]
125 **Taketomi A**, Sanefuji K, Soejima Y, Yoshizumi T, Uhciyama H, Ikegami T, Harada N, Yamashita Y, Sugimachi K, Kayashima H, Iguchi T, Maehara Y. Impact of des-gamma-carboxy prothrombin and tumor size on the recurrence of hepatocellular carcinoma after living donor liver transplantation. *Transplantation* 2009; **87**: 531-537 [PMID: 19307789 DOI: 10.1097/TP.0b013e3181943bee]
126 **Shindoh J**, Sugawara Y, Nagata R, Kaneko J, Tamura S, Aoki T, Sakamoto Y, Hasegawa K, Tanaka T, Kokudo N. Evaluation methods for pretransplant oncologic markers and their prognostic impacts in patient undergoing living donor liver transplantation for hepatocellular carcinoma. *Transpl Int* 2014; **27**: 391-398 [PMID: 24472068 DOI: 10.1111/tri.12274]
127 **Lee JH**, Cho Y, Kim HY, Cho EJ, Lee DH, Yu SJ, Lee JW, Yi NJ, Lee KW, Kim SH, Kim JM, Joh JW, Teperman LW, Park JS, Kim YJ, Suh KS, Yoon JH. Serum Tumor Markers Provide Refined Prognostication in Selecting Liver Transplantation Candidate for Hepatocellular Carcinoma Patients Beyond the Milan Criteria. *Ann Surg* 2016; **263**: 842-850 [PMID: 26779979 DOI: 10.1097/SLA.0000000000001578]
128 **Wang YL**, Zhu ZJ, Teng DH, Yao Z, Gao W, Shen ZY. Glypican-3 expression and its relationship with recurrence of HCC after liver transplantation. *World J Gastroenterol* 2012; **18**: 2408-2414 [PMID: 22654434 DOI: 10.3748/wjg.v18.i19.2408]
129 **Jing JS**, Ye W, Jiang YK, Ma J, Zhu MQ, Ma JM, Zhou H, Yu LQ, Yang YF, Wang SC. The Value of GPC3 and GP73 in Clinical Diagnosis of Hepatocellular Carcinoma. *Clin Lab* 2017; **63**: 1903-1909 [PMID: 29226636]
130 **Oya H**, Sato Y, Yamamoto S, Nakatsuka H, Kobayashi T, Hara Y, Waguri N, Suda T, Aoyagi Y, Hatakeyama K. Comparison between human-telomerase reverse transcriptase mRNA and alpha-fetoprotein mRNA as a predictive value for recurrence of hepatocellular carcinoma in living donor liver transplantation. *Transplant Proc* 2006; **38**: 3636-3639 [PMID: 17175353 DOI: 10.1016/j.transproceed.2006.10.172]
131 **Sato Y**, Yamamoto S, Oya H, Nakatsuka H, Kobayashi T, Takeishi T, Hirano K, Hara Y, Watanabe T, Waguri N, Suda T, Ichida T, Aoyagi Y, Hatakeyama K. Preoperative human-telomerase reverse transcriptase mRNA in peripheral blood and tumor recurrence in living-related liver transplantation for hepatocellular carcinoma. *Hepatogastroenterology* 2005; **52**: 1325-1328 [PMID: 16201066]
132 **Kim YD**, Hwang S, Lee YJ, Kim KH, Ahn CS, Park KM, Moon DB, Ha TY, Song GW, Jung DH, Park SR, Hong HN, Lee SG. Preoperative peripheral blood human telomerase reverse transcriptase mRNA concentration is not a prognostic factor for resection of hepatocellular carcinoma. *Hepatogastroenterology* 2012; **59**: 1512-1515 [PMID: 22683968 DOI: 10.5754/hge10342]
133 **Nart D**, Yaman B, Yilmaz F, Zeytunlu M, Karasu Z, Kiliç M. Expression of matrix metalloproteinase-9 in predicting prognosis of hepatocellular carcinoma after liver transplantation. *Liver Transpl* 2010; **16**: 621-630 [PMID: 20440771 DOI: 10.1002/lt.22028]
134 **Zhang Q**, Chen X, Zhou J, Zhang L, Zhao Q, Chen G, Xu J, Qian F, Chen Z. CD147, MMP-2, MMP-9 and MVD-CD34 are significant predictors of recurrence after liver transplantation in hepatocellular carcinoma patients. *Cancer Biol Ther* 2006; **5**: 808-814 [PMID: 16775432]
135 **Kuyvenhoven JP**, Verspaget HW, Gao Q, Ringers J, Smit VT, Lamers CB, van Hoek B. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. *Transplantation* 2004; **77**: 1646-1652 [PMID: 15201662]
136 **Lempinen M**, Lyytinen I, Nordin A, Tervahartiala T, Mäkisalo H, Sorsa T, Isoniemi H. Prognostic value of serum MMP-8, -9 and TIMP-1 in patients with hepatocellular carcinoma. *Ann Med* 2013; **45**: 482-487 [PMID: 23962148 DOI: 10.3109/07853890.2013.823779]
137 **Lorente L**, Martín MM, Ramos L, Cáceres JJ, Solé-Violán J, Argueso M, Jiménez A, Borreguero-León JM, Orbe J, Rodríguez JA, Páramo JA. Serum tissue inhibitor of matrix metalloproteinase-1 levels are associated with mortality in patients with malignant middle cerebral artery infarction. *BMC Neurol* 2015; **15**: 111 [PMID: 26162891 DOI: 10.1186/s12883-015-0364-7]
138 **Lorente L**, Martín MM, López P, Ramos L, Blanquer J, Cáceres JJ, Solé-Violán J, Solera J, Cabrera J, Argueso M, Ortiz R, Mora ML, Lubillo S, Jiménez A, Borreguero-León JM, González A, Orbe J, Rodríguez JA, Páramo JA. Association between serum tissue inhibitor of matrix metalloproteinase-1 levels and mortality in patients with severe brain trauma injury. *PLoS One* 2014; **9**: e94370 [PMID: 24728097 DOI: 10.1371/journal.pone.0094370]
139 **Lorente L**, Martín MM, Solé-Violán J, Blanquer J, Labarta L, Díaz C, Borreguero-León JM, Orbe J, Rodríguez JA, Jiménez A, Páramo JA. Association of sepsis-related mortality with early increase of TIMP-1/MMP-9 ratio. *PLoS One* 2014; **9**: e94318 [PMID: 24727739 DOI: 10.1371/journal.pone.0094318]
140 **Lorente L**, Martín MM, Labarta L, Díaz C, Solé-Violán J, Blanquer J, Orbe J, Rodríguez JA, Jiménez A, Borreguero-León JM, Belmonte F, Medina JC, Llimiñana MC, Ferrer-Agüero JM, Ferreres J, Mora ML, Lubillo S, Sánchez M, Barrios Y, Sierra A, Páramo JA. Matrix metalloproteinase-9, -10, and tissue inhibitor

of matrix metalloproteinases-1 blood levels as biomarkers of severity and mortality in sepsis. *Crit Care* 2009; **13**: R158 [PMID: 19799791 DOI: 10.1186/cc8115]

141 **Soyama A**, Eguchi S, Takatsuki M, Kawashita Y, Hidaka M, Tokai H, Nagayoshi S, Mochizuki S, Matsumoto S, Hamasaki K, Tajima Y, Kanematsu T. Significance of the serum level of soluble E-cadherin in patients with HCC. *Hepatogastroenterology* 2008; **55**: 1390-1393 [PMID: 18795696]

142 **Fiorentino M**, Altimari A, Ravaioli M, Gruppioni E, Gabusi E, Corti B, Vivarelli M, Bringuier PP, Scoazec JY, Grigioni WF, D' Errico-Grigioni A. Predictive value of biological markers for hepatocellular carcinoma patients treated with orthotopic liver transplantation. *Clin Cancer Res* 2004; **10**: 1789-1795 [PMID: 15014033]

143 **Ataide EC**, Perales SR, Silva MG, Filho FC, Sparapani AC, Latuf Filho PF, Stucchi RSB, Vassallo J, Escanhoela CAF, Boin IFSF. Immunoexpression of Heat Shock Protein 70, Glypican 3, Glutamine Synthetase, and Beta-Catenin in Hepatocellular Carcinoma After Liver Transplantation: Association Between Positive Glypican 3 and Beta-Catenin With the Presence of Larger Nodules. *Transplant Proc* 2017; **49**: 858-862 [PMID: 28457411 DOI: 10.1016/j.transproceed.2017.01.048]

144 **Hakeem AR**, Young RS, Marangoni G, Lodge JP, Prasad KR. Systematic review: the prognostic role of alpha-fetoprotein following liver transplantation for hepatocellular carcinoma. *Aliment Pharmacol Ther* 2012; **35**: 987-999 [PMID: 22429190 DOI: 10.1111/j.1365-2036.2012.05060.x]

145 **Pommergaard HC**, Burcharth J, Rosenberg J, Rasmussen A. Serologic and molecular biomarkers for recurrence of hepatocellular carcinoma after liver transplantation: A systematic review and meta-analysis. *Transplant Rev* (Orlando) 2016; **30**: 171-177 [PMID: 27118303 DOI: 10.1016/j.trre.2016.03.001]

146 **Shen Q**, Fan J, Yang XR, Tan Y, Zhao W, Xu Y, Wang N, Niu Y, Wu Z, Zhou J, Qiu SJ, Shi YH, Yu B, Tang N, Chu W, Wang M, Wu J, Zhang Z, Yang S, Gu J, Wang H, Qin W. Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicentre study. *Lancet Oncol* 2012; **13**: 817-826 [PMID: 22738799 DOI: 10.1016/S1470-2045(12)70233-4]

147 **Erdal H**, Gül Utku Ö, Karatay E, Çelik B, Elbeg Ş, Doğan İ. Combination of DKK1 and AFP improves diagnostic accuracy of hepatocellular carcinoma compared with either marker alone. *Turk J Gastroenterol* 2016; **27**: 375-381 [PMID: 27458854 DOI: 10.5152/tjg.2016.15523]

148 **Kim SU**, Park JH, Kim HS, Lee JM, Lee HG, Kim H, Choi SH, Baek S, Kim BK, Park JY, Kim DY, Ahn SH, Lee JD, Han KH. Serum Dickkopf-1 as a Biomarker for the Diagnosis of Hepatocellular Carcinoma. *Yonsei Med J* 2015; **56**: 1296-1306 [PMID: 26256972 DOI: 10.3349/ymj.2015.56.5.1296]

149 **Ge T**, Shen Q, Wang N, Zhang Y, Ge Z, Chu W, Lv X, Zhao F, Zhao W, Fan J, Qin W. Diagnostic values of alpha-fetoprotein, dickkopf-1, and osteopontin for hepatocellular carcinoma. *Med Oncol* 2015; **32**: 59 [PMID: 25652109 DOI: 10.1007/s12032-014-0367-z]

150 **Fouad YM**, Mohamed HI, Kamal EM, Rasek MA. Clinical significance and diagnostic value of serum dickkopf-1 in patients with hepatocellular carcinoma. *Scand J Gastroenterol* 2016; **51**: 1133-1137 [PMID: 27161933 DOI: 10.3109/00365521.2016.1172337]

151 **Jang ES**, Jeong SH, Kim JW, Choi YS, Leissner P, Brechot C. Diagnostic Performance of Alpha-Fetoprotein, Protein Induced by Vitamin K Absence, Osteopontin, Dickkopf-1 and Its Combinations for Hepatocellular Carcinoma. *PLoS One* 2016; **11**: e0151069 [PMID: 26986465 DOI: 10.1371/journal.pone.0151069]

152 **Liu Y**, Tang W, Xie L, Wang J, Deng Y, Peng Q, Zhai L, Li S, Qin X. Prognostic significance of dickkopf-1 overexpression in solid tumors: a meta-analysis. *Tumour Biol* 2014; **35**: 3145-3154 [PMID: 24258111 DOI: 10.1007/s13277-013-1411-x]

153 **Sakabe T**, Azumi J, Umekita Y, Toriguchi K, Hatano E, Hirooka Y, Shiota G. Expression of Cancer Stem Cell-associated DKK1 mRNA Serves as Prognostic Marker for Hepatocellular Carcinoma. *Anticancer Res* 2017; **37**: 4881-4888 [PMID: 28870909 DOI: 10.21873/anticanres.11897]

154 **Huang Y**, Yang X, Zhao F, Shen Q, Wang Z, Lv X, Hu B, Yu B, Fan J, Qin W. Overexpression of Dickkopf-1 predicts poor prognosis for patients with hepatocellular carcinoma after orthotopic liver transplantation by promoting cancer metastasis and recurrence. *Med Oncol* 2014; **31**: 966 [PMID: 24878698 DOI: 10.1007/s12032-014-0966-8]

155 **Zhan P**, Qian Q, Yu LK. Serum VEGF level is associated with the outcome of patients with hepatocellular carcinoma: a meta-analysis. *Hepatobiliary Surg Nutr* 2013; **2**: 209-215 [PMID: 24570945 DOI: 10.3978/j.issn.2304-3881.2013.06.07]

156 **Zhang W**, Kim R, Quintini C, Hashimoto K, Fujiki M, Diago T, Eghtesad B, Miller C, Fung J, Tan A, Menon KV, Aucejo F. Prognostic role of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma undergoing liver transplantation. *Liver Transpl* 2015; **21**: 101-111 [PMID: 25283528 DOI: 10.1002/lt.24013]

157 **Chu Q**, Jiang Y, Zhang W, Xu C, Du W, Tuguzbaeva G, Qin Y, Li A, Zhang L, Sun G, Cai Y, Feng Q, Li G, Li Y, Du Z, Bai Y, Yang B. Pyroptosis is involved in the pathogenesis of human hepatocellular carcinoma. *Oncotarget* 2016; **7**: 84658-84665 [PMID: 27705930 DOI: 10.18632/oncotarget.12384]

158 **Wan L**, Yuan X, Liu M, Xue B. miRNA-223-3p regulates NLRP3 to promote apoptosis and inhibit proliferation of hep3B cells. *Exp Ther Med* 2018; **15**: 2429-2435 [PMID: 29467847 DOI: 10.3892/etm.2017.5667]

159 **Kofahi HM**, Taylor NG, Hirasawa K, Grant MD, Russell RS. Hepatitis C Virus Infection of Cultured Human Hepatoma Cells Causes Apoptosis and Pyroptosis in Both Infected and Bystander Cells. *Sci Rep* 2016; **6**: 37433 [PMID: 27974850 DOI: 10.1038/srep37433]

160 **Sonohara F**, Inokawa Y, Kanda M, Nishikawa Y, Yamada S, Fujii T, Sugimoto H, Kodera Y, Nomoto S. Association of Inflammasome Components in Background Liver with Poor Prognosis After Curatively-resected Hepatocellular Carcinoma. *Anticancer Res* 2017; **37**: 293-300 [PMID: 28011505 DOI: 10.21873/anticanres.11320]

161 **Scholz A**, Plate KH, Reiss Y. Angiopoietin-2: a multifaceted cytokine that functions in both angiogenesis and inflammation. *Ann N Y Acad Sci* 2015; **1347**: 45-51 [PMID: 25773744 DOI: 10.1111/nyas.12726]

162 **Faillaci F**, Marzi L, Critelli R, Milosa F, Schepis F, Turola E, Andreani S, Vandelli G, Bernabucci V, Lei B, D'Ambrosio F, Bristot L, Cavalletto L, Chemello L, Sighinolfi P, Manni P, Maiorana A, Caporali C, Bianchini M, Marsico M, Turco L, de Maria N, Del Buono M, Todesca P, di Lena L, Romagnoli D, Magistri P, di Benedetto F, Bruno S, Taliani G, Giannelli G, Martinez-Chantar ML, Villa E. Liver Angiopoietin-2 is a key predictor of de novo or recurrent hepatocellular cancer after HCV direct-acting antivirals. *Hepatology* 2018; **68**: 1010-1024 [PMID: 29604220 DOI: 10.1002/hep.29911]

163 **Ogura Y**, Hori T, Uemoto S. Intentional portal pressure control is key to improving the outcome of living donor liver transplantation: the Kyoto University Hospital experience. *Clin Transpl* 2008; 143-149 [PMID: 19708453]

164 **Hidaka M**, Takatsuki M, Soyama A, Tanaka T, Muraoka I, Hara T, Kuroki T, Kanematsu T, Eguchi S. Intraoperative portal venous pressure and long-term outcome after curative resection for hepatocellular carcinoma. *Br J Surg* 2012; **99**: 1284-1289 [PMID: 22864890 DOI: 10.1002/bjs.8861]

165 **Hernandez-Gea V**, Turon F, Berzigotti A, Villanueva A. Management of small hepatocellular carcinoma in cirrhosis: focus on portal hypertension. *World J Gastroenterol* 2013; **19**: 1193-1199 [PMID: 23482437 DOI: 10.3748/wjg.v19.i8.1193]

166 **Waziry R**, Hajarizadeh B, Grebely J, Amin J, Law M, Danta M, George J, Dore GJ. Hepatocellular carcinoma risk following direct-acting antiviral HCV therapy: A systematic review, meta-analyses, and meta-regression. *J Hepatol* 2017; **67**: 1204-1212 [PMID: 28802876 DOI: 10.1016/j.jhep.2017.07.025]

167 **Cancer Genome Atlas Research Network.** Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

168 **Chiang DY**, Villanueva A. Progress towards molecular patient stratification of hepatocellular carcinoma: Lost in translation? *J Hepatol* 2017; **67**: 893-895 [PMID: 28837834 DOI: 10.1016/j.jhep.2017.07.031]

P- Reviewer: Gorrell MD, Wang K, Zhu X **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4243-4253

DOI: 10.3748/wjg.v24.i37.4243

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

MINIREVIEWS

Colonoscopy attachments for the detection of precancerous lesions during colonoscopy: A review of the literature

Paraskevas Gkolfakis, Georgios Tziatzios, Eleftherios Spartalis, Ioannis S Papanikolaou, Konstantinos Triantafyllou

Paraskevas Gkolfakis, Georgios Tziatzios, Ioannis S Papanikolaou, Konstantinos Triantafyllou, Hepato-gastroenterology Unit, Second Department of Internal Medicine -Propaedeutic, Research Institute and Diabetes Center, Medical School, National and Kapodistrian University of Athens, "Attikon" University General Hospital, Athens 12462, Greece

Eleftherios Spartalis, Laboratory of Experimental Surgery and Surgical Research, National and Kapodistrian University of Athens, Athens 12462, Greece

ORCID number: Paraskevas Gkolfakis (0000-0002-9677-4013); Georgios Tziatzios (0000-0002-2945-6007); Eleftherios Spartalis (0000-0003-4451-8074); Ioannis S Papanikolaou (0000-0002-7368-6168); Konstantinos Triantafyllou (0000-0002-5183-9426).

Author contributions: Triantafyllou K conceived the idea, revised and finally approved the manuscript; Gkolfakis P and Tziatzios G searched the literature and drafted the manuscript; Papanikolaou IS and Spartalis E revised and finally approved the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest related to this publication.

Manuscript source: Invited manuscript

Correspondence to: Konstantinos Triantafyllou, MD, PhD, Associate Professor, Hepato-gastroenterology Unit, Second Department of Internal Medicine - Propaedeutic, Research Institute and Diabetes Center, Medical School, National and Kapodistrian University of Athens, "Attikon" University General Hospital, Rimini 1, Athens 12462, Greece. ktriant@med.uoa.gr

Telephone: +30-210-5832087
Fax: +30-210-58326454

Received: June 11, 2018
Peer-review started: June 12, 2018
First decision: August 1, 2018
Revised: August 6, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

Although colonoscopy has been proven effective in reducing the incidence of colorectal cancer through the detection and removal of precancerous lesions, it remains an imperfect examination, as it can fail in detecting up to almost one fourth of existing adenomas. Among reasons accounting for such failures, is the inability to meticulously visualize the colonic mucosa located either proximal to haustral folds or anatomic curves, including the hepatic and splenic flexures. In order to overcome these limitations, various colonoscope attachments aiming to improve mucosal visualization have been developed. All of them - transparent cap, Endocuff, Endocuff Vision and Endorings - are simply mounted onto the distal tip of the scope. In this review article, we introduce the rationale of their development, present their mode of action and discuss in detail the effect of their implementation in the detection of lesions during colonoscopy.

Key words: Adenoma detection rate; Adenoma miss rate; Colonoscopy; Cup; Endocuff; Endocuff Vision; Endorings

Core tip: Colonoscopy is the modality of choice for the detection and removal of precancerous lesions. However,

almost one fourth of adenomas can be lost during conventional colonoscopy. Their location proximal to the colonic folds or in proximity to anatomic flexures is one of the reasons for this particular detection failure. To overcome this caveat, various single-use devices mounted onto the tip of the scope have been developed. They facilitate lesions' detection by manipulating and flattening the haustral folds. In this Minireview we present the development of these devices (Cap, Endocuff, Endocuff Vision and Endorings) and their effectiveness in improving detection rates of lesions during colonoscopy.

Gkolfakis P, Tziatzios G, Spartalis E, Papanikolaou IS, Triantafyllou K. Colonoscopy attachments for the detection of precancerous lesions during colonoscopy: A review of the literature. *World J Gastroenterol* 2018; 24(37): 4243-4253 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4243.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4243

INTRODUCTION

Colorectal cancer (CRC) is the second most lethal cancer among common cancers and more than 140200 new CRC cases are expected to be diagnosed in the United States by the end of 2018[1]. Colonoscopy has been proven efficient for both diagnosis and screening of colorectal cancer. It allows the detection and consequent removal of adenomas, the most well-known precancerous lesions, preventing CRC-associated death[2]. Adenoma detection rate (ADR)-the percentage of colonoscopies with at least one adenoma- has been associated with both decreased risk of interval CRC (*i.e.*, CRC that is diagnosed in the meantime between a screening colonoscopy and the next recommended surveillance examination) incidence and death[3-5]. Thus, ADR has been established as the core quality indicator for colonoscopy[6]. However, colonoscopy stands far from being the perfect examination. Back-to-back studies have shown that endoscopists fail to detect almost 25% of existing polyps and adenomas[7,8]. These miss rates are higher in the right colon, where a variant of precancerous lesions (the sessile serrated adenomas) that does not follow the classic adenoma-carcinoma pathway of carcinogenesis occurs more frequently[9,10]. To a great extent, missed lesions like these have been held responsible for the aforementioned interval cancers[11]. Inadequate bowel preparation, lack of physician's expertise, inability to accurately visualize the colonic mucosa located proximal to the haustral folds or in proximity to anatomic flexures have been listed among the main reasons such lesions can be missed during a colonoscopy[12]. Lately, several devices-ranging from complex endoscopic systems to simple plastic attachments- have been developed, in an attempt to address this problem[13]. They promise to flatten the mucosa during scope withdrawal and facilitate maneuverability around anatomic flexures offering meticulous mucosal visualization and detection of "hidden" lesions. In this review we aim to present the rationale that led to the development of these detachable devices, their evolution through time, their main mode of action and technical characteristics, as well as their impact on various patient-related colonoscopy outcomes. A comprehensive review of English literature published in MEDLINE until May 2018 was conducted. We aimed to identify high quality studies (randomized controlled trials and meta-analyses) using the following key words: "Cap", "Cap-assisted colonoscopy", "Endocuff", "Endocuff-Vision", "Endocuff-assisted colonoscopy" and "Endorings". Apart from ADR the following measures were assessed: polyp detection rate (PDR), *i.e.*, the percentage of colonoscopies with at least one polyp, mean number of adenomas detected per colonoscopy (MAC), adenoma miss rate-the percentage of adenomas missed by the index examination and detected by the tandem colonoscopy- and advanced ADR (the percentage of colonoscopies with at least one advanced adenoma).

THE CAP

The transparent cap is a simple, single-use device made of thermoplastic elastomer (Figure 1). It was initially designed to facilitate endoscopic mucosectomy, since it enables an optimal field of view by maintaining an appropriate distance between the endoscope tip and the intervention site[14]. Originally launched by Olympus (Olympus America Inc., Center Valley, PA, United States), it is available in various sizes, in order to accommodate all types of endoscopes. Its rounded edge prevents tissue damage during contact, while its side hole allows fluid draining. Endoscope functions such as suction and air insufflation remain undisturbed. The distal end of the cap protrudes from the scope's tip (protruding length ranges from 2 mm to 7 mm). Its basic characteristics are outlined in Table 1. It is the protruding edge that allows manipulation and flattening of the colonic folds in the field of view. During the last 10 years numerous randomized control trials[15-27] have been conducted to evaluate the usefulness of cap-assisted colonoscopy (CAC) in improving colonoscopy outcomes, including potential augmentation of the detection of precancerous lesions. Beyond ADR and PDR, additional outcomes such as cecal intubation rate and cecal intubation time were assessed as well.

Kondo *et al*[15] evaluated colonoscopy with two types of caps (2 mm-short or 4 mm-transparent) *vs* conventional colonoscopy without a cap. More than 200 patients undergoing colonoscopy for various indications were randomized in each of the three groups. The use of the transparent 4mm cap was associated with decreased cecal intubation time compared to the 2 mm-short cap and the control (11.5 min *vs* 13.5 min *vs* 15 min; $P = 0.008$). At the same time, PDR was significantly increased in the transparent cap group (49.3%) compared to the controls (39.1%; $P = 0.04$)[15]. In another Japanese randomized controlled trial (RCT) with 592 patients, CAC with a 2 mm

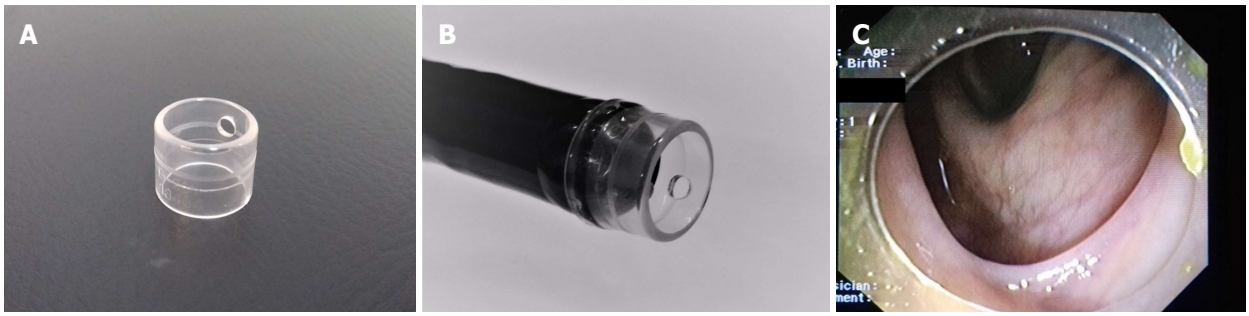


Figure 1 Cap (A) mounted on the tip of the scope (B) and the endoscopic view (C) (photos from the authors' archive).

short cap was also related to a shorter cecal intubation rate, but without any difference in the rate of polyp detection[17]. Horiuchi *et al*[16] randomized 60 patients diagnosed with colonic adenomas to repeat colonoscopy in three months, with or without a 7 mm cap; all lesions were removed during the second examination. Cap-assisted colonoscopy detected 20% more adenomas compared to a 4% increase in adenoma detection without the cap[16]. Moreover, a small back-to-back RCT of 67 screening/surveillance patients demonstrated a reduced adenoma miss rate associated to cap-assisted colonoscopy compared to conventional colonoscopy (21% *vs* 33%; $P = 0.04$)[19].

In terms of ADR and MAC, evidence remains controversial. A study from Japan[21], evaluating the efficacy of autofluorescence imaging with a transparent cap in a cancer referral center, found that CAC leads to an increased ADR (62% *vs* 56%; $P = 0.023$) compared to conventional white-light imaging. A few years later, in the first USA. study, Rastogi *et al*[23] randomized 420 screening/surveillance individuals (210 in each group) to undergo either CAC using a 4 mm transparent cap or a conventional examination. Investigators concluded that CAC not only shortened the cecal intubation time (3.3 min *vs* 4 min; $P < 0.001$), but also increased ADR (69% *vs* 56%; $P = 0.009$) and MAC (2.3 *vs* 1.4; $P < 0.001$) compared to colonoscopy without the cap[23]. In a study that randomized 1113 patients with various indications to undergo either CAC (4 mm cap) or conventional colonoscopy[24], cecal intubation was faster in the CAC arm (4.9 min *vs* 5.8 min; $P < 0.001$), but both arms had similar ADR (42% *vs* 40%; $P = 0.452$) and MAC (0.89 *vs* 0.82; $P = 0.432$)[24]. It is of great interest that among the 10 participating endoscopists the effect of CAC in terms of ADR ranged from a 15% decrease to a 20% increase[24]. Looking at individual endoscopists' performance, the authors concluded that CAC may be beneficial especially for endoscopists who spend more time during scope withdrawal since the cap may further enhance their already meticulous examination[24]. Recently, Othman *et al*[26] showed that CAC compared to conventional colonoscopy is related to an increased advanced ADR - the detection rate of advanced adenomas- (9.9% *vs* 4.6%; $P = 0.049$) and detection of more polyps larger than 9mm (9.5% *vs* 3.7%; $P = 0.026$)[26]. However, in this RCT of 440 screening/surveillance participants no difference between the two groups in terms of ADR and PDR was found[26].

In two RCTs[20,22], 400 individuals of mixed indications[20] and 1380 screening participants[22] were allocated either to CAC or conventional examination. A 4-mm cap was used in both studies. The first study[20] did not show any benefit of CAC in terms of PDR (32.8% *vs* 31.3%; $P = 0.75$) and cecal intubation time (9.9 min *vs* 10.3 min; $P = 0.21$), while in the second study[22] CAC was associated with a shorter intubation time (7.7 min *vs* 8.9 min; $P < 0.001$), but ADR (29% *vs* 29%; $P = 0.96$) and MAC (0.52 *vs* 0.50; $P = 0.83$) did not differ between the two groups.

Two studies involving endoscopy trainees provided similar results; trainees had a higher cecal intubation rate (CIR) and reached the cecum faster using the cap[27,28]. However, CAC did not improve trainees' detection rates (ADR and advanced ADR)[27,28].

Paradoxically, in a large RCT (1000 patients recruited) from Hong Kong, ADR was lower in the cap-assisted arm compared to the standard one (30.5% *vs* 37.5%; $P = 0.018$), but there was no difference regarding advanced lesions[18]. Shorter withdrawal times and inadequate bowel preparation in the CAC arm were postulated by the authors as potential explanations for this finding[18]. In accordance with previous results, cecal intubation time was shorter in the cap arm (6 min *vs* 7.2 min; $P < 0.001$) with no difference in the CIR[18].

Data from meta-analyses

Seven meta-analyses[29-35] that attempt to summarize the role of CAC in improving colonoscopy outcomes have been published so far (Table 2). Despite their different designs and inclusion criteria, one can figure out a couple of mutual conclusions. Four[30,31,34,35] out of five meta-analyses reporting on cecal intubation time, conclude that CAC significantly shortens it (mean difference ranging from -0.93 min to -0.64 min), while three of them[30,34,35] did not show any increase of CIR associated to CAC. Moreover, six[29-32,34,35] and four[30,32,34,35] meta-analyses examined PDR and ADR, respectively. The majority of these[29-31,34] link CAC to a higher PDR. On the contrary, none of the relevant meta-analyses showed a benefit in terms of ADR with CAC[29,30,32,34,35]. However, in the most

Table 1 Add-on devices' main characteristics

	Cap	Endocuff	Endocuff Vision	Endorings
Manufacturer	Olympus, Centre Valley, Pennsylvania	Arc Medical Leeds, United Kingdom	Norgine Pharmaceuticals Ltd, Uxbridge, United Kingdom	EndoAid, Caesarea, Israel
Launched in market	1993	2011	2016	2015
Short description	Transparent, single-use distal attachment with side hole for draining of fluid	Single-use, soft, radiopaque, 2 cm long cylindrical sleeve with flexible projections arranged in 2 rows of 8, emerging from gaps on the shaft of the device	Single-use, device with single row of 8 flexible 15 mm spikes	Single-use device composed of 2 layers of flexible, soft circular rings, placed on a cylindrical cuff
Material	Thermoplastic elastomer	Core: Non-latex, biocompatible polymer; Projections: thermoplastic elastomer	Latex free, polypropylene	Silicone
Dimensions	Outer diameter ranging from 13.9-16.1 mm according to each type of cap	Finger projections: proximal 8.15 mm, distal 5mm; core length: 23.8 mm; diameter: 16.1, 16.7, 17.2, and 18.5 mm (hairs folded back) and 32.6, 33.1, 33.6, and 34.8 mm (hairs opened out)	Diameter: 16.1, 16.7, 17.2, and 18.5mm (spikes folded back) and 39.07, 39.07, 39.07, and 39.66 mm (spikes opened out)	22-50 mm diameter
Mode of action	Protruding cap manipulates and flattens haustral folds to inspect the mucosa on the proximal side of the fold maintaining optimal field of view	Hinged projections flatten and spread mucosa and folds	Hinged projections flatten and improve visibility behind the colon folds	Sequential rings stretches out the folds of the colon during withdrawal for a clear view
Interfere with view of field	Edge of the hood comes into the vision field of the colonoscope, but lesions can be seen through the transparent wall	No interference of vision	No interference with vision	No interference with vision
Compatible scopes	Adult, pediatric: Ten different sizes, to fit all scopes	Adult, pediatric: 4 color-coded sizes (purple, orange, green and blue) to fit all scopes	Adult, pediatric: 4 color-coded sizes (purple, orange, green and blue) to fit all scopes	Scope Distal End Diameter [mm]; Adult colonoscope 12.8-14.5 mm; Slim Adult colonoscope 11.5-13.0 mm
Advantages	Resection of wider areas; Suction and insufflation of air unaffected	Folds movement provides a dynamic picture - even the smallest polyps can be identified; Centers the scope in the middle of the lumen preventing sudden slip back and "red-out"; Projections allow traction to avoid sudden slippage around turns and flexures, improving scope's stability; Helps perform EMR	Delivers more tip control without compromising intubation - improving loop management; Early and controlled view of the upstream surface of large folds - no need for repeated intubation; Prevents sudden slip back and red out; Optimizes tip position during therapy and polyp retrieval	Maintains position during loop reduction, decreases slippage, anchoring during endoscopic therapy; Maintains identical depth and breadth of scope's viewing field; Minimal resistance on insertion; Easy ileum intubation
Disadvantages	Interfere with the field of view	Petechial marks on colon; Potential dislodgement; Larger model more effective than smaller; Ileum intubation may be difficult	Potential dislodgement	Ileum intubation may be difficult

recent meta-analysis that included 23 RCTs and almost 13000 participants[34], sensitivity analysis showed that the exclusion of one large study[18]-in which the quality of bowel preparation was significantly worse in the CAC arm- not only eliminated the existing heterogeneity, but also altered the synthetic outcome direction, showing a significant benefit of CAC *vs* conventional colonoscopy regarding ADR [OR (95%CI): 1.17 (1.04-1.33)][34]. Finally, one meta-analysis[33] has evaluated the effect of CAC on the ADR of the right colon. Pooled data from 4 studies (2546 and 2547 patients in the CAC and the conventional arms, respectively) associated CAC with an increased right colon ADR [OR (95%CI): 1.49 (1.08-2.05)] compared to conventional colonoscopy[33].

THE ENDOCUFF

The first generation Endocuff

Endocuff (Arc Medical Design, Leeds, United Kingdom) is a single-use soft, radiopaque device that consists of a cylindrical polypropylene core and 2 rows of flexible thermoplastic elastomer-made projections. Each row counts 8 projections that emerge from gaps on the shaft of the device (Figure 2A and B). It is available in 4 different color-coded sizes to fit all scopes and its technical characteristics are presented in Table 1. Its designers were inspired through the practical difficulties that occur during a conventional colonoscopy, including the scope slipping back, difficulties in tip stabilization

Table 2 Meta-analyses evaluating the effect of accessories on colonoscopy outcomes

Author (yr)	Device *vs* comparator	Included Studies (*n*)	Included studies' design	Patients (*n*)	ADR	PDR	MAC	CIR	CIT
Westwood 2012	CAC *vs* CC	12 (9 FP, 3 AB)	RCTs	6185	NR	[a]OR (95%CI): 1.13 (1.02-1.26)	NR	[a]OR (95%CI): 1.36 (1.06-1.74)	MD (95%CI): 0.04 (-0.03 to 0.12) min
Ng 2012	CAC *vs* CC	16 (13 FP, 3 AB)	RCTs	8991	RR (95%CI): 1.04 (0.90-1.19)	[a]RR (95%CI): 1.08 (1.00-1.17)	NR	RR (95%CI): 1.00 (0.90-1.02)	[a]MD (95%CI): -0.64 (-1.19 to -0.10) min
He 2012	CAC *vs* CC	19 (14 FP, 5 AB)	RCTs	9235	NR	[a]OR (95%CI): 1.12 (1.02-1.22)	NR	[a]OR (95%CI): 1.36 (1.13-1.64)	[a]MD (95%CI): -0.65 (-0.85 to −0.44) min
Omata 2014	CAC *vs* CC	10 (10 FP)	RCTs	5219	RR (95%CI): 1.07 (0.94-1.23)	RR (95%CI): 1.00 (0.86-1.16)	NR	NR	NR
Desai 2017	CAC *vs* CC	4 (4 FP)	2 RCTs; 2 retrospective	5093	[a,1]OR (95%CI): 1.49 (1.08-2.05)	NR	NR	NR	NR
Mir 2017	CAC *vs* CC	23 (18 FP, 5 AB)	RCTs	12947	OR (95%CI): 1.11 (0.95-1.30)	[a]OR (95%CI): 1.17 (1.06-1.29)	NR	OR (95%CI): 1.32 (0.94-1.87)	[a]MD (95%CI): -0.82 (-1.20 to -0.44) min
Chin 2016	[2]EAC *vs* CC	9 (4FP, 5 AB)	4 RCTs; 1 prospective observational; 4 retrospective	5624	[a]OR (95%CI): 1.49 (1.23-1.80)	NR	NR	OR (95%CI): 1.26 (0.70-2.27)	NR
Williet 2018	[2]EAC *vs* CC	12 (7 FP, 5 AB)	RCTs	8376	[a]RR (95%CI): 1.20 (1.06-1.36)	[a]RR (95%CI): 1.20 (1.06-1.36)	MD (95%CI): 0.11 (-0.17-0.38)	RR (95%CI): 0.99 (0.97- 1.00)	MD (95%CI): -0.57 (-1.43 to 0.28) min
[3]Facciorusso 2017	CAC *vs* CC	14 (14 FP)	RCTs	8306	RR (95%CI): 1.07 (0.96-1.19)	RR (95%CI): 1.08 (0.99-1.18)	NR	RR (95%CI): 1.00 (1.00- 1.01)	[a]MD (95%CI): -0.68 (-1.11 to -0.24) min
	[2]EAC *vs* CC	9 (4FP, 5 AB)	RCTs	7072	[a]RR (95%CI): 1.21 (1.03-1.41)	[a]RR (95%CI): 1.22 (1.07-1.40)	NR	RR (95%CI): 1.00 (0.98- 1.01)	[a]MD (95%CI): -0.93 (-1.55 to -0.30) min
	Endorings *vs* CC	1 (1 FP)	RCTs	116	RR (95%CI): 1.70 (0.86-3.36)	RR (95%CI): 1.68 (0.94-2.99)	NR	NR	MD (95%CI): 0.90 (-1.47 to 3.27) min

[1]refers to right colon ADR; [2]refers to both first generation Endocuff and Endocuff Vision; [3]network meta-analysis. [a]Statistical significant. ADR: Adenoma detection rate; AMR: Adenoma miss rate; PDR: Polyp detection rate; MAC: Mean adenomas detected per colonoscopy; CIR: Cecal intubation rate; CIT: Cecal intubation time; CAC: Cap-assisted colonoscopy; CC: Conventional colonoscopy; EAC: Endocuff-assisted colonoscopy; FP: Full paper; AB: Abstract; RCT: Randomized controlled trial; NR: Not reported; OR: Odds ratio; 95%CI: 95% confidence intervals; RR: Relative risk; MD: Mean difference.

Figure 2 Endocuff (A) mounted on the tip of the scope (B) and the endoscopic view of the hinged projections during the withdrawal phase (C) (photos from the authors' archive).

and inability to inspect the mucosa located behind folds, to mention a few. Endocuff was launched in 2012 and its use was reported for the first time in a small retrospective feasibility study where it facilitated endoscopic access for complex polypectomy and scar assessment in the sigmoid colon[36]. The projections move independently from another in a passive way when in contact with the mucosa and during withdrawal, they extend radially manipulating colonic folds away from the field of view, allowing a more meticulous mucosa inspection (Figure 2C). Moreover, the device stabilizes the scope in the middle of the lumen and allows traction against sudden slippage around flexures. Moreover, the examiner's visibility is not affected, since the device does

not extend beyond the tip of the scope and thus does not interfere with suction, flushing or the working channel.

There are enough data regarding the effect of Endocuff on colonoscopy outcomes, since seven RCTs of parallel[37-43] and one of tandem[44] design have been published. The first German studies[37,38]-each recruiting almost 500 patients who underwent colonoscopy for various indications (screening included)-showed a significant benefit of Endocuff-assisted colonoscopy (EAC) compared to the conventional one regarding ADR (35.4% *vs* 20.7%, $P < 0.0001$ and 36% *vs* 28%, $P = 0.043$, respectively)[37,38]. Similar results regarding PDR were also achieved (55.4% *vs* 38.4%, $P < 0.0001$ and 56% *vs* 42%, $P = 0.001$, respectively), while only the second study[38] detected a difference in the mean number of adenomas detected per colonoscopy [2 (IQR: 1-3) *vs* 1 (IQR: 1-2), $P = 0.002$]. Regarding polyp location, both studies identified a superiority of EAC for the detection of polyps located in the sigmoid and the cecum. Moreover, no major adverse events related to EAC were reported and there were no differences between overall procedure and withdrawal times[37,38].

Similar positive results associated to Endocuff use were reported from Japan[39] (477 patients, mixed indications for colonoscopy) and Mexico[40] (337 screening individuals), where two single-centre RCTs demonstrated increase of ADR (55.2% *vs* 39.2%, $P = 0.0002$ and 22.4% *vs* 13.5%, $P = 0.02$, respectively), PDR (61.9% *vs* 49.2%, $P = 0.003$ and 29.9% *vs* 16%, $P = 0.002$, respectively) and MAC (1.11 *vs* 0.66, $P < 0.01$ and 0.29 *vs* 0.22, $P = 0.04$) in the device arms. An Italian single-centre study by De Palma *et al*[41] enrolled 288 patients with mixed indications and reported that EAC increased ADR by 3.3% (29.6% *vs* 26.3%) compared to the conventional colonoscopy. However, use of Endocuff was associated with mucosal erosions in 7 (2.5%) cases, with one of them needing to be treated with adrenaline solution injection at the site of bleeding[41].

Additionally, in a recently published 4-arm multicenter parallel-group study comparing Endocuff, Endorings, FUSE and conventional colonoscopy, 299 and 295 patients underwent Endocuff-assisted and conventional high definition colonoscopy, respectively[42]. EAC performed significantly better compared to conventional colonoscopy in terms of ADR (64% *vs* 56%, $P = 0.003$), PDR (83% *vs* 77%, $P = 0.001$) and MAC (1.82 ± 2.58 *vs* 1.53 ± 2.33, $P = 0.014$)[42]. However, Endocuff did not enhance the detection rate of sessile serrated polyps (11% *vs* 12%, $P = 0.047$)[42]. There were no differences between the mean insertion time (354 s ± 216 s *vs* 422 s ± 319 s) for Endocuff-assisted and conventional colonoscopy respectively and no adverse events were reported[42]. On the contrary, a benefit regarding sessile serrated adenoma/polyp detection was shown in a retrospective veterans' study[45] which included almost 500 participants: Endocuff detected 50 sessile serrated adenomas/polyps compared to 8 detected by conventional colonoscopy (detection rate 15% *vs* 3%, $P < 0.0001$).

So far, the largest parallel RCT[43] failed to confirm the positive results reported in the abovementioned studies. In this multicentre study from the Netherlands[43] more than 1000 patients of various indications were randomized to undergo either Endocuff-assisted or conventional colonoscopy; MAC and ADR consisted the primary outcomes. ADR was the same in both groups (52%, $P = 0.92$), whereas the higher number of adenomas per patient in the Endocuff group (1.36 ± 2.10 *vs* 1.17 ± 1.65) did not reach statistical significance ($P = 0.08$). Interestingly, detection rates did not differ either according to indication or between academic and non-academic centres[43]. Cecal intubation time was significantly shorter in the Endocuff arm [median (IQR) 7 min (5-10) *vs* 8.3 min (6-12), $P < 0.001$] and there were no Endocuff-associated adverse events[43].

Finally, a multicentre back-to-back study[44] assessed Endocuff in terms of adenoma miss rates. Two hundred patients (86.5% were screening and surveillance cases) were randomized (1:1) to undergo either initial EAC followed by a conventional one or vice versa[44]. EAC was associated with lower adenoma miss rates, both overall and in the proximal colon compared to conventional colonoscopy (14.7% *vs* 38.4% and 10.4% *vs* 38.9%, respectively)[44]. It is worthy to note that all examinations were performed by endoscopists with an historical ADR > 35%, suggesting that the device could enhance detection ability even of experienced and skilled endoscopists. Despite the fact that there were no serious adverse events, in three index Endocuff examinations cecal intubation failed to be achieved, compared to none with the conventional scope ($P = 0.08$)[44].

The Endocuff Vision

Despite its revolutionary design, Endocuff was associated with a couple of drawbacks (mucosal erosions and difficulties in terminal ileum intubation) that paved to way for its descendant, namely Endocuff Vision (Norgine Pharmaceuticals Ltd, Uxbridge, United Kingdom). This single-use device is made of a polypropylene cylinder and a single row of 8-longer than in the first generation Endocuff-thermoplastic elastomer-made "spikes" (Figure 3). There are 4 different sizes with respective colors to fit in all scopes ranging from pediatric to adult ones (Table 1). Endocuff Vision is also mounted onto the tip of the scope before insertion and its "spikes" fold around the scope while it advances in the colon due to a hinge at the base of each spike that thins progressively. On the other hand, the "spikes" evert during withdrawal (Figure 3). This leads to an early and controlled view of the upstream surface of the large colonic folds in the right colon and prevents sudden scope slip-back. Moreover, when in the sigmoid colon, the device facilitates the opening of contracted folds, permitting a clearer view of the in-between mucosa. Similar to the first generation Endocuff it optimizes the tip's position during endoscopically applied therapy (*e.g.*, polypectomy).

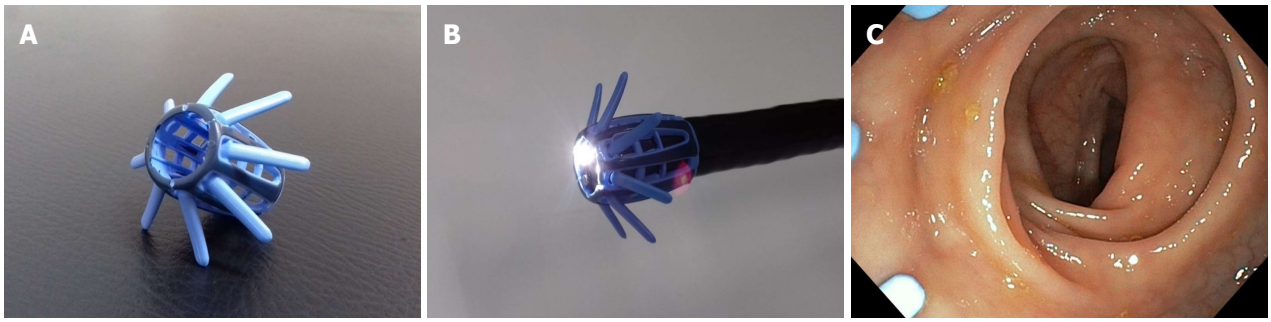


Figure 3 Endocuff-Vision (A), illustration (B) and endoscopic view (C) of the opened-out projections during the withdrawal phase (photos from the authors' archive).

Endocuff Vision has been evaluated only in two parallel multicenter RCTs from the United Kingdom[46,47]. The "ADENOMA" study[46] recruited 1772 adult patients (45% screening). Of them, 884 underwent conventional colonoscopy and 886 Endocuff Vision-assisted colonoscopy. ADR was significantly higher with EAC compared to conventional colonoscopy (40.9% *vs* 36.2%, $P = 0.02$). The benefit of Endocuff Vision was even higher in patients participating in the screening program, where ADR was 61.5% for EAC compared to 50.9% ($P < 0.001$) for the conventional colonoscopy arm. Similar results in favor of EAC were also reported regarding PDR (54.1% *vs* 48%; $P = 0.005$ and 73.9% *vs* 63.3%; $P < 0.001$ for the whole and the screening cohorts, respectively). Of note, EAC showed a statistically significant increase in the detection rate in the left colon (26.1% *vs* 2.2%; $P = 0.03$), of small (10.6% *vs* 7.7%; $P = 0.02$) and of diminutive adenomas (34.6% *vs* 30.8%; $P = 0.04$). It should be underlined that in this study[46] EAC detected significantly more cancers both in the whole cohort (4.1% *vs* 2.3%; $P = 0.02$) as well as in the screening participants (6.6% *vs* 3.7%; $P = 0.03$). Moreover, median insertion time was shorter with Endocuff Vision compared to conventional colonoscopy (8 min *vs* 9 min; $P = 0.001$). The investigators did not report any adverse event related to use of Endocuff Vision; however the device had to be removed in 4.1% of the cases mostly due to acute angulation in a fixed sigmoid colon.

On the contrary, the "E-cap" study failed to show any benefit in terms of ADR (60.9% *vs* 63%, $P = 0.85$), PDR (70.3% *vs* 69.8%, $P = 0.93$) and MAC (1.3 ± 1.8 *vs* 1.4 ± 1.5, $P = 0.54$)[47]. This single center study had PDR as the primary endpoint. Only patients attending the national screening program with a positive FOBT test were enrolled and all four participating endoscopists had an extremely high pre-study ADR (58.9%)[47]. All these reasons may attribute to the lack of any significant benefit deriving from application of Endocuff Vision and should be considered in the design of future "real-life" studies, which should possibly include both endoscopists with an average or even a low ADR and patients with various indications for colonoscopy.

Finally, a pilot evaluation study[48] demonstrated that Endocuff Vision was associated with an improvement in endoscopists' performance measured as increased ADR, increased MAC and decreased insertion time. In this non-randomized study[48], the investigators performed 410 screening colonoscopies in three periods (137 pre-Endocuff, 136 using Endocuff Vision and 137 post-Endocuff). Overall, an increase in ADR (16%, $P < 0.03$) and MAC (83%, $P = 0.007$) was noted between the pre-Endocuff and the Endocuff period; this benefit was maintained in the post-Endocuff period, where the device was not available. A potential explanation could be that during the Endocuff period the endoscopists had the chance to comprehend their flaws during the withdrawal phase, look for adenomas in more detail and improve their skills[49]. Interestingly, insertion time was statistically lower during the Endocuff period compared to pre- and post-Endocuff one (7 min *vs* 8 min, $P = 0.002$ and 7 min *vs* 9 min, $P = 0.002$, respectively)[48]; no adverse events were reported[48].

Data from meta-analyses

To date, three meta-analyses attempting to summarize the impact of Endocuff devices on colonoscopy outcomes have already been published[35,50,51] (Table 2).

The earliest one[50] meta-analyzed data from three published papers and six studies presented as abstracts, four of which with a prospective and five with a retrospective design. Eight studies ($n = 4387$) of mixed populations reported on ADR, which was measured to be higher for the Endocuff group [OR (95%CI): 1.49 (1.23-1.80), $I^2 = 50\%$][50]. In this pooled analysis, 27 patients (2.3%) in the Endocuff group experienced superficial mucosal lacerations[50].

A recently published meta-analysis updated these data by including only RCTs (7 published and 5 presented as abstracts)[51]. Regarding ADR, data from more than 8370 patients demonstrated a benefit of EAC compared to conventional colonoscopy [RR (95%CI): 1.20 (1.06-1.36), $P = 0.003$, $I^2 = 79\%$]. Of interest, this benefit was lower in the subgroup of studies with a mean conventional arm ADR > 45% [RR (95%CI): 1.01 (0.93-1.09), $P = 0.087$, $I^2 = 0$], while it was maximized in the subgroup of studies with a respective ADR lower than 35% [RR (95%CI): 1.51 (1.35-1.69), $P < 0.001$, $I^2 = 43\%$]. These data imply a potential ancillary role of

Figure 4 Endorings (A) mounted on the tip of the scope (B) and illustration of rings stretching during withdrawal phase (C) (photos courtesy of Endoaid).

the device especially for lower detectors[51]. Furthermore, a numerical higher MAC was detected in the Endocuff-assisted colonoscopy group, but this difference did not reach statistical significance [mean difference (95%CI): 0.11(-0.17 to 0.38][51]. Mean insertion times did not differ between the two groups and 4% of the Endocuff patients experienced adverse events (exclusively minor lacerations)[51]. This meta-analysis reported on additional outcomes such as advanced ADR and right colon ADR, with no difference detected between the two groups [RR (95%CI): 0.93 (0.76-1.13), P = 0.47 and RR (95%CI): 1.36 (0.80-2.34), P = 0.26, respectively]. However, the small number of studies included in the analysis regarding these outcomes warrants caution when attempting to generalize the respective results.

Finally, similar results were shown in a network meta-analysis investigating the comparative efficacy of distal attachments in increasing detection rates during colonoscopy[35]. The mixed effect estimate (including both pairwise and indirect treatment effects) supported that ADR increased significantly with EAC compared to the conventional examination [RR (95%CI): 1.21 (1.03-1.41)][35]. Interestingly and contrary to the meta-analysis from Williet *et al*[51] this network meta-analysis[35] calculated a very modest benefit of Endocuff regarding low (baseline ADR 10%) detectors [anticipated ADR (95%CI): 11 (10-12)%] compared to a more considerable effect [anticipated ADR (95%CI): 48 (14-56)%] on ADR of high detectors (baseline ADR 40%).

THE ENDORINGS

EndoRings (EndoAid Ltd., Caesarea, Israel) is a single-use scope attachment consisting of 2 layers of flexible, soft circular silicon rings placed on a cylindrical cuff (Figure 4A and B). Endorings fit on scopes of an outer diameter ranging from 12.8 mm to 14.5 mm and two sizes for adult and slim adult scopes are available (Table 1). The flexible rings deflect to the opposite direction during scope manipulation. In that way scope insertion is not affected -minimal resistance may be noted- as the rings fold at the side of the scope's shaft without projecting beyond the distal end of the scope. During withdrawal, the two rings deploy with the proximal-most circular ring creating a wider lumen fenestration by stretching the mucosa and colonic folds (Figure 4C) assisting the detection of otherwise "hidden" lesions. Moreover, the device maintains identical depth and width of scope viewing by stabilizing the scope, maintaining position during loop reduction, deceasing slippage and finally by anchoring during application of endoscopic therapy. Terminal ileum intubation is reported not to be limited by use of the device. Endorings has been evaluated in two RCTs[42,52]. In the first one, a multicentre back-to-back study[52], 116 patients of mixed indications were randomized to undergo initial examination using the Endorings followed by conventional colonoscopy or vice versa. Applying Endorings on the tip of the scope was associated with a statistically significant lower adenoma miss rate compared to conventional colonoscopy (10.4% *vs* 48.3%, P < 0.001). A similar benefit was also noted for polyp miss rates (9.1% *vs* 52.8%, P < 0.001). Endorings significantly decreased adenoma miss rates both in the proximal (10.6% *vs* 58.1%, P < 0.001) and the distal colon (10% *vs* 37%, P < 0.001). Cecal intubation time was shorter with conventional colonoscopy compared to EndoRings-assisted colonoscopy (8.4 min *vs* 9.3 min), but this difference did not achieve statistical significance.

In the aforementioned 4-arm parallel group multicenter study[42], 295 and 295 patients were allocated in the in Endorings and the conventional colonoscopy arms respectively. ADR did not differ between the two groups (57% *vs* 56%). Moreover, in one of the participating centers, insertion time was significantly increased when Endorings was used (251 s *vs* 170 s, P = 0.003), but this finding was not uniform for all participating centers (overall time to cecum: 263 s *vs* 319 s, no statistical difference). Of note, the device was not able to pass the sigmoid colon in 6 patients, thus authors commented that the larger the diameter of Endorings, the greater the difficulty of scope insertion.

CONCLUSION

Detecting and removing precancerous lesions remains the mainstay of screening colonoscopy. In this review, data on how 4 different colonoscope attachments influence detection rates were presented. The transparent

cap was the first device launched in the market. CAC achieves a better orientation during insertion by pushing away the folded mucosa and reduces the time needed to reach the cecum. During scope withdrawal, the cap opens and manipulates colonic folds, revealing otherwise obscured lesions[23]. CAC has been validated extensively and pooled data from numerous studies have linked it with a significant increase in polyp detection[34]. Taking into account its simplicity in use, it can be an interesting and accessible solution for colonoscopy outcome improvement.

Endocuff and Endocuff Vision - two similar detachable attachments - have been commercialized during the last years. They are fitted at the tip of the scope and enhance mucosa visualization through their opened-out projections that flatten the colonic folds. Data from studies conclude that their application increases ADR[35,50,51]. Furthermore, they can prove to be beneficial for both low adenoma detectors[51] (increase in ADR) as well as in high ones[44] (decrease in miss rates). Moreover, Endocuff and Endocuff Vision are safe, user-friendly and affordable devices. Taking this into account, Endocuff-assisted colonoscopy should be considered as a pivotal part of the physicians' arsenal in their effort to diminish missed precancerous lesions during colonoscopy.

Endorings is the last device that was presented. It functions in a -more or less- similar way to EAC. Its double-layered rings stretch the mucosa and optimize visualization. While significantly decreased adenoma miss rates have been reported[52], this device has been validated in only two studies[42,52] and more data are definitely needed to reach safe and generalizable conclusions regarding its effectiveness.

An issue that remains to be highlighted is the efficacy of these devices in terms of screening colonoscopy. To the best of our knowledge, no study has been conducted in an exclusively screening population yet. Thus, conclusions are extrapolated only from studies enrolling mixed populations (screening, symptomatic, surveillance). Although promising, more robust data are definitely needed in order to systematically assess the performance of these mechanical novelties overall and particularly in specific populations. Moreover, it is still unknown whether all levels of endoscopists are to benefit from their implementation. Finally, optimizing several useful and simultaneously low-cost existing alternatives (*e.g.*, water-aided colonoscopy, second observer, dynamic position change) may be as effective as these add-on devices in improving colonoscopy and patient outcomes. All the aforementioned reasons probably prevent these devices from being integrated into current everyday clinical practice worldwide.

In conclusion, technological advancements offer physicians various choices to improve their performance during colonoscopy. Undoubtedly, they can be considered an adjuvant approach to improve colonoscopy outcomes as they are feasible, easy-to-use, affordable and effective. At the same time, these devices should not be considered a panacea and physicians should always pay attention to other well-established quality measures such as high level education, adequate bowel preparation, and adequate withdrawal time to ensure that they offer their patients optimal clinical services.

REFERENCES

1 SEER Cancer Stat Facts: Colorectal Cancer. 2018
2 **Zauber AG**, Winawer SJ, O'Brien MJ, Lansdorp-Vogelaar I, van Ballegooijen M, Hankey BF, Shi W, Bond JH, Schapiro M, Panish JF, Stewart ET, Waye JD. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. *N Engl J Med* 2012; **366**: 687-696 [PMID: 22356322 DOI: 10.1056/NEJMoa1100370]
3 **Corley DA**, Jensen CD, Marks AR, Zhao WK, Lee JK, Doubeni CA, Zauber AG, de Boer J, Fireman BH, Schottinger JE, Quinn VP, Ghai NR, Levin TR, Quesenberry CP. Adenoma detection rate and risk of colorectal cancer and death. *N Engl J Med* 2014; **370**: 1298-1306 [PMID: 24693890 DOI: 10.1056/NEJMoa1309086]
4 **Kaminski MF**, Regula J, Kraszewska E, Polkowski M, Wojciechowska U, Didkowska J, Zwierko M, Rupinski M, Nowacki MP, Butruk E. Quality indicators for colonoscopy and the risk of interval cancer. *N Engl J Med* 2010; **362**: 1795-1803 [PMID: 20463339 DOI: 10.1056/NEJMoa0907667]
5 **Kaminski MF**, Wieszczy P, Rupinski M, Wojciechowska U, Didkowska J, Kraszewska E, Kobiela J, Franczyk R, Rupinska M, Kocot B, Chaber-Ciopinska A, Pachlewski J, Polkowski M, Regula J. Increased Rate of Adenoma Detection Associates With Reduced Risk of Colorectal Cancer and Death. *Gastroenterology* 2017; **153**: 98-105 [PMID: 28428142 DOI: 10.1053/j.gastro.2017.04.006]
6 **Kaminski MF**, Thomas-Gibson S, Bugajski M, Bretthauer M, Rees CJ, Dekker E, Hoff G, Jover R, Suchanek S, Ferlitsch M, Anderson J, Roesch T, Hultcranz R, Racz I, Kuipers EJ, Garborg K, East JE, Rupinski M, Seip B, Bennett C, Senore C, Minozzi S, Bisschops R, Domagk D, Valori R, Spada C, Hassan C, Dinis-Ribeiro M, Rutter MD. Performance measures for lower gastrointestinal endoscopy: a European Society of Gastrointestinal Endoscopy (ESGE) Quality Improvement Initiative. *Endoscopy* 2017; **49**: 378-397 [PMID: 28268235 DOI: 10.1055/s-0043-103411]
7 **Heresbach D**, Barrioz T, Lapalus MG, Coumaros D, Bauret P, Potier P, Sautereau D, Boustière C, Grimaud JC, Barthélémy C, Sée J, Serraj I, D'Halluin PN, Branger B, Ponchon T. Miss rate for colorectal neoplastic polyps: a prospective multicenter study of back-to-back video colonoscopies. *Endoscopy* 2008; **40**: 284-290 [PMID: 18389446 DOI: 10.1055/s-2007-995618]
8 **Leufkens AM**, van Oijen MG, Vleggaar FP, Siersema PD. Factors influencing the miss rate of polyps in a back-to-back colonoscopy study. *Endoscopy* 2012; **44**: 470-475 [PMID: 22441756 DOI: 10.1055/s-0031-1291666]
9 **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 DOI: 10.1016/S0016-5085(97)70214-2]
10 **Lee J**, Park SW, Kim YS, Lee KJ, Sung H, Song PH, Yoon WJ, Moon JS. Risk factors of missed colorectal lesions after colonoscopy. *Medicine* (Baltimore) 2017; **96**: e7468 [PMID: 28682916 DOI: 10.1097/MD.0000000000007468]
11 **Robertson DJ**, Lieberman DA, Winawer SJ, Ahnen DJ, Baron JA, Schatzkin A, Cross AJ, Zauber AG, Church TR, Lance P, Greenberg ER, Martínez ME. Colorectal cancers soon after colonoscopy: a pooled multicohort analysis. *Gut* 2014; **63**: 949-956 [PMID: 23793224 DOI: 10.1136/gutjnl-2012-303796]
12 **Xiang L**, Zhan Q, Zhao XH, Wang YD, An SL, Xu YZ, Li AM, Gong W, Bai Y, Zhi FC, Liu SD. Risk factors associated with missed colorectal flat adenoma: a multicenter retrospective tandem colonoscopy study. *World J Gastroenterol* 2014; **20**: 10927-10937 [PMID: 25152596 DOI: 10.3748/wjg.v20.i31.10927]
13 **Gkolfakis P**, Tziatzios G, Dimitriadis GD, Triantafyllou K. New endoscopes and add-on devices to improve colonoscopy performance. *World J Gastroenterol* 2017; **23**: 3784-3796 [PMID: 28638218 DOI:

10.3748/wjg.v23.i21.3784]
14 **Tada M**, Inoue H, Yabata E, Okabe S, Endo M. Colonic mucosal resection using a transparent cap-fitted endoscope. *Gastrointest Endosc* 1996; **44**: 63-65 [PMID: 8836719 DOI: 10.1016/S0016-5107(96)70231-6]
15 **Kondo S**, Yamaji Y, Watabe H, Yamada A, Sugimoto T, Ohta M, Ogura K, Okamoto M, Yoshida H, Kawabe T, Omata M. A randomized controlled trial evaluating the usefulness of a transparent hood attached to the tip of the colonoscope. *Am J Gastroenterol* 2007; **102**: 75-81 [PMID: 17100978 DOI: 10.1111/j.1572-0241.2006.00897.x]
16 **Horiuchi A**, Nakayama Y. Improved colorectal adenoma detection with a transparent retractable extension device. *Am J Gastroenterol* 2008; **103**: 341-345 [PMID: 18076740 DOI: 10.1111/j.1572-0241.2007.01555.x]
17 **Harada Y**, Hirasawa D, Fujita N, Noda Y, Kobayashi G, Ishida K, Yonechi M, Ito K, Suzuki T, Sugawara T, Horaguchi J, Takasawa O, Obana T, Oohira T, Onochi K, Kanno Y, Kuroha M, Iwai W. Impact of a transparent hood on the performance of total colonoscopy: a randomized controlled trial. *Gastrointest Endosc* 2009; **69**: 637-644 [PMID: 19251004 DOI: 10.1016/j.gie.2008.08.029]
18 **Lee YT**, Lai LH, Hui AJ, Wong VW, Ching JY, Wong GL, Wu JC, Chan HL, Leung WK, Lau JY, Sung JJ, Chan FK. Efficacy of cap-assisted colonoscopy in comparison with regular colonoscopy: a randomized controlled trial. *Am J Gastroenterol* 2009; **104**: 41-46 [PMID: 19098847 DOI: 10.1038/ajg.2008.56]
19 **Hewett DG**, Rex DK. Cap-fitted colonoscopy: a randomized, tandem colonoscopy study of adenoma miss rates. *Gastrointest Endosc* 2010; **72**: 775-781 [PMID: 20579648 DOI: 10.1016/j.gie.2010.04.030]
20 **Tee HP**, Corte C, Al-Ghamdi H, Prakoso E, Darke J, Chettiar R, Rahman W, Davison S, Griffin SP, Selby WS, Kaffes AJ. Prospective randomized controlled trial evaluating cap-assisted colonoscopy vs standard colonoscopy. *World J Gastroenterol* 2010; **16**: 3905-3910 [PMID: 20712051 DOI: 10.3748/wjg.v16.i31.3905]
21 **Takeuchi Y**, Inoue T, Hanaoka N, Higashino K, Iishi H, Chatani R, Hanafusa M, Kizu T, Ishihara R, Tatsuta M, Shimokawa T, Uedo N. Autofluorescence imaging with a transparent hood for detection of colorectal neoplasms: a prospective, randomized trial. *Gastrointest Endosc* 2010; **72**: 1006-1013 [PMID: 21034901 DOI: 10.1016/j.gie.2010.06.055]
22 **de Wijkerslooth TR**, Stoop EM, Bossuyt PM, Mathus-Vliegen EM, Dees J, Tytgat KM, van Leerdam ME, Fockens P, Kuipers EJ, Dekker E. Adenoma detection with cap-assisted colonoscopy versus regular colonoscopy: a randomised controlled trial. *Gut* 2012; **61**: 1426-1434 [PMID: 22187070 DOI: 10.1136/gutjnl-2011-301327]
23 **Rastogi A**, Bansal A, Rao DS, Gupta N, Wani SB, Shipe T, Gaddam S, Singh V, Sharma P. Higher adenoma detection rates with cap-assisted colonoscopy: a randomised controlled trial. *Gut* 2012; **61**: 402-408 [PMID: 21997547 DOI: 10.1136/gutjnl-2011-300187]
24 **Pohl H**, Bensen SP, Toor A, Gordon SR, Levy LC, Berk B, Anderson PB, Anderson JC, Rothstein RI, MacKenzie TA, Robertson DJ. Cap-assisted colonoscopy and detection of Adenomatous Polyps (CAP) study: a randomized trial. *Endoscopy* 2015; **47**: 891-897 [PMID: 26126162 DOI: 10.1055/s-0034-1392261]
25 **Huang RX**, Xiao ZL, Li F, Ji DN, Zhou J, Xiang P, Bao ZJ. Black hood assisted colonoscopy for detection of colorectal polyps: a prospective randomized controlled study. *Eur Rev Med Pharmacol Sci* 2016; **20**: 3266-3272 [PMID: 27467003]
26 **Othman MO**, Zhang D, Elhanafi S, Eloliby M, Davis B, Guererro R, Alvarado L, Sanchez L, Dwivedi A, Zuckerman MJ. Cap-Assisted Colonoscopy Increases Detection of Advanced Adenomas and Polyps. *Am J Med Sci* 2017; **353**: 367-373 [PMID: 28317624 DOI: 10.1016/j.amjms.2017.01.015]
27 **Tang Z**, Zhang DS, Thrift AP, Patel KK. Impact of cap-assisted colonoscopy on the learning curve and quality in colonoscopy: a randomized controlled trial. *Gastrointest Endosc* 2018; **87**: 723-732.e3 [PMID: 28648577 DOI: 10.1016/j.gie.2017.06.011]
28 **Park SM**, Lee SH, Shin KY, Heo J, Sung SH, Park SH, Choi SY, Lee DW, Park HG, Lee HS, Jeon SW, Kim SK, Jung MK. The cap-assisted technique enhances colonoscopy training: prospective randomized study of six trainees. *Surg Endosc* 2012; **26**: 2939-2943 [PMID: 22538693 DOI: 10.1007/s00464-012-2288-2]
29 **Westwood DA**, Alexakis N, Connor SJ. Transparent cap-assisted colonoscopy versus standard adult colonoscopy: a systematic review and meta-analysis. *Dis Colon Rectum* 2012; **55**: 218-225 [PMID: 22228167 DOI: 10.1097/DCR.0b013e31823461ef]
30 **Ng SC**, Tsoi KK, Hirai HW, Lee YT, Wu JC, Sung JJ, Chan FK, Lau JY. The efficacy of cap-assisted colonoscopy in polyp detection and cecal intubation: a meta-analysis of randomized controlled trials. *Am J Gastroenterol* 2012; **107**: 1165-1173 [PMID: 22664471 DOI: 10.1038/ajg.2012.135]
31 **He Q**, Li JD, An SL, Liu SD, Xiao B, Zhang YL, Jiang B, Bai Y, Zhi FC. Cap-assisted colonoscopy versus conventional colonoscopy: systematic review and meta-analysis. *Int J Colorectal Dis* 2013; **28**: 279-281 [PMID: 22460306 DOI: 10.1007/s00384-012-1466-y]
32 **Omata F**, Ohde S, Deshpande GA, Kobayashi D, Masuda K, Fukui T. Image-enhanced, chromo, and cap-assisted colonoscopy for improving adenoma/neoplasia detection rate: a systematic review and meta-analysis. *Scand J Gastroenterol* 2014; **49**: 222-237 [PMID: 24328858 DOI: 10.3109/00365521.2013.863964]
33 **Desai M**, Sanchez-Yague A, Choudhary A, Pervez A, Gupta N, Vennalaganti P, Vennelaganti S, Fugazza A, Repici A, Hassan C, Sharma P. Impact of cap-assisted colonoscopy on detection of proximal colon adenomas: systematic review and meta-analysis. *Gastrointest Endosc* 2017; **86**: 274-281.e3 [PMID: 28365356 DOI: 10.1016/j.gie.2017.03.1524]
34 **Mir FA**, Boumitri C, Ashraf I, Matteson-Kome ML, Nguyen DL, Puli SR, Bechtold ML. Cap-assisted colonoscopy versus standard colonoscopy: is the cap beneficial? A meta-analysis of randomized controlled trials. *Ann Gastroenterol* 2017; **30**: 640-648 [PMID: 29118558 DOI: 10.20524/aog.2017.0180]
35 **Facciorusso A**, Del Prete V, Buccino RV, Della Valle N, Nacchiero MC, Monica F, Cannizzaro R, Muscatiello N. Comparative Efficacy of Colonoscope Distal Attachment Devices in Increasing Rates of Adenoma Detection: A Network Meta-analysis. *Clin Gastroenterol Hepatol* 2018; **16**: 1209-1219.e9 [PMID: 29133257 DOI: 10.1016/j.cgh.2017.11.007]
36 **Tsiamoulos ZP**, Saunders BP. A new accessory, endoscopic cuff, improves colonoscopic access for complex polyp resection and scar assessment in the sigmoid colon (with video). *Gastrointest Endosc* 2012; **76**: 1242-1245 [PMID: 23164515 DOI: 10.1016/j.gie.2012.08.019]
37 **Floer M**, Biecker E, Fitzlaff R, Röming H, Ameis D, Heinecke A, Kunsch S, Ellenrieder V, Ströbel P, Schepke M, Meister T. Higher adenoma detection rates with endocuff-assisted colonoscopy-a randomized controlled multicenter trial. *PLoS One* 2014; **9**: e114267 [PMID: 25470133 DOI: 10.1371/journal.pone.0114267]
38 **Biecker E**, Floer M, Heinecke A, Ströbel P, Böhme R, Schepke M, Meister T. Novel endocuff-assisted colonoscopy significantly increases the polyp detection rate: a randomized controlled trial. *J Clin Gastroenterol* 2015; **49**: 413-418 [PMID: 24921209 DOI: 10.1097/MCG.0000000000000166]
39 **Wada Y**, Fukuda M, Ohtsuka K, Watanabe M, Fukuma Y, Wada Y, Wada M. Efficacy of Endocuff-assisted colonoscopy in the detection of colorectal polyps. *Endosc Int Open* 2018; **6**: E425-E431 [PMID: 29607395 DOI: 10.1055/s-0044-101142]
40 **González-Fernández C**, García-Rangel D, Aguilar-Olivos NE, Barreto-Zúñiga R, Romano-Munive AF, Grajales-Figueroa G, Zamora-Nava LE, Téllez-Avila FI. Higher adenoma detection rate with the endocuff: a randomized trial. *Endoscopy* 2017; **49**: 1061-1068 [PMID: 28898920 DOI: 10.1055/s-0043-117879]
41 **De Palma GD**, Giglio MC, Bruzzese D, Gennarelli N, Maione F, Siciliano S, Manzo B, Cassese G, Luglio G. Cap cuff-assisted colonoscopy versus standard colonoscopy for adenoma detection: a randomized back-to-back study. *Gastrointest Endosc* 2018; **87**: 232-240 [PMID: 28082115 DOI: 10.1016/j.gie.2016.12.027]
42 **Rex DK**, Repici A, Gross SA, Hassan C, Ponugoti PL, Garcia JR, Broadley HM, Thygesen JC, Sullivan AW, Tippins WW, Main SA, Eckert GJ, Vemulapalli KC. High-definition colonoscopy versus Endocuff versus EndoRings versus full-spectrum endoscopy for adenoma detection at colonoscopy: a multicenter randomized trial.

Gastrointest Endosc 2018; **88**: 335-344.e2 [PMID: 29530353 DOI: 10.1016/j.gie.2018.02.043]

43 **van Doorn SC**, van der Vlugt M, Depla A, Wientjes CA, Mallant-Hent RC, Siersema PD, Tytgat K, Tuynman H, Kuiken SD, Houben G, Stokkers P, Moons L, Bossuyt P, Fockens P, Mundt MW, Dekker E. Adenoma detection with Endocuff colonoscopy versus conventional colonoscopy: a multicentre randomised controlled trial. *Gut* 2017; **66**: 438-445 [PMID: 26674360 DOI: 10.1136/gutjnl-2015-310097]

44 **Triantafyllou K**, Polymeros D, Apostolopoulos P, Lopes Brandao C, Gkolfakis P, Repici A, Papanikolaou IS, Dinis-Ribeiro M, Alexandrakis G, Hassan C. Endocuff-assisted colonoscopy is associated with a lower adenoma miss rate: a multicenter randomized tandem study. *Endoscopy* 2017; **49**: 1051-1060 [PMID: 28763808 DOI: 10.1055/s-0043-114412]

45 **Baek MD**, Jackson CS, Lunn J, Nguyen C, Shah NK, Serrao S, Juma D, Strong RM. Endocuff assisted colonoscopy significantly increases sessile serrated adenoma detection in veterans. *J Gastrointest Oncol* 2017; **8**: 636-642 [PMID: 28890813 DOI: 10.21037/jgo.2017.03.07]

46 **Ngu WS**, Bevan R, Tsiamoulos ZP, Bassett P, Hoare Z, Rutter MD, Clifford G, Totton N, Lee TJ, Ramadas A, Silcock JG, Painter J, Neilson LJ, Saunders BP, Rees CJ. Improved adenoma detection with Endocuff Vision: the ADENOMA randomised controlled trial. *Gut* 2018; Epub ahead of print [PMID: 29363535]

47 **Bhattacharyya R**, Chedgy F, Kandiah K, Fogg C, Higgins B, Haysom-Newport B, Gadeke L, Thursby-Pelham F, Ellis R, Goggin P, Longcroft-Wheaton G, Bhandari P. Endocuff-assisted vs. standard colonoscopy in the fecal occult blood test-based UK Bowel Cancer Screening Programme (E-cap study): a randomized trial. *Endoscopy* 2017; **49**: 1043-1050 [PMID: 28614895 DOI: 10.1055/s-0043-111718]

48 **Tsiamoulos ZP**, Misra R, Rameshshanker R, Elliott TR, Beintaris I, Thomas-Gibson S, Haycock A, Suzuki N, Rees C, Saunders BP. Impact of a new distal attachment on colonoscopy performance in an academic screening center. *Gastrointest Endosc* 2018; **87**: 280-287 [PMID: 28412271 DOI: 10.1016/j.gie.2017.04.001]

49 **Bugajski M**, Kaminski MF. Devices for adenoma detection rate: Holy Grail or training tool? *Gastrointest Endosc* 2018; **87**: 241-242 [PMID: 29241852 DOI: 10.1016/j.gie.2017.07.041]

50 **Chin M**, Karnes W, Jamal MM, Lee JG, Lee R, Samarasena J, Bechtold ML, Nguyen DL. Use of the Endocuff during routine colonoscopy examination improves adenoma detection: A meta-analysis. *World J Gastroenterol* 2016; **22**: 9642-9649 [PMID: 27920485 DOI: 10.3748/wjg.v22.i43.9642]

51 **Williet N**, Tournier Q, Vernet C, Dumas O, Rinaldi L, Roblin X, Phelip JM, Pioche M. Effect of Endocuff-assisted colonoscopy on adenoma detection rate: meta-analysis of randomized controlled trials. *Endoscopy* 2018; **50**: 846-860 [PMID: 29698990 DOI: 10.1055/a-0577-3500]

52 **Dik VK**, Gralnek IM, Segol O, Suissa A, Belderbos TD, Moons LM, Segev M, Domanov S, Rex DK, Siersema PD. Multicenter, randomized, tandem evaluation of EndoRings colonoscopy--results of the CLEVER study. *Endoscopy* 2015; **47**: 1151-1158 [PMID: 26220283 DOI: 10.1055/s-0034-1392421]

P- Reviewer: Arya V, Qayed E, Rerknimitr R **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4254-4262

DOI: 10.3748/wjg.v24.i37.4254

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

ORIGINAL ARTICLE

Basic Study

VSL#3 can prevent ulcerative colitis-associated carcinogenesis in mice

Chun-Sai-Er Wang, Wen-Bin Li, Hong-Ying Wang, Yi-Ming Ma, Xin-Hua Zhao, Hong Yang, Jia-Ming Qian, Jing-Nan Li

Chun-Sai-Er Wang, Wen-Bin Li, Hong Yang, Jia-Ming Qian, Jing-Nan Li, Department of Gastroenterology, PUMC Hospital, CAMS and PUMC, Beijing 100730, China

Hong-Ying Wang, Yi-Ming Ma, Xin-Hua Zhao, National Cancer Center/Cancer Hospital, CAMS and PUMC, Beijing 100021, China

ORCID number: Chun-Sai-Er Wang (0000-0002-4410-0834); Wen-Bin Li (0000-0003-0003-9768); Hong-Ying Wang (0000-0001-8443-3839); Yi-Ming Ma (0000-0002-4654-5642); Xin-Hua Zhao (0000-0002-4523-9416); Hong Yang (0000-0002-9475-1256); Jia-Ming Qian (0000-0001-6570-9262); Jing-Nan Li (0000-0002-9578-2580).

Author contributions: Wang CS and Li WB are the co-first author; Li JN conceived and designed the experiments; Wang CS and Li WB performed the experiments, and analyzed and interpreted the data; Wang CS and Li JN drafted the paper and revised it critically for important intellectual content; Wang HY, Ma YM, Zhao XH, Yang H, and Qian JM offered help during the experiments; all authors approved the final version of the manuscript.

Supported by the National Natural Science Foundation of China, No. 81370500 and No. 81770559.

Institutional animal care and use committee statement: All animal experiments were conducted in accordance with the recommendations of the Animal Care Ethics and Use Committee of Peking Union Medical College Hospital and approved by the same Committee (XHDW-2015-0032).

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Jing-Nan Li, MD, Doctor, Department of Gastroenterology, PUMC Hospital, CAMS and PUMC, No.1 Shuai Fu Yuan, Beijing 100730, China. lijn2008@126.com
Telephone: +86-10-69155019
Fax: +86-10-69155017

Received: May 27, 2018
Peer-review started: May 27, 2018
First decision: July 6, 2018
Revised: August 7, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

AIM

To investigate the effects of VSL#3 on tumor formation, and fecal and intestinal mucosal microbiota in azoxymethane/dextran sulfate sodium (AOM/DSS) induced mice model.

METHODS

C57BL/6 mice were administered AOM/DSS to develop the ulcerative colitis (UC) carcinogenesis model. Mice were treated with 5-ASA (75 mg/kg/d), VSL#3 (1.5×10^9 CFU/d), or 5-ASA combined with VSL#3 by gavage from the day of AOM injection for three months (five days/week). The tumor load was compared in

each group, and tumor necrosis factor (TNF-α) and interleukin (IL)-6 levels were evaluated in colon tissue. The stool and intestinal mucosa samples were collected to analyze the differences in the intestinal microbiota by 16s rDNA sequencing method.

RESULTS

VSL#3 significantly reduced the tumor load in AOM/DSS-induced mice model and decreased the level of TNF-α and IL-6 in colon tissue. The model group had a lower level of *Lactobacillus* and higher level of *Oscillibacter* and *Lachnoclostridium* in fecal microbiota than the control group. After the intervention with 5-ASA and VSL#3, *Bacillus* and *Lactococcus* were increased, while *Lachnoclostridium* and *Oscillibacter* were reduced. 5-ASA combined with VSL#3 increased the *Lactobacillus* and decreased the *Oscillibacter*. The intestinal mucosal microbiota analysis showed a lower level of *Bifidobacterium* and *Ruminococcaceae*_UCG-014 and higher level of *Alloprevotella* in the model group as compared to the control group. After supplementation with VSL#3, *Bifidobacterium* was increased. 5-ASA combined with VSL#3 increased the level of both *Lachnoclostridium* and *Bifidobacterium*.

CONCLUSION

VSL#3 can prevent UC-associated carcinogenesis in mice, reduce the colonic mucosal inflammation levels, and rebalance the fecal and mucosal intestinal microbiota.

Key words: Tumor necrosis factor-α; VSL#3; Ulcerative colitis carcinogenesis; Interleukin-6; Intestinal microbiota

Core tip: Microbiota and chronic inflammation play an important role in the process of ulcerative colitis (UC)-associated carcinogenesis. Our study found VSL#3 could effectively prevent UC-associated carcinogenesis in azoxymethane/dextran sulfate sodium induced mice and decrease the level of tumor necrosis factor-α and IL-6 in colon tissue. The intestinal microbiota dysbiosis exists in UC-associated carcinogenesis. Supplementary VSL#3 is beneficial for rebalancing the fecal and mucosal intestinal microbiota. Based on the data presented here, VSL#3 may be a potential therapeutic agent for UC-associated carcinogenesis prevention.

Wang CS, Li WB, Wang HY, Ma YM, Zhao XH, Yang H, Qian JM, Li JN. VSL#3 can prevent ulcerative colitis-associated carcinogenesis in mice. *World J Gastroenterol* 2018; 24(37): 4254-4262 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4254.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4254

INTRODUCTION

Recently, the incidence of ulcerative colitis (UC) has shown an upward trend, leading to increased clinical attention on UC-associated carcinogenesis. A recent meta-analysis encompassing eight population-based cohort studies reported a 1.6% prevalence of colorectal cancer (CRC) in patients with UC, and the rate of CRC was 2.4-fold higher than that in the general population[1]. Moreover, the existing treatment for UC is not satisfactory for the prevention of carcinogenesis, involving several risks and side effects with long-term usage. Thus, finding new treatment regimens are essential.

Although the etiology of UC is yet to be elucidated, several studies have indicated that the host intestinal microbiota triggers an immune response that is requisite for the onset of the disease[2]. Microbiota also plays a major role in promoting UC-associated carcinogenesis. It downregulates the host immune response, improves the epithelial barrier function, and increases the mucus production[3]. Previous studies demonstrated that in the sterile intestinal environment, *i.e.*, the lack of intestinal microbiota, a significant reduction in carcinogenic mutations and intestinal tumor formation was observed[4]. Chronic inflammation plays a crucial role in UC-associated tumorigenesis *via* cellular DNA damage, telomere shortening, and senescence[5]. Previous studies demonstrated that probiotics exert a superior therapeutic effect on inflammation and UC[6]. VSL#3 is a mixture of *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis*, and *Streptococcus salivarius*[7]. It proved to be beneficial in the treatment of UC, including remission and relief of the relapse in mild to moderate disease[8-10]. Thus, we speculated that probiotic treatment or adjuvant treatment of UC could prevent carcinogenesis. One study demonstrated that VSL#3 can inhibit UC-associated carcinogenesis in a mouse model[11]. However, the mechanism underlying the VSL#3 treatment of UC carcinogenesis is yet to be elucidated.

Therefore, in the present study, VSL#3 was selected to investigate the effect of prevention on UC-associated carcinogenesis and the differences between fecal and mucosal microbiota were analyzed to gain a theoretical insight for the prevention of UC-associated carcinogenesis.

MATERIALS AND METHODS

Animals

Eight week old C57BL/6 male mice were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), housed under 12 h light/dark cycle conditions (temperature 22 ± 1 ℃, humidity 40%-60%) in the National Cancer Center/Cancer Hospital animal facilities, and fed a standard diet for the duration of the study. All animal experiments were conducted in accordance with the recommendations of the Animal Care Ethics and Use Committee of Peking Union Medical College Hospital and approved by the same Committee

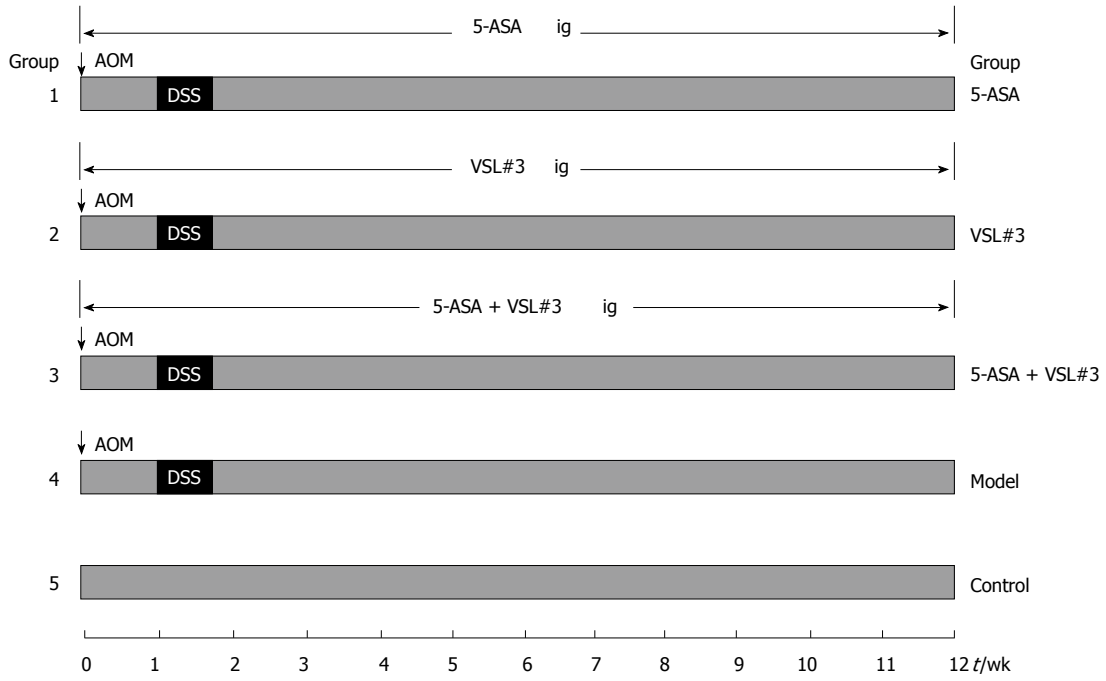


Figure 1 Experimental protocol for ulcerative colitis-associated carcinogenesis model and treatment.

(XHDW-2015-0032).

Development of UC-associated carcinogenesis model and in vivo treatment

All mice (n = 90) were initially housed together (5 animals/cage) for adaption one week before randomization into five experimental groups: control (no induction of UC-associated carcinogenesis, n = 10), model (no treatment) (n = 20), 5-ASA treatment (n = 20), VSL#3 treatment (n = 20), and 5-ASA + VSL#3 treatment (n = 20). In order to establish the UC-associated carcinogenesis model, mice were injected with 12.5 mg/kg body weight (BW) Azoxymethane (AOM) intraperitoneally, and after one week, 2.5% dextran sulfate sodium (DSS) (Mpbio, Solon, OH, United States) was added to their drinking water for five days, followed by ten weeks and two days of regular drinking water. This modeling method was based on a method described previously with some changes[12]. The three treatment groups, including 5-ASA, VSL#3, and 5-ASA + VSL#3 were gavaged 5-ASA (75 mg/kg BW, QD, Ferring Pharmaceuticals Ltd, solubilized in drinking water), VSL#3 (1.5×10^9 CFU/mice, QD, Sigma-Tau Pharmaceuticals Ltd, solubilized in drinking water), and 5-ASA + VSL#3 (75 mg/kg BW + 1.5×10^9 CFU/mice, QD) from the day of AOM injection. The control and model group were not subjected to gavage (Figure 1).

Specimen collection

The mice were sacrificed by the 12th week *via* transcardiac perfusion, and colon tissues were removed. The colons were slit longitudinally along the main axis and washed with 0.9% saline. The long and short diameter of each tumor was measured using sliding calipers, and the total tumor load of each colon was calculated (sum of the product of long and short diameter of each tumor). Subsequently, the whole colon was divided into four sections. The section near the anus washed with 0.9% saline to remove the non-adherent bacteria were flash-frozen in liquid nitrogen and stored at -80 ℃ for subsequent microbiota analysis. The remaining sections were used for enzyme-linked immunosorbent assays (ELISA) and histopathological examinations. A stool sample was collected just before AOM injection and sacrifice. A total of six mice were randomly selected from each group, and their stool and intestinal mucosa samples were sent to Allwegene (Beijing, China) for analyzing the differences in intestinal microbiota by 16S rDNA sequencing method.

Fecal DNA extraction and pyrosequencing

Microbial genomic DNA was isolated using a QIAamp DNA Micro Kit according to the manufacturer's instructions. The final quantity and quality of the DNA were assessed at 260 nm and 280 nm using an ultraviolet spectrophotometer and stored at -20 ℃ before further analysis. The V3-V4 hypervariable regions of the 16S rDNA gene were subjected to high-throughput sequencing by Allwegene using the Illumina Miseq PE300 sequencing platform (Illumina Inc., CA, United States).

ELISA for tumor necrosis factor-α and interleukin-6 in colon mucosa

The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the colon mucosa were measured using commercial mouse TNF-α and IL-6 ELISA Kits (eBioscience, United States), according to the manu-

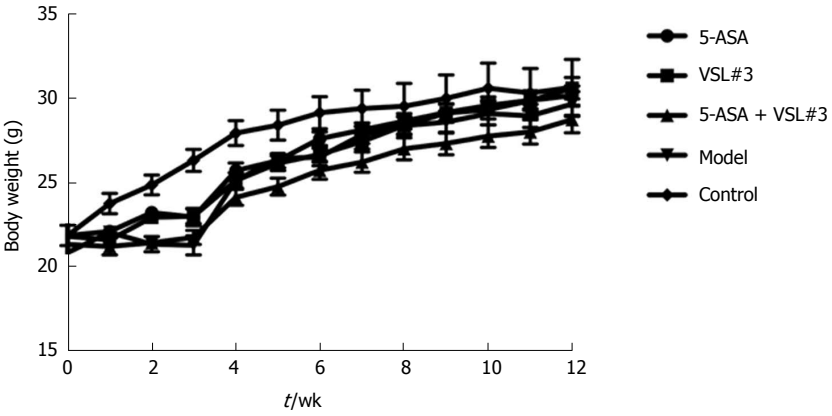


Figure 2 Body weight in each group.

facturer's protocols. The absorbance was measured at 450 nm. The results were expressed as pg/mg tissue. A total of eight mice were selected randomly from each group for ELISA.

Statistical analysis

Data are presented as mean ± SE. All statistical analyses were performed using GraphPad Prism Software Version 6.0 (GraphPad Software Inc., La Jolla, CA, United States). Statistical differences between experimental variants were assessed by two-tailed independent *t*-test, and data from more than two groups were analyzed by one-way ANOVA. Anosim and metastats analysis were used for microbiota analysis. $P < 0.05$ was considered statistically significant.

RESULTS

General health of mice in each group

As shown in Figure 2, compared to the control mice, the body weight loss was significantly higher in mice treated with azoxymethane/dextran sulfate sodium (AOM/DSS) after day 10 of DSS administration, which was accompanied by colitis symptoms, such as loose and bloody stool and dim body hair, fatigue, and less movement. These symptoms were alleviated when the mice received ordinary drinking water. In week 9, some mice treated with AOM/DSS presented bloody stool again, as well as, anal prolapse in week 10. However, no apparent weight loss was observed in the control mice, and no significant differences were detected among the five groups at the end of week 12.

Establishment of UC-associated carcinogenesis mice model

The mice were sacrificed by week 12, and the colorectal tumors were observed in the model and treatment groups (5-ASA, VSL#3, and 5-ASA + VSL#3). Strikingly, the tumor was primarily localized in the distal two-thirds of the colon. Anal tumor fusion and ring growth at the end of the rectum were observed in mice with anal prolapse (Figure 3). The pathological analysis showed mucosal carcinoma or high-grade intraepithelial neoplasia in mice treated with AOM/DSS. They were manifested with colonic gland structure disorder, large nuclei, deep staining, and nucleoplasmic ratio imbalance (Figure 4).

Effects of VSL#3 on UC-associated carcinogenesis

Treatment with AOM and DSS led to 100% (19/19, one mouse died during the experiment due to fighting) incidence of colonic neoplasms in the model group with the mean tumor load of 0.97 ± 0.19 cm. 5-ASA and VSL#3 administration significantly reduced both the tumor formation rate and the tumor load (Table 1 and Figure 5). Furthermore, no colonic tumor was detected in the control group.

Colonic TNF-α and IL-6 level comparison

As illustrated in Figure 6 and Tables 2 and 3, the levels of colonic tissue TNF-α and IL-6 in the model group were significantly higher than that in the control group. The increased levels of these inflammatory factors induced by AOM/DSS were attenuated by 5-ASA and VSL#3 treatment.

VSL#3 treatment alters the composition of fecal microbiota in AOM/DSS treated mice

In order to characterize the diversity of fecal-associated community in UC-associated carcinogenesis, we used Chao 1 and the observed species indexes, as well as the Shannon and Simpson indexes. No significant difference was detected in the diversity and composition of fecal microbiota in each group at the beginning of the experiment. After the 12-wk experiment, although no statistically significant difference was detected in the diversity among groups, the microbiota composition was altered considerably. The change in the composition of fecal microbiota induced by AOM/DSS administration was characterized by a decrease in *Lactobacillus* coupled with an increase in *Oscillibacter* and *Lachnoclostridium* as indicated by metastats analysis ($P < 0.05$). Both 5-ASA and VSL#3 supplementation was associated with a significant increase in *Bacillus* and *Lactococcus* and a decrease in *Oscillibacter* and

Table 1 Tumor formation rate and tumor load in each group

Group	*n*	Tumor formation rate (%)	Tumor load (cm)	*P* value (*vs* model group)
5-ASA	20	65.0 (13/20)	0.43 ± 0.14	0.0269
VSL#3	20	65.0 (13/20)	0.25 ± 0.07	0.0009
5-ASA + VSL#3	19	63.2 (12/19)	0.46 ± 0.11	0.0261
Model	19	100.0 (19/19)	0.97 ± 0.19	-
Control	10	0	0	-

Table 2 Level of tumor necrosis factor-α in colon tissue in each group

Group	*n*	TNF-α (pg/mg tissue)	*P* value (*vs* model group)
5-ASA	8	14.66 ± 0.72	< 0.05
VSL#3	8	25.89 ± 5.25	< 0.05
5-ASA + VSL#3	8	21.33 ± 4.55	< 0.05
Model	8	68.38 ± 18.73	-
Control	8	10.49 ± 0.30	< 0.01

TNF-α: Tumor necrosis factor.

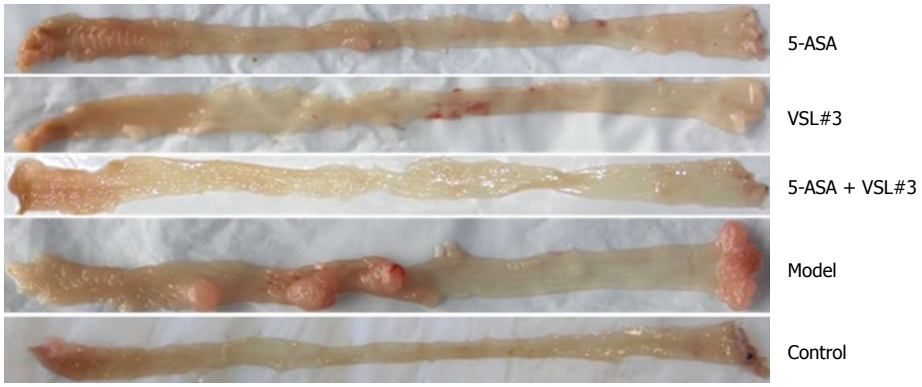


Figure 3 Representative image of colonic tumor in each group that was examined under naked eye.

Lachnoclostridium as compared to the model group ($P < 0.05$). 5-ASA combined with VSL#3 increased the level of *Lactobacillus* and decreased that of *Oscillibacter* ($P < 0.05$) (Table 4).

VSL#3 treatment alters the composition of mucosal microbiota in AOM/DSS treated mice

For the mucosal microbiota, no difference was observed in the community diversity among the groups after the 12-wk experiment. However, the distinct shift in the microbiota composition was observed by PCA and Anosim analysis ($R > 0$, $P < 0.05$). Further investigation into the discrete bacterial taxa revealed that *Ruminococcaceae* UCG-014 and *Bifidobacterium* decreased, while *Alloprevotella* increased in the model group compared to the control group. After supplementation with VSL#3, *Bifidobacterium* was increased. Although 5-ASA alone did not alter the mucosal microbiota, the combination with VSL#3 increased *Lachnoclostridium* and *Bifidobacterium* in the mucosa (Table 5).

DISCUSSION

The current study found that the rate of tumor formation and tumor load decreased after VSL#3 treatment compared to the model group, while the levels of TNF-α and IL-6 in the colon tissue in the model group were significantly higher than the control group. After the 12 wk treatment of VSL#3, the increase in TNF-α and IL-6 caused by AOM/DSS declined significantly. These findings were consistent with that of previous studies[11,13,14]. The major risk of long-term chronic inflammation is tumor occurrence[2]. Thus, we speculated that VSL#3 could prevent UC carcinogenesis by inhibiting the inflammatory response.

Herein, we found differences between the fecal and mucosal microbiota. In the case of fecal microbiota, the model group mice possessed less *Lactobacillus* and more *Oscillibacter* and *Lachnoclostridium* as compared to the control group. Previous studies have shown that *Lactobacillus bulgaricus* can reduce colitis[15], and *Lactobacillus rhamnosus* can effectively maintain UC remission[16]. *Oscillibacter* and *Lachnoclostridium* are newly discovered genera with respect to digestive diseases. In the case of mucosal microbiota, the level of the genus UCG-014 of Ruminococcaceae and Bifidobacterium decreased, while that of *Alloprevotella* increased in the model group as compared to the control

Table 3 Level of interleukin-6 in colon tissue in each group

Group	*n*	IL-6 (pg/mg tissue)	*P* value (*vs* model group)
5-ASA	8	28.19 ± 6.80	< 0.0001
VSL#3	8	99.71 ± 31.14	< 0.0500
5-ASA+VSL#3	8	81.43 ± 26.98	< 0.0100
Model	8	254.20 ± 32.49	-
Control	8	25.47 ± 5.50	< 0.0001

IL-6: Interleukin 6.

Table 4 Comparison of fecal microbiota (abundance)

Genus	Control (%)	Model (%)	5-ASA (%)	VSL#3 (%)	5-ASA + VSL#3 (%)
Lactobacillus	4.77	3.26[1]	2.65	4.06	9.86[4]
Oscillibacter	0.64	1.30[1]	0.54[2]	0.52[3]	0.79[4]
Lachnoclostridium	0.45	1.19[1]	0.48[2]	0.39[3]	1.08
Bacillus	1.01	0.98	24.00[2]	23.34[3]	0.66
Lactococcus	2.30	2.29	8.58[2]	7.86[3]	1.59

[1]$P < 0.05$ between the model and control groups; [2]$P < 0.05$ between the model and 5-ASA groups; [3]$P < 0.05$ between the model and VSL#3 groups; [4]$P < 0.05$ between the model and 5-ASA + VSL#3 groups.

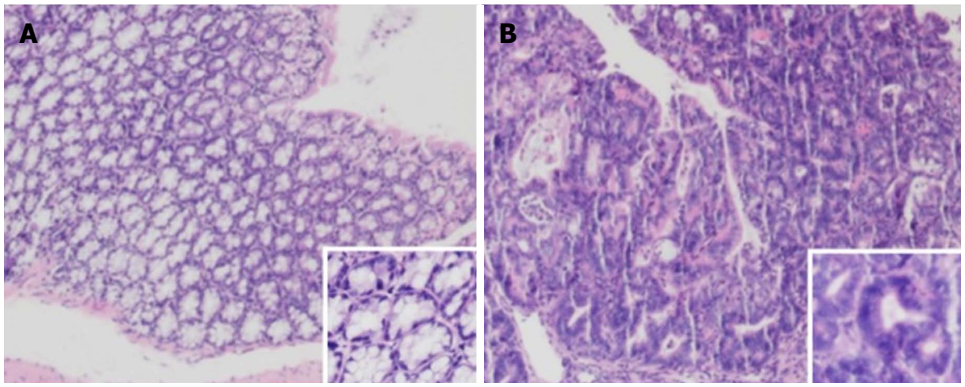


Figure 4 Representative image of hematoxylin-eosin staining of colon tissue examined under a microscope (40 × and 100 ×). A: Control group, the colonic mucosa glands were normal in the control group, the structure was regular, and the opening was good; B: Model group, the colonic gland structure presented disorder, large nuclei, deep staining, and nucleoplasmic ratio imbalance.

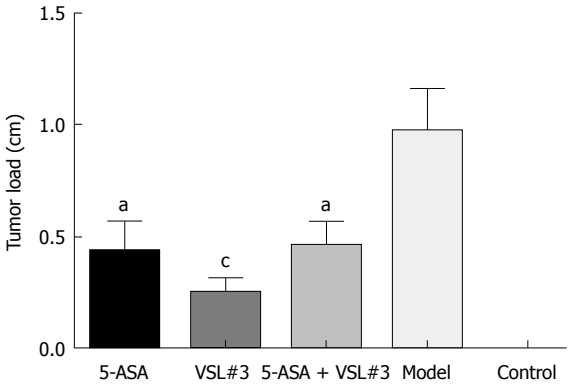


Figure 5 Tumor load in each group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$.

group. Some genus of *Ruminococcaceae* can consume hydrogen to produce acetate, which is subsequently used by *Roseburia* to produce butyrate that is not only the main source of energy for intestinal epithelial cells but can also inhibit the signaling pathway of proinflammatory cytokines[17]. *Bifidobacterium* can produce bacteriocin and organic acids against pathogens on intestinal mucosal invasion[18]. It regulates the intestinal mucosal immunity and prevents the colonization of pathogens. The role of *Alloprevotella* is not yet clarified as it is not reported frequently in the digestive disease. Therefore, we hypothesize that dysbiosis occurs during UC-associated carcinogenesis, which reduces the beneficial types and increases the detrimental types.

Previous studies have shown that supplementation of probiotics can balance the intestinal microbiota of UC patients[6], which led us to speculate that supplementation of probiotics can also balance the intestinal microbiota of UC-associated carcinogenesis. The current study demonstrated that *Bacillus* and *Lactococcus* were increased, while *Oscillibacter* and *Lachnoclostridium* were decreased in the feces following VSL#3 treatment as compared to the model group. Some species of *Bacillus* and *Lactococcus* are widely used as probiotics. For example, *Bacillus subtilis* can significantly reduce

Table 5 Comparison of mucosal microbiota (abundance)

Genus	Control (%)	Model (%)	5-ASA (%)	VSL#3 (%)	5-ASA + VSL#3 (%)
Alloprevotella	0.26	1.57[1]	1.16	0.95	1.22
*Ruminococcaceae*_UCG-014	6.63	1.49[1]	1.64	1.50	1.15
Bifidobacterium	3.45	0.24[1]	0.19	3.34[2]	1.90[3]
Lachnoclostridium	0.24	0.40	2.05	0.25	2.03[3]

[1]$P < 0.05$ between the model and the control groups; [2]$P < 0.05$ between the model and the VSL#3 groups; [3]$P < 0.05$ between the model and the 5-ASA + VSL#3 groups.

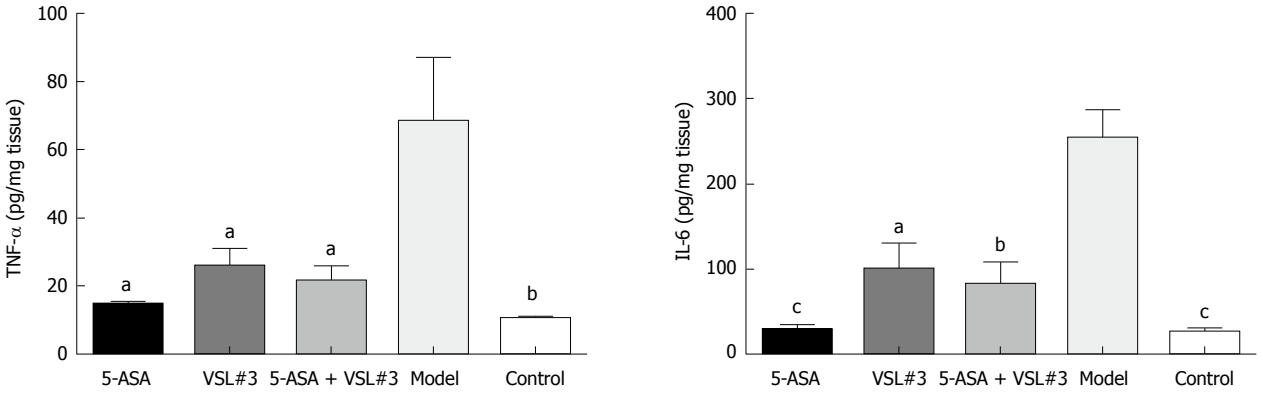


Figure 6 Colonic tumor necrosis factor-α and interleukin-6 levels in different groups. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.

DSS-induced colonic mucosal injury and inflammatory factors in mice and improve the levels of short-chain fatty acids[19]. *Lactococcus lactis* exerts a protective effect on DSS-induced colitis model mice[20].

Furthermore, *Bifidobacterium* increased in the mucosa after VSL#3 supplementation, thereby suggesting that VSL#3 supplementation, following the onset of AOM/DSS-induced colitis, promotes a healthy gastrointestinal bacterial community. Interestingly, VSL#3 is composed of eight strains, including one *Streptococcus*, three *Bifidobacterium*, and four *Lactobacillus*. However, none of the above strains increased significantly in the fecal intestinal microbiota after three-month gavage, suggesting that the positive effect of probiotics on the intestinal microbiota of the host is by regulating the proportion of beneficial and harmful bacteria.

For the differences between fecal and mucosal microbiota, we make the following explanation. There are three kinds of *Bifidobacterium* in VSL#3, and *Bifidobacterium* increased in mucosal microbiota but not in fecal. This phenomenon indicated that *Bifidobacterium* is easily colonized in the mucosa. Conversely, *Bacillus* and *Lactococcus* increased in fecal microbiota after VSL#3 intervention but not in the mucosa, indicating that *Bacillus* and *Lactococcus* can colonize easily in the feces. Strikingly, the four types of *Lactobacillus* in VSL#3 did not increase either in the fecal or mucosal microbiota, thereby suggesting that the intestinal environment of UC-associated carcinogenesis is not optimal for the growth of *Lactobacillus*. Only in the 5-ASA + VSL#3 group, the increase in *Lactobacillus* was observed in feces, which might be attributed to the low luminal pH. However, these hypotheses necessitate further studies for substantiation.

5-ASA is the first-line treatment for mild-to-moderate UC, and studies have found that 5-ASA $\geqslant$ 1.2 g/d could reduce the risk of carcinogenesis in patients with mild-to-moderate UC[21]. Thus, considering the clinical significance, we designed the 5-ASA monotherapy group and the 5-ASA + VSL#3 group. Interestingly, the change in the fecal microbiota in the 5-ASA group was similar to that in the VSL#3 monotherapy group. The potential mechanisms regulating the microbiota by 5-ASA are as follows: (1) Change in the colonic luminal pH: 5-ASA is released in the colon and translated into acetylsalicylic acid, which in turn, can decrease the luminal pH[22]. Low luminal pH is optimal for the growth of *Bifidobacteria* and *Lactobacilli*[23]; (2) improvement in the anoxia environment: 5-ASA can inhibit the production of chemotactic eicosanoids and cyclooxygenase 2 (COX2), which induces anoxia and can inactivate the oxygen-derived free radicals, improving the anoxia situation, which might affect the composition of intestinal microbiota[22]; and (3) 5-ASA can downregulate the expression of genes that are involved in bacterial metabolism, invasiveness, and antibiotic/stress resistance[24].

Nevertheless, the present study has some limitations. Herein, we only observed the phenomenon of gut microbiota changes while the specific role of flora is yet to be explored. Our future *in vitro* studies would focus on the underlying mechanisms.

In conclusion, the current study demonstrated that VSL#3 prevented UC-associated carcinogenesis in the AOM/DSS-induced mice model and decreased the level of

TNF-α and IL-6 in colon tissue. The intestinal microbiota dysbiosis was exhibited in UC-associated carcinogenesis mice. Supplementary VSL#3 was beneficial for a balanced fecal and mucosal microbiota in UC-associated carcinogenesis mice. Taken together, VSL#3 may serve as a potential therapeutic agent for the prevention of UC-associated carcinogenesis. Ongoing studies in our group are focused on the underlying mechanisms.

ARTICLE HIGHLIGHTS

Research background

Recently, an upward trend has been observed in the incidence of ulcerative colitis (UC) leading to increased clinical attention on UC-associated carcinogenesis.

Research motivation

Existing treatment for UC in the prevention of carcinogenesis involves several risks and side effects with long-term usage. Finding new treatment regimens are essential.

Research objectives

To investigate the effects of VSL#3 on tumor formation, and fecal and intestinal mucosal microbiota in the azoxymethane/dextran sulfate sodium (AOM/DSS) induced mice model.

Research methods

C57BL/6 mice were administered AOM/DSS to develop the UC-associated carcinogenesis model. The treatment group was gavaged with 5-ASA (75 mg/kg/d), VSL#3 (1.5×10^9 CFU/d), and 5-ASA + VSL#3 from the day of AOM injection for three months (five days/week). The tumor load was compared in each group, and tumor necrosis factor (TNF-α) and interleukin (IL)-6 levels evaluated in colon tissue. The stool and intestinal mucosa samples were collected to analyze the differences in the intestinal microbiota by 16s rDNA sequencing.

Research results

VSL#3 significantly reduced the tumor load in the AOM/DSS-induced mice model, and decreased the level of TNF-α and IL-6 in colon tissue. The model group had a lower level of *Lactobacillus* and higher level of *Oscillibacter* and *Lachnoclostridium* in fecal microbiota than the control group (UC-associated carcinogenesis not induced). *Bacillus* and *Lactococcus* were increased after the intervention with 5-ASA and VSL#3, while *Lachnoclostridium* and *Oscillibacter* were reduced. 5-ASA + VSL#3 increased the *Lactobacillus* and decreased the *Oscillibacter*. The intestinal mucosal microbiota analysis showed a lower level of *Bifidobacterium* and *Ruminococcaceae*_UCG-014 and higher level of *Alloprevotella* in the model group compared to the control group. *Bifidobacterium* was increased after supplementation with VSL#3. 5-ASA + VSL#3 increased the level of both *Lachnoclostridium* and *Bifidobacterium*.

Research conclusions

In mice, VSL#3 can prevent UC-associated carcinogenesis, reduce the colonic mucosal inflammation levels, and is beneficial for rebalancing the fecal and mucosal intestinal microbiota.

Research perspectives

VSL#3 may be a potential therapeutic agent for UC-associated carcinogenesis prevention based on the data presented here.

ACKNOWLEDGMENTS

We thank the staff at the National Cancer Center/Cancer Hospital of the Chinese Academy of Medical Sciences and Peking Union Medical College.

REFERENCES

1 **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.2012.01.010]
2 **Uronis JM**, Mühlbauer M, Herfarth HH, Rubinas TC, Jones GS, Jobin C. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. *PLoS One* 2009; **4**: e6026 [PMID: 19551144 DOI: 10.1371/journal.pone.0006026]
3 **Derikx LA**, Dieleman LA, Hoentjen F. Probiotics and prebiotics in ulcerative colitis. *Best Pract Res Clin Gastroenterol* 2016; **30**: 55-71 [PMID: 27048897 DOI: 10.1016/j.bpg.2016.02.005]
4 **Rakoff-Nahoum S**, Medzhitov R. Role of toll-like receptors in tissue repair and tumorigenesis. *Biochemistry* (Mosc) 2008; **73**: 555-561 [PMID: 18605980]
5 **Risques RA**, Lai LA, Himmetoglu C, Ebaee A, Li L, Feng Z, Bronner MP, Al-Lahham B, Kowdley KV, Lindor KD, Rabinovitch PS, Brentnall TA. Ulcerative colitis-associated colorectal cancer arises in a field of short telomeres, senescence, and inflammation. *Cancer Res* 2011; **71**: 1669-1679 [PMID: 21363920 DOI: 10.1158/0008-5472.CAN-10-1966]
6 **Verna EC**, Lucak S. Use of probiotics in gastrointestinal disorders: what to recommend? *Therap Adv Gastroenterol* 2010; **3**: 307-319 [PMID: 21180611 DOI: 10.1177/1756283X10373814]
7 **Liang J**, Sha SM, Wu KC. Role of the intestinal microbiota and fecal transplantation in inflammatory bowel diseases. *J Dig Dis* 2014; **15**: 641-646 [PMID: 25389085 DOI: 10.1111/1751-2980.12211]
8 **Shen J**, Zuo ZX, Mao AP. Effect of probiotics on inducing remission and maintaining therapy in ulcerative colitis, Crohn's disease, and pouchitis: meta-analysis of randomized controlled trials. *Inflamm Bowel Dis* 2014; **20**: 21-35 [PMID: 24280877 DOI: 10.1097/01.MIB.0000437495.30052.be]
9 **Sood A**, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, Tandon RK. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. *Clin Gastroenterol Hepatol* 2009; **7**: 1202-1209, 1209.e1 [PMID: 19631292 DOI: 10.1016/j.cgh.2009.07.016]
10 **Tursi A**, Brandimarte G, Papa A, Giglio A, Elisei W, Giorgetti GM, Forti G, Morini S, Hassan C, Pistoia MA, Modeo ME, Rodino' S, D'Amico T, Sebkova L, Sacca' N, Di Giulio E, Luzza F, Imeneo M, Larussa T, Di Rosa S, Annese V, Danese S, Gasbarrini A. Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2010; **105**: 2218-2227 [PMID: 20517305 DOI: 10.1038/ajg.2010.218]
11 **Talero E**, Bolivar S, Ávila-Román J, Alcaide A, Fiorucci S, Motilva V. Inhibition of chronic ulcerative colitis-associated adenocarcinoma development in mice by VSL#3. *Inflamm Bowel Dis* 2015; **21**: 1027-1037 [PMID: 25793324 DOI: 10.1097/MIB.0000000000000346]
12 **Tanaka T**, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* 2003; **94**: 965-973 [PMID: 14611673]
13 **Bromberg J**, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. *Cancer Cell* 2009; **15**: 79-80 [PMID: 19185839 DOI: 10.1016/j.ccr.2009.01.009]
14 **Bassaganya-Riera J**, Viladomiu M, Pedragosa M, De Simone C, Hontecillas R. Immunoregulatory mechanisms underlying prevention of colitis-associated colorectal cancer by probiotic bacteria. *PLoS One* 2012; **7**: e34676 [PMID: 22511958 DOI: 10.1371/journal.pone.0034676]
15 **Takamura T**, Harama D, Fukumoto S, Nakamura Y, Shimokawa N, Ishimaru K, Ikegami S, Makino S, Kitamura M, Nakao A. Lactobacillus bulgaricus OLL1181 activates the aryl hydrocarbon receptor pathway and inhibits colitis. *Immunol Cell Biol* 2011; **89**:

817-822 [PMID: 21321579 DOI: 10.1038/icb.2010.165]
16 **Zocco MA**, dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, Novi M, Rigante D, Cazzato IA, Ojetti V, Armuzzi A, Gasbarrini G, Gasbarrini A. Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther* 2006; **23**: 1567-1574 [PMID: 16696804 DOI: 10.1111/j.1365-2036.2006.02927.x]
17 **Morgan XC**, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, Bousvaros A, Korzenik J, Sands BE, Xavier RJ, Huttenhower C. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 2012; **13**: R79 [PMID: 23013615 DOI: 10.1186/gb-2012-13-9-r79]
18 **Zhiwei L**, Xuequn W, tiantian L. The clinical significance of intestinal flora changes in patients with ulcerative colitis. *Chin J Gastroenterol Hepatol* 2016; **25**: 554-556
19 **Zhang HL**, Li WS, Xu DN, Zheng WW, Liu Y, Chen J, Qiu ZB, Dorfman RG, Zhang J, Liu J. Mucosa-reparing and microbiota-balancing therapeutic effect of Bacillus subtilis alleviates dextrate sulfate sodium-induced ulcerative colitis in mice. *Exp Ther Med* 2016; **12**: 2554-2562 [PMID: 27698758 DOI: 10.3892/etm.2016.3686]
20 **Berlec A**, Perše M, Ravnikar M, Lunder M, Erman A, Cerar A, Štrukelj B. Dextran sulphate sodium colitis in C57BL/6J mice is alleviated by Lactococcus lactis and worsened by the neutralization of Tumor necrosis Factor α. *Int Immunopharmacol* 2017; **43**: 219-226 [PMID: 28039805 DOI: 10.1016/j.intimp.2016.12.027]
21 **Zhao LN**, Li JY, Yu T, Chen GC, Yuan YH, Chen QK. 5-Aminosalicylates reduce the risk of colorectal neoplasia in patients with ulcerative colitis: an updated meta-analysis. *PLoS One* 2014; **9**: e94208 [PMID: 24710620 DOI: 10.1371/journal.pone.0094208]
22 **Xue L**, Huang Z, Zhou X, Chen W. The possible effects of mesalazine on the intestinal microbiota. *Aliment Pharmacol Ther* 2012; **36**: 813-814 [PMID: 22984958 DOI: 10.1111/apt.12034]
23 **Kerr BJ**, Weber TE, Ziemer CJ, Spence C, Cotta MA, Whitehead TR. Effect of dietary inorganic sulfur level on growth performance, fecal composition, and measures of inflammation and sulfate-reducing bacteria in the intestine of growing pigs. *J Anim Sci* 2011; **89**: 426-437 [PMID: 20952529 DOI: 10.2527/jas.2010-3228]
24 **Kaufman J**, Griffiths TA, Surette MG, Ness S, Rioux KP. Effects of mesalamine (5-aminosalicylic acid) on bacterial gene expression. *Inflamm Bowel Dis* 2009; **15**: 985-996 [PMID: 19202572 DOI: 10.1002/ibd.20876]

P- Reviewer: Gardlik R, Suzuki H **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4263-4271

DOI: 10.3748/wjg.v24.i37.4263

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

ORIGINAL ARTICLE

Basic Study

Potential involvement of heat shock proteins in pancreatic-duodenal homeobox-1-mediated effects on the genesis of gastric cancer: A 2D gel-based proteomic study

Juan Ma, Bei-Bei Wang, Xiao-Yan Ma, Wei-Ping Deng, Li-Shu Xu, Wei-Hong Sha

Juan Ma, Bei-Bei Wang, Wei-Ping Deng, Li-Shu Xu, Wei-Hong Sha, Department of Gastroenterology and Hepatology, Guangdong General Hospital (Guangdong Academy of Medical Sciences), Guangdong Geriatrics Institute, Guangzhou 510080, Guangdong Province, China

Xiao-Yan Ma, Forensic Identification Institute, Key Laboratory of Reproduction and Genetics of Guangdong Higher Education Institutes, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou 510150, Guangdong Province, China

ORCID number: Juan Ma (0000-0003-1256-4914); Bei-Bei Wang (0000-0002-7950-3641); Xiao-Yan Ma (0000-0001-6101-1318); Wei-Ping Deng (0000-0003-0624-9846); Li-Shu Xu (0000-0002-2629-2684); Wei-Hong Sha (0000-0001-6615-422X).

Author contributions: Ma J and Wang BB contributed equally to this work; Ma J and Sha WH conceived the study; Ma J and Ma XY performed the research; Wang BB and Deng WP analyzed the data; Ma J wrote this manuscript; Xu LS revised this manuscript; Sha WH supervised the report; all authors gave intellectual input to the study and approved the final version of the manuscript.

Supported by Guangdong Natural Science Fund, No. 2016A030313765; and Guangdong medical scientific research fund, No. A2017070 and No. A2017122.

Institutional review board statement: The institutional review board at the Guangdong General Hospital approved the study protocol. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Wei-Hong Sha, MD, PhD, Chief Doctor, Professor, Department of Gastroenterology and Hepatology, Guangdong General Hospital (Guangdong Academy of Medical Sciences), Guangdong Geriatrics Institute, 106 Zhongshan Er Road, Guangzhou 510080, Guangdong Province, China. shaweihong@gdghospital.com.cn
Telephone: +86-20-83827812
Fax: +86-20-83827812

Received: May 30, 2018
Peer-review started: May 30, 2018
First decision: July 18, 2018
Revised: August 9, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

AIM

To identify functional proteins involved in pancreatic-duodenal homeobox-1 (PDX1)-mediated effects on gastric carcinogenesis.

METHODS

A PDX1-overexpressed model was established by transfecting gastric cancer cell line SGC7901 with pcDNA3.1(+)-PDX1 vector (SGC-PDX1). Transfection with empty pcDNA3.1 vector (SGC-pcDNA) served as control. Comparative protein profiles of the two groups

were analyzed by two-dimensional electrophoresis based-proteomics (2DE gel-based proteomics). The differential proteins identified by 2DE were further validated by qRT-PCR and immunoblotting. Finally, co-immunoprecipitation was used to determine any direct interactions between PDX1 and the differential proteins.

RESULTS

2DE gel proteomics identified seven differential proteins in SGC-PDX1 when compared with those in SGC-pcDNA. These included four heat shock proteins (HSPs; HSP70p1B, HSP70p8, HSP60, HSP27) and three other proteins (ER60, laminin receptor 1, similar to epsilon isoform of 14-3-3 protein). Immunoblotting validated the expression of the HSPs (HSP70, HSP60, HSP27). Furthermore, their expressions were lowered to 80%, 20% and 24%, respectively, in SGC-PDX1, while PDX1 exhibited a 9-fold increase, compared to SGC-pcDNA. However, qRT-PCR analysis revealed that mRNA levels of the *HSP*s were increased in SGC-PDX1, suggesting that the expression of the *HSP*s was post-translationally regulated by the PDX1 protein. Finally, co-immunoprecipitation failed to identify any direct interaction between PDX1 and HSP70 proteins.

CONCLUSION

This study demonstrates the potential involvement of HSPs in PDX1-mediated effects on the genesis of gastric cancer.

Key words: Pancreatic-duodenal homeobox-1; Heat shock proteins; Gastric cancer; Proteomics; Two-dimensional electrophoresis

Core tip: Using a pcDNA3.1(+)-pancreatic-duodenal homeobox-1 (PDX1) vector, a PDX1-overexpressed model was built. Seven differential proteins were identified in SGC-PDX1 by two-dimensional electrophoresis gel proteomics compared with those in SGC-pcDNA. Four heat shock proteins were identified and confirmed by immunoblotting. qRT-PCR analysis further revealed that the expression of the *HSP*s was post-translationally regulated by the PDX1 protein. This study suggests the potential involvement of HSPs in PDX1 mediated effects on gastric carcinogenesis.

Ma J, Wang BB, Ma XY, Deng WP, Xu LS, Sha WH. Potential involvement of heat shock proteins in pancreatic-duodenal homeobox-1-mediated effects on the genesis of gastric cancer: A 2D gel-based proteomic study. *World J Gastroenterol* 2018; 24(37): 4263-4271 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4263.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4263

INTRODUCTION

Homeobox genes greatly contribute to the pattern formation of embryos by encoding many homeodomain transcriptional regulators[1,2]. The human gene of pancreatic-duodenal homeobox-1 (PDX1) is located at chromosomal locus 13q12.1[3]. In adults, PDX1 is expressed in Brunner's glands of the duodenum, pancreatic β cells and gastric pyloric gland cells. As one of the key homeodomain transcription regulators, PDX1 plays an important role in the development of the digestive system, including antrum, duodenum, and pancreas[4,5]. PDX1 is also a biomarker of pancreatic stem cells and regulates normal islet function[6]. In addition, PDX1 is expressed in the distal stomach and is involved in the secretion of hormones, such as somatostatin, serotonin, and gastrin[4]. Dysregulation of PDX1 may lead to pancreatic and gastric carcinogenesis. For example, overexpression of PDX1 was shown to be associated with the development of pancreatic cancer as it promoted proliferation, invasion, and colony formation in cancer cells[7]. Moreover, we earlier reported downregulation of PDX1 in gastric cancer, which suggests its potential role as a tumor suppressor. Further, overexpression of PDX1 induced apoptosis and inhibited proliferation, clone formation, and migration of gastric cancer cells. In addition, stable transfection with PDX1 was shown to suppress development of gastric cancer *in vivo*[8]. We have also shown that silencing of PDX1 in gastric cancer is likely caused by promoter hypermethylation and histone hypoacetylation[9]. However, the downstream mechanism by which PDX1 mediates gastric tumorigenesis remains elusive.

Proteins are the fundamental molecules that perform cellular functions. Changes in the protein expression profile under pathological conditions may reflect potential pathogenic mechanisms. Proteomic approaches represent a powerful tool to explore the underlying mechanism of tumorigenesis by characterizing the cellular events related to tumor development, angiogenesis, and progression. A number of proteomic approaches have been used to investigate the pathogenesis of gastric cancer[10-12]. Among these, 2D gel electrophoresis is a conventional approach and has been the principal step in the development of proteomics. Subsequent to 2D gel electrophoresis, protein expression profiles have been elucidated by computational image analysis and identified by mass spectrometry[13]. Although a number of different proteomics techniques have evolved in recent years, 2DE-based proteomics is still widely used to identify cancer-associated proteins[14].

To clarify the role of downstream mediators of PDX1 in gastric tumorigenesis, we established a PDX1-overexpressed gastric cancer cell model by transfection. After validation of the PDX1 overexpressed model, we used a 2D gel-based proteomic approach to determine the differentially expressed proteins as compared to that in the empty vector transfected gastric cancer cells. The expressions of identified candidate proteins were further assessed by real-time qRT-PCR and/or immunoblotting. Finally, co-immunoprecipitation was used to examine whether a differential protein, HSP70 has direct inter-

actions with PDX1 protein. Our results will greatly extend our understanding of the mechanisms of PDX1 mediated effects on gastric tumorigenesis.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cell line SGC7901 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 μg/mL penicillin. The cells were maintained in a constant temperature incubator at 37 ℃ and 5% CO_2. The cells were harvested and passaged as required.

Cell transfection and protein sample preparation

The vector of pcDNA3.1(+)-PDX1 was established according to the methods previously published[15]. The SGC7901 cells were cultured in 6-well plates and transfected with pcDNA3.1(+)-PDX1 vector (SGC-PDX1) or pcDNA3.1(+) control vector (SGC-pcDNA) using liposome transfection reagent (Lipofectamine™ 2000 Transfection Reagent, ThermoFisher Scientific, United States). After six hours, fresh complete medium was used to replace the transfection medium and the cells of each group were aliquoted in triplicate. After 24-h incubation, the transfected SGC7901 cells were harvested for protein extraction. Initially, the culture plates were placed on ice, followed by addition of 250 μL lysis buffer for 10 min. The lysed cells were scraped off and sonicated for 10 min. Subsequently, the cell lysates were centrifuged at 20000 g for 30 min, and the supernatant containing cell proteins was collected. The whole cell lysate proteins were purified using a Cleanup kit (ProteoPrep® Total Extraction Sample Kit, Sigma-Aldrich, United States) to remove salt and lipid impurities. Protein concentration was determined by bicinchoninic acid (BCA) method. Finally, 600 μg protein from each sample was used for 2D gel electrophoresis.

2D gel electrophoresis, image analysis, and mass spectroscopy

2D gel electrophoresis was performed according to the method described elsewhere[16]. The first-dimensional isoelectric focusing of protein sample was conducted on Immobiline™ pH 3-10 IPG linear strips (Amersham, Pharmacia Biotech Inc.), and focused by an IPGphor electrophoresis system (Ettan IPGphor Isoelectric Focusing System, Amersham Biosciences, United States) by following the manufacturer's instructions. During second dimensional separation, focused strips were subjected onto the top of 12.5% SDS-PAGE gradient gels and overlaid with 1% agarose gel buffer. The gels were run under 20 ℃ at a speed of 15 mA/gel in stacking gel and 30 mA/gel in resolving gel until bromophenol blue front was within 0.5-1 cm of the gel bottom. The 2D gels were then stained with silver and scanned with UMax Powerlook 2110XL (GE Amersham). Image analysis was conducted using Image Master 2D platinum 5.0 software (Amersham pharmcia, Biotech) to identify differentially expressed protein profiles.

The differential protein spots between the two groups were excised using an automated Spot Handling Workstation (Amersham pharmcia, Biotech) and were subjected to discoloration and dehydration. The proteins were digested by trypsin and the peptides were obtained using peptide extraction buffer. The peptide suspension was vacuum-dried and resuspended in D/W. The resuspended peptide extracts were then spotted on a matrix-assisted laser-desorption ionization (MALDI) target. MALDI-MS analysis was conducted through a MALDI-time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems, United States) and peptide mass mapping was performed by searching the NCBInr database.

qRT-PCR analysis

Total RNA from the cells was isolated using a Mini-RNease RNA extract kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was reverse-transcribed from total RNA using a Thermoscript RT-PCR system (Gibco BRL, Gaithersburg, MD, United States). Applied Biosystems Sequence Detection System 7900 (Applied Biosystems, United States) was used to perform the qRT-PCR analysis. The reaction was performed using 10 μL mixture of 300 ng cDNA templates, 500 nmol of each primer and Power SYBR GREEN PCR Master Mix (Applied Biosystems, United States) as previously reported[8]. The generated melting curves and CT values were used to calculate the copy numbers of *PDX1* or *HSP*s mRNA. Online tool was utilized to design the PCR primers and the corresponding sequences, which are shown in Table 1.

Immunoblotting

As described in our previous study[8], the whole cell lysate protein was mixed with SDS-PAGE sample buffer and boiled for five minutes. Prepared protein samples were separated by SDS-PAGE electrophoresis and electrotransferred onto polyvinylidene fluoride membranes. After blocking, the membranes were blotted with primary antibodies against PDX1, HSP27, HSP60, and HSP70 (Santa Cruz Biotechnology). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using an enhanced chemiluminescence system (Amersham, Piscataway, NJ, United States).

Co-immunoprecipitation

SGC7901 cells were co-transfected with both pcDNA3.1(+)-PDX1 and pcDNA3.1(+)-HSP70 plasmids and cultured for 24 h. The cells were subsequently washed with PBS and lysed using lysis buffer (1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L 2-mercaptoethanol, 2 mmol/L $MgCl_2$, 20 mmol/L HEPES, 150 mmol/L NaCl, 10 μg/mL leupeptin and 10 μg/mL aprotinin). The lysates were transferred onto protein A beads (Thermo Fisher Scientific) and incubated overnight with primary antibodies for PDX1 or HSP70 (Santa Cruz Biotechnology) at 4 ℃.

Table 1 Primer sequences of pancreatic-duodenal homeobox-1 (*PDX1*) and heat shock protein (*HSP*) genes used in the RT-PCR analysis

Gene name	Forward	Reverse	Product size (bp)
PDX1	5′-ATCTCCCCATACGAAGTGCC-3′	5′-CGTGAGCTTTGGTGGATTTCAT-3′	92
GAPDH	5′-ATGGGGAAGGTGAAGGTCG-3′	5′-GGGGTCATTGATGGCAACAATA-3	108
HSPA2	5′-CACCACCTATTCGTGCGTC-3′	5′-TTTCCGTCCAATCAGCCTCTT-3′	196
HSPA6	5′-CAAGGTGCGCGTATGCTAC-3′	5′-GCTCATTGATGATCCGCAACAC-3′	224
HSPA8	5′-GGAGGTGGCACTTTTGATGTG-3′	5′-CAAGCAGTACGGAGGCGTCT-3′	200
HSPA1B	5′-TTTGAGGGCATCGACTTCTACA-3	5′-CCAGGACCAGGTCGTGAATC-3′	148
HSPA1L	5′-CTACTGCCAAGGGAATCGCC-3′	5′GCCGATCAGACGTTTAGCATC-3′	227
HSP27	5′-GGACGAGCATGGCTACATCT-3′	5′-CTTTACTTGGCGGCAGTCTC-3′	237
HSP60	5′-CACCGTAAGCCTTTGGTCAT-3′	5′-CCCTCTTCTCCAAACACTGC-3′	188

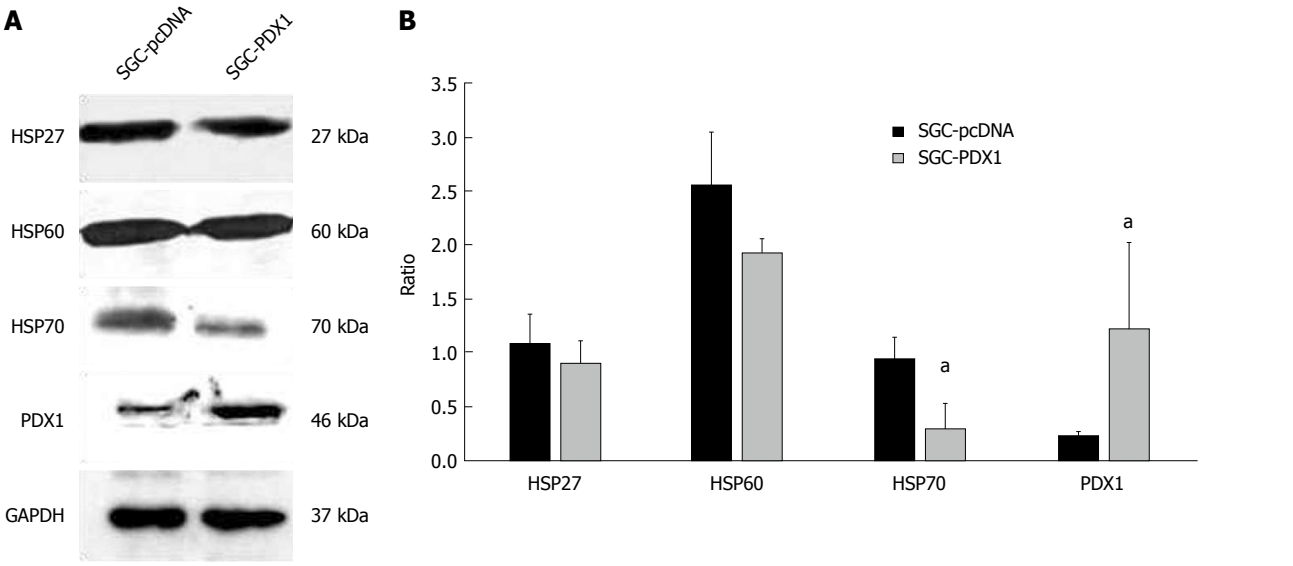


Figure 1 Pancreatic-duodenal homeobox-1 overexpression regulated the expression of heat shock proteins in gastric cancer SGC7901 cells. A: Immunoblotting analysis confirmed the enhanced expression of PDX1 protein after PDX1-pcDNA transfection in SGC7901 cells. It also revealed that PDX1 overexpression could downregulate the expression of HSP27, HSP60, and HSP70 proteins in SGC-PDX1 cells; B: Quantitative analysis of the results of immunoblotting indicated that HSP70 protein was significantly decreased in SGC-PDX1 cells. $^{a}P < 0.05$.

The beads were washed twice using lysis buffer to remove unbound proteins. To elute the bound proteins, the beads were then resuspended in sample buffer and boiled at 95 ℃ for three minutes. The collected unbound and bound proteins were stored at -80 ℃ for further use during immunoblotting.

Statistical analysis

Data are presented as mean ± SD, and the differences in continuous variables were assessed by Mann-Whitney *U* test (Student *t* test). *P*-values < 0.05 were considered statistically significant and all statistical tests were two-tailed.

RESULTS

Establishment of PDX1 overexpressed model using gastric cell line

To study the downstream mechanisms of PDX1 in gastric carcinogenesis, we established a PDX1 overexpressed model of gastric cancer cell line SGC7901 using a vector of pcDNA3.1(+)-PDX1 (SGC-PDX1). The whole cell lysate proteins were extracted and subjected to immunoblotting to confirm the establishment of PDX1 overexpressed model. As shown in Figure 1A, the expression of PDX1 in pSGC-PDX1 cells was significantly increased compared to that in the SGC-pcDNA cells. These results indicated the successful establishment of a PDX1 overexpressed model, which was further used for 2D gel-based proteomic analysis.

Differentially expressed protein profiles in PDX1 overexpressed model revealed by 2D gel-based proteomics

The comparative protein profile of PDX1 overexpressed model and control group was determined using 2D PAGE coupled with MALDI-TOF-MS. The 2-DE maps were displayed between a pH range of 3-10. Figure 2A and 2B shows the representative 2-DE maps of SGC-PDX1 and SGC-pcDNA. Compared with SGC-pcDNA control cells, seven proteins were found to be downregulated about two-fold in SGC-PDX1 cells (Figure 2C). The seven

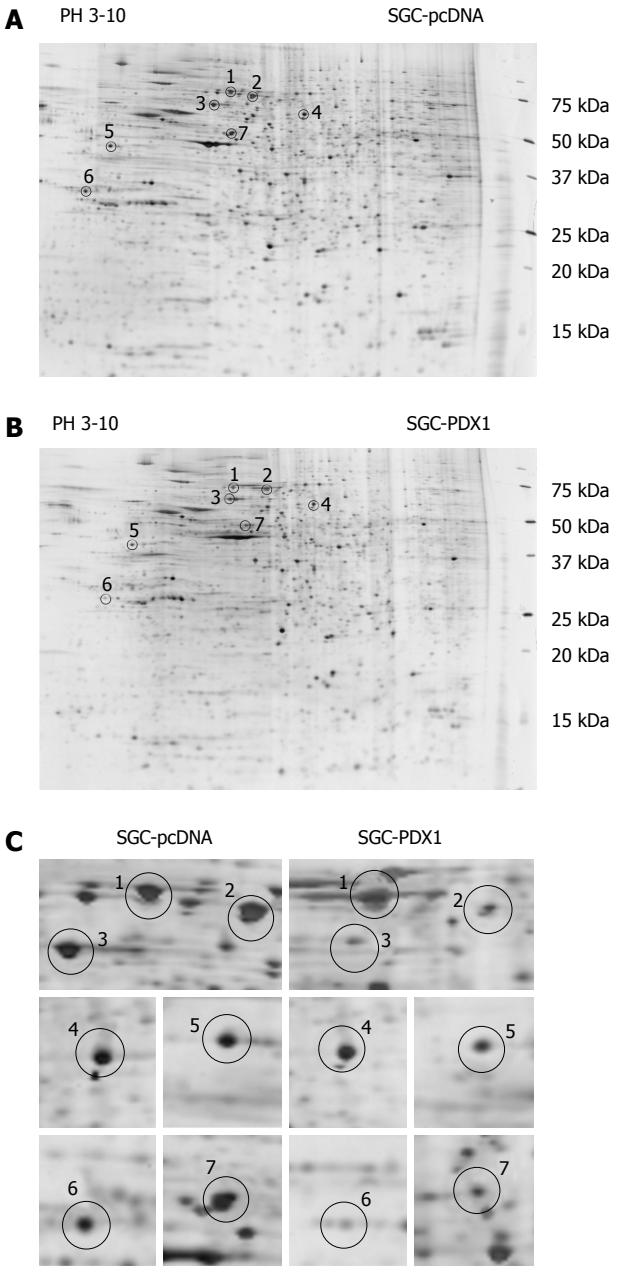


Figure 2 Two-dimensional electrophoresis-gel images of proteins from SGC7901 with pcDNA3.1(+)-pancreatic-duodenal homeobox-1 (PDX1) vector and SGC7901-pcDNA cells. A and B: Representative 2DE-gel images of SGC-PDX1 and SGC-pcDNA groups. The differentially expressed proteins are indicated by circles; C: Close-up images of the differential protein spots between SGC-PDX1 and SGC-pcDNA groups. 2DE: 2-dimensional electrophoresis.

differentially expressed protein spots were excised from replicate 2D-gels and the corresponding peptides were obtained by proteolytic digestion. Extracted peptides were subjected to MALDI-TOF-MS analysis and then the database search was performed. The seven differentially expressed proteins were identified as, heat shock protein (70 kDa) and its isoforms, heat shock protein (60 kDa), heat shock protein (27 kDa), glucose regulated protein (58 kDa), laminin receptor 1, epsilon isoform of 14-3-3 protein. The detailed information of the seven differentially expressed proteins is shown in Table 2.

Validation of differentially expressed proteins by qRT-PCR and immunoblotting

Through literature search and review, we found that the heat shock proteins (HSPs) were shown to play an important role in the regulation of tumorigenesis, including proliferation, invasion, and colony formation of cancer cells[17-19]. This finding suggested that the HSPs might also be involved in gastric carcinogenesis because they also showed differential protein expressions in SGC-PDX1 as revealed by 2D gel-based proteomics. Therefore, we selected the heat shock proteins for further evaluation.

SGC7901 cells were maintained under the same culture conditions and transfected with corresponding vectors as mentioned earlier. Total RNA and whole cell proteins were extracted for qRT-PCR and immunoblotting, respectively. The expression of *PDX1* mRNA was significantly upregulated in SGC-PDX1 cells as compared to that in the control group (Figure 3). This indicated the successful transfection of SGC7901 cells with the *PDX1* gene. In addition, the mRNA levels of the *HSP*70 isoforms (*HSPA1B*, *HSPA1L*, *HSPA2*, *HSPA6*), *HSP27*, and *HSP60* were found to have increased after *PDX1* overexpression (Figure 3) in SGC7901 cells. Immunoblotting confirmed that the expression of the PDX1 protein was significantly increased in SGC-PDX1 cells. Moreover, the expression of HSP70, HSP27, and HSP60 were downregulated in SGC-PDX1 cells (reduced to 80%, 24%, and 20%, respectively), which was consistent with the proteomic results (Figure 1). However, these results demonstrated the opposite patterns of mRNA and protein expression of HSP70, HSP27, and HSP60 in SGC-PDX1 cells, which suggests that the expressions of these proteins were post-translationally regulated in SGC-PDX1 cells.

Interaction between PDX1 and HSP70 evaluated by co-immunoprecipitation

Results of immunoblotting showed that the difference in the expression of HSP70 between the two groups was much higher than that for the other two HSP proteins, which emphasized the significance of the HSP70 protein in PDX1-mediated effects on gastric tumorigenesis. Therefore, we further evaluated whether PDX1 and HSP70 proteins have direct interactions. SGC7901 cells were co-transfected with both pcDNA3.1(+)-PDX1 and pcDNA3.1(+)-HSP70 plasmids and cultured for 24 h. The whole cell lysate protein of co-transfected SGC7901 cells were then subjected to co-immunoprecipitation. As illustrated in Figure 4A, both PDX1 and HSP70 proteins were identified in the cell lysate proteins of co-transfected SGC7901 cells, which confirmed the input of PDX1 and HSP70 proteins in co-immunoprecipitation assay. The whole cell lysate proteins were then subjected to co-immunoprecipitation assay using precipitating antibodies against PDX1 and HSP70. The corresponding immunoprecipitated proteins were further detected by immunoblotting using primary antibodies against HSP70 and PDX1. Interestingly, no significant binding between PDX1 and HSP70 proteins was observed (Figure

Table 2 Differential protein spots between SGC7901 with pcDNA3.1(+)-pancreatic-duodenal homeobox-1 (PDX1) vector and SGC7901-pcDNA cells analyzed by matrix-assisted laser desorption ionization-time of flight-mass spectrometry

Spot No.	Rank protein name	Accession No.	Protein score	Protein score CI%	Protein MW
S1	Heat shock 70 kDa protein 8 isoform 2 (homo sapiens)	gi 24234686	595	100	53598.4
	HSPA8 protein (homo sapiens)	gi 48257068	546	100	54804.2
	Heat shock 70 kDa protein 2 (homo sapiens)	gi 3287 9973	290	100	70263.0
	Heat shock 70 kDa protein 6 (HSP70B') (homo sapiens)	gi 55960611	81	100	71440.4
S2	DNAK-type molecular chaperone HSPA1L-human	gi 2119712	559	100	70110.0
	HSPA1A protein (homo sapiens)	gi 14414588	558	100	70294.1
	Heat shock 10 kDa protein 1-like (homo sapiens)	gi 55961919	372	100	70730.5
	Heat shock 70 kDa protein 1-like (homo sapiens)	gi 21759781	361	100	70748.4
	Heat shock 70 kDa protein 1B (homo sapiens)	gi 55962554	296	100	52199.8
S3	Chaperonin 60 (Hsp60) (homo sapiens)	gi 6996447	375	100	61187.4
S4	ER-60 protein (homo sapiens)	gi 2245365	223	100	57146.9
	Glucose regulated protein 58 kDa (Bos taurus)	gi 27805905	113	100	57293.0
S5	Ribosomal protein SA (laminin receptor 1) (homo sapiens)	gi 47125390	204	100	32933.5
S6	PREDICTED: Similar to epsilon isoform of 14-3-3 protein	gi 57091321	53	37.851	29326.5
S7	Heat shock 70 kDa protein 1B (homo sapiens)	gi 55962554	67	97.468	52199.8

4B), which implied that there was no direct interaction between PDX1 and HSP70 proteins.

DISCUSSION

Gastric cancer is a leading cause of cancer-associated mortality across the world[20]. Despite years of extensive research, the molecular mechanisms involved in the pathogenesis of gastric cancer are not completely understood. Recent advances in proteomics display its great potential for use in cancer diagnosis, prognostic assessment, and to understand the molecular mechanism of carcinogenesis. Proteomic approaches have been widely applied in research on gastric cancer[21-23]. Liu *et al*[24] used 2D gel-based proteomics to compare the differential serum protein profiles between gastric cancer patients and healthy controls, and identified several serum biomarkers for gastric cancer. Poon *et al*[22] also demonstrated that an exclusive serum proteomic fingerprint, identified by SELDI-based proteomics, can be used for noninvasive diagnosis of gastric cancer. In addition, many prognostic biomarkers of gastric cancer were identified by proteomic approaches, such as S100P[25] and S100A9[25] proteins. Chen *et al*[26] utilized a quantitative proteomic technique to identify the differentially expressed proteins in metastatic gastric cancer cells as compared to that in noninvasive gastric cancer cells. After downstream validation, they found that vimentin and galectin-1 were potential markers of gastric cancer metastasis and suggested their involvement in cell-cell and cell-ECM adhesion interactions.

PDX1 is a member of the homeobox family and plays an important role in the development of embryonic digestive system. A previous study demonstrated that PDX1 knockout mice showed abnormal growth of gastroduodenal junction, which increased the difficulty in emptying gastric contents and ultimately led to gastric retention[27]. Aberrant expression of the *PDX1* gene has been associated with carcinogenesis. PDX1 was shown to function as a tumor promoter by enhancing the proliferation, invasion[28], and induction of acinar-to-ductal metaplasia[29] in pancreatic cancer. Hence, there is a growing interest to develop a novel therapy for pancreatic cancer by targeting PDX1. Interestingly, PDX1 was also found to be associated with the tumorigenesis of gastric cancer. Both Faller *et al*[30] and Sakai *et al*[31] have reported abnormal expression of PDX1 protein in pseudo-pyloric glandular metaplasia. In a previous study, we found that PDX1 protein was downregulated in *Helicobacter pylori* infection, incisural antralisation, and intestinal metaplasia[32]. We further identified significantly decreased *PDX1* mRNA and PDX1 protein expression in gastric cancer cells. Downregulation of PDX1 in gastric cancer tissues might be caused by promoter hypermethylation and histone hypoacetylation[9]. We also observed that the transient overexpression of the *PDX1* gene could inhibit proliferation and induce apoptosis of gastric cancer cells. Moreover, stable overexpression of the *PDX1* gene suppressed colony formation, wound healing, migration of gastric cancer cells, and decreased tumor incidence in nude mice; these findings imply that PDX1 may act as a tumor suppressor in the context of gastric cancer[8].

In the present study, we sought to clarify the downstream mediators of PDX1-mediated effects on gastric cancer tumorigenesis. We used 2D gel-based proteomics to identify differentially expressed proteins between PDX1-overexpressed gastric cancer cells and the control empty vector transfected gastric cells. Seven differentially expressed proteins were identified by proteomic analysis, which included three heat shock proteins. The three HSPs proteins, HSP70, HSP60, and HSP27, were significantly decreased in gastric cells after *PDX1* overexpression. The results of immunoblotting analysis were consistent with those of proteomics analysis. However, the mRNA levels of *HSP70*, *HSP60*, and *HSP27* were significantly increased after *PDX1* overexpression. These results indicated that overexpression of PDX1

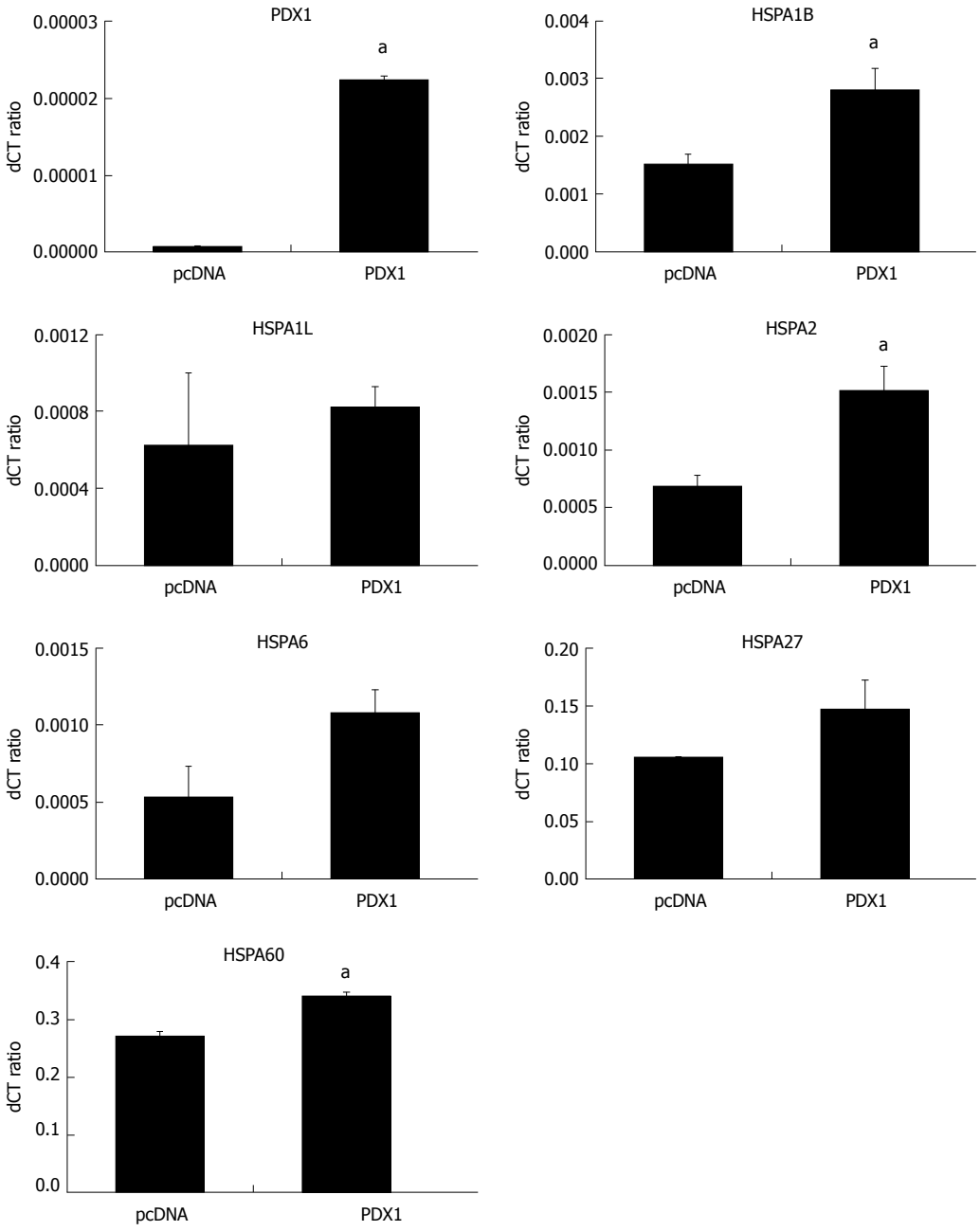


Figure 3 Comparisons of mRNA levels of the heat shock proteins in SGC7901 with pcDNA3.1(+)-pancreatic-duodenal homeobox-1 (PDX1) vector and SGC7901-pcDNA cells. The mRNA expressions of HSPs in SGC-PDX1 and SGC-pcDNA cells were compared by qRT-PCR. The relative mRNA levels of two of HSP70 isoforms (*HSPA1B and HSPA2*) and *HSP60* were significantly increased in the SGC-PDX1 group, while no significant differences were identified for the other HSP genes. ($^{a}P < 0.05$). HSPs: Heat shock proteins.

could downregulate HSP70, HSP60, and HSP27 proteins through post-translational regulation pathways, such as mRNA degradation, glycosylation, degradation, or phosphorylation.

HSPs are a group of chaperone proteins that play dual roles in tumorigenesis[33]. HSP70, HSP60, and HSP27 proteins were reported to be upregulated in gastric cancer and associated with tumor progression and poor prognosis[34-36]. Our immunoblotting analysis revealed that HSP70 proteins showed the greatest differential expression in PDX1-overexpressed gastric cells, which emphasized its significance in PDX1 mediated effects on gastric cancer tumorigenesis. Besides, HSP70 protein was shown to serve as a tumor promoter in gastric cancer by reducing apoptosis[37,38]. Therefore, PDX1 might affect the apoptosis of gastric cancer cells by regulating the expression of HSP70. To further evaluate the interaction between PDX1 and HSP70, we performed a co-immunoprecipitation assay. However, no direct interactions could be found between the two proteins, which indicates that there may be some intermediate mediators that link PDX1 and HSP70 proteins in the regulation of gastric cancer tumorigenesis. Hence, further studies are warranted to elucidate the relationship

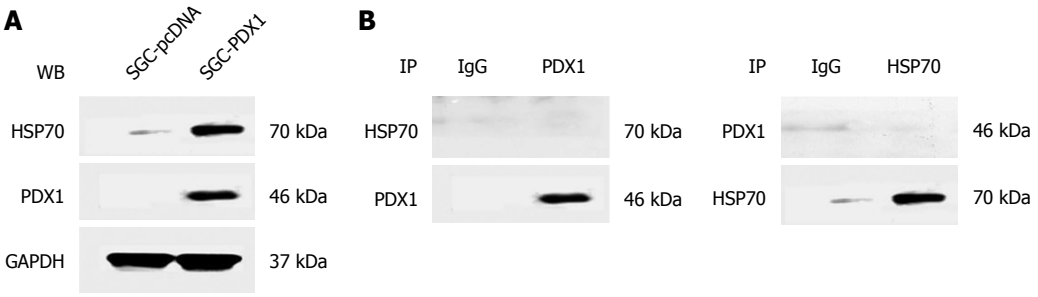


Figure 4 Results of co-immunoprecipitation analysis showing no direct interaction of pancreatic-duodenal homeobox-1 (PDX1) and HSP70 in SGC7901 with pcDNA3.1(+)-PDX1 cells. A: Both PDX1 and HSP70 proteins were detected in the whole cell lysate proteins of co-transfected SGC7901 cells, ensuring the input of PDX1 and HSP70 proteins in the co-immunoprecipitation experiment; B: The immunoprecipitated proteins obtained by precipitating antibodies against PDX1 or HSP70 were immunoblotted by HSP70 or PDX1, respectively. No direct interactions between PDX1 and HSP70 were detected.

between PDX1 and HSP70, along with a detailed mechanism regarding their collaboration to regulate gastric tumorigenesis.

In conclusion, our study showed that proteomics is a powerful tool to study the molecular mechanisms involved in the genesis of gastric cancer. In addition, the inhibition of PDX1 in gastric cancers may contribute to the upregulation of HSPs, especially HSP70.

ARTICLE HIGHLIGHTS

Research background

As one of the homeobox genes that play critical roles in the pattern formation of embryos, pancreatic-duodenal homeobox-1 (PDX1) is widely expressed in Brunner's glands of the duodenum, pancreatic β cells, and gastric pyloric gland cells. PDX1 plays a key role in the development of the digestive system, including antrum, duodenum, and pancreas. Downregulation of PDX1 has been observed in gastric cancer, which suggests its potential role in gastric tumorigenesis. Nevertheless, the downstream mechanisms that mediate the effect of PDX1 on gastric tumorigenesis are still poorly understood.

Research motivation

Although PDX1 has been found to be involved in gastric tumorigenesis, its downstream regulating mechanism is still unclear.

Research objectives

To clarify the differential protein profile in PDX1-overexpressed gastric cell line and explore functional proteins involved in PDX1-mediated effects on gastric tumorigenesis.

Research methods

A PDX1-overexpressed model was established using gastric cancer cell line SGC7901 (SGC-PDX1). As a method that is still widely used to identify cancer-associated proteins, 2DE-based proteomics was applied to determine the differential protein profile between SGC-PDX1 and SGC-pcDNA. The differential proteins were then subjected to qRT-PCR and immunoblotting for further confirmation. Finally, direct interactions between PDX1 and the identified differential proteins were evaluated by co-immunoprecipitation.

Research results

Seven proteins were found to be differentially expressed in SGC-PDX1 using 2-DE proteomics. Immunoblotting confirmed that the three differential HSPs (HSP70, HSP60, HSP27) were downregulated in SGC-PDX1. However, qRT-PCR analysis identified increased *HSP* mRNA in SGC-PDX1, which indicates that PDX1 may post-translationally regulate the expression of the *HSP*s. Further study is warranted to elucidate the relationship of HPS70 and PDX1, as co-immunoprecipitation did not identify direct interaction between them.

Research conclusions

HSPs are involved in PDX1-mediated effects on the genesis of gastric cancer and the interaction between HSP70 and PDX1 is indirect.

Research perspectives

This study demonstrates the involvement of HSPs in PDX1-mediated effects on gastric tumorigenesis. Further study is warranted to elucidate the downstream regulating mechanism of PDX1 on HSPs.

ACKNOWLEDGMENTS

HSP70 plasmid was kindly provided by Dr. BCY Wong, Hong Kong University, Hong Kong.

REFERENCES

1 **Melton DA**. Pattern formation during animal development. *Science* 1991; **252**: 234-241 [PMID: 1672778 DOI: 10.1126/science.1672778]
2 **Alappat S**, Zhang ZY, Chen YP. Msx homeobox gene family and craniofacial development. *Cell Res* 2003; **13**: 429-442 [PMID: 14728799 DOI: 10.1038/sj.cr.7290185]
3 **Brooke NM**, Garcia-Fernàndez J, Holland PW. The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. *Nature* 1998; **392**: 920-922 [PMID: 9582071 DOI: 10.1038/31933]
4 **Larsson LI**, Madsen OD, Serup P, Jonsson J, Edlund H. Pancreatic-duodenal homeobox 1 -role in gastric endocrine patterning. *Mech Dev* 1996; **60**: 175-184 [PMID: 9025070 DOI: 10.1016/S0925-4773(96)00609-0]
5 **Offield MF**, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996; **122**: 983-995 [PMID: 8631275]
6 **Edlund H**. Pancreatic organogenesis--developmental mechanisms and implications for therapy. *Nat Rev Genet* 2002; **3**: 524-532 [PMID: 12094230 DOI: 10.1038/nrg841]
7 **Liu T**, Gou SM, Wang CY, Wu HS, Xiong JX, Zhou F. Pancreas duodenal homeobox-1 expression and significance in pancreatic cancer. *World J Gastroenterol* 2007; **13**: 2615-2618 [PMID: 17552012 DOI: 10.3748/wjg.v13.i18.2615]
8 **Ma J**, Chen M, Wang J, Xia HH, Zhu S, Liang Y, Gu Q, Qiao L, Dai Y, Zou B, Li Z, Zhang Y, Lan H, Wong BC. Pancreatic duodenal homeobox-1 (PDX1) functions as a tumor suppressor in gastric cancer. *Carcinogenesis* 2008; **29**: 1327-1333 [PMID: 18477649 DOI: 10.1093/carcin/bgn112]
9 **Ma J**, Wang JD, Zhang WJ, Zou B, Chen WJ, Lam CS, Chen MH, Pang R, Tan VP, Hung IF, Lan HY, Wang QY, Wong BC. Promoter hypermethylation and histone hypoacetylation contribute to pancreatic-duodenal homeobox 1 silencing in gastric cancer. *Carcinogenesis* 2010; **31**: 1552-1560 [PMID: 20622005 DOI: 10.1093/carcin/bgq140]
10 **Hu HD**, Ye F, Zhang DZ, Hu P, Ren H, Li SL. iTRAQ quantitative

analysis of multidrug resistance mechanisms in human gastric cancer cells. *J Biomed Biotechnol* 2010; **2010**: 571343 [PMID: 20625496 DOI: 10.1155/2010/571343]
11 **Lin LL**, Huang HC, Juan HF. Discovery of biomarkers for gastric cancer: a proteomics approach. *J Proteomics* 2012; **75**: 3081-3097 [PMID: 22498886 DOI: 10.1016/j.jprot.2012.03.046]
12 **Chen J**, Kähne T, Röcken C, Götze T, Yu J, Sung JJ, Chen M, Hu P, Malfertheiner P, Ebert MP. Proteome analysis of gastric cancer metastasis by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of metastasis-related proteins. *J Proteome Res* 2004; **3**: 1009-1016 [PMID: 15473690 DOI: 10.1021/pr049916l]
13 **Gygi SP**, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci USA* 2000; **97**: 9390-9395 [PMID: 10920198 DOI: 10.1073/pnas.160270797]
14 **Rogowska-Wrzesinska A**, Le Bihan MC, Thaysen-Andersen M, Roepstorff P. 2D gels still have a niche in proteomics. *J Proteomics* 2013; **88**: 4-13 [PMID: 23353020 DOI: 10.1016/j.jprot.2013.01.010]
15 **Ma J**, Liu QH, Sha WH, Wang QY. The construction and expression of the PDX1 vector (in Chinese). *Guangdong Medical Journal* 2010; **31**: 3030-3031
16 **Görg A**, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 2004; **4**: 3665-3685 [PMID: 15543535 DOI: 10.1002/pmic.200401031]
17 **Arata S**, Hamaguchi S, Nose K. Inhibition of colony formation of NIH 3T3 cells by the expression of the small molecular weight heat shock protein HSP27: involvement of its phosphorylation and aggregation at the C-terminal region. *J Cell Physiol* 1997; **170**: 19-26 [PMID: 9012781 DOI: 10.1002/(SICI)1097-4652(199701)170:1<19::AID-JCP3>3.0.CO;2-O]
18 **Yang Y**, Rao R, Shen J, Tang Y, Fiskus W, Nechtman J, Atadja P, Bhalla K. Role of acetylation and extracellular location of heat shock protein 90alpha in tumor cell invasion. *Cancer Res* 2008; **68**: 4833-4842 [PMID: 18559531 DOI: 10.1158/0008-5472.CAN-08-0644]
19 **Park JW**, Yeh MW, Wong MG, Lobo M, Hyun WC, Duh QY, Clark OH. The heat shock protein 90-binding geldanamycin inhibits cancer cell proliferation, down-regulates oncoproteins, and inhibits epidermal growth factor-induced invasion in thyroid cancer cell lines. *J Clin Endocrinol Metab* 2003; **88**: 3346-3353 [PMID: 12843186 DOI: 10.1210/jc.2002-020340]
20 **Wadhwa R**, Song S, Lee JS, Yao Y, Wei Q, Ajani JA. Gastric cancer-molecular and clinical dimensions. *Nat Rev Clin Oncol* 2013; **10**: 643-655 [PMID: 24061039 DOI: 10.1038/nrclinonc.2013.170]
21 **Ryu JW**, Kim HJ, Lee YS, Myong NH, Hwang CH, Lee GS, Yom HC. The proteomics approach to find biomarkers in gastric cancer. *J Korean Med Sci* 2003; **18**: 505-509 [PMID: 12923326 DOI: 10.3346/jkms.2003.18.4.505]
22 **Poon TC**, Sung JJ, Chow SM, Ng EK, Yu AC, Chu ES, Hui AM, Leung WK. Diagnosis of gastric cancer by serum proteomic fingerprinting. *Gastroenterology* 2006; **130**: 1858-1864 [PMID: 16697748 DOI: 10.1053/j.gastro.2006.02.011]
23 **Bai Z**, Ye Y, Liang B, Xu F, Zhang H, Zhang Y, Peng J, Shen D, Cui Z, Zhang Z, Wang S. Proteomics-based identification of a group of apoptosis-related proteins and biomarkers in gastric cancer. *Int J Oncol* 2011; **38**: 375-383 [PMID: 21165559 DOI: 10.3892/ijo.2010.873]
24 **Liu W**, Liu B, Cai Q, Li J, Chen X, Zhu Z. Proteomic identification of serum biomarkers for gastric cancer using multi-dimensional liquid chromatography and 2D differential gel electrophoresis. *Clin Chim Acta* 2012; **413**: 1098-1106 [PMID: 22446497 DOI: 10.1016/j.cca.2012.03.003]
25 **Jia SQ**, Niu ZJ, Zhang LH, Zhong XY, Shi T, Du H, Zhang GG, Hu Y, Su XL, Ji JF. Identification of prognosis-related proteins in advanced gastric cancer by mass spectrometry-based comparative proteomics. *J Cancer Res Clin Oncol* 2009; **135**: 403-411 [PMID: 18830628 DOI: 10.1007/s00432-008-0474-3]
26 **Chen YR**, Juan HF, Huang HC, Huang HH, Lee YJ, Liao MY, Tseng CW, Lin LL, Chen JY, Wang MJ, Chen JH, Chen YJ. Quantitative proteomic and genomic profiling reveals metastasis-related protein expression patterns in gastric cancer cells. *J Proteome Res* 2006; **5**: 2727-2742 [PMID: 17022644 DOI: 10.1021/pr060212g]
27 **Gu G**, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* 2002; **129**: 2447-2457 [PMID: 11973276]
28 **Liu S**, Ballian N, Belaguli NS, Patel S, Li M, Templeton NS, Gingras MC, Gibbs R, Fisher W, Brunicardi FC. PDX-1 acts as a potential molecular target for treatment of human pancreatic cancer. *Pancreas* 2008; **37**: 210-220 [PMID: 18665085 DOI: 10.1097/MPA.0b013e31816a4a33]
29 **Miyatsuka T**, Kaneto H, Shiraiwa T, Matsuoka TA, Yamamoto K, Kato K, Nakamura Y, Akira S, Takeda K, Kajimoto Y, Yamasaki Y, Sandgren EP, Kawaguchi Y, Wright CV, Fujitani Y. Persistent expression of PDX-1 in the pancreas causes acinar-to-ductal metaplasia through Stat3 activation. *Genes Dev* 2006; **20**: 1435-1440 [PMID: 16751181 DOI: 10.1101/gad.1412806]
30 **Faller G**, Kirchner T. Immunological and morphogenic basis of gastric mucosa atrophy and metaplasia. *Virchows Arch* 2005; **446**: 1-9 [PMID: 15583929 DOI: 10.1007/s00428-004-1157-3]
31 **Sakai H**, Eishi Y, Li XL, Akiyama Y, Miyake S, Takizawa T, Konishi N, Tatematsu M, Koike M, Yuasa Y. PDX1 homeobox protein expression in pseudopyloric glands and gastric carcinomas. *Gut* 2004; **53**: 323-330 [PMID: 14960508 DOI: 10.1136/gut.2003.026609]
32 **Zhu S**, Xia HH, Yang Y, Ma J, Chen M, Hu P, Gu Q, Liang Y, Lin H, Wong BC. Alterations of gastric homeoprotein expression in Helicobacter pylori infection, incisural antralisation, and intestinal metaplasia. *Dig Dis Sci* 2009; **54**: 996-1002 [PMID: 18754095 DOI: 10.1007/s10620-008-0459-8]
33 **Calderwood SK**, Khaleque MA, Sawyer DB, Ciocca DR. Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem Sci* 2006; **31**: 164-172 [PMID: 16483782 DOI: 10.1016/j.tibs.2006.01.006]
34 **Li XS**, Xu Q, Fu XY, Luo WS. Heat shock protein 60 overexpression is associated with the progression and prognosis in gastric cancer. *PLoS One* 2014; **9**: e107507 [PMID: 25207654 DOI: 10.1371/journal.pone.0107507]
35 **Ge H**, He X, Guo L, Yang X. Clinicopathological significance of HSP27 in gastric cancer: a meta-analysis. *Onco Targets Ther* 2017; **10**: 4543-4551 [PMID: 28979146 DOI: 10.2147/OTT.S146590]
36 **Bodoor K**, Jalboush SA, Matalka I, Abu-Sheikha A, Waq RA, Ebwaini H, Abu-Awad A, Fayyad L, Al-Arjat J, Haddad Y. Heat Shock Protein Association with Clinico-Pathological Characteristics of Gastric Cancer in Jordan : HSP70 is Predictive of Poor Prognosis. *Asian Pac J Cancer Prev* 2016; **17**: 3929-3937 [PMID: 27644641]
37 **Xiang TX**, Li Y, Jiang Z, Huang AL, Luo C, Zhan B, Wang PL, Tao XH. RNA interference-mediated silencing of the Hsp70 gene inhibits human gastric cancer cell growth and induces apoptosis in vitro and in vivo. *Tumori* 2008; **94**: 539-550 [PMID: 18822691 DOI: 10.1177/030089160809400416]
38 **Arora N**, Alsaied O, Dauer P, Majumder K, Modi S, Giri B, Dudeja V, Banerjee S, Von Hoff D, Saluja A. Downregulation of Sp1 by Minnelide leads to decrease in HSP70 and decrease in tumor burden of gastric cancer. *PLoS One* 2017; **12**: e0171827 [PMID: 28192510 DOI: 10.1371/journal.pone.0171827]

P- Reviewer: Aurello P, Christodoulidis G, Matowicka-Karna J
S- Editor: Wang XJ **L- Editor**: Filipodia **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4272-4280

DOI: 10.3748/wjg.v24.i37.4272

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

ORIGINAL ARTICLE

Case Control Study

Evaluation of elastography combined with serological indexes for hepatic fibrosis in patients with chronic hepatitis B

Bin Xu, Ning-Ming Zhou, Wei-Tian Cao, Xiao-Jing Li

Bin Xu, Ning-Ming Zhou, Wei-Tian Cao, Department of ultrasound, Fudan University affiliated Shanghai fifth people's hospital, Shanghai 200240, China

Xiao-Jing Li, Department of pathology, Fudan University affiliated Shanghai fifth people's hospital, Shanghai 200240, China

ORCID number: Bin Xu (0000-0002-9184-107X); Ning-Ming Zhou (0000-0001-8424-7825); Wei-Tian Cao (0000-0002-4880-7589); Xiao-Jing Li (0000-0001-6370-9146).

Author contributions: Xu B designed research; Xu B, Cao WT, Li XJ performed research; Zhou NM contributed new reagents or analytic tools; Li XJ analyzed data; Xu B wrote the paper.

Supported by the Natural Science Research Project of Minhang District, No. 2013MHZ003.

Institutional review board statement: The study was approved by the ethics committee of Fudan University affiliated Shanghai fifth people's hospital.

Informed consent statement: All patients gave informed consent.

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Data sharing statement: Data are available from the corresponding author at zhouningming@5thhospital.com.

STROBE Statement: The STROBE Statement have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Ning-Ming Zhou, MD, Chief Doctor, Department of ultrasound, Fudan University affiliated Shanghai fifth people's hospital, 128 Ruili road, Minhang district, Shanghai 200240, China. zhouningming@5thhospital.com
Telephone: +86-21-24289356
Fax: +86-21-24289356

Received: June 22, 2018
Peer-review started: June 22, 2018
First decision: July 25, 2018
Revised: August 6, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

AIM

To investigate the value of ultrasound elastography combined with serological indexes in diagnosing liver fibrosis and assessing its severity.

METHODS

A total of 338 chronic hepatitis B (CHB) patients were divided into a disease group (patients with hepatic fibrosis) and control group (subjects without hepatic fibrosis). The disease group was further divided into S1-S4 according to the degree of fibrosis. Independent risk factors for hepatic fibrosis were analyzed using multivariate logistic regression. The diagnostic values of hepatic fibrosis from different indicators were compared using receiver operating characteristic (ROC) curves. The combination of elastography and serological indexes was explored to assess the severity of hepatic fibrosis.

RESULTS

The multivariate logistic regression analysis results revealed that shear wave velocity (SWV), hyaluronic acid (HA), type Ⅳ collagen (CⅣ) and aspartate aminotransferase-to-platelet ratio index (APRI) significantly affected the occurrence of hepatic fibrosis. The ROC curve revealed that the accuracy of the diagnosis of hepatic fibrosis for SWV and HA were 87.3% and 84.8%, respectively. The accuracy of SWV combined with HA was 88.9%. The multiple linear regression analysis revealed that SWV, aspartate aminotransferase (AST)/alanine aminotransferase (ALT), HA, CⅣ, APRI and fibrosis index based on the 4 factor (FIB-4) were screened as statistically significant independent factors. The established regression equation was: Fibrosis level = -4.046 + 1.024 × SWV + 1.170 × AST/ALT + 0.011 × HA + 0.020 × CⅣ + 0.719 × APRI + 0.379 × FIB-4.

CONCLUSION

SWV combined with serological indexes can improve the accuracy of diagnosis for CHB hepatic fibrosis. Serum indexes can help diagnose the degree of hepatic fibrosis.

Key words: Elastography; Serology; Hepatic fibrosis; Non-invasive diagnosis

Core tip: Hepatic fibrosis affects the physiological function of the liver. The current assessment method for the degree of hepatic fibrosis is still unreliable. This study found that the shear wave velocity of ultrasound elastography can improve the accuracy of the diagnosis of hepatic fibrosis. Its combination with serological indicators (aspartate aminotransferase/alanine aminotransferase, hyaluronic acid, type Ⅳ collagen, aspartate aminotransferase-to-platelet ratio index and fibrosis index based on the 4 factor) can further help in the clinical assessment of the degree of hepatic fibrosis.

Xu B, Zhou NM, Cao WT, Li XJ. Evaluation of elastography combined with serological indexes for hepatic fibrosis in patients with chronic hepatitis B. *World J Gastroenterol* 2018; 24(37): 4272-4280 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4272.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4272

INTRODUCTION

Hepatic fibrosis is a pathological change caused by chronic liver injury, which in turn affects the physiological function of the liver[1-4]. Pathological examination is the gold standard for the diagnosis of hepatic fibrosis, which enables a definitive diagnosis of hepatic fibrosis[5-8]. However, pathological examination mainly relies on biopsy. Biopsy is a kind of invasive examination with the drawbacks of poor reproducibility and sampling errors. Therefore, non-invasive diagnostic methods that seek repeatable measurements have presently become research hotspots. At present, serological indexes are the main clinical methods to assess hepatic fibrosis, although the accuracy needs to be improved[9-11]. The latest research has shown that ultrasound elastography can measure the hardness of liver tissue to determine the degree of hepatic fibrosis with features of non-invasiveness, simplicity, speed and repeatability[12,13]. However, its diagnostic accuracy is not high, and the accuracy of different studies are different[14,15]. Hence, we still need to explore the diagnostic methods of hepatic fibrosis, as well as search for a reliable method to assess the degree of hepatic fibrosis. The investigators therefore collected patients who were admitted to our hospital with chronic hepatitis B (CHB) as subjects in the present study. Their final pathological results were used as a basis for the diagnosis of hepatic fibrosis, and the serological indexes and ultrasound elastography data of these patients were analyzed. The aim of the present study was to search for an optimal method for the combined diagnosis of hepatic fibrosis, and establish an optimal non-invasive assessment model for the severity of hepatic fibrosis.

MATERIALS AND METHODS

Research object

A total of 338 CHB patients were randomly enrolled in our hospital from January 2015 to June 2017. Among these patients, 200 patients were male and 138 patients were female. Inclusion criteria: (1) Patients who underwent liver biopsy; and (2) patients who received ultrasound elastography and serological detection before the biopsy. Exclusion criteria: (1) Patients combined with other types of liver disease; (2) patients with severe heart, liver and kidney insufficiency, coma, or puncture site infection; and (3) patients associated with liver cancer, immune system disease, or active bleeding and other diseases. These patients were divided into two groups according to the presence of hepatic fibrosis *via* biopsy: disease group (patients with hepatic fibrosis) and control group (subjects without hepatic fibrosis). The disease group was further divided into four subgroups, according to the degree of fibrosis: S1, S2, S3 and S4. All patients or their families provided a signed informed consent. The present study met the requirements of the hospital ethics committee and received their approval.

Research methods

Detection of hepatitis B hepatic fibrosis *via* acoustic radiation force impulse: The acoustic radiation force impulse (ARFI) test was performed using an ACOUSON S2000 Color Ultrasound Scanner (Siemens). (1) The patient underwent fasting and was placed in the left lateral decubitus position, with the right-hand on the head. Then, the right hepatic tissue of the liver was detected; (2) the elastic sampling frame was placed perpendicular to the

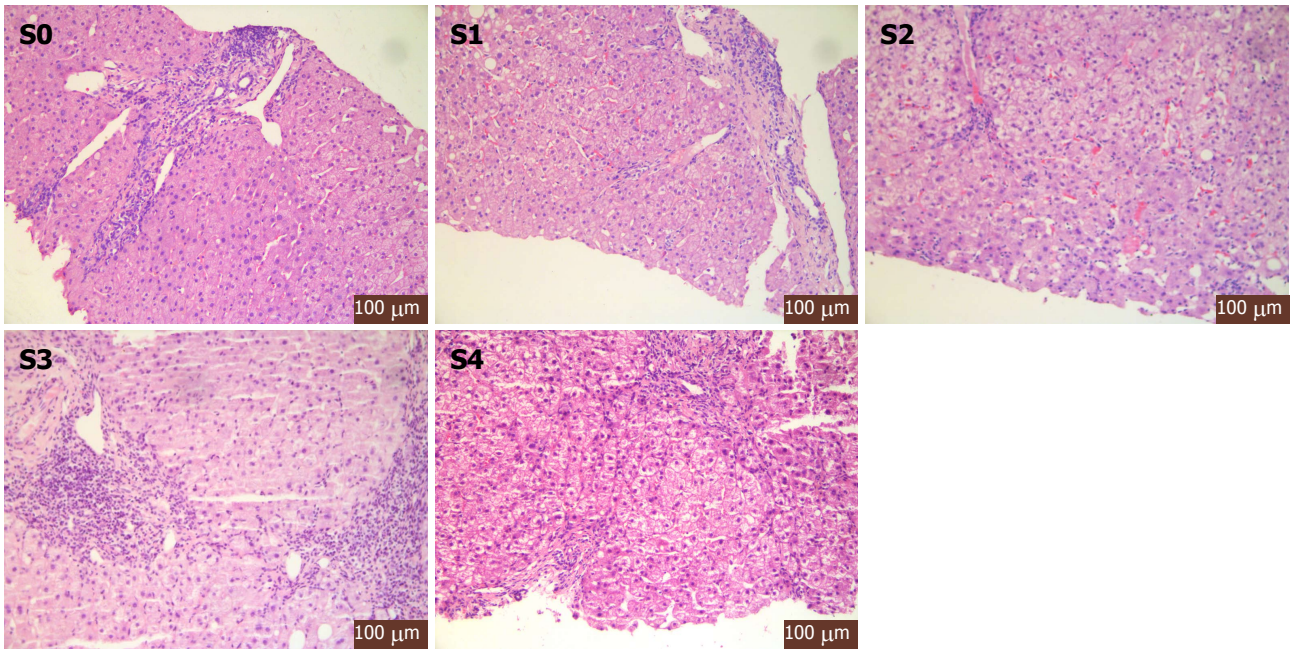


Figure 1 Histopathology of different pathological stages of hepatic fibrosis. S0 phase: No fibrosis; S1 phase: Fibrous enlargement in the portal area, but no fibrillary septum formation; S2 phase: Fibrosis enlargement in the portal area, a few fibrous septae formed; S3 stage: Most fibrous septae formed but no hardened nodules; S4 stage: Liver cirrhosis.

liver surface, and then placed in the liver parenchyma at approximately 4 cm away from the probe surface in order to avoid the surrounding blood vessels. The patient was instructed to hold their breath; and (3) the update key was pressed, a high-intensity low-frequency pulse wave was launched, the transverse shear wave velocity (SWV) was received in m/s, and the value was recorded. The measurement was repeated three times, and the SWV value was taken as the SWV value of the liver parenchyma.

Serological examination: On the next day of admission, 3 mL of fasting venous blood was collected in the morning. Alanine aminotransferase (ALT), aspartate transaminase (AST), blood platelet (PLT), total bilirubin (TBIL), hyaluronic acid (HA) levels, laminin (LN), type Ⅳ collagen (CⅣ), and type Ⅲ procollagen (PⅢNP) were measured. Serum ALT, AST, PLT and TBIL were detected using an automatic biochemical analyzer, while HA, LN, CⅣ and PⅢNP were measured by photochemiluminescence. APRI score: the AST and platelet (PLT) ratio index (aspartate aminotransferase-to-platelet ratio index, APRI). APRI = [(AST/ULN) × 100/PLT (10^9/L)][16]. FIB-4 index: FIB-4 = (age × AST) ÷ (platelet ×√ALT).

Liver biopsy: Liver tissue biopsies were simultaneously performed with ultrasound elastography and serological tests. The subjects were placed in the supine position, the preoperative ultrasound was localized, and the liver puncture was performed under ultrasound guidance. The puncture gun was an automatic biopsy gun obtained from Bard Inc. (United States), with a 16 G disposable biopsy. The needle biopsy was performed in the ARFI sampling frame area. Liver biopsy was conducted with routine disinfection, which was covered with a towel, and local anesthesia with 5% lidocaine was given to avoid the visible pipeline in the liver. A tissue length of 1-2 cm was removed. The degree of hepatic fibrosis in patients with CHB was determined based on histological staging criteria, according to the "Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2015 Update version)". In particular, S0 phase refers to patients with no fibrosis, the S1 phase refers to patients with enlarged fibrosis in the portal area but no fibril septum formation, the S2 phase refers to patient with a fibrous enlargement in the portal area and minimal fibrillary septae formation, the S3 phase refers to patients with the most fibrillary septae formed but without hardened nodules, and the S4 phase refers to patients with cirrhosis (Figure 1).

Statistical analysis

Statistical analysis was performed using SPSS 19.0 and MedCalc software. Measurement data were expressed as mean ± SD. The *t*-test was used for comparisons between the two groups. The rate of adoption of count data was expressed using a Chi-square test to compare the two groups. Independent risk factors of fibrotic liver were analyzed by multivariate logistic regression analysis, while ROC curve analysis was conducted to determine the accuracy in the diagnosis of hepatic fibrosis. Spearman correlation analysis was used to compare the degree of hepatic fibrosis with serological markers and elastography. Multiple linear regression was used to establish a hepatic fibrosis assessment model and determine its degree of fit. $P < 0.05$ was considered

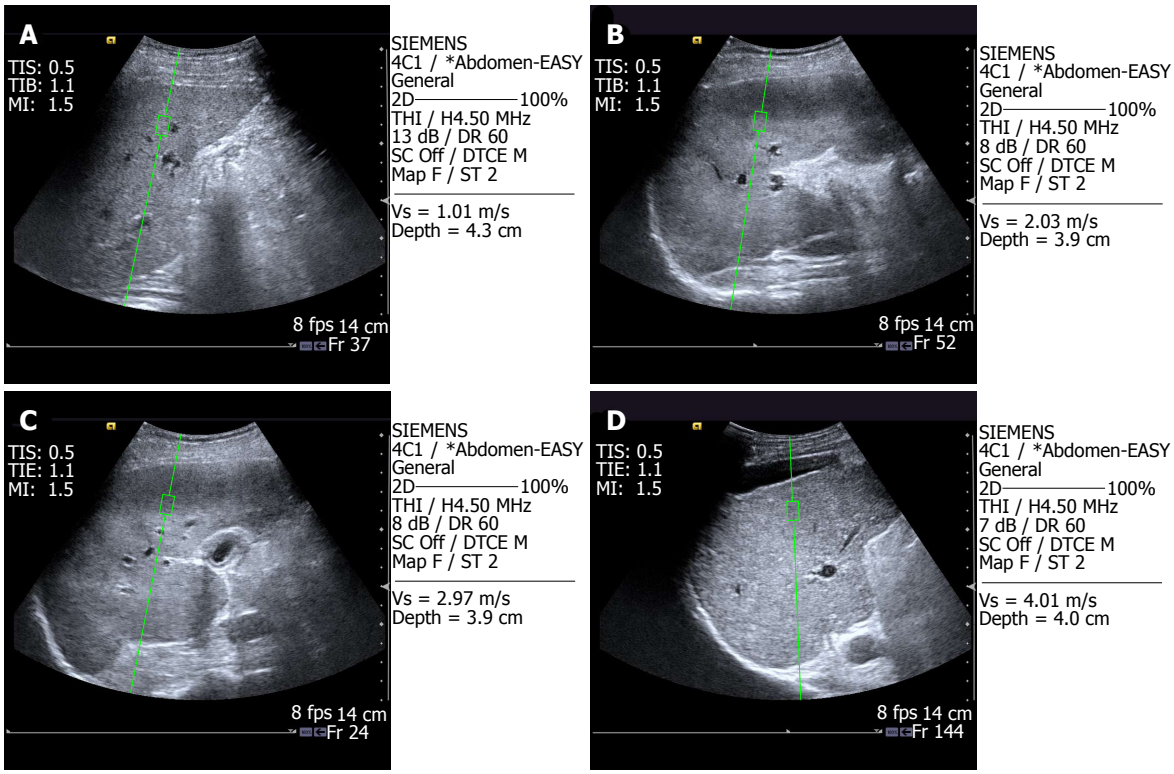


Figure 2 Image of hepatic fibrosis assessed by acoustic radiation force impulse to assess liver tissue elasticity. A: Normal liver tissue, SWV = 1.01 m/s; B: Mild hepatic fibrosis, SWV = 2.03 m/s; C: Moderate hepatic fibrosis, SWV = 2.97 m/s; D: Severe hepatic fibrosis, SWV = 4.01m/s. ARFI: Acoustic radiation force impulse.

Table 1 Comparison of clinical data in the two groups

	Hepatic fibrosis group (n = 245)	Control group (n = 93)	t/χ^2 value	P value
Sex (male/%)	136/55.51	45/48.39	1.375	0.241
Age	40.31 ± 10.47	37.82 ± 13.72	1.785	0.075
SWV (m/s)	3.02 ± 0.80	1.52 ± 0.62	16.310	0.000
ALT (U/L)	52.23 ± 26.02	46.87 ± 87.45	1.487	0.138
AST (U/L)	41.12 ± 12.72	36.14 ± 14.52	1.607	0.109
AST/ALT	0.93 ± 0.41	0.74 ± 0.23	4.221	0.000
PLT (10^9/L)	184.02 ± 02.21	192.37 ± 37.42	1.220	0.223
TBIL (μmol/L)	19.83 ± 9.83	18.75 ± 8.72	1.570	0.117
HA (μg/L)	113.24 ± 24.78	63.23 ± 23.54	15.748	0.000
LN (μg/L)	36.21 ± 21.34	34.21 ± 10.28	1.485	0.139
CⅣ (μg/L)	33.28 ± 28.20	20.89 ± 9.56	4.144	0.000
PⅢNP (μg/L)	36.29 ± 29.45	32.87 ± 2.32	1.100	0.272
APRI	0.85 ± 0.61	0.62 ± 0.52	3.219	0.001
FIB-4	1.63 ± 0.89	1.17 ± 0.62	4.578	0.000

SWV: Shear wave velocity; ALT: Alanine aminotransferase; AST: Aspartate transaminase; PLT: Blood platelet; TBIL: Total bilirubin; HA: Hyaluronic acid; LN: Laminin; CⅣ: Type Ⅳ collagen; PⅢNP: Type Ⅲ procollagen; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis index based on the 4 factor.

statistically significant.

RESULTS

Comparison of clinical data

A total of 338 patients were enrolled in the present study. Among these patients, 93 subjects were assigned to the control group, while 245 patients were assigned to the disease group. Among the patients in the disease group, 72 patients were in the S1 phase, 65 patients were in the S2 phase, 58 patients were in the S3 phase, and 50 patients were in the S4 phase. Furthermore, among the 245 patients in the disease group, 62 patients had mild hepatic fibrosis, 176 patients had moderate hepatic fibrosis, and seven patients had severe hepatic fibrosis (Figure 2). The serological indexes, such as AST/ALT, HA, CⅣ, APRI and FIB-4, were significantly greater in the disease group than in the control group, and the differences were statistically significant (P < 0.05). For the elastography, SWV was significantly greater in the disease group than in the control group, and the difference was statistically significant (P < 0.05). The remaining indicators were similar between the two groups, and the difference was not statistically significant (P > 0.05) (Table 1).

Multivariate analysis of hepatic fibrosis

Indicators with significant differences (SWV, AST/ALT, HA, CⅣ, APRI and FIB-4) were used as independent variables. The occurrence of fibrosis was a dependent variable, and a multivariate logistic regression analysis was conducted. These results revealed that SWV, HA, CⅣ and APRI had a significant effect on hepatic fibrosis (P < 0.05). According to the OR value, the sequence was SWV, HA, APRI and CⅣ (Table 2).

Diagnosis of different indicators in hepatic fibrosis

The ROC curve for the diagnosis of hepatic fibrosis by

Table 2 Binary logistic regression analysis of risk factors associated with hepatic fibrosis

Influencing factors	B	SE	Wald	OR	95%CI	*P* value
SWV	0.931	0.325	5.024	2.537	1.342-4.797	0.025
AST/ALT	0.561	0.286	2.765	1.752	0.896-3.069	0.056
HA	0.838	0.127	5.352	2.311	1.802-2.964	0.021
CⅣ	0.466	0.183	4.042	1.593	1.113-2.280	0.046
APRI	0.719	0.312	4.642	2.053	1.114-3.784	0.037
FIB-4	0.433	0.287	2.973	1.542	0.879-2.706	0.063

SWV: Shear wave velocity; ALT: Alanine aminotransferase; AST: Aspartate transaminase; HA: Hyaluronic acid; CⅣ: Type Ⅳ collagen; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis index based on the 4 factor.

Table 3 Diagnostic efficacy of various indicators in the diagnosis of hepatic fibrosis

Index	AUC	Best diagnostic point	Sensitivity, %	Specificity, %
SWV	0.873	1.66 m/s	86.90	88.20
AST/ALT	0.803	0.920	55.90	95.70
HA	0.848	87.79 μg/L	91.00	79.60
CⅣ	0.784	30.36 μg/L	52.70	98.90
APRI	0.789	0.787	57.60	86.00
FIB-4	0.797	1.157	80.00	65.60

SWV: Shear wave velocity; ALT: Alanine aminotransferase; AST: Aspartate transaminase; HA: Hyaluronic acid; CⅣ: Type Ⅳ collagen; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis index based on the 4 factor.

Table 4 Correlation between serological data and the degree of hepatic fibrosis

Index	r	*P* value
SWV (m/s)	0.767	0.000
HA (μg/L)	0.711	0.000
AST/ALT	0.684	0.000
CⅣ (μg/L)	0.681	0.000
APRI	0.634	0.000
FIB-4	0.702	0.000

SWV: Shear wave velocity; HA: Hyaluronic acid; ALT: Alanine aminotransferase; AST: Aspartate transaminase; CⅣ: Type Ⅳ collagen; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis index based on the 4 factor.

each index is illustrated in Figure 3. The area under the curve (AUC) for hepatic fibrosis diagnosed by SWV was the highest (0.873), followed by HA (0.848). The remaining AUC rankings were as follows: AST/ALT, APRI, FIB-4, and CⅣ (Figure 3). The combined diagnosis of SWV and HA with the highest AUC indicated that diagnostic accuracy was further improved with an AUC of 0.889 (sensitivity: 95.92% and specificity: 72.04%) (Table 3 and Figure 4).

Association of serological markers with elastography and hepatic fibrosis

Spearman correlation analysis revealed that hepatic fibrosis was positively correlated with SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 levels. The R values were 0.767, 0.684, 0.711, 0.681, 0.634 and 0.702, respectively, and the difference was statistically significant (all $P < 0.05$) (Table 4). The statistically significant indicators in the correlation analysis were included in the multiple linear regression analysis. The results revealed that SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 were selected as statistically significant independent factors, and the constant analysis was statistically significant. The following regression equation was established: degree of fibrosis = -4.046 + 1.024 × SWV + 1.170 × AST/ALT + 0.011 × HA + 0.020 × CⅣ + 0.719 × APRI + 0.379 × FIB-4 (Table 5).

DISCUSSION

CHB is one of the most common causes of liver-related diseases[17-21], which can gradually develop into hepatic fibrosis, cirrhosis and liver cancer[22-30]. At present, hepatic fibrosis remains a reversible process. Its early diagnosis, as well as its timely and effective treatment, can delay or avoid the development of irreversible cirrhosis stages. Developing an approach to simply and correctly evaluate the severity of hepatic fibrosis has become a clinical challenge that needs to be solved[31]. The literature revealed that liver pathology biopsy is the most important diagnostic basis for the diagnosis of hepatic fibrosis[32-34]. Although it is the "gold standard" for the diagnosis of hepatic fibrosis, it requires immense invasiveness and demonstrates poor reproducibility. Imaging and serological examination can reflect hepatic fibrosis. However, neither of them can be used as an independent diagnostic indicator. Elastography has been used to measure shear waves in liver tissues by ultrasound. The speed of ultrasound propagation is used to calculate the hardness of the liver and determine the degree of hepatic fibrosis. Changes in serological indexes reflect the progression of the disease in patients with hepatic fibrosis[35-44]. In this study, in order to search for non-invasive methods for the diagnosis of hepatic fibrosis, 245 patients with hepatic fibrosis and 93 subjects without hepatic fibrosis were used as observation subjects. The general data, elastography and serological indicators of these subjects were used to analyze the feasibility of ultrasound elastography combined with serological markers for the diagnosis of hepatic fibrosis and the degree of hepatic fibrosis.

The present study first analyzed the clinical data of these two groups. The results revealed that SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 were significantly greater in the disease group than in the control group. This suggests that SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 are the six indicators that can help in the clinical screening for patients with hepatic fibrosis, which is consistent with previous studies[45-50]. Subsequently, in the present study, a multivariate logistic regression analysis was performed on these indicators, which showed significant differences. These results revealed that SWV, HA, CⅣ and APRI

Table 5 Multiple linear regression analysis of the degree of hepatic fibrosis

	Non-standardized coefficient		Standard coefficient	*t*	Sig.
	B value	Standard error	*B* value		
Constant	-4.046	0.209	-	-19.365	0.000
SWV	1.024	0.148	0.2200	6.930	0.000
AST/ALT	1.170	0.190	0.3231	7.861	0.000
HA	0.011	0.005	0.2010	7.126	0.000
CⅣ	0.020	0.003	0.1980	5.749	0.000
APRI	0.719	0.102	0.1880	7.040	0.000
FIB-4	0.379	0.088	0.1490	4.304	0.000

SWV: Shear wave velocity; ALT: Alanine aminotransferase; AST: Aspartate transaminase; HA: Hyaluronic acid; CⅣ: Type Ⅳ collagen; APRI: Aspartate amino-transferase-to-platelet ratio index; FIB-4: Fibrosis index based on the 4 factor.

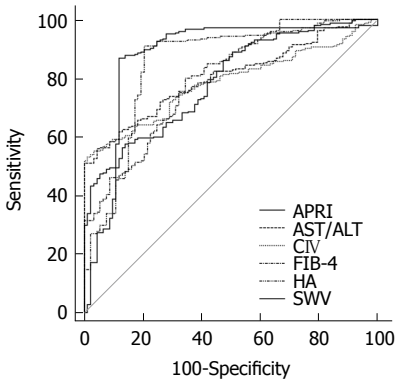


Figure 3 Receiver operating characteristic curve for the diagnosis of hepatic fibrosis based on different indicators. APRI: Aspartate amino-transferase-to-platelet ratio index; AST: Aspartate transaminase; ALT: Alanine aminotransferase; CⅣ: Type Ⅳ collagen; FIB-4: Fibrosis index based on the 4 factor; HA: Hyaluronic acid; SWV: Shear wave velocity.

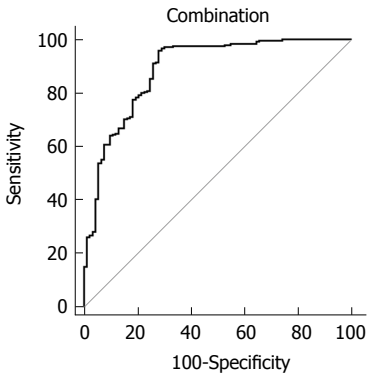


Figure 4 Receiver operating characteristic curve for the diagnosis of hepatic fibrosis based on different combined indicators.

had a significant effect on the development of hepatic fibrosis, suggesting that clinical attention should be given to patients with high levels of SWV, HA, CⅣ and APRI. In order to further explore the clinical significance of these indicators, an ROC curve analysis was performed. Among these four indicators, the maximum area under the ROC curve for SWV was 0.873, suggesting that SWV may be used as an ideal indicator for hepatic fibrosis screening. After these indicators were combined, it was noted that the accuracy of the diagnosis was further enhanced, suggesting that the clinical accuracy of hepatic fibrosis can be improved by combining SWV with serological indexes.

In order to fully explain the effects of SWV and serological indexes on hepatic fibrosis in patients with clinical hepatic fibrosis, correlation analyses and multiple linear regression analyses were performed. The results revealed that the degree of hepatic fibrosis and SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 were positively correlated. After multiple linear regression analysis, the results revealed that SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 were independent factors that affected the degree of hepatic fibrosis, and these were further established. Multiple linear regression equation: Degree of fibrosis = -4.046 + 1.024 × SWV + 1.170 × AST/ALT + 0.011 × HA + 0.020 × CⅣ + 0.719 × APRI + 0.379 × FIB-4. A non-invasive clinical tool was provided for assessing hepatic fibrosis. The SPSS software can be used in clinic to assess the extent of the hepatic fibrosis in a patient by entering the above parameters. Although the richness degree of data collected in the present study can be further improved, the present single-center study was not sufficient to fully guarantee the reliability of the study. Hence, the equation cannot be used as a clinical tool to predict lymph node metastasis. However, this method is worthy of further clinical validation and promotion. In addition, for serological indexes that can reflect the degree of hepatic fibrosis, further review of the literature is needed to explore the mechanism of the degree of fibrosis of the indicator response. This will allow us to obtain a deeper understanding of the significance of serological indexes in the diagnosis of hepatic fibrosis.

In summary, SWV can improve the accuracy of hepatic fibrosis diagnosis, and overcomes the invasive and poor reproducibility shortcomings associated with liver biopsy. At the same time, SWV in combination with serological indexes can further help in the clinical assessment of the extent of hepatic fibrosis in patients.

ARTICLE HIGHLIGHTS

Research background

Pathological examination is known to be the gold standard for diagnosing

liver fibrosis, as it enables a clear diagnosis of liver fibrosis grading. However, pathological examination is an invasive examination and cannot be used as a screening tool. At present, the degree of liver fibrosis is mainly evaluated by serological indicators in the clinic, however the accuracy is relatively low. With advances in technology, ultrasound elastography can be used to assess liver tissue stiffness, although the accuracy is not high. Therefore, it is necessary to explore reliable methods for diagnosing liver fibrosis and assessing the degree of liver fibrosis.

Research motivation

The motivation of this study is to find a more suitable method for the combined diagnosis of liver fibrosis and to establish an optimal non-invasive model for assessing the severity of liver fibrosis. This will provide a reference for non-invasive screening of liver fibrosis.

Research objectives

This study enrolled patients with chronic hepatitis B (CHB) as the research subjects. The aim of this study is to analyze serum markers and ultrasound elastography indicators for diagnosing liver fibrosis and liver fibrosis grading based on pathological results.

Research methods

According to the results of liver biopsy, 338 patients with CHB admitted to our hospital were divided into a diseased group and control group. The diseased group continued to be divided into four groups according to the degree of fibrosis. General data, shear wave velocity (SWV), and serological markers were compared between the two groups. Further independent risk factors for liver fibrosis in patients were analyzed by logistic regression. The accuracy of different indicators in diagnosing liver fibrosis was compared by receiver operating characteristic (ROC) curves. The correlation between different fiber levels and serum indicators or elastography indicators was analyzed. Finally, a multivariate linear regression was used to establish a mathematical model for assessing the severity of liver fibrosis with elastography combined with serological markers.

Research results

SWV, aspartate aminotransferase (AST)/alanine aminotransferase (ALT), hyaluronic acid (HA), type-Ⅳ collagen (CⅣ), aspartate aminotransferase-to-platelet ratio index (APRI) and fibrosis index based on the 4 factor (FIB-4) were significantly higher in the disease group than in the control group ($P < 0.05$). The multivariate logistic regression analysis results revealed that SWV, HA, CⅣ and APRI significantly affected the occurrence of hepatic fibrosis. The ROC curve revealed that the accuracy of the diagnosis of hepatic fibrosis for SWV and HA were 87.3% and 84.8%, respectively. The accuracy of SWV combined with HA was 88.9%. Spearman correlation analysis revealed that hepatic fibrosis was positively correlated with SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 levels. The R values were 0.767, 0.684, 0.711, 0.681, 0.634 and 0.702, respectively, and the difference was statistically significant (all $P < 0.05$). The multiple linear regression analysis revealed that SWV, AST/ALT, HA, CⅣ, APRI and FIB-4 were screened as statistically significant independent factors. The established model was: fibrosis level = -4.046 + 1.024 × SWV + 1.170 × AST/ALT + 0.011 × HA + 0.020 × CⅣ + 0.719 × APRI + 0.379 × FIB-4.

Research conclusions

SWV can non-invasively and effectively diagnose liver fibrosis. SWV combined with serological indicators can further improve the accuracy of diagnosing liver fibrosis. The multiple linear regression equation established by SWV combined with serological indicators is expected to be a non-invasive tool for assessing the degree of liver fibrosis.

Research perspectives

This study is a single-center study, and the sample size is limited and insufficient to fully guarantee the reliability of the study. Therefore, the equation we established cannot be used as an accurate tool for clinical prediction of lymph node metastasis, but it is worthy of further clinical validation and promotion. In addition, for serological indicators that can reflect the degree of liver fibrosis, we can further consult the literature to explore the mechanism of the degree of fibrosis. This would help us understand the diagnostic significance of serological markers with respect to the degree of liver fibrosis.

REFERENCES

1 **Friedman SL**. Liver fibrosis - from bench to bedside. *J Hepatol* 2003; **38** Suppl 1: S38-S53 [PMID: 12591185 DOI: 10.1016/S0168-8278(02)00429-4]

2 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218 [PMID: 15690074 DOI: 10.1172/JCI24282]

3 **Wallace K**, Burt AD, Wright MC. Liver fibrosis. *Biochem J* 2008; **411**: 1-18 [PMID: 18333835 DOI: 10.1042/BJ20071570]

4 **Tsukada S**, Parsons CJ, Rippe RA. Mechanisms of liver fibrosis. *Clin Chim Acta* 2006; **364**: 33-60 [PMID: 16139830 DOI: 10.1016/j.cca.2005.06.014]

5 **Lefkowitch JH**. Liver biopsy assessment in chronic hepatitis. *Arch Med Res* 2007; **38**: 634-643 [PMID: 17613355 DOI: 10.1016/j.arcmed.2006.08.005]

6 **Pinzani M**, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: diagnosis and management. *J Hepatol* 2005; **42** Suppl: S22-S36 [PMID: 15777570 DOI: 10.1016/j.jhep.2004.12.008]

7 **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]

8 **Sun Y**, Zhou J, Wang L, Wu X, Chen Y, Piao H, Lu L, Jiang W, Xu Y, Feng B, Nan Y, Xie W, Chen G, Zheng H, Li H, Ding H, Liu H, Lv F, Shao C, Wang T, Ou X, Wang B, Chen S, Wee A, Theise ND, You H, Jia J. New classification of liver biopsy assessment for fibrosis in chronic hepatitis B patients before and after treatment. *Hepatology* 2017; **65**: 1438-1450 [PMID: 28027574 DOI: 10.1002/hep.29009]

9 **Lee HH**, Seo YS, Um SH, Won NH, Yoo H, Jung ES, Kwon YD, Park S, Keum B, Kim YS, Yim HJ, Jeen YT, Chun HJ, Kim CD, Ryu HS. Usefulness of non-invasive markers for predicting significant fibrosis in patients with chronic liver disease. *J Korean Med Sci* 2010; **25**: 67-74 [PMID: 20052350 DOI: 10.3346/jkms.2010.25.1.67]

10 **Imbert-Bismut F**, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T; MULTIVIRC Group. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069-1075 [PMID: 11297957 DOI: 10.1016/S0140-6736(00)04258-6]

11 **Bottero J**, Lacombe K, Guéchot J, Serfaty L, Miailhes P, Bonnard P, Wendum D, Molina JM, Lascoux-Combe C, Girard PM. Performance of 11 biomarkers for liver fibrosis assessment in HIV/HBV co-infected patients. *J Hepatol* 2009; **50**: 1074-1083 [PMID: 19398234 DOI: 10.1016/j.jhep.2009.01.022]

12 **Khallafi H**, Qureshi K. Imaging Based Methods of Liver Fibrosis Assessment in Viral Hepatitis: A Practical Approach. *Interdiscip Perspect Infect Dis* 2015; **2015**: 809289 [PMID: 26779260 DOI: 10.1155/2015/809289]

13 **Shima H**, Igarashi G, Wakisaka M, Hamano S, Nagae H, Koyama M, Kitagawa H. Noninvasive acoustic radiation force impulse (ARFI) elastography for assessing the severity of fibrosis in the post-operative patients with biliary atresia. *Pediatr Surg Int* 2012; **28**: 869-872 [PMID: 22864589 DOI: 10.1007/s00383-012-3140-4]

14 **Zeng J**, Liu GJ, Huang ZP, Zheng J, Wu T, Zheng RQ, Lu MD. Diagnostic accuracy of two-dimensional shear wave elastography for the non-invasive staging of hepatic fibrosis in chronic hepatitis B: a cohort study with internal validation. *Eur Radiol* 2014; **24**: 2572-2581 [PMID: 25027837 DOI: 10.1007/s00330-014-3292-9]

15 **Samir AE**, Dhyani M, Vij A, Bhan AK, Halpern EF, Méndez-Navarro J, Corey KE, Chung RT. Shear-wave elastography for the estimation of liver fibrosis in chronic liver disease: determining accuracy and ideal site for measurement. *Radiology* 2015; **274**: 888-896 [PMID: 25393946 DOI: 10.1148/radiol.14140839]

16 **Wai CT**, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518-526 [PMID: 12883497 DOI:

10.1053/jhep.2003.50346]
17 **Chu CM**. Natural history of chronic hepatitis B virus infection in adults with emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. *J Gastroenterol Hepatol* 2000; **15** Suppl: E25-E30 [PMID: 10921378 DOI: 10.1046/j.1440-1746.2000.02097.x]
18 **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 DOI: 10.1016/j.jhep.2012.02.010]
19 **European Association for the Study of the Liver**. Electronic address: easloffice@easloffice.eu; European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]
20 **Papatheodoridis GV**, Manolakopoulos S. EASL clinical practice guidelines on the management of chronic hepatitis B: the need for liver biopsy. *J Hepatol* 2009; **51**: 226-227 [PMID: 19410321 DOI: 10.1016/j.jhep.2009.02.017]
21 **Yapali S**, Talaat N, Lok AS. Management of hepatitis B: our practice and how it relates to the guidelines. *Clin Gastroenterol Hepatol* 2014; **12**: 16-26 [PMID: 23660419 DOI: 10.1016/j.cgh.2013.04.036]
22 **Huang CF**, Sun CC, Zhao F, Zhang YD, Li DJ. miR-33a levels in hepatic and serum after chronic HBV-induced fibrosis. *J Gastroenterol* 2015; **50**: 480-490 [PMID: 25155445 DOI: 10.1007/s00535-014-0986-3]
23 **Alam MM**, Mahtab MA, Akbar SM, Kamal M, Rahman S. Hepatic necroinflammation and severe liver fibrosis in patients with chronic hepatitis B with undetectable HBV DNA and persistently normal alanine aminotransferase. *Bangladesh Med Res Counc Bull* 2014; **40**: 92-96 [PMID: 26402972 DOI: 10.3329/bmrcb.v40i3.25229]
24 **Chan HL**, Tsang SW, Leung NW, Tse CH, Hui Y, Tam JS, Chan FK, Sung JJ. Occult HBV infection in cryptogenic liver cirrhosis in an area with high prevalence of HBV infection. *Am J Gastroenterol* 2002; **97**: 1211-1215 [PMID: 12014730 DOI: 10.1111/j.1572-0241.2002.05706.x]
25 **Hu J**, Ludgate L. HIV-HBV and HIV-HCV coinfection and liver cancer development. *Cancer Treat Res* 2007; **133**: 241-252 [PMID: 17672044 DOI: 10.1007/978-0-387-46816-7_9]
26 **Morales-Romero J**, Vargas G, García-Román R. Occult HBV infection: a faceless enemy in liver cancer development. *Viruses* 2014; **6**: 1590-1611 [PMID: 24717680 DOI: 10.3390/v6041590]
27 **Liu S**, Zhang H, Gu C, Yin J, He Y, Xie J, Cao G. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J Natl Cancer Inst* 2009; **101**: 1066-1082 [PMID: 19574418 DOI: 10.1093/jnci/djp180]
28 **Yang HI**, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, Wang LY, Lu SN, You SL, Chen DS, Liaw YF, Chen CJ; REVEAL-HBV Study Group. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 1134-1143 [PMID: 18695135 DOI: 10.1093/jnci/djn243]
29 **El-Serag HB**. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; **142**: 1264-1273.e1 [PMID: 22537432 DOI: 10.1053/j.gastro.2011.12.061]
30 **Liu CJ**, Kao JH. Hepatitis B virus-related hepatocellular carcinoma: epidemiology and pathogenic role of viral factors. *J Chin Med Assoc* 2007; **70**: 141-145 [PMID: 17475593 DOI: 10.1016/S1726-4901(09)70346-6]
31 **Standish RA**, Cholongitas E, Dhillon A, Burroughs AK, Dhillon AP. An appraisal of the histopathological assessment of liver fibrosis. *Gut* 2006; **55**: 569-578 [PMID: 16531536 DOI: 10.1136/gut.2005.084475]
32 **Chevallier M**, Guerret S, Chossegros P, Gerard F, Grimaud JA. A histological semiquantitative scoring system for evaluation of hepatic fibrosis in needle liver biopsy specimens: comparison with morphometric studies. *Hepatology* 1994; **20**: 349-355 [PMID: 8045495 DOI: 10.1002/hep.1840200213]
33 **Mani H**, Kleiner DE. Liver biopsy findings in chronic hepatitis B. *Hepatology* 2009; **49**: S61-S71 [PMID: 19399798 DOI: 10.1002/hep.22930]
34 **Cholongitas E**, Senzolo M, Standish R, Marelli L, Quaglia A, Patch D, Dhillon AP, Burroughs AK. A systematic review of the quality of liver biopsy specimens. *Am J Clin Pathol* 2006; **125**: 710-721 [PMID: 16707372 DOI: 10.1309/W3XC-NT4H-KFBN-2G0B]
35 **Serra C**, Grasso V, Conti F, Felicani C, Mazzotta E, Lenzi M, Verucchi G, D'errico A, Andreone P. A New Two-Dimensional Shear Wave Elastography for Noninvasive Assessment of Liver Fibrosis in Healthy Subjects and in Patients with Chronic Liver Disease. *Ultraschall Med* 2018; **39**: 432-439 [PMID: 29458217 DOI: 10.1055/s-0043-119356]
36 **Rockey DC**, Bissell DM. Noninvasive measures of liver fibrosis. *Hepatology* 2006; **43**: S113-S120 [PMID: 16447288 DOI: 10.1002/hep.21046]
37 **Friedrich-Rust M**, Koch C, Rentzsch A, Sarrazin C, Schwarz P, Herrmann E, Lindinger A, Sarrazin U, Poynard T, Schäfers HJ, Zeuzem S, Abdul-Khaliq H. Noninvasive assessment of liver fibrosis in patients with Fontan circulation using transient elastography and biochemical fibrosis markers. *J Thorac Cardiovasc Surg* 2008; **135**: 560-567 [PMID: 18329470 DOI: 10.1016/j.jtcvs.2007.09.039]
38 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713 [PMID: 14698338 DOI: 10.1016/j.ultrasmedbio.2003.07.001]
39 **Zhang D**, Li P, Chen M, Liu L, Liu Y, Zhao Y, Wang R. Non-invasive assessment of liver fibrosis in patients with alcoholic liver disease using acoustic radiation force impulse elastography. *Abdom Imaging* 2015; **40**: 723-729 [PMID: 24811766 DOI: 10.1007/s00261-014-0154-5]
40 **Chen SH**, Li YF, Lai HC, Kao JT, Peng CY, Chuang PH, Su WP, Chiang IP. Noninvasive assessment of liver fibrosis via spleen stiffness measurement using acoustic radiation force impulse sonoelastography in patients with chronic hepatitis B or C. *J Viral Hepat* 2012; **19**: 654-663 [PMID: 22863270 DOI: 10.1111/j.1365-2893.2012.01588.x]
41 **Gangadharan B**, Antrobus R, Chittenden D, Rossa J, Bapat M, Klenerman P, Barnes E, Dwek RA, Zitzmann N. New approaches for biomarker discovery: the search for liver fibrosis markers in hepatitis C patients. *J Proteome Res* 2011; **10**: 2643-2650 [PMID: 21410221 DOI: 10.1021/pr101077c]
42 **Lau GK**, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, Gane E, Fried MW, Chow WC, Paik SW, Chang WY, Berg T, Flisiak R, McCloud P, Pluck N; Peginterferon Alfa-2a HBeAg-Positive Chronic Hepatitis B Study Group. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005; **352**: 2682-2695 [PMID: 15987917 DOI: 10.1056/NEJMoa043470]
43 **Bonnard P**, Sombié R, Lescure FX, Bougouma A, Guiard-Schmid JB, Poynard T, Calès P, Housset C, Callard P, Le Pendeven C, Drabo J, Carrat F, Pialoux G. Comparison of elastography, serum marker scores, and histology for the assessment of liver fibrosis in hepatitis B virus (HBV)-infected patients in Burkina Faso. *Am J Trop Med Hyg* 2010; **82**: 454-458 [PMID: 20207872 DOI: 10.4269/ajtmh.2010.09-0088]
44 **Moreno S**, García-Samaniego J, Moreno A, Ortega E, Pineda JA, del Romero J, Tural C, von Wichmann MA, Berenguer J, Castro A, Espacio R. Noninvasive diagnosis of liver fibrosis in patients with HIV infection and HCV/HBV co-infection. *J Viral Hepat* 2009; **16**: 249-258 [PMID: 19215579 DOI: 10.1111/j.1365-2893.2009.01088.x]
45 **Pinto J**, Matos H, Nobre S, Cipriano MA, Marques M, Pereira JM, Gonçalves I, Noruegas MJ. Comparison of acoustic radiation force impulse/serum noninvasive markers for fibrosis prediction in liver transplant. *J Pediatr Gastroenterol Nutr* 2014; **58**: 382-386 [PMID: 24164902 DOI: 10.1097/MPG.0000000000000226]
46 **Ala-Kokko L**, Pihlajaniemi T, Myers JC, Kivirikko KI, Savolainen ER. Gene expression of type I, Ⅲ and Ⅳ collagens in hepatic fibrosis induced by dimethylnitrosamine in the rat. *Biochem J* 1987; **244**: 75-79 [PMID: 3663119 DOI: 10.1042/bj2440075]
47 **Petersen JR**, Stevenson HL, Kasturi KS, Naniwadekar A, Parkes J, Cross R, Rosenberg WM, Xiao SY, Snyder N. Evaluation of the aspartate aminotransferase/platelet ratio index and enhanced liver fibrosis tests to detect significant fibrosis due to chronic hepatitis

C. *J Clin Gastroenterol* 2014; **48**: 370-376 [PMID: 24045284 DOI: 10.1097/MCG.0b013e3182a87e78]

48 **Vallet-Pichard A**, Mallet V, Nalpas B, Verkarre V, Nalpas A, Dhalluin-Venier V, Fontaine H, Pol S. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology* 2007; **46**: 32-36 [PMID: 17567829 DOI: 10.1002/hep.21669]

49 **Parés A**, Deulofeu R, Giménez A, Caballería L, Bruguera M, Caballería J, Ballesta AM, Rodés J. Serum hyaluronate reflects hepatic fibrogenesis in alcoholic liver disease and is useful as a marker of fibrosis. *Hepatology* 1996; **24**: 1399-1403 [PMID: 8938169 DOI: 10.1002/hep.510240615]

50 **Liu J**, Ji Y, Ai H, Ning B, Zhao J, Zhang Y, Dun G. Liver Shear-Wave Velocity and Serum Fibrosis Markers to Diagnose Hepatic Fibrosis in Patients with Chronic Viral Hepatitis B. *Korean J Radiol* 2016; **17**: 396-404 [PMID: 27134527 DOI: 10.3348/kjr.2016.17.3.396]

P- Reviewer: Apisarnthanarax S, Eun Sun K, Hayes MJ, Tomiyasu A, Ward J
S- Editor: Wang XJ **L- Editor**: Filipodia **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4281-4290

DOI: 10.3748/wjg.v24.i37.4281

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

ORIGINAL ARTICLE

Observational Study

Risk factors for liver disease among adults of Mexican descent in the United States and Mexico

Yvonne N Flores, Zuo-Feng Zhang, Roshan Bastani, Mei Leng, Catherine M Crespi, Paula Ramírez-Palacios, Heather Stevens, Jorge Salmerón

Yvonne N Flores, Paula Ramírez-Palacios, Jorge Salmerón, Unidad de Investigación Epidemiológica y en Servicios de Salud, Delegación Morelos, Instituto Mexicano del Seguro Social, Cuernavaca, Morelos 62000, México

Yvonne N Flores, Roshan Bastani, UCLA Department of Health Policy and Management and Kaiser Permanente Center for Health Equity, Fielding School of Public Health, Los Angeles, CA 90095, United States

Yvonne N Flores, Roshan Bastani, Catherine M Crespi, UCLA Cancer Prevention and Control Research Center, Fielding School of Public Health and Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095, United States

Zuo-Feng Zhang, UCLA Department of Epidemiology, Fielding School of Public Health, Los Angeles, CA 90095, United States

Mei Leng, UCLA Division of General Internal Medicine and Health Services Research, Los Angeles, CA 90095, United States

Catherine M Crespi, UCLA Department of Biostatistics, Fielding School of Public Health, Los Angeles, CA 90095, United States

Heather Stevens, University of Washington, School of Medicine, Seattle, WA 98195, United States

Jorge Salmerón, Universidad Nacional Autónoma de México, Academic Epidemiology Research Unit, Avenida Universidad 3000, Ciudad Universitaria, Coyoacán, Mexico City 04510, México

Jorge Salmerón, Centro de Investigación en Salud Poblacional, Instituto Nacional de Salud Pública, Cuernavaca, Morelos 62100, México

ORCID number: Yvonne N Flores (0000-0002-0601-357X); Zuo-Feng Zhang (0000-0002-4669-3995); Roshan Bastani (0000-0001-6594-9231); Mei Leng (0000-0002-2758-7599); Catherine M Crespi (0000-0002-6150-2181); Paula Ramírez-Palacios (0000-0002-2586-4396); Heather Stevens (0000-0002-8261-2005); Jorge Salmerón (0000-0001-8654-2393).

Author contributions: Flores YN, Zhang ZF, Bastani R, Leng M, Crespi CM, Ramírez-Palacios P, Stevens H, Salmerón J contributed to the study design; statistical analysis; interpretation of the findings; writing the article and approval of the final draft.

Supported by the Programa de Investigación en Migracion y Salud (PIMSA), No. 2015-2106; the Instituto Mexicano del Seguro Social (IMSS), No. 2005/1/ I /093; and the Consejo Nacional de Ciencia y Tecnología (CONACYT), No. 26267M and No. SALUD-2011-01-161930; the NIH, No. UL1TR000124 to Crespi CM, and NIH/NCI No. K07CA197179 to Flores YN.

Institutional review board statement: Approval for both the Health Worker Cohort Study and this bi-national investigation was obtained from the Internal Review Boards of IMSS and UCLA.

Conflict-of-interest statement: All authors declare no potential conflicts of interest relevant to this article.

Data sharing statement: No additional data are available.

STROBE statement: The guidelines of the STROBE Statement have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Yvonne N Flores, PhD, Unidad de Investigación Epidemiológica y en Servicios de Salud, Delegación Morelos, Instituto Mexicano del Seguro Social, Blvd. Juárez #31, Colonia Centro, Cuernavaca, Morelos 62000, México. ynflores@ucla.edu
Telephone: +52-777-1001364

Received: June 20, 2018
Peer-review started: June 20, 2018
First decision: July 4, 2018
Revised: August 1, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

AIM

To compare the prevalence of chronic liver disease (CLD) risk factors in a representative sample of Mexican-Americans born in the United States (US) or Mexico, to a sample of adults in Mexico.

METHODS

Data for Mexican-Americans in the US were obtained from the 1999-2014 National Health and Nutrition Examination Survey (NHANES), which includes persons of Mexican origin living in the US (n = 4274). The NHANES sample was restricted to Mexican-American participants who were 20 years and older, born in the US or Mexico, not pregnant or breastfeeding, and with medical insurance. The data in Mexico were obtained from the 2004-2013 Health Worker Cohort Study in Cuernavaca, Mexico (n = 9485). The following known risk factors for liver disease/cancer were evaluated: elevated aminotransferase levels (elevated alanine aminotransferase was defined as > 40 IU/L for males and females; elevated aspartate aminotransferase was defined as > 40 IU/L for males and females), infection with hepatitis B or hepatitis C, metabolic syndrome, high total cholesterol, diabetes, obesity, abdominal obesity, and heavy alcohol use. The main independent variables for this study classified individuals by country of residence (*i.e.*, Mexico *vs* the US) and place of birth (*i.e.*, US-born *vs* Mexico-born). Regression analyses were used to investigate CLD risk factors.

RESULTS

After adjusting for socio-demographic characteristics, Mexican-American males were more likely to be obese, diabetic, heavy/binge drinkers or have abdominal obesity than males in Mexico. The adjusted multivariate results for females also indicate that Mexican-American females were significantly more likely to be obese, diabetic, be heavy/binge drinkers or have abdominal obesity than Mexican females. The prevalence ratios and prevalence differences mirror the multivariate analysis findings for the aforementioned risk factors, showing a greater risk among US-born as compared to Mexico-born Mexican-Americans.

CONCLUSION

In this study, Mexican-Americans in the US had more risk factors for CLD than their counterparts in Mexico. These findings can be used to design and implement more effective health promotion policies and programs to address the specific factors that put Mexicans at higher risk of developing CLD in both countries.

Key words: Liver disease; Risk factors; Health disparities; Mexico; Mexican Americans; Latinos

Core tip: United States (US) Latinos have greater morbidity and mortality from liver disease than non-Hispanic whites, and liver disease is the fifth leading cause of death in Mexico. Known risk factors for chronic liver disease include hepatitis B or C infection, heavy/binge drinking, obesity, diabetes, and metabolic syndrome. We found that Mexican-Americans in the US have a greater risk of obesity, diabetes and heavy/binge drinking than their counterparts in Mexico. The prevalence of heavy/binge drinking was alarmingly high among Mexican-Americans, with over 70% among males and over 50% among US-born females. Our results identify a high prevalence of specific risk factors that should be targeted to reduce the high rates of liver disease-related mortality in this population.

Flores YN, Zhang ZF, Bastani R, Leng M, Crespi CM, Ramírez-Palacios P, Stevens H, Salmerón J. Risk factors for liver disease among adults of Mexican descent in the United States and Mexico. *World J Gastroenterol* 2018; 24(37): 4281-4290 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4281.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4281

INTRODUCTION

Over 30 million people, or one in ten, are likely to have some form of chronic liver disease (CLD) in the United States (US). This number includes the approximately 850000 to 2.2 million cases of chronic hepatitis B (HBV), 3.5 million cases of chronic hepatitis C (HCV)[1], the estimated 30% of Americans who have non-alcoholic fatty liver disease (NAFLD), and the 10% with advanced fibrosis[2,3]. Also included are the nearly 20000 individuals who die from alcoholic liver disease in the US each year[4]. In 2017, there were an estimated 40000 incident cases of liver cancer and 30000 deaths from liver cancer[5]. New cases of liver cancer have more than tripled since 1980 and liver cancer mortality has increased by nearly 3% each year since 2000[5]. CLD is the 12th leading cause of general mortality in the US[4], the fifth among individuals between 45-54 years, and the sixth among 25-44 year olds and those aged 55-64 years[6].

Latinos in the US have disproportionately higher rates of CLD. Since 2002, CLD has consistently been the sixth leading cause of mortality for Latinos[7], and the third cause of death among Latino males ages 55-64[8]. They are twice as likely to have CLD and 1.7 times more likely to die from liver cancer than non-Hispanic

whites (whites)[9]. The prevalence of earlier stage liver disease, such as steatohepatitis, is also higher among Latinos (45%) than whites (33%) or blacks (24%)[10]. In Mexico, cirrhosis and other forms of CLD were the fourth leading cause of general mortality in 2016, and the third among males aged 35-65 years[11]. An estimated 3 million individuals are infected with HBV and 400000 to 1400000 people are infected with HCV[12]. In 2016, there were 38755 deaths due to CLD in Mexico and 14029 (36%) were attributed to alcoholic liver disease[11]. By 2050, an estimated 90% of cases of CLD in Mexico will be attributable to obesity and excessive alcohol consumption, as compared to other populations with high rates of CLD due to infection with HBV or HCV[13].

Although infection with HBV or HCV and heavy alcohol use are well known risk factors for CLD and liver cancer, a significant proportion of cases (15% to 50%) do not present with these risk factors[14]. Other risk factors for CLD include obesity and diabetes, and the proposed mechanism is through the development of NAFLD and non-alcoholic steatohepatitis (NASH)[15-18]. NAFLD is found in up to 80-90% of obese adults, in 30-50% of patients with diabetes, and in up to 90% of patients with hyperlipidemia[19]. In the US, the prevalence of NAFLD and NASH is highest among Latinos, followed by whites and blacks[10,15,20]. Rates of obesity, diabetes, and hyperlipidemia are also higher among Latinos than whites in the US[21-23]. In Mexico, over 70% of the population is overweight or obese, and this figure is predicted to rise to 90% by 2050[24]. Additionally, the prevalence of metabolic syndrome is estimated to be 40%[25], and in 2015, diabetes was the second cause of general mortality in Mexico, which has one of the highest incidence rates of diabetes in the world[11,26].

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are common clinical measures used to assess liver health. Elevated aminotransferase levels can indicate sudden or acute liver injury, or they can be persistently elevated, suggesting ongoing liver disease. The leading cause of mild aminotransferase levels is NAFLD, but other common causes include excessive alcohol consumption, medication-associated liver injury, infection with HVB or HCV, and hemochromatosis[27]. While not all individuals with elevated aminotransferase levels have liver damage or disease, these measures can be used to detect asymptomatic disease[27]. Several US studies report a higher prevalence of elevated ALT among Mexican-Americans (17.4%) than among whites (8.2%)[28-30].

The aim of this study was to compare the prevalence of known risk factors for CLD in a representative sample of Mexican-Americans, who were born in the US or Mexico, to a sample of adults in Mexico. We examined data from the 1999-2014 National Health and Nutrition Examination Survey (NHANES), which includes persons of Mexican origin living in the US, and data from the 2004-2013 Health Worker Cohort Study (HWCS) in Cuernavaca, Mexico. We hypothesized that Mexican-Americans in the US would have a higher prevalence of CLD risk factors than their counterparts in Mexico. This hypothesis is based on studies suggesting that immigrant Mexican-Americans have better health outcomes than more acculturated, US-born Mexican-Americans[31-33]. We tested this hypothesis by analyzing the independent association between country of residence (*i.e.*, Mexico *vs* the US) and place of birth, sex, and the following risk factors for CLD: Elevated ALT or AST levels, infection with HBV or HCV, metabolic syndrome, high cholesterol, diabetes, obesity, abdominal obesity, and heavy or binge drinking, while also controlling for potential confounders.

MATERIALS AND METHODS

Ethical considerations

Ethical approval for the HWCS and this bi-national study was granted from the Internal Review Boards of the Mexican Social Security Institute (IMSS) and the University of California, Los Angeles (UCLA). Informed consent was obtained from all the HWCS subjects prior to their participation in any study activities.

Data sources

This observational study used existing data from Mexican-Americans who participated in NHANES, a cross-sectional, representative, examination survey of the total civilian non-institutionalized population residing in the continental US and Hawaii. This continuous survey is conducted by the National Center for Health Statistics to assess and track the health and nutritional status of Americans over time. The survey collects health data through standardized questionnaires, physical examinations and a series of laboratory tests. The design of NHANES over-samples Mexican-Americans to allow for analyses of this subgroup. The 1999-2014 NHANES data includes a total of 3929 male and 4182 female Mexican-Americans, for a total sample size of 8111[34].

The data in Mexico came from the HWCS, a longitudinal study of workers and their immediate family members from two large health care institutions in Cuernavaca, Mexico: the IMSS and the National Institute of Public Health. Briefly, the HWCS collects information using physical examinations, self-reported questionnaires, and laboratory tests in order to prospectively evaluate risk factors and the incidence of chronic diseases, including heart disease, diabetes, and liver disease (CLD). From 2004 to 2006 (Wave 1), approximately 9000 health workers enrolled in the HWCS. During 2011 to 2013 (Wave 2), a total of 1855 participants were followed-up. Details about the design and methods of the HWCS are described elsewhere[35]. The clinical and anthropometric procedures that were used for the HWCS are comparable to those used for the NHANES surveys[36].

Study samples in the United States and Mexico

The 1999-2014 NHANES sample was restricted to Mexican-American participants who were 20 years and older, born in the US or Mexico, and had medical insurance. Females who were pregnant at the time of

data collection were excluded. The final NHANES sample consisted of 2097 males and 2177 females 20 years and older with completed questionnaire data. Of these individuals, 4075 also underwent physical examinations including laboratory studies, and of the individuals with laboratory studies, 1775 provided fasting blood samples.

The HWCS sample was limited to participants 20 years and older who reside and were born in Mexico, and had medical insurance. Of the 10035 participants in the HWCS sample with questionnaire and laboratory data, 193 females were excluded because they were pregnant or breastfeeding at the time of the survey. An additional 48 individuals were excluded because they were not born in Mexico, and 309 were excluded because they did not report a place of birth. The final HWCS sample consisted of 3010 males and 6475 females 20 years and older who reside and were born in Mexico, with completed questionnaire and laboratory data. The total study sample of 13798 individuals consisted of 9485 Mexican subjects who currently reside in Mexico, 2324 US-born Mexican-Americans who live in the US, and 1989 Mexican-Americans who were born in Mexico and now live in the US.

Definition of chronic liver disease risk factors

Elevated aminotransferase levels: Elevated ALT was defined as > 40 IU/L for males and females; elevated AST was defined as > 40 IU/L for males and females[29,30].

Hepatitis B or hepatitis C infection: HBV infection was identified by having a positive hepatitis B core antibody serology and a positive hepatitis B surface antigen serology. We identified an HCV infection by a positive antibody titer[36]. Individuals infected with HBV and HCV were combined into one category due to their small sample sizes.

Metabolic syndrome: We used the definition of metabolic syndrome from the Third Report of the National Cholesterol Education Program's Adult Treatment Panel Ⅲ (NCEP/ATPⅢ) criteria[37].

High Total Cholesterol: Following NCEP/ATPⅢ recommendations, high total cholesterol was defined as > 200 mg/dL for males and females[37].

Diabetes: Type 2 diabetes in males and females was defined as having one of the following: a plasma glucose level > 125 mg/dL after > 8 h of fasting, a medical history of diabetes (other than during pregnancy), currently taking medication for diabetes, or a random glucose test > 200 mg/dL[38].

Obesity: Subjects were categorized according to body mass index (BMI) following the recommendations of the National Heart, Lung and Blood Institute: Normal (BMI 18.5-24.9 kg/m^2), overweight (BMI 25.0-29.9 kg/m^2), and obese (BMI $\geqslant$ 30.0 kg/m^2)[39].

Abdominal obesity: Abdominal obesity was defined as a waist circumference > 102 cm for males and a waist circumference > 88 cm for females[40].

Alcohol consumption: Heavy drinking was defined as two to four drinks per day for females and three to four drinks per day for males, and binge drinking was defined as having five or more drinks at one time for both males and females[41].

Definition of independent variables

The main independent variables for this study classified individuals by country of residence (*i.e.*, Mexico *vs* the US) and place of birth (*i.e.*, US-born *vs* Mexico-born). The HWCS participants represent Mexicans who were born and currently live in Mexico. Individuals from the NHANES sample were further classified by birthplace (US-born *vs* Mexico-born). The following three groups were compared: (1) HWCS (Mexico resident, Mexico-born), (2) NHANES (US resident, Mexico-born), and (3) NHANES (US resident, US-born). Other independent variables included age, sex, marital status, and education level. Approximately 18% of the subjects in the HWCS sample had missing education data, which was imputed using a three-step procedure. There were no missing data for the other independent variables.

Statistical analysis

Descriptive analyses were performed to characterize the study population and examine the study variables. Chi-square tests were used to compare the socio-demographic characteristics of the study sample by country of birth/residence, which were stratified by sex. Age-adjusted means and prevalence rates were calculated for each CLD risk factor, which were stratified by sex and country of birth/residence. Separate multiple logistic regression models were estimated for males and females to evaluate the independent associations of each liver disease risk factor to country of birth/residence. Adjusted odds ratios with 95% confidence intervals (95%CI) are reported. Marginal standardization was used to calculate the predicted probability as well as the prevalence ratios and prevalence differences with their corresponding 95%CIs. This allowed us to compare the prevalence of CLD risk factors between the three groups. For all analyses, a two-sided *P*-value < 0.05 was considered statistically significant. The data analyses were conducted using SAS software, version 9.4 for Windows, and STATA 14.

RESULTS

Sample characteristics

The socio-demographic characteristics of the bi-national study sample are presented in Table 1. One third of the HWCS participants in Mexico are male, as compared to the US NHANES sample, which is 49% male. There are no differences between the US and Mexico samples in

Table 1 Socio-demographic characteristics of the study sample *n* (%)

	Male			*P* value[1]		Female			*P* value[1]	
	Mexico cohort (REF)	NHANES Mexico born	NHANES US born	Mexico born	US born	Mexico cohort (REF)	NHANES Mexico born	NHANES US born	Mexico born	US born
Total sample sizes	3010 (58.9)	1021 (20.0)	1076 (21.1)	0.925	0.897	6475 (68.3)	944 (10.9)	1233 (14.3)	0.923	0.845
Age (yr)										
20-44	1680 (55.8)	380 (58.6)	377 (59.8)			3357 (51.8)	356 (55.5)	460 (56.9)		
45-59	951 (31.6)	239 (25.6)	222 (22.7)			2031 (31.4)	196 (24.3)	256 (23.4)		
60+	379 (12.6)	402 (15.9)	477 (17.5)			1087 (16.8)	392 (20.2)	517 (19.8)		
Marital status				0.074	0.804				0.088	0.423
Never married/single	458 (15.2)	73 (9.8)	162 (22.4)			1489 (23.0)	81 (10.0)	169 (19.5)		
Married/living together	2390 (79.4)	843 (83.)	746 (65.2)			3701 (57.2)	580 (67.8)	697 (57.3)		
Divorced/separated/widowed	162 (5.4)	105 (6.6)	167 (12.4)			1285 (19.8)	283 (22.2)	367 (23.2)		
Education										
Less than high school	706 (23.5)	724 (61.7)	381 (24.8)	0.000	0.463	2073 (32.0)	660 (61.3)	436 (24.8)	0.002	0.705
High school graduate	574 (19.1)	136 (17.6)	246 (26.2)			1121 (17.3)	108 (14.6)	279 (23.6)		
More than high school	1730 (57.5)	160 (20.7)	447 (48.9)			3281 (50.7)	173 (24.1)	513 (51.5)		

[1]The Chi square test was used to determine differences between groups.

terms of age or marital status. Approximately half the total sample is between 20 to 44 years, nearly 30% is 45 to 59 years, and roughly 20% is 60 years or older. Most of the study subjects are married/living together (65%), almost 20% are never married/single, and the rest are divorced/separated/widowed. There are no differences in the levels of education observed between the HWCS participants in Mexico and the Mexican-Americans who were born in the US. Approximately half of the participants in both groups have an education beyond high school and less than a third did not finish high school. The only significant difference observed between the two samples is that over 60% of the Mexico-born NHANES participants did not graduate from high school, compared to 29% of the participants in Mexico.

Chronic liver disease risk factors

Table 2 reports the age-adjusted means and prevalence of CLD risk factors for males and females by country of birth/residence. Elevated ALT levels are more common among males (22%-27%) than females (8%-10%), with a mean ALT ranging from 35-36 IU/L among males and 23-25 IU/L among females. There are no differences in mean AST levels among males (range 28-30 IU/L), but a significantly higher mean AST is observed among the Mexico-born (23.6 IU/L) and US-born (25.9 IU/L) females, as compared to the mean AST of 22.2 IU/L found among females in Mexico. The prevalence of diabetes, obesity, abdominal obesity, and heavy/binge drinking is higher among the NHANES participants than among the HWCS subjects. Conversely, more HWCS participants are current smokers and have lower levels of HDL cholesterol and elevated triglycerides compared with their NHANES counterparts.

Mexican-American males born in Mexico have a lower rate of HBV or HCV infection (0.4%) than either US-born Mexican-Americans (3.0%) or the HWCS participants (2.6%). A greater proportion of Mexico-born Mexican-American males have high cholesterol (49%) compared with males in Mexico (40%) and US-born Mexican-Americans (39%). Males in Mexico are more likely to have elevated triglycerides (57.5%) compared with Mexico-born (35.4%) and US-born Mexican-Americans (41%). The prevalence of diabetes is significantly lower among males in Mexico (6%) when compared with the 11% and 16% rates among the Mexico- and US-born NHANES participants, respectively. The proportion of obese males in Mexico is 17%, compared to 30% and 45% among Mexico- and US-born males in the US, respectively. Males in Mexico have a lower prevalence of abdominal obesity (16%) than Mexico- and US-born Mexican-American males (36% and 49%, respectively), with mean waist circumferences of 91.4 cm, 98.7 cm and 103.1 cm, respectively. Heavy or binge drinking is more common among the Mexico- and US-born Mexican-American males (75% and 71%, respectively) compared with their counterparts in Mexico (38%).

Rates of HBV or HCV infection are lower among Mexico-born and US-born Mexican-American females (0.3% and 0.8%, respectively) when compared to females in Mexico (1.7%). A lower percentage of females in Mexico have high cholesterol (36%) compared to the Mexico-born (40.5%) and US-born (40.2%) Mexican-American females. US-born females in the US are less likely to have elevated triglycerides (23.8%) than Mexico-born females (31.9%) and females living in Mexico (34.5%). The prevalence of diabetes among the females in the Mexican sample is 7%, compared to 14% and 11% among Mexico- and US-born Mexican-American females, respectively. Obesity rates are also lower among females in Mexico than among Mexican-American females (19% *vs* 39% and 47%, respectively). Abdominal obesity among females in Mexico is 48%, compared to a prevalence of 68% among their Mexican-American counterparts. Rates of heavy or binge drinking are substantially higher among Mexican-American females born in Mexico or the US (34% and 54%, respectively), as compared to females in Mexico (10%)

Table 2 Age-adjusted means and prevalence of chronic liver disease risk factors

	Male			Female		
	Mexico cohort (REF)	NHANES Ⅶ Mexico-born	NHANES Ⅶ US-born	Mexico cohort (REF)	NHANES Ⅶ Mexico-born	NHANES Ⅶ US-born
Elevated ALT (> 40 IU/L)[1], %	26.7	22.1	25.2	8.7	8.3	10.2
ALT (IU/L), mean	35.9	35.0	36.0	23.0	23.7	25.3[a]
Elevated AST (> 40 IU/L)[2], %	11.2	8.4	12.2	5.2	4.6	5.2
AST (IU/L), mean	27.9	30.1	29.5	22.2	23.6[a]	25.9[b]
Hepatitis B or C, %	2.6	0.4[b]	3.0	1.7	0.3[b]	0.8[a]
Metabolic syndrome[3], %	27.5	23.5	33.4	30.8	28.6	27.6
Elevated total cholesterol (mg/dL)[4], %	39.8	48.8[b]	39.1	36.0	40.5[a]	40.2[a]
Total cholesterol (mg/dL), mean	193.6	199.1[a]	192.7	189.6	193.1[a]	193.6[a]
Low HDL cholesterol (HDL < 40)[5], %	62.9	35.4[b]	27.7[b]	50.1	14.1[b]	12.0[b]
HDL cholesterol (mg/dL), mean	37.6	45.1[b]	46.7[b]	40.7	52.7[b]	55.1[b]
LDL cholesterol (mg/dL), mean	116.5	124.1[b]	117.2	115.2	110.9	11.5[a]
Elevated triglycerides[6] (mg/dL), %	57.5	35.4[b]	41.0[b]	34.5	31.9	23.8[b]
Triglycerides (mg/dL), mean	202.4	150.0[b]	173.9[a]	143.5	132.0[a]	123.8[b]
Diabetic[7], %	6.3	11.0[a]	16.1[b]	6.9	14.4[b]	11.0[a]
Overweight (BMI ⩾ 25)[8], %	48.1	46.3	37.3[b]	37.2	31.1[a]	26.7[b]
Obesity (BMI ⩾ 30)[9], %	17.1	30.0[b]	45.0[b]	19.2	39.2[b]	46.6[b]
BMI (kg/m^2), mean	26.7	28.3[b]	30.1[b]	26.4	29.0[b]	30.2[b]
Abdominal obesity[10], %	15.8	36.0[b]	49.0[b]	48.3	68.3[b]	67.9[b]
Waist circumference (cm), mean	91.4	98.7[a]	103.1[b]	88.5	94.7[b]	97.2[b]
Heavy or binge drinker[11], %	38.0	75.1[b]	71.3[b]	9.6	33.8[b]	54.0[b]
Current smoker[12], %	36.9	21.6[b]	21.9[b]	20.8	8.2[b]	14.2[b]

[1]Elevated alanine aminotransferase (ALT) was defined as ALT > 40 IU/L for males and females; [2]Elevated alanine aminotransferase (AST) was defined as AST > 40 IU/L for males and females; [3]Metabolic syndrome was defined base on the Third Report of the National Cholesterol Education Program's Adult Treatment Panel Ⅲ criteria; [4]Elevated total cholesterol was defined as ⩾ 200 mg/dL; [5]Low High Density Lipoprotein-Cholesterol (HDL-C) was defined as < 40 mg/dL; [6]Elevated triglycerides was defined as ⩾ 150 mg/dL; [7]Diabetes was defined as having a plasma glucose level > 125 mg/dL after a more than 8 h fast, and/or a medical history of diabetes, and/or currently taking medication for diabetes, and/or a random glucose test >200 mg/dL; [8]Overweight was defined as having a body mass index (BMI) of ⩾ 25.0 kg/m^2; [9]Obesity was defined as having a BMI of ⩾ 30.0 kg/m^2; [10]Abdominal obesity was defined as having a waist circumference > 102 cm for males, and a waist circumference > 88 cm for females; [11]Heavy drinking was defined as two to four drinks per day for females and three to four drinks per day for males, and binge drinking was defined as having five or more drinks at one time for both males and females; [12]Current cigarette smoking was defined as having smoked at least 100 cigarettes and being a current smoker. [a]*P* value ⩽ 0.05; [b]*P* value ⩽ 0.001.

(Table 2).

Multivariate analyses and other effect measures

After controlling for age, marital status, and education level, the logistic regression results indicate that Mexico-born Mexican-American males are less likely to have HBV or HCV (OR: 0.2, 95%CI: 0.1-0.6), but are more likely to have high cholesterol (OR: 1.4, 95%CI: 1.1-1.8) than their counterparts in Mexico. US-born Mexican-American males are more likely to have metabolic syndrome (OR: 1.4, 95%CI: 1.1-1.9) and diabetes (OR: 3.0, 95%CI: 1.9-4.8) than males in Mexico. Regardless of where they were born, Mexican-American males are more likely to be obese, diabetic, have abdominal obesity, or be heavy/binge drinkers than Mexican males. The prevalence ratios and predicted probabilities confirm the results of our multivariate analyses and may provide more precise estimates of the increased risk of diabetes, obesity, abdominal obesity, and heavy/binge drinking observed among Mexican-American males, as compared with males in Mexico. The probability of having any of the aforementioned risk factors is significantly greater among US-born Mexican-Americans than among their Mexico-born counterparts (Table 3).

The adjusted multivariate results presented in Table 4 also indicate that Mexico-born Mexican-American females are significantly more likely to be diabetic (OR: 2.2, 95%CI: 1.4-3.4), obese (OR: 2.5, 95%CI: 1.8-3.5), have abdominal obesity (OR: 2.1, 95%CI: 1.2-3.6), or be heavy/binge drinkers (OR: 5.6, 95%CI: 4.2-7.3) than females in Mexico. The same is true for US-born Mexican-American females, who are also more likely to be diabetic (OR: 1.7, 95%CI: 1.2-2.6), obese (OR: 3.5, 95%CI: 2.5-4.9), have abdominal obesity (OR: 2.3, 95%CI: 1.4-3.8), or be heavy/binge drinkers (OR: 12.8, 95%CI: 10.0-16.3) than their counterparts in Mexico. The prevalence ratios and predicted probabilities mirror the multivariate analysis findings for the aforementioned risk factors, showing a greater risk among US-born as compared to Mexico-born Mexican-American females. However, the prevalence ratios indicate that Mexican-American females are significantly less likely to be infected with HBV or HCV than females in Mexico (Table 4).

Supplemental Tables 1 and 2 report the odds ratios and 95% confidence intervals for CLD risk factors among males and females, respectively, by country of birth/residence, age, marital status and education.

DISCUSSION

This epidemiological study is the first to compare the risk factors for CLD in a cohort of Mexican health workers with nationally representative samples of US- and Mexico-born Mexican-Americans living in the US. The findings of our

Table 3 Effect measures comparing prevalence of risk factors among males by country of residence/birth

	Elevated ALT or AST[1]	Hepatitis B or C	Metabolic syndrome[2]	High cholesterol	Diabetes[3]	Obesity[4]	Abdominal obesity[5]	Heavy/binge drinker[6]
Odds ratios								
HWCS (Mexico)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NHANES- Mexico-born	0.8 (0.5-1.2)	0.2 (0.1-0.6)[a]	0.8 (0.6-1.1)	1.4 (1.1-1.8)[a]	1.7 (1.0-2.8)	2.1 (1.7-2.6)[a]	3.3 (1.8-6.2)[a]	3.9 (3.2-4.7)[a]
NHANES- US-born	0.9 (0.6-1.4)	1.3 (0.7-2.4)	1.4 (1.1-1.9)[a]	0.9 (0.7-1.2)	3.0 (1.9-4.8)[a]	3.9 (3.1-4.9)[a]	5.4 (2.9-10.1)[a]	4.1 (3.4-5.1)[a]
Predicted probabilities								
HWCS (Mexico)	25.0	2.4	30.2	40.9	8.5	17.4	16.8	36.4
NHANES- Mexico-born	20.8	0.5	25.8	49.3	13.0	30.6	39.3	67.1
NHANES- US-born	24.0	3.2	38.0	39.6	20.4	44.7	51.1	68.5
Prevalence ratios (95%CI)								
NHANES- Mexico-born *vs* HWCS	0.8 (0.6, 1.1)	0.2 (0.0, 0.4)[a]	0.9 (0.7, 1.1)	1.2 (1.0, 1.4)[a]	1.5 (0.9, 2.2)[a]	1.8 (1.5, 2.0)[a]	2.3 (1.2, 3.5)[a]	1.8 (1.7, 2.0)[a]
NHANES- US-born *vs* HWCS	1.0 (0.7, 1.3)	1.3 (0.6, 2.0)	1.3 (1.0, 1.5)[a]	1.0 (0.8, 1.1)	2.4 (1.5, 3.3)[a]	2.6 (2.2, 2.9)[a]	3.0 (1.6, 4.5)[a]	1.9 (1.7, 2.0)[a]
Prevalence differences (95%CI)								
NHANES- Mexico-born *vs* HWCS	-4.2 (-11.5, 3.1)	-1.9 (-3.0, -0.9)[a]	-4.3 (-10.4, 1.7)	8.5 (2.4, 14.5)[a]	4.5 (0.0, 8.9)[a]	13.2 (9.0, 17.4)[a]	22.6 (13.2, 32.0)[a]	30.7 (26.4, 35.0)[a]
NHANES- US-born *vs* HWCS	-1.0 (-8.5, 6.5)	0.7 (-0.8, 2.3)	7.8 (1.4, 14.2)[a]	-1.3 (-6.9, 4.3)	11.8 (7.2, 16.5)[a]	27.3 (22.1, 32.4)[a]	34.4 (25.3, 43.5)[a]	32.1 (28.2, 36.0)[a]

Logistic regression models adjusted for age, marital status and education. Predicted probabilities, prevalence ratios and prevalence differences were produced using marginal standardization. [1]Elevated alanine aminotransferase (ALT) and elevated alanine aminotransferase (AST) were defined as > 40 IU/L; [2]Metabolic syndrome was defined base on the Third Report of the National Cholesterol Education Program's Adult Treatment Panel Ⅲ criteria; [3]Diabetes was defined as having a plasma glucose level > 125 mg/dL after a more than 8 h fast, a medical history of diabetes, currently taking medication for diabetes, and/or a random glucose test > 200 mg/dL; [4]Obesity was defined as having a body mass index (BMI) of ≥ 30.0 kg/m^2; [5]Abdominal obesity was defined as having a waist circumference > 102 cm for males, and a waist circumference > 88 cm for females; [6]Heavy drinking was defined as two to four drinks per day for females and three to four drinks per day for males, and binge drinking was defined as having five or more drinks at one time for both males and females. [a]P< 0.05 for testing the null hypothesis of no difference between groups.

bi-national study indicate that the HWCS participants in Mexico have fewer CLD risk factors than their counterparts in the US. Specifically, we found that Mexican-American males who were born in the US are more likely to be infected with HBV or HCV, have metabolic syndrome, diabetes, obesity, abdominal obesity, or being heavy/binge drinkers when compared to immigrant Mexican-American males or their counterparts in Mexico. Similar trends are observed among females, with US-born Mexican-American females having a greater probability of having elevated AST, obesity, abdominal obesity and heavy/binge drinking. Mexican-American females who were born in Mexico are more likely to have elevated total cholesterol or diabetes when compared to those born in the US or the HWCS participants in Mexico.

Our results are consistent with other studies that report high rates of obesity, diabetes, and excessive alcohol consumption among Mexicans in both countries[29,33,42-44]. The high prevalence of obesity (30%-47%), diabetes (11%-16%), as well as heavy/binge drinking (34%-75%) we found among Mexican-Americans in this binational study are of particular concern, and are likely contributing to the liver disease disparities observed among Latinos in the US. Additionally, having a combination of certain factors, such as obesity and excessive drinking, or diabetes and HCV, has been shown to increase the risk of elevated aminotransferase levels and liver cancer[45,46]. More studies are needed to evaluate how the accumulation of specific risk factors may be contributing to the increased risk of CLD among Mexican-Americans.

Latinos are the largest ethnic or racial minority in the US. In 2016, an estimated 57.5 million Americans identified as Hispanic or Latino, representing 17.8% of the US population[47]. By 2060, the number of Latinos is projected to increase to 119 million and make up 29% of the US population. Mexican-Americans are the largest group of Latinos in the US (63%)[47]. Identifying ways to prevent CLD in this rapidly growing population is very important. As the Mexican-American population continues to grow, the challenges to address the high rates of CLD in this group will also increase. A keener awareness and deeper understanding of CLD risk factors is needed to help policy makers anticipate how changes in immigration policy, coupled with health trends in Mexico, are likely to affect the health and health care needs of the growing number of Mexican-Americans in the US. We hope our findings can be used to develop health policy strategies and programs to prevent CLD by addressing the specific risk factors that affect Mexicans in both countries.

This study has some limitations. First, unlike NHANES,

Table 4 Effect measures comparing prevalence of risk factors among females by country of residence/birth

	Elevated ALT or AST[1]	Hepatitis B or C	Metabolic syndrome[2]	High cholesterol	Diabetes[3]	Obesity[4]	Abdominal obesity[5]	Heavy/binge drinker[6]
Odds ratios								
HWCS (Mexico)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NHANES- Mexico-born	0.8 (0.5, 1.1)	0.3 (0.1, 1.8)	0.7 (0.5, 1.1)	1.2 (1.0, 1.5)	2.2 (1.4, 3.4)[a]	2.5 (1.8, 3.5)[a]	2.1 (1.2, 3.6)[a]	5.6 (4.2, 7.3)[a]
NHANES- US-born	1.0 (0.8, 1.3)	0.4 (0.2, 1.1)	0.8 (0.6, 1.2)	1.1 (0.9, 1.4)	1.7 (1.2, 2.6)[a]	3.5 (2.5, 4.9)[a]	2.3 (1.4, 3.8)[a]	12.8 (10.0, 16.3)[a]
Predicted probabilities								
HWCS (Mexico)	10.2	2.2	34.9	41.2	8.3	20.0	51.1	8.4
NHANES- Mexico-born	8.2	0.7	28.8	45.8	15.4	38.4	67.8	32.1
NHANES- US-born	10.6	1.0	31.2	43.3	12.9	46.3	69.6	50.3
Prevalence ratios (95%CI)								
NHANES- Mexico-born *vs* HWCS	0.8 (0.5, 2.1)	0.3 (0.0, 0.9)[a]	0.8 (0.6, 1.0)	1.1 (1.0, 1.2)	1.9 (1.2, 2.5)[a]	1.9 (1.4, 2.4)[a]	1.3 (1.0, 1.6)[a]	3.8 (3.0, 4.6)[a]
NHANES- US-born *vs* HWCS	1.0 (0.8, 1.2)	0.4 (0.0, 0.8)[a]	0.9 (0.7, 1.1)	1.1 (0.9, 1.2)	1.6 (1.1, 2.1)[a]	2.3 (1.7, 2.9)[a]	1.4 (1.0, 1.7)[a]	6.0 (4.9, 7.1)[a]
Prevalence differences (95%CI)								
NHANES- Mexico-born *vs* HWCS	-2.1 (-4.7, 0.6)	-1.5 (-2.8, -0.2)[a]	-6.1 (-13.0, 0.8)	4.6 (-0.7, 9.8)	7.1 (1.8, 12.4)[a]	18.4 (12.5, 24.3)[a]	16.8 (4.6, 29.0)[a]	23.7 (19.4, 28.0)[a]
NHANES- US-born *vs* HWCS	0.3 (-1.7, 2.3)	-1.2 (-2.7, 0.0)	-3.7 (-10.2, 2.7)	2.2 (-2.9, 7.3)	4.7 (1.3, 8.0)[a]	26.2 (20.5, 31.9)[a]	18.5 (6.5, 30.6)[a]	41.9 (38.0, 45.9)[a]

Logistic regression models adjusted for age, marital status and education. Predicted probabilities, prevalence ratios and prevalence differences were produced using marginal standardization. [1]Elevated alanine aminotransferase (ALT) and elevated alanine aminotransferase (AST) were defined as > 40 IU/L; [2]Metabolic syndrome was defined base on the Third Report of the National Cholesterol Education Program's Adult Treatment Panel Ⅲ criteria; [3]Diabetes was defined as having a plasma glucose level > 125 mg/dL after a more than 8 h fast, a medical history of diabetes, currently taking medication for diabetes, and/or a random glucose test > 200 mg/dL; [4]Obesity was defined as having a body mass index (BMI) of ≥ 30.0 kg/m^2; [5]Abdominal obesity was defined as having a waist circumference > 102 cm for males, and a waist circumference > 88 cm for females; [6]Heavy drinking was defined as two to four drinks per day for females and three to four drinks per day for males, and binge drinking was defined as having five or more drinks at one time for both males and females. [a]$P < 0.05$ for testing the null hypothesis of no difference between groups.

the HWCS is not a population-based study that is representative of the Mexican population. The HWCS participants are health workers who are younger, more educated, and predominantly female. However, to the best of our knowledge, the HWCS is the only longitudinal study in Mexico that includes ALT and AST measures as well as HBV and HCV results for a large number of Mexican adults, which is why we used the HWCS data for this binational study. In order to address this limitation, we compared some of the HWCS results to the findings of the 2012 Encuesta Nacional de Salud y Nutrición (ENSANUT)[48], a larger, population-based study that is representative of the Mexican population and can be considered a simplified version of NHANES. The prevalence of diabetes reported in the 2012 ENSANUT was 9.2%[48], while the prevalence of diabetes among the HWCS was 6.7%. Obesity was also higher among the male (26.8%) and female (37.5%) ENSANUT participants[48], as compared to the HWCS participants. The prevalence of heavy/binge drinking (42%) was also more common among the ENSANUT participants[48]. Nonetheless, even when compared to the ENSANUT results, the prevalence of these risk factors remains greater among the Mexican-Americans from the NHANES sample. Due to the limited generalizability of our study's results, they should be viewed as exploratory and preliminary.

Another limitation was our ability to control for confounding variables in the comparisons between Mexico and the US. To address this issue, all analyses were stratified by sex and controlled for age, marital status, and educational level in the regression analyses. We also limited the US sample to individuals with health insurance, since all the HWCS participants have health insurance. Our ability to control for potential confounders was restricted by the available data, and it is therefore possible that other unobserved differences between the two samples may be confounding our results.

In conclusion, the results of this bi-national study indicate that Mexican-Americans in the US have more risk factors for CLD than their counterparts in Mexico, and point to a critical need for prevention programs. Of particular concern are the high rates of heavy/binge drinking observed among Mexican-Americans. We hope these findings can be used by health professionals in Mexico and the US to tailor screening and prevention strategies to help reduce the risk of CLD among their patients. Our results could also be used to design and implement more effective health promotion programs to address the specific factors that put Mexicans at higher risk for developing CLD in both countries. These findings add to the relatively scarce literature on bi-national research, and provide preliminary data for future studies of migrant health in the US and Mexico. Other bi-national primary data collection projects with representative samples and comparable

demographic, socioeconomic and health status measures are needed to further investigate the growing problem of CLD among Mexicans in both countries. The results of this bi-national analysis indicate that Mexican-Americans in the US have more risk factors for CLD than their counterparts in Mexico. These results can be used to design and implement more effective health promotion programs and policies to address the specific factors that put Mexicans at higher risk of developing CLD in both countries. Our findings add to the relatively scarce literature on bi-national research, and provide preliminary data for future studies of migrant health in the US and Mexico. Other bi-national primary data collection projects with representative samples and comparable demographic, socioeconomic and health status measures are needed to further investigate the growing problem of CLD among Mexicans in both countries.

ARTICLE HIGHLIGHTS

Research background

United States (US) Latinos have greater morbidity and mortality from liver disease than non-Hispanic whites. Liver disease is the fifth leading cause of death in Mexico. In the US, Mexican-Americans have a greater risk of obesity, diabetes and heavy/binge drinking than in Mexico.

Research motivation

Over 30 million people are likely to have some form of chronic liver disease (CLD) in the US. CLD is the 12th leading cause of general mortality in the US.

Research objectives

To compare the prevalence of CLD risk factors in a representative sample of Mexican-Americans, born in the US or Mexico, to a sample of adults in Mexico.

Research methods

The main independent variables for this study classified individuals by country of residence and place of birth. Regression analyses were used to investigate CLD risk factors.

Research results

There is a greater risk among US-born *vs* Mexico-born Mexican-Americans.

Research conclusions

Mexican-Americans in the US had more risk factors for CLD.

Research perspectives

Our findings add to the relatively scarce literature on bi-national research, providing preliminary data for future studies of migrant health in the US and Mexico. Other bi-national primary data collection projects with representative samples and comparable demographic, socioeconomic and health status measures are needed to further investigate the growing problem of CLD among Mexicans in both countries.

REFERENCES

1 Surveillance for Viral Hepatitis-United States. 2017. Available from: URL: https://www.cdc.gov/hepatitis/statistics/2015surveillance/commentary.htm

2 **Le MH**, Devaki P, Ha NB, Jun DW, Te HS, Cheung RC, Nguyen MH. Prevalence of non-alcoholic fatty liver disease and risk factors for advanced fibrosis and mortality in the United States. *PLoS One* 2017; **12**: e0173499 [PMID: 28346543 DOI: 10.1371/journal.pone.0173499]

3 **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]

4 **Kochanek KD**, Murphy SL, Xu J, Tejada-Vera B. Deaths: Final Data for 2014. *Natl Vital Stat Rep* 2016; **65**: 1-122 [PMID: 27378572]

5 **American Cancer Society**. Key Statistics about Liver Cancer. 2017. Available from: URL: https://www.cancer.org/cancer/liver-cancer/about/what-is-key-statistics.html

6 Ten Leading Causes of Death by Age Group, United States, 2014. Available from: URL: https://www.cdc.gov/injury/wisqars/leadingcauses.html

7 **Heron M**. Deaths: Leading Causes for 2014. *Natl Vital Stat Rep* 2016; **65**: 1-96 [PMID: 27376998]

8 Leading Causes of Death in Males, 2014. 2017. Available from: URL: https://www.cdc.gov/men/lcod/2014/hispanic/index.htm

9 **US Department of Health and Human Services**. Office of Minority Health. Chronic Liver Disease and Hispanic Americans. 2015. Available from: URL: http://minorityhealth.hhs.gov/omh/browse.aspx?lvl=4lvlid=62

10 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570 DOI: 10.1002/hep.20466]

11 **Instituto Nacional de Estadística y Geografía (INEGI)**. Principales causas de mortalidad por residencia habitual, grupos de edad y sexo. 2016. Available from: URL: http://www.inegi.org.mx/est/contenidos/proyectos/registros/vitales/mortalidad/tabulados/ConsultaMortalidad.asp

12 **Panduro A**, Escobedo Meléndez G, Fierro NA, Ruiz Madrigal B, Zepeda-Carrillo EA, Román S. Epidemiology of viral hepatitis in Mexico. *Salud Publica Mex* 2011; **53** Suppl 1: S37-S45 [PMID: 21877071]

13 **Méndez-Sánchez N**, Villa AR, Chávez-Tapia NC, Ponciano-Rodriguez G, Almeda-Valdés P, González D, Uribe M. Trends in liver disease prevalence in Mexico from 2005 to 2050 through mortality data. *Ann Hepatol* 2005; **4**: 52-55 [PMID: 15798662]

14 **El-Serag HB**. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004; **127**: S27-S34 [PMID: 15508094 DOI: 10.1053/j.gastro.2004.09.013]

15 **Clark JM**. The epidemiology of nonalcoholic fatty liver disease in adults. *J Clin Gastroenterol* 2006; **40** Suppl 1: S5-10 [PMID: 16540768]

16 **Ascha MS**, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972-1978 [PMID: 20209604 DOI: 10.1002/hep.23527]

17 **Davila JA**. Diabetes and hepatocellular carcinoma: what role does diabetes have in the presence of other known risk factors? *Am J Gastroenterol* 2010; **105**: 632-634 [PMID: 20203644 DOI: 10.1038/ajg.2009.715]

18 **Regimbeau JM**, Colombat M, Mognol P, Durand F, Abdalla E, Degott C, Degos F, Farges O, Belghiti J. Obesity and diabetes as a risk factor for hepatocellular carcinoma. *Liver Transpl* 2004; **10**: S69-S73 [PMID: 14762843 DOI: 10.1002/lt.20033]

19 **Bellentani S**, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* 2010; **28**: 155-161 [PMID: 20460905 DOI: 10.1159/000282080]

20 **Weston SR**, Leyden W, Murphy R, Bass NM, Bell BP, Manos MM, Terrault NA. Racial and ethnic distribution of nonalcoholic fatty liver in persons with newly diagnosed chronic liver disease. *Hepatology* 2005; **41**: 372-379 [PMID: 15723436 DOI: 10.1002/hep.20554]

21 **Flegal KM**, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA* 2016; **315**: 2284-2291 [PMID: 27272580 DOI: 10.1001/jama.2016.6458]

22 National Diabetes Statistics Report, 2017. Estimates of Diabetes and Its Burden in the United States. Atlanta, GA: Centers for Disease

Control and Prevention, US Dept of Health and Human Services; 2017. Available from: URL: https://www.cdc.gov/diabetes/data/statistics-report/index.html

23 **Mozaffarian D**, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation* 2015; **131**: e29-e322 [PMID: 25520374 DOI: 10.1161/CIR.0000000000000152]

24 **Gutiérrez JP,** Rivera-Dommarco J, Shamah-Levy T, Villalpando-Hernández S, Franco A, Cuevas-Nasu L, Romero-Martínez M, Hernández-Ávila M. Encuesta Nacional de Salud y Nutrición 2012. Resultados Nacionales. Cuernavaca, México: Instituto Nacional de Salud Pública (MX), 2012. Available from: URL: https://ensanut.insp.mx/informes/ENSANUT2012ResultadosNacionales.pdf

25 **Rojas R**, Aguilar-Salinas CA, Jiménez-Corona A, Shamah-Levy T, Rauda J, Avila-Burgos L, Villalpando S, Ponce EL. Metabolic syndrome in Mexican adults: results from the National Health and Nutrition Survey 2006. *Salud Publica Mex* 2010; **52** Suppl 1: S11-S18 [PMID: 20585723 DOI: 10.1590/S0036-36342010000700004]

26 **González-Villalpando C**, Dávila-Cervantes CA, Zamora-Macorra M, Trejo-Valdivia B, González-Villalpando ME. Incidence of type 2 diabetes in Mexico: results of the Mexico City Diabetes Study after 18 years of follow-up. *Salud Publica Mex* 2014; **56**: 11-17 [PMID: 24912516 DOI: 10.21149/spm.v56i1.7318]

27 **Oh RC**, Hustead TR. Causes and evaluation of mildly elevated liver transaminase levels. *Am Fam Physician* 2011; **84**: 1003-1008 [PMID: 22046940]

28 **Tsai J**, Ford ES, Li C, Zhao G. Past and current alcohol consumption patterns and elevations in serum hepatic enzymes among US adults. *Addict Behav* 2012; **37**: 78-84 [PMID: 21975024 DOI: 10.1016/j.addbeh.2011.09.002]

29 **Flores YN**, Yee HF Jr, Leng M, Escarce JJ, Bastani R, Salmerón J, Morales LS. Risk factors for chronic liver disease in Blacks, Mexican Americans, and Whites in the United States: results from NHANES IV, 1999-2004. *Am J Gastroenterol* 2008; **103**: 2231-2238 [PMID: 18671818 DOI: 10.1111/j.1572-0241.2008.02022.x]

30 **Ioannou GN**, Boyko EJ, Lee SP. The prevalence and predictors of elevated serum aminotransferase activity in the United States in 1999-2002. *Am J Gastroenterol* 2006; **101**: 76-82 [PMID: 16405537 DOI: 10.1111/j.1572-0241.2005.00341.x]

31 **Abraído-Lanza AF**, Dohrenwend BP, Ng-Mak DS, Turner JB. The Latino mortality paradox: a test of the "salmon bias" and healthy migrant hypotheses. *Am J Public Health* 1999; **89**: 1543-1548 [PMID: 10511837 DOI: 10.2105/AJPH.89.10.1543]

32 **Rubalcava LN**, Teruel GM, Thomas D, Goldman N. The healthy migrant effect: new findings from the Mexican Family Life Survey. *Am J Public Health* 2008; **98**: 78-84 [PMID: 18048791 DOI: 10.2105/AJPH.2006.098418]

33 **Morales LS**, Flores YN, Leng M, Sportiche N, Gallegos-Carrillo K, Salmerón J. Risk factors for cardiovascular disease among Mexican-American adults in the United States and Mexico: a comparative study. *Salud Publica Mex* 2014; **56**: 197-205 [PMID: 25014426 DOI: 10.21149/spm.v56i2.7335]

34 National Health and Nutrition Examination Survey, 2013-2014. Available from: URL: http://www.cdc.gov/nchs/nhanes/about_nhanes.htm

35 **Denova-Gutiérrez E**, Flores YN, Gallegos-Carrillo K, Ramírez-Palacios P, Rivera-Paredez B, Muñoz-Aguirre P, Velázquez-Cruz R, Torres-Ibarra L, Meneses-León J, Méndez-Hernández P, Hernández-López R, Salazar-Martínez E, Talavera JO, Tamayo J, Castañón S, Osuna-Ramírez I, León-Maldonado L, Flores M, Macías N, Antúnez D, Huitrón-Bravo G, Salmerón J. Health workers cohort study: methods and study design. *Salud Publica Mex* 2016; **58**: 708-716 [PMID: 28225947 DOI: 10.21149/spm.v58i6.8299]

36 **National Health and Nutrition Examination Survey (NHANES).** 2013-2014 MEC Laboratory Procedures Manual. Available from: URL: https://wwwn.cdc.gov/nchs/data/nhanes/2013-2014/manuals/2013_MEC_Laboratory_Procedures_Manual.pdf

37 Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel Ⅲ). *JAMA* 2001; **285**: 2486-2497 [PMID: 11368702 DOI: 10.1001/jama.285.19.2486]

38 American Diabetes Association. Screening for type 2 diabetes. *Diabetes Care* 2004; **27** Suppl 1: S11-S14 [PMID: 14693922 DOI: 10.2337/diacare.27.2007.S11]

39 Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary. Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults. *Am J Clin Nutr* 1998; **68**: 899-917 [PMID: 9771869 DOI: 10.1093/ajcn/68.4.899]

40 **Sundquist J**, Winkleby MA, Pudaric S. Cardiovascular disease risk factors among older black, Mexican-American, and white women and men: an analysis of NHANES Ⅲ, 1988-1994. Third National Health and Nutrition Examination Survey. *J Am Geriatr Soc* 2001; **49**: 109-116 [PMID: 11207863 DOI: 10.1046/j.1532-5415.2001.49030.x]

41 **Jiles R**, Hughes E, Murphy W, Flowers N, McCracken M, Roberts H, Ochner M, Balluz L, Mokdad A, Elam-Evans L, Giles W. Surveillance for certain health behaviors among states and selected local areas--Behavioral Risk Factor Surveillance System, United States, 2003. *MMWR Surveill Summ* 2005; **54**: 1-116 [PMID: 16319816]

42 **Flores YN**, Lang CM, Salmerón J, Bastani R. Risk factors for liver disease and associated knowledge and practices among Mexican adults in the US and Mexico. *J Community Health* 2012; **37**: 403-411 [PMID: 21877109 DOI: 10.1007/s10900-011-9457-4]

43 **Vega WA**, Rodriguez MA, Gruskin E. Health disparities in the Latino population. *Epidemiol Rev* 2009; **31**: 99-112 [PMID: 19713270 DOI: 10.1093/epirev/mxp008]

44 **Islam N**, Flores YN, Ramirez P, Bastani R, Salmerón J. Hepatitis and liver disease knowledge and preventive practices among health workers in Mexico: a cross-sectional study. *Int J Public Health* 2014; **59**: 381-394 [PMID: 24097058 DOI: 10.1007/s00038-013-0515-9]

45 **Mahli A**, Hellerbrand C. Alcohol and Obesity: A Dangerous Association for Fatty Liver Disease. *Dig Dis* 2016; **34** Suppl 1: 32-39 [PMID: 27548267 DOI: 10.1159/000447279]

46 **Hammerstad SS**, Grock SF, Lee HJ, Hasham A, Sundaram N, Tomer Y. Diabetes and Hepatitis C: A Two-Way Association. *Front Endocrinol* (Lausanne) 2015; **6**: 134 [PMID: 26441826 DOI: 10.3389/fendo.2015.00134]

47 **United States Census Bureau.** Facts for Features: Hispanic Heritage Month 2017. Available from: URL: https://www.census.gov/newsroom/facts-for-features/2017/hispanic-heritage.html

48 **Encuesta Nacional de Salud y Nutrición.** Resultados Nacionales. 2012. Available from: URL: http://ensanut.insp.mx/informes/ENSANUT2012ResultadosNacionales.pdf

P- Reviewer: Abenavoli L, Trovato GM **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 7; 24(37): 4291-4296

DOI: 10.3748/wjg.v24.i37.4291

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

CASE REPORT

Cerebral lipiodol embolism related to a vascular lake during chemoembolization in hepatocellular carcinoma: A case report and review of the literature

Hideki Ishimaru, Minoru Morikawa, Takayuki Sakugawa, Ichiro Sakamoto, Yasuhide Motoyoshi, Yohei Ikebe, Masataka Uetani

Hideki Ishimaru, Minoru Morikawa, Department of Radiology, Nagasaki University Hospital, Nagasaki 852-8501, Japan

Takayuki Sakugawa, Department of Radiology, University of the Ryukyus, Okinawa 903-0213, Japan

Ichiro Sakamoto, Department of Radiology, Nagasaki Harbor Medical Cancer, Nagasaki 850-8555, Japan

Yasuhide Motoyoshi, Department of Gastroenterology, Nagasaki Harbor Medical Cancer, Nagasaki 850-8555, Japan

Yohei Ikebe, Masataka Uetani, Department of Radiological Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8501, Japan

ORCID number: Hideki Ishimaru (0000-0002-7817-0507); Minoru Morikawa (0000-0003-3423-3008); Takayuki Sakugawa (0000-0003-3593-8739); Ichiro Sakamoto (0000-0002-4512-4950); Yasuhide Motoyoshi (0000-0002-7295-8758); Yohei Ikebe (0000-0002-4130-1446); Masataka Uetani (0000-0001-5922-0508).

Author contributions: Ishimaru H, Morikawa M, and Sakamoto I designed the report; Sakugawa T and Motoyoshi Y collected the patient's clinical data; Ishimaru H, Ikebe Y, and Uetani M wrote the paper.

Informed consent statement: Consent was obtained from relatives of the patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

CARE Checklist (2013): The guidelines of the CARE Checklist (2013) were adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Minoru Morikawa, MD, Assistant Professor, Department of Radiology, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. m-minoru@nagasaki-u.ac.jp
Telephone: +81-95-8197354
Fax: +81-95-8197357

Received: July 16, 2018
Peer-review started: July 16, 2018
First decision: August 1, 2018
Revised: August 4, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 7, 2018

Abstract

A male patient underwent conventional transcatheter chemoembolization for advanced recurrent hepatocellular carcinoma (HCC). Even after the injection of 7 mL of lipiodol followed by gelatin sponge particles, the flow of feeding arteries did not slow down. A repeat angiography revealed a newly developed vascular lake draining into systemic veins; however, embolization was continued without taking noticing of the vascular lake. The patient's level of consciousness deteriorated immediately after the procedure, and non-contrast computed tomography revealed pulmonary and cerebral lipiodol embolisms. The patient's level of consciousness gradually improved after 8 wk in intensive care. In this

case, a vascular lake emerged during chemoembolization and drained into systemic veins, offering a pathway carrying lipiodol to pulmonary vessels, the most likely cause of this serious complication. We should be aware that vascular lakes in HCC may drain into systemic veins and can cause intratumoral arteriovenous shunts.

Key words: Transcatheter arterial chemoembolization; Arteriovenous shunt; Hepatocellular carcinoma; Vascular lake; Cerebral embolism

Core tip: Vascular lakes that resemble extravasation within hepatocellular carcinomas occasionally emerge during chemoembolization. To date, the drainage routes from vascular lakes are not well understood. We present a patient with a recurrent large hepatocellular carcinoma in which a vascular lake emerged during conventional chemoembolization, draining into systemic veins and causing pulmonary and cerebral lipiodol embolism.

Ishimaru H, Morikawa M, Sakugawa T, Sakamoto I, Motoyoshi Y, Ikebe Y, Uetani M. Cerebral lipiodol embolism related to a vascular lake during chemoembolization in hepatocellular carcinoma: A case report and review of the literature. *World J Gastroenterol* 2018; 24(37): 4291-4296 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i37/4291.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i37.4291

INTRODUCTION

Transcatheter arterial chemoembolization (TACE) is utilized worldwide for the treatment of patients with unresectable hepatocellular carcinoma (HCC). Although various complications of TACE have been reported[1], cerebral lipiodol embolism (CLE) after TACE is very rare. To our knowledge, 27 cases have been reported in the English literature, and possible pathways for carrying lipiodol from HCCs to systemic arteries have been hypothesized[2-18]. This is the first report of CLE, in which the vascular lake phenomenon emerged during the TACE procedure and caused an intratumoral arteriovenous shunt, playing the most important role in its occurrence. We also discussed the mechanism of CLE and technical considerations to avoid this serious complication. We also provided a review of the literature.

CASE REPORT

A 63-year-old man with a large recurrent HCC replacing most of the lateral segment of the liver and expanding to the left diaphragm was admitted to our hospital for a second TACE. He had a history of type-B cirrhosis for 3 years. Eight months prior to admission, he had undergone TACE for the same lesion *via* the left hepatic artery (LHA), and the postprocedural course was uneventful. Laboratory tests before second TACE revealed a serum total bilirubin level of 1.4 mg/dL, serum albumin level of 3.0 mg/dL, and prothrombin activity level of 81%. Neither ascites nor hepatic encephalopathy were found, which corresponded to Child-Pugh class A. The α-fetoprotein level was 900 ng/mL. At angiography, the large HCC was supplied by the LHA and the left inferior phrenic artery (LIPA) (Figure 1A) without an apparent arteriovenous (AV) shunt. For arterial redistribution to convert multiple feeding arteries into a single arterial supply, distal branches of the LIPA supplying HCC were embolized initially. To avoid migration of embolic materials into pulmonary vessels, the LIPA was embolized with 0.6 mL of a 33% mixture of n-butyl cyanoacrylate (Histoacryl; B. Braun, Melsungen, Germany) and lipiodol (Lipiodol; Terumo Corporation, Tokyo, Japan) (Figure 1B). Subsequently, we infused 140 mg of miriplatin (Miripla; Dainippon Sumitomo Pharma, Osaka, Japan) suspended in 7 mL of lipiodol *via* the LHA (Figure 2A). This was followed by the injection of 1-mm-diameter gelatin sponge particles (Gelpart; Nippon Kayaku Co. Ltd., Tokyo, Japan). However, the flow of the LHA did not slow down. A repeated left hepatic angiography showed contrast material pooling associated with an aberrant intratumoral space newly developed during embolization, called the "vascular lake phenomenon" (Figure 2B). Late-phase imaging revealed that the pericardiacophrenic vein was the draining vein (Figure 2C), which was not recognized at the time of the procedure but rather retrospectively after remasking and pixel shifting were performed. Epirubicin (10 mg) (Farmorubicin; Kyowa Hakko, Tokyo, Japan) emulsified in 9 mL of lipiodol was additionally infused into the LHA, followed by the injection of a 2-mm block gelatin sponge (Spongel; Astellas, Tokyo, Japan) until the flow slowed down. Immediately after TACE, the patient became drowsy and disoriented. Unenhanced CT of the brain obtained 30 minutes after the procedure revealed multiple lesions of increased attenuation in the cerebral cortex, basal ganglia, thalami and cerebellum (Figure 3). Simultaneously, a CT of the chest revealed hyperattenuation at both lung bases (Figure 4). The patient's oxygen saturation was 88%, indicating hypoxia. The patient was monitored in our intensive care unit on mechanical ventilator support. Echocardiography revealed no atrial septal defect or other intracardiac shunts. The patient's level of consciousness gradually improved, but his limb weakness persisted. He was discharged 8 wk later requiring the use of a wheelchair and assistance to eat meals.

DISCUSSION

TACE has been widely accepted as an effective therapy for HCC. Lipiodol is the most common embolic material used in TACE; it is usually mixed with anticancer drugs dissolved in non-ionic contrast medium. Lipiodol can enter the microcirculation of the tumor and flow into the surrounding portal vein, which is the main drainage route from the hypervascular HCC[19]. However, lipiodol

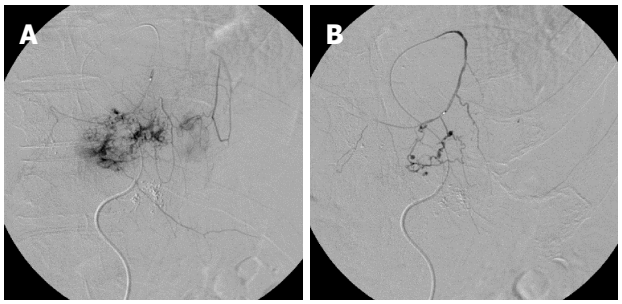


Figure 1 Angiography. A: Selective angiography of the left inferior phrenic artery shows feeding of the anterior part of the tumor without a shunt to the pulmonary vasculature; B: Angiography during glue embolization shows no influx into the pulmonary vasculature.

can reportedly flow into systemic circulation, causing pulmonary embolism and, rarely, cerebral embolism. We found 27 reports of CLE following TACE in 17 English studies (Table 1). To reach the cerebral arteries, lipiodol must pass through two pathways, one from the tumor to the pulmonary artery (PA) and the other from the pulmonary artery to the left atrium (LA). Lipiodol accumulation in the lung was identified simultaneously in 17 of 27 previously reported cases[2-4,6,8-10,14,16,18].

Localized pooling of the contrast medium emerges occasionally during chemoembolization using drug eluting beads (DEB-TACE), resembling extravasation within the tumor. This angiographic finding is known as the vascular lake phenomenon (VLP). As we reported herein, this phenomenon may also appear during chemoembolization using lipiodol, although it is difficult to distinguish a vascular lake from lipiodol accumulating in the tumor by fluoroscopy. Concerning the etiology of VLP during chemoembolization, Seki *et al*[20] speculated that rapid occlusion of most of the tumor vessels increased the pressure inside the fragile tumor microvasculature, causing disruption of the tumor architecture and a new blood space. VLP is thought to indicate a good local response in HCC patients undergoing DEB-TACE[20,21]. Nevertheless, the drainage routes from vascular lakes are unknown. To the best of our knowledge, this is the first report observing a vascular lake draining into systemic veins, resulting in an intratumoral AV shunt and affording lipiodol pathways to pulmonary vessels.

Intratumoral AV shunts can be the pathway to pulmonary vessels[5,7,10], and this was confirmed by angiography in only one CLE case[10]. On hepatic arteriography, the incidence of intratumoral AV shunts was reported to be 2.4%[22]. According to one study using technetium-99-m-labeled macroaggregated albumin[23], lung shunt fractions exceeding 10% were identified in 50 of 125 HCC patients (40%). The study also reported a direct correlation between angiographic tumor vascularity and lung shunt fractions[23]. The particles of lipiodol emulsion were less than 30 μm in size[24], within the range of the size of technetium-99-m-labeled macroaggregated albumin (10-60 μm in size). Lipiodol redistribution in the lungs may be more frequent than that previously thought.

TACE *via* IPA frequently results in pulmonary complications due to the existence of an AV shunt between the IPA and PA[25]. Some authors speculated that communication between the IPA and pulmonary vessels *via* adhesive pleurae or tumor invasion is the most likely pathway from the tumor to the PA[4,6]. In the present case, we performed IPA embolization using glue under DSA guidance, and the glue fragment never flowed into the pulmonary vessels.

In most previously reported cases of CLE, including the case described herein, a large dose of lipiodol was infused (Table 1). Cerebral and pulmonary lipiodol embolisms have been reported in patients receiving more than 20 mL of iodized oil after TACE of HCC[4,6,16,18]. According to Kishi *et al*[26], when lipiodol was infused into the dog's hepatic artery, the amount of lipiodol oil deposition in the lungs was proportional to the lipiodol dose infused. They also found lipiodol deposits in the brain and pancreas[26]. These findings suggested a dose-dependent circulation of oil droplets *via* hepatic sinusoids to pulmonary capillaries and then into the systemic circulation. It was suggested that the lipiodol dose should not exceed 15-20 mL to prevent the risk of an extrahepatic embolism[4,6]. Nevertheless, in 7 previous cases, CLE occurred when the lipiodol dose was < 15 mL[2,5,7,14,18]. The required lipiodol dose was determined by multiple factors, including the blood supply to the tumor, tumor size, catheter position and liver function reserve. When lipiodol goes through an intratumoral AV shunt, the dose of lipiodol required to accomplish HCC flow stasis increases. If there had not been an intratumoral AV shunt in the presented case, we could have accomplished flow stoppage with a lower dose of lipiodol.

CLE is thought to be associated with intrapulmonary or intracardiac shunts[4,7,10,16]. Evidence of an underlying intracardiac right-to-left shunt was proven by transesophageal echocardiogram in one previous case[7]; however, the pathway from the PA to the LA was not verified in most previously reported cases of CLE, including the present case. Wu *et al*[9] speculated that an intra-pulmonary arteriovenous shunt might appear during pulmonary lipiodol embolization due to increasing pulmonary artery pressure or hypoxia. Wu *et al*[10] added that communication between the systemic and pulmonary vessels might develop *via* adhesive pleurae or tumor invasion of the diaphragm, leading to a right-to-left shunt. In a case report of delayed CLE, Wu *et al*[8] concluded that the rapid flow of the feeding artery washed out the lipiodol, and the lipiodol deposited in the lungs was washed out again upon entering the systemic circulation. Since it has been verified that fat globules < 7 μm in diameter can pass directly through the pulmonary arteriolar network[2], lipiodol can enter the systemic circulation in the absence of a right-to-left shunt to cause cerebrovascular complications.

In summary, we presented a case in which a vascular lake draining into systemic veins caused a lipiodol cerebral embolism. As intratumoral AV shunts *via* vas-

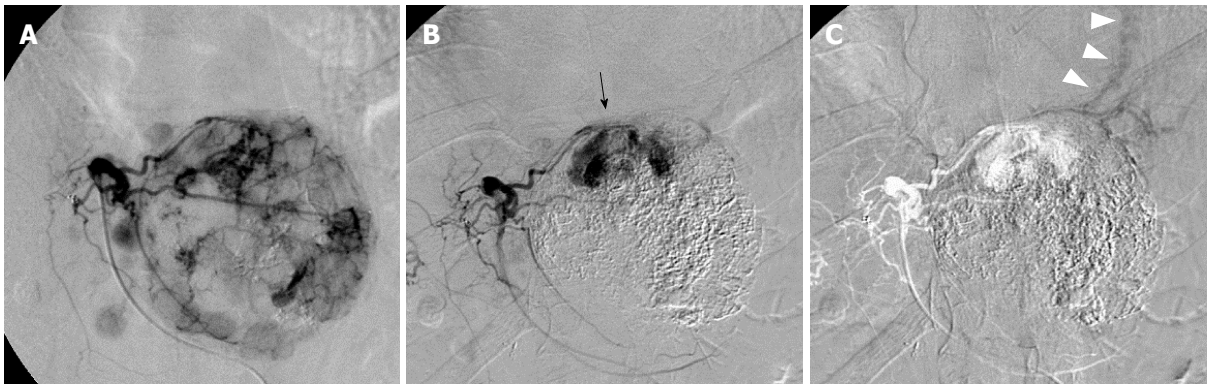


Figure 2 Infused 140 mg of miriplatin suspended in 7 mL of lipiodol *via* the left hepatic artery. A: Left hepatic angiography shows a large hypervascular tumor in the left hepatic lobe and intrahepatic metastases neighborhood without an intratumoral AV shunt; B: Repeated left hepatic angiography shows a vascular lake in the superior portion of the tumor (arrow) that developed after chemoembolization; C: Venous phase shows drainage into the pericardiacophrenic vein (arrow heads), which was unrecognized until remasking and pixel shifting were performed. AV: Arteriovenous.

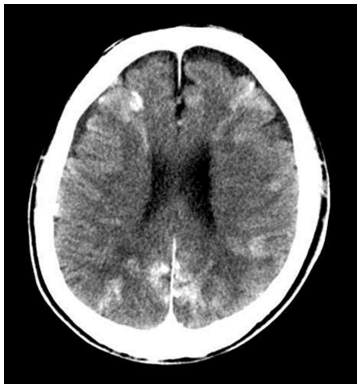


Figure 3 Non-contrast brain computed tomography scan obtained 30 min after chemoembolization shows multiple increased attenuated lesions in the bilateral cerebral hemisphere, consistent with lipiodol embolism.

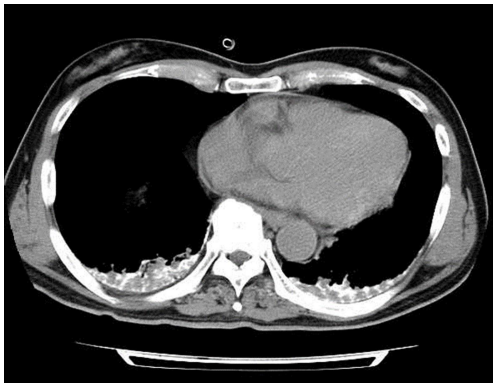


Figure 4 Chest computed tomography scan obtained 30 min after chemoembolization shows lipiodol deposition at both lung bases.

cular lakes may develop during chemoembolization, we recommend performing repeated angiography during TACE procedures, especially when obtaining a decreased blood flow is difficult. When an intratumoral AV shunt is identified by angiography, embolization of the feeding artery using coils or glue should be considered instead of an additional injection of lipiodol or embolic particles. We should try to prevent lipiodol accumulation in the lungs because the pathways from the PA to the LA cannot be blocked regardless of the mechanism by which they are developed.

ARTICLE HIGHLIGHTS

Case characteristics

A 63-year-old man with a large hepatocellular carcinoma underwent transcatheter arterial chemoembolization (TACE), but his level of consciousness deteriorated immediately after the procedure.

Clinical diagnosis

CT scan of the brain revealed multiple lesions of increased attenuation, and cerebral lipiodol embolism (CLE) was confirmed.

Differential diagnosis

There is no differential diagnosis.

Laboratory diagnosis

No specific finding was obtained by laboratory testing.

Imaging diagnosis

An angiography during TACE procedure revealed a newly developed vascular lake draining into systemic veins, which offered a pathway carrying lipiodol to pulmonary vessels and was the most likely cause of this serious complication.

Pathological diagnosis

No pathological examination was performed.

Treatment

The patient was treated in our intensive care unit.

Related reports

This is the first report of CLE, in which vascular lake phenomenon emerging during the procedure caused intratumoral arteriovenous shunt and played the most important role for its occurrence.

Term explanation

The term CLE describes cerebral lipiodol embolism.

Experiences and lessons

Arteriovenous shunt *via* vascular lake may develop during chemoembolization, repeated angiography during TACE procedures should be performed to prevent

Table 1 Characteristics of 28 cases of cerebral lipiodol embolism

Age	Sex	Tumor characteristics	Pulmonary involvement	Embolization *via* extrahepatic artery supply	Dose of lipiodol	AV shunt	RL shunt	Ref.
52	M	Advanced	+	-	35		ND	[2]
58	M		+		8		ND	[2]
56	M		+				ND	[2]
76	M	Large	+	IPA				[3]
81	F	Large	+	IPA	20		ND	[4]
70	F	Large			12	ND		[5]
62	F	15.0 cm	+	IPA	30	ND	ND	[6]
67	M			IPA	5		PFO	[7]
63	F			IPA	10		+	[7]
36	M	Huge	+		40	ND		[8]
51	F	Huge	+		40	ND		[9]
41	M	Multiple and PVTT	+		30	+	ND	[10]
71	M						ND	[11]
44	M	Large		BA				[12]
54	M	13.0 cm		IPA	30			[13]
66	M	11.5 cn	+	-	4		ND	[14]
62	M	16.0 cm					ND	[15]
52	M	18.0 cm	+	IPA	50	ND	Pulmonary AV shunt	[16]
66	F			IPA	20			[17]
39	M	8.0 cm		IPA	13	PV		[18]
51	M	13.0 cm	+	-	30	PV		[18]
73	F	19.0 cm	+	IPA	15	PV		[18]
67	F	6.0 cm	+	LGA	10			[18]
54	F	3.0 cm		IPA	90			[18]
63	M	14.0 cm	+	RSGA	50			[18]
52	M	17.0 cm	+	-	30			[18]
72	M	10.0 cm	+	-	20			[18]
63	M	9.0 cm	+	IPA	16	+	ND	Present case

ND: Not detected; IPA: Inferior phrenic artery; AV: Arteriovenous; RL: Right-to-left; PFO: Patent foramen ovale; PVTT: Portal vein tumor thrombus; BA: Bronchial artery; PV: Shunt to pulmonary vein; LGA: Left gastric artery; RSGA: Right superior gluteal artery.

CLE, especially when it is difficult to obtain a decrease of blood flow.

REFERENCES

1 **Sakamoto I**, Aso N, Nagaoki K, Matsuoka Y, Uetani M, Ashizawa K, Iwanaga S, Mori M, Morikawa M, Fukuda T, Hayashi K, Matsunaga N. Complications associated with transcatheter arterial embolization for hepatic tumors. *Radiographics* 1998; **18**: 605-619 [PMID: 9599386 DOI: 10.1148/radiographics.18.3.9599386]

2 **Yoo KM**, Yoo BG, Kim KS, Lee SU, Han BH. Cerebral lipiodol embolism during transcatheter arterial chemoembolization. *Neurology* 2004; **63**: 181-183 [PMID: 15249637 DOI: 10.1212/01.WNL.0000132645.23611.2D]

3 **Takao H**, Makita K, Doi I, Watanabe T. Cerebral lipiodol embolism after transcatheter arterial chemoembolization of hepatocellular carcinoma. *J Comput Assist Tomogr* 2005; **29**: 680-682 [PMID: 16163042 DOI: 10.1097/01.rct.0000165906.83160.7d]

4 **Wu RH**, Tzeng WS, Chang CM. Iodized oil embolization to brain following transcatheter arterial embolization of liver. *J Gastroenterol Hepatol* 2005; **20**: 1465-1467 [PMID: 16105143 DOI: 10.1111/j.1440-1746.2005.03412.x]

5 **Matsumoto K**, Nojiri J, Takase Y, Egashira Y, Azama S, Kato A, Kitahara K, Miyazaki K, Kudo S. Cerebral lipiodol embolism: a complication of transcatheter arterial chemoembolization for hepatocellular carcinoma. *Cardiovasc Intervent Radiol* 2007; **30**: 512-514 [PMID: 17171304 DOI: 10.1007/s00270-006-0092-x]

6 **Choi CS**, Kim KH, Seo GS, Cho EY, Oh HJ, Choi SC, Kim TH, Kim HC, Roh BS. Cerebral and pulmonary embolisms after transcatheter arterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 4834-4837 [PMID: 18720550 DOI: 10.3748/wjg.14.4834]

7 **Kim JT**, Heo SH, Choi SM, Lee SH, Park MS, Kim BC, Kim Y, Kim MK, Cho KH. Cerebral embolism of iodized oil (lipiodol) after transcatheter arterial chemoembolization for hepatocellular carcinoma. *J Neuroimaging* 2009; **19**: 394-397 [PMID: 19496902 DOI: 10.1111/j.1552-6569.2009.00380.x]

8 **Wu JJ**, Chao M, Zhang GQ, Li B. Delayed cerebral lipiodol embolism after transcatheter arterial chemoembolization of hepatocellular carcinoma. *Chin Med J* (Engl) 2009; **122**: 878-880 [PMID: 19493407]

9 **Wu JJ**, Chao M, Zhang GQ, Li B, Dong F. Pulmonary and cerebral lipiodol embolism after transcatheter arterial chemoembolization [corrected] in hepatocellular carcinoma. *World J Gastroenterol* 2009; **15**: 633-635 [PMID: 19195069 DOI: 10.3748/wjg.15.633]

10 **Wu L**, Yang YF, Liang J, Shen SQ, Ge NJ, Wu MC. Cerebral lipiodol embolism following transcatheter arterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2010; **16**: 398-402 [PMID: 20082490 DOI: 10.3748/wjg.v16.i3.398]

11 **Karapanayiotides T**, Goulis J, Theodorou A, Anastasiou A, Georgiadis G, Ilonidis G. Lipiodol brain embolism during hepatic transcatheter arterial chemoembolization. *J Neurol* 2009; **256**: 1171-1173 [PMID: 19252772 DOI: 10.1007/s00415-009-5066-x]

12 **Lee CS**, Kim SJ, Choi JW, Choi CG, Lee DH. Cerebral lipiodol embolism proven by dual-energy computed tomography: a case report. *J Comput Assist Tomogr* 2010; **34**: 105-106 [PMID: 20118731 DOI: 10.1097/RCT.0b013e3181b382f8]

13 **Jia ZZ**, Tian F, Jiang GM. Cerebral lipiodol embolism after transarterial chemoembolization for hepatic carcinoma: a case report. *World J Gastroenterol* 2012; **18**: 4069-4070 [PMID: 22912560 DOI: 10.3748/wjg.v18.i30.4069]

14 **Zach V**, Rapaport B, Yoo JY, Goldfeder L, Weinberger J. Multiple

ischemic strokes after transcatheter arterial chemoembolization for hepatocellular carcinoma with a radiographic and pathological correlate. *J Stroke Cerebrovasc Dis* 2012; **21**: 217-224 [PMID: 21036627 DOI: 10.1016/j.jstrokecerebrovasdis.2010.08.001]

15 **Ishikawa T**, Kubota T, Abe H, Toduka Y, Horigome R, Watanabe Y, Kimura N, Honda H, Iwanaga A, Seki K, Honma T, Yoshida T, Sato M. Case of cerebral lipiodol embolism after repeated transcatheter arterial chemoembolization of hepatocellular carcinoma. *Hepatol Res* 2013; **43**: 1251-1252 [PMID: 24580681 DOI: 10.1111/hepr.12074]

16 **Bánsághi Z**, Kaposi PN, Lovas G, Szentmártoni G, Várallyay G, Bata P, Kalina I, Futácsi B, Bérczi V. Cerebral iodized lipid embolization via a pulmonary arteriovenous shunt: rare complication of transcatheter arterial embolization for hepatocellular carcinoma. *World J Surg Oncol* 2013; **11**: 122 [PMID: 23721061 DOI: 10.1186/1477-7819-11-122]

17 **Wan CC**, Liu KL. Cerebral Lipiodol embolism. *Liver Int* 2015; **35**: 673 [PMID: 24571486 DOI: 10.1111/liv.12511]

18 **Chu HJ**, Lee CW, Yeh SJ, Tsai LK, Tang SC, Jeng JS. Cerebral Lipiodol Embolism in Hepatocellular Carcinoma Patients Treated with Transarterial Embolization/Chemoembolization. *PLoS One* 2015; **10**: e0129367 [PMID: 26107693 DOI: 10.1371/journal.pone.0129367]

19 **Ueda K**, Matsui O, Kawamori Y, Nakanuma Y, Kadoya M, Yoshikawa J, Gabata T, Nonomura A, Takashima T. Hypervascular hepatocellular carcinoma: evaluation of hemodynamics with dynamic CT during hepatic arteriography. *Radiology* 1998; **206**: 161-166 [PMID: 9423667 DOI: 10.1148/radiology.206.1.9423667]

20 **Seki A**, Hori S, Shimono C. Management of vascular lake phenomenon on angiography during chemoembolization with superabsorbent polymer microspheres. *Jpn J Radiol* 2015; **33**: 741-748 [PMID: 26493810 DOI: 10.1007/s11604-015-0486-2]

21 **Cavalcante RN**, Nasser F, Motta-Leal-Filho JM, Affonso BB, Galastri FL, De Fina B, Garcia RG, Wolosker N. Occurrence of Vascular Lake Phenomenon as a Predictor of Improved Tumor Response in HCC Patients That Underwent DEB-TACE. *Cardiovasc Intervent Radiol* 2017; **40**: 1044-1051 [PMID: 28500458 DOI: 10.1007/s00270-017-1678-1]

22 **Ngan H**, Peh WC. Arteriovenous shunting in hepatocellular carcinoma: its prevalence and clinical significance. *Clin Radiol* 1997; **52**: 36-40 [PMID: 9022578 DOI: 10.1016/S0009-9260(97)80303-0]

23 **Leung WT**, Lau WY, Ho SK, Chan M, Leung NW, Lin J, Metreweli C, Johnson PJ, Li AK. Measuring lung shunting in hepatocellular carcinoma with intrahepatic-arterial technetium-99m macroaggregated albumin. *J Nucl Med* 1994; **35**: 70-73 [PMID: 8271063]

24 **Kanematsu M**. Transcatheter arterial chemoembolization therapy with epirubicin hydrochloride, mitomycin C-iohexol-Lipiodol emulsion (EMILE) for hepatocellular carcinoma. *J Gastroenterol* 1995; **30**: 215-223 [PMID: 7773353 DOI: 10.1007/BF02348668]

25 **Tajima T**, Honda H, Kuroiwa T, Yabuuchi H, Okafuji T, Yosimitsu K, Irie H, Aibe H, Masuda K. Pulmonary complications after hepatic artery chemoembolization or infusion via the inferior phrenic artery for primary liver cancer. *J Vasc Interv Radiol* 2002; **13**: 893-900 [PMID: 12354823 DOI: 10.1016/S1051-0443(07)61772-2]

26 **Kishi K**, Sonomura T, Satoh M, Nishida N, Terada M, Shioyama Y, Yamada R. Acute toxicity of lipiodol infusion into the hepatic arteries of dogs. *Invest Radiol* 1994; **29**: 882-889 [PMID: 7852039 DOI: 10.1097/00004424-199410000-00004]

P- Reviewer: Pompili M, Xiao E, Yao DF **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 October 14; 24(38): 4297-4418

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 38 October 14, 2018

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 38 October 14, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Yasuhito Tanaka, MD, PhD, Professor, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
October 14, 2018

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4297-4303

DOI: 10.3748/wjg.v24.i38.4297

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

EDITORIAL

Circadian rhythms in the pathogenesis of gastrointestinal diseases

Pilar Codoñer-Franch, Marie Gombert

Pilar Codoñer-Franch, Marie Gombert, Department of Pediatrics, Obstetrics and Ginecology, University of Valencia, Valencia 46010, Spain

Pilar Codoñer-Franch, Department of Pediatrics, Dr. Peset University Hospital, Valencia 46017, Spain

Marie Gombert, Department of Biotechnology, University of La Rochelle, La Rochelle 17000, France

ORCID number: Pilar Codoñer-Franch (0000-0002-1549-1573); Marie Gombert (0000-0003-0577-215X).

Author contributions: Codoñer-Franch P and Gombert M conceived the study and drafted the manuscript; both authors approved the final version of the article.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Pilar Codoñer-Franch, MD, PhD, Professor, Department of Pediatrics, Dr. Peset University Hospital, Avenida Gaspar Aguilar 90, Valencia 46017, Spain. pilar.codoner@uv.es
Telephone: +34-96-3864170
Fax: +34-96-3864815

Received: July 27, 2018
Peer-review started: July 27, 2018
First decision: August 27, 2018
Revised: August 31, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018

Published online: October 14, 2018

Abstract

The etiology of digestive pathologies such as irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD) and cancer is not yet fully understood. In recent years, several studies have evidenced circadian variations in mechanisms involved in digestive health. In situations of disturbed circadian rhythms (chronodisruption) where the central clock and the peripheral clocks receive incoherent signals, the synchronicity is lost producing implications for health. This lack of coordination could alter the tissue function and cause long term damage to the organs. Life habits such as sleep, physical exercise, social interaction, and feeding times are determinants for stability and integrity of circadian rhythms. In recent years, experimental and clinical studies have consistently evidenced that the alteration of circadian rhythms is associated with the development of digestive pathologies mainly linked to dismotility or changes in microbiota composition. Likewise, it seems reasonable to deep into the importance of chronodisruption as a factor that may participate in the development of pathologies such as IBS, IBD and digestive cancers. Moreover, life habits respecting circadian rhythms should be promoted for the prevention of these diseases. Further studies will allow us a better understanding of the mechanisms acting at molecular level, and the development of new therapeutic targets.

Key words: Circadian rhythms; Gastrointestinal diseases; Irritable bowel syndrome; Inflammatory bowel disease; Digestive cancers

Core tip: Chronodisruption, or circadian rhythm disruption, has been associated with impairments in metabolic functions and organ physiology. In this review

we focus on the link between circadian rhythms and digestive pathologies such as irritable bowel syndrome, inflammatory bowel diseases and cancers of the digestive tract. The purpose of this review is to highlight what is known about the negative impact of chronodisruption on pathogenesis of these digestive diseases and provide reasons for future research.

Codoñer-Franch P, Gombert M. Circadian rhythms in the pathogenesis of gastrointestinal diseases. *World J Gastroenterol* 2018; 24(38): 4297-4303 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4297.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4297

INTRODUCTION

Light and dark cycles of 24 h, which are due to rotation of the earth, serve as the dominant environmental factor affecting living organisms. Temporal organization within an organism is critical for maintenance of homeostasis as well as adaptation to changing external conditions. The term «circadian rhythms» describes endogenously generated rhythms that occur approximately every 24 h and play a fundamental role in the survival and evolution of life by ensuring that an organism's internal physiology remains synchronized with the external environment.

In mammals, several mechanisms contribute to circadian variations. While the suprachiasmatic nucleus in the hypothalamus is the master circadian pacemaker, most tissues, including other brain regions and different organs, harbor self-sustained cellular circadian clocks. The suprachiasmatic nucleus is aligned with the light-dark cycle *via* the retina; this serves as the primary stimulus for melatonin production in the pineal gland[1] and coordinates all other oscillators throughout the body with external time by binding to melatonin receptors in the organs[2]. At the molecular level, circadian rhythms emerge from Circadian Locomotor Output Cycles Protein Kaput (*Clock*) genes and proteins, comprising a network of interconnected autoregulatory transcriptional-translational molecular clocks present in virtually every cell of the body. These "clock genes" form a regulation loop with a period lasting approximately twenty-four hours. By regulating the expression of other genes coding for proteins, enzymes and factors related to metabolic homeostasis, the clock genes cooperate to induce circadian variations all over the organism[3]. Circadian clocks are self-sustained and intrinsic, but their rhythm can be entrained by environmental signals, called "Zeitgeber" (timing cue), including light, temperature, and quality of lifestyle habits, such as sleep, physical exercise, social interaction, and very importantly, feeding times[3-6]. A lack of coordination of these elements leads to desynchronization of the circadian rhythms and impacts health.

In the digestive tract, a broad range of vital functions and mechanisms display circadian variations. In the gut, circadian clocks could regulate digestive physiology and maintain intestinal barrier function. For example, in the mouth, the volume of saliva produced is more important during the day than the night[7]. In association, mouth microbiota composition and diversity varies and is influenced by meal times[8]. Further, there is evidence of circadian rhythms in the peristalsis of the digestive tube. Thus, gastric emptying rates are longer in the evening than in the morning, nocturnal propagation velocities of the migrating motor complex are slower, and colonic motor activity is minimal during sleep[9]. The gastrointestinal tract is the most important source of the chemicals melatonin and serotonin outside the central nervous system. Both of these chemicals play an important role in gastrointestinal motility. Furthermore, melatonin plays a major role in the synchronization of central and peripheral oscillators allowing the adaptation of the internal milieu to external environment. Recently, the role of melatonin in the host-microbiota communication within the gut has been emphasized[10]. Both permeability of the digestive tract[11] and the secretion of mucus and digestive enzymes[12] are different at nighttime. Moreover, immune parameters ensuring digestive health follow a 24-h period. Recent studies highlight circadian regulation of innate and adaptive gut immunity[13]. These daily changes are reflected in the gut microbiota diversity and composition[14]. Gastrointestinal ecosystem has a diurnal variation according to the state of food/fasting and the time of day. But the microbiota also impacts the host circadian rhythms[15]. A change in the diet can rapidly shift the composition of the gut microbiome that in turn might be responsible for the reprogramming of circadian rhythmicity[16,17].

Circadian regulation also plays a large role in liver metabolism, as maintenance of plasma glucose, regulation of lipids, including triglycerides, cholesterol and free fatty acids follow circadian rhythms. Bile acids are also under circadian regulation to synchronize with periods of feeding and fasting[18].

It should be highlighted the specificity in the response of distinct peripheral clocks to food challenges[19]. Diet macronutrient variations (high-fat diet, ketogenic diet) trigger differential effects on liver and intestine clocks.

Altogether, these parameters contribute to digestive health, and their dysfunction is related to several pathologies. Given their association with circadian rhythms, it is logical to suspect that chronodisruption may play a part in gastrointestinal diseases pathogenesis (Figure 1).

In this editorial, we focus on the link between circadian rhythms and three types of pathologies: irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and cancer.

IBS

IBS is a functional gastrointestinal disorder diagnosed clinically and affecting approximately 11% of the population. Patients suffer abdominal pain and altered

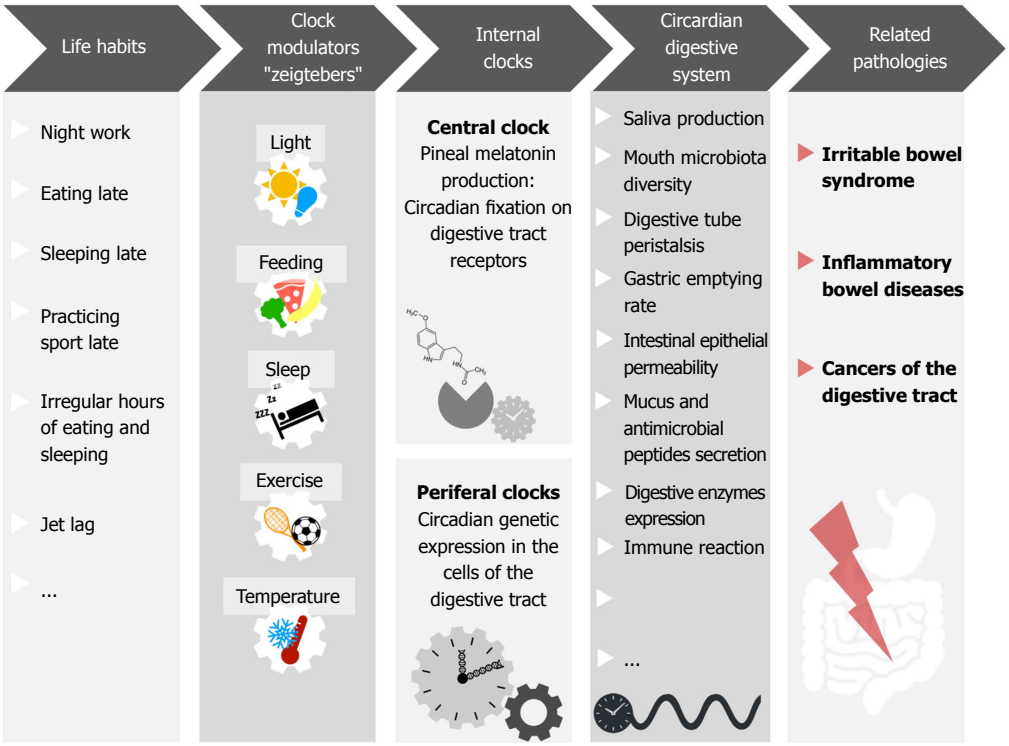


Figure 1 Numerous mechanisms involved in gastrointestinal homeostasis display daily rhythms. For example, saliva production, mouth microbiota diversity, digestive tube peristalsis, gastric emptying rate, intestinal epithelial permeability, mucus and antimicrobial peptides secretion, digestive enzymes expression, immune reaction and gut microbiota diversity. Night shift work, light at night, late evening food, late sleep or late physical exercise, as well as irregular meal schedules and jet lag, have been shown to affect the coherence/synchronization of internal circadian rhythms with the environment, affecting the homeostatic rhythms of digestive health parameters. This sequence favors the development of gastrointestinal pathologies such as irritable bowel syndrome, inflammatory bowel diseases, and cancers of the digestive tract.

bowel habits[20]. The causes and mechanistic basis of this disease are not fully understood. Currently, IBS is considered a multifactorial disease that implies visceral hypersensitivity, an alteration in the relationship between the enteric nervous system and the central nervous system, a modification of the gut microflora, increased intestinal permeability and probably minimal intestinal inflammation. Several studies suggest that circadian rhythms play a role in the development and severity of IBS.

Human studies have demonstrated that lifestyle factors, such as disturbed sleep and working the night shift, are associated with a higher prevalence of IBS[21-24]. Consistent with this observation, significantly altered melatonin excretion was detected in the urine of women with IBS[24]. According to the hypothesis that circadian rhythm disruption directly impacts motility, melatonin could be potentially useful in IBS, especially for pain symptoms and bowel motility in constipation-predominant IBS. Some evidence supports the hypothesis that melatonin is involved in the modulation of pain and has analgesic effects; patients displayed an improvement in symptoms with melatonin supplementation[25,26]. However, the high rate of placebo effect observed in patients with IBS means that therapeutic uses of melatonin for pain need further investigation. In another study, IBS patients experienced a decrease in abdominal pain duration and distension intensity, along with an increase in rectal distension pain threshold, with probiotic supplementation (VSL3#). Such improvement occurred in parallel with modulation of melatonin profiles, with patients showing increased morning melatonin levels with VSL#3 treatment. It is possible that probiotic treatment regulates melatonin, leading to an improvement in symptoms[27].

Sleep impairment is not the only risk factor for impaired circadian rhythms. Alimentation schedules participate actively in circadian variations by mobilizing the mechanisms involved in digestion at different times of the day. Many hormones oscillate in a daily fashion in the anticipation of feeding, including ghrelin, leptin, corticosterone, insulin, glucagon, and glucagon-like peptide-1, suggesting the circadian rhythm plays an important role in the regulation of metabolic processing of food. IBS has been associated with habits such as snacking between main meals[28] and eating irregularly[29]. Beyond IBS, disturbed sleep is associated with fewer meals per day and more frequent snacking. Future studies should investigate the benefits of eating and sleeping at fixed hours on the symptoms of IBS.

IBD

IBD comprises both ulcerative colitis (UC), which is characterized by continuous damage located in the colon, and Crohn's disease (CD), which is characterized by dis-

continuous alterations that can be located anywhere in the digestive tract, from the mouth to the anus. IBD is typically diagnosed at a young age (20-30 years old), has a relapsing and remitting disease course, and has no known cure. This combination of factors leads to a significant healthcare burden.

Genetics, immune responses and environment have been considered as the major etiologic factors of IBD. Available evidence suggests that both deregulated innate and adaptive immune pathways contribute to the aberrant intestinal inflammatory response in patients with IBD. There are multiple aspects of immune function that are under circadian control, such as host-pathogen interactions, trafficking of leukocytes, and the activation of innate and adaptive immunity[30]. Among the environmental risk factors, dietary elements have received considerable attention, particularly with the spread of the "Western" diet, which is high in fat and protein but low in fruit and vegetables. The hypothesis that dietary factors influence gut inflammation may be explained through several biological mechanisms, including antigen presentation, change in prostaglandin balance, and alteration of the microflora. Emerging evidence suggests that sleep also plays an important role, as circadian rhythms and melatonin could act as regulators of inflammation in the gastrointestinal tract. With prolonged sleep loss, there are elevations in monocytes and natural killer cells which form the source for the secretion of inflammatory cytokines[31]. Thus, disturbed sleep and chronic inflammation in IBD could form a self-perpetuating feedback loop, with the chronic inflammation of IBD worsening sleep and decreased sleep exacerbating the production of inflammatory cytokines and the inflammatory milieu. In mice, it has been demonstrated that functional circadian rhythms are necessary to maintain the enteric epithelial barrier. Impairment of circadian rhythms by genetic mutation of the clock genes that control circadian rhythms, including the *3 Period* genes (*Per1–3*), or desynchronization of environmental signals result in more severe colitis, epithelial homeostasis alteration, increase of necrosis and diminution of secretory cells[32]. Recent studies have found an alteration in the expression of circadian genes in patients with IBD. Almost all circadian genes were reduced in both intestinal biopsies and peripheral blood mononuclear cells and showed a negative correlation with activity score. Furthermore, greater sensitivity to inflammatory damage and exacerbation of colitis were seen in mice who went through a phase shift in the light-dark cycle, resulting in increased secretion of pro-inflammatory cytokines and activation of inflammatory-related signaling pathways[33].

In recent years, several studies have linked IBD to intestinal dysbiosis. Likewise, there is evidence of the induction of a modification of the microbiota produced for chronodisruption. Thus, the alteration of the microbiota can be one of the mechanisms by which circadian rhythms disruption favors IBD development[34].

Several human studies support the hypothesis that IBD is associated with circadian rhythms. In a prospective study of women who were enrolled in the Nurses' Health Study Ⅰ and Ⅱ, the association between sleep duration an incidence of CD and UC was examined. The authors found that both short sleep duration (less than 6 h) or long sleep duration (more than 9 h) were associated with an increased risk of UC but not with CD[35].

At the genetic level, the influence of the rs2797685 variant of the clock gene *Per3* on susceptibility and behavior of IBD has been suggested. Allele and genotype frequencies of rs2797685 were significantly increased in both CD and UC patients. Moreover, the rs2797685 variant of the *Per3* gene is associated with both early onset and more aggressive forms of CD, highlighted by increased use of immunosuppressants and more frequent stricturing and fistulizing disease requiring surgery[36].

Several studies have assessed genes associated with circadian rhythms in IBD patients. By genome-wide cDNA microarray analysis, the transcriptome of endoscopic mucosal biopsies of patients with IBD) were analyzed, focusing on the expression of circadian genes in CD and UC. Damaged tissue and adjacent healthy tissue were compared. This study revealed an alteration in the expression of 50 genes associated with circadian rhythms in IBD damaged tissues when compared to adjacent tissues. Some of these genetic alterations were different between UC and CD patients. In CD specimens, the core clock genes *ARNTL2* and *RORA* were up-regulated, while *CSNK2B, NPAS2, Per1* and *Per3* were down-regulated. Conversely, in UC patients, *ARNTL2, CRY1, CSNK1E, RORA* and *TIPIN* were up-regulated, while *NR1D2* and *Per3* were down-regulated[37]. Consistent with these findings, another study demonstrated a decrease in the expression of clock genes in the biopsies of damaged tissue in IBD patients compared to healthy controls. Interestingly, this decrease was more important in UC patients than in CD patients. The reduced clock genes expression was not only evidenced in intestinal biopsies but also in circulating mononucleated cells[33].

Taken together, these results suggest that genes associated with circadian rhythms are implicated in the physiopathology of IBD.

DIGESTIVE CANCERS

In recent decades, the number of digestive cancers has increased worldwide. Although digestive cancer is a multifactorial disease, the marked geographic variation, time trends, and migratory effects on cancer incidence suggest that environmental or lifestyle factors are major contributors to the etiology of this disease. Recently, the alteration of circadian rhythms has emerged as a suspected factor in this increase. Indeed, several studies have associated night shift work and induced chronodisruption with colorectal[38] and stomach cancer[39]. However, other studies failed to demonstrate this association[40]. Another study demonstrated a link between circadian variations, measured with wrist

temperature variation, and colorectal cancer survival[41]. Thus, circadian rhythms are associated to prognostic factors.

The alteration of the microbiota that occurs with circadian rhythms disruption has been hypothesized as one of the causes of colorectal cancer. In mice, abnormal microbial community structure was found to be associated with inflammation and tumorigenesis in the colon[42].

It has been shown that in the colon cellular proliferation follows a circadian rhythm[43]. At some points of the cell cycle, the DNA is less protected and therefore more susceptible to damage. Thus, circadian variation of cell proliferation timing in the colon could limit DNA exposure to potential mutagen agents during digestion, serving as a protective factor. Furthermore, the low expression levels of the mitotic and anti-apoptotic gene *Birc5/survivin* significantly and specifically increased the sensitivity of colon epithelial cells to cyclin-dependent kinase inhibitors. This dynamic establishes a link between cell cycle, circadian rhythms and cellular sensitivity to cyclin-dependent kinase inhibitors, making this gene a potential target in anti-cancer treatment[43].

In patients affected by colorectal cancer, cancerous cells show a differential expression of clock genes such as *Clock* and *Per* when compared to healthy cells in the same patient[44-46], suggesting that clock genes may be interesting biomarkers in colorectal cancer[46]. Other clock genes, namely, *Clock* and *Bmal1*, have been shown to interfere with the cell cycle. They are over-expressed in human colorectal cancer cells and can suppress cell growth. Moreover, clock genes may suppress CyclinD1 expression, inhibiting the cell cycle between phases G1 and S[47]. Clock genes have also been shown to modulate the expression of the rat sarcoma viral oncogene (*RAS*)[48]. Alteration in clock genes expression could play a role in these respective pathways during the development of cancers and affect resistance to treatment.

It has been observed that tumor markers in the blood of colon cancer patients display different circadian variations, according to the stage of cancer (from Ⅰ to Ⅳ)[49]. The expression of clock genes have been analyzed in metastases cells of colon cancer stage Ⅳ, and are disrupted in comparison to the expression of the same genes in healthy cells[50]. Following these findings, alterations were found in clock gene expression in the organs (kidney and liver) where colorectal cancer metastases had developed[51]. The possibility that tumors may modify peripheral clocks and induce variations from ordinary circadian rhythms in nearby cells suggests putative systemic participation in the chronodisruption. This phenomenon may explain the development of fatigue and weakness characteristically felt by cancer patients.

All of these genetic mechanisms are currently being investigated to determine the best way to treat cancers by taking circadian variations into consideration. Interesting associations between clock genes polymorphisms and colorectal cancer severity have been found[52]; in gastric cancer, survival prognosis is associated with the version of the allele of the clock gene[53]. Finally, several authors suggest using the analysis of clock genes polymorphisms to predict the response to chemotherapy[54].

CONCLUSION

In conclusion, the parameters implicated in digestive health display circadian variations. In recent decades, there has been an increase in lifestyle habits that interfere with circadian rhythms and often induce chronodisruption. When these internal clocks receive incoherent signals, they lose synchronization, and the mechanisms that they control are affected. This phenomenon likely plays an important role in the development of pathologies such as IBS, IBD and cancers. Strong evidence of the connection between circadian rhythm impairment and digestive pathologies have been published; however, additional studies are necessary to understand the molecular mechanisms involved.

The insights from the studies of circadian oscillations of innate and adaptive gut immunity joint to the host-microbial interactions may incorporate the chronopharmacology to increase the effectiveness of the agents used to modulate the immune response, *i.e.*, to indicate the time-of-day-specific for the administration of antimicrobial and antiinflammatory therapy. Likewise, the potential benefits of melatonin as a co-adjuvant treatment in gastrointestinal diseases, especially IBS and IBD should be explored. These considerations open novel perspectives in preventive and therapeutic applications of chronobiology.

REFERENCES

1 **Jenwitheesuk A**, Nopparat C, Mukda S, Wongchitrat P, Govitrapong P. Melatonin regulates aging and neurodegeneration through energy metabolism, epigenetics, autophagy and circadian rhythm pathways. *Int J Mol Sci* 2014; **15**: 16848-16884 [PMID: 25247581 DOI: 10.3390/ijms150916848]

2 **Slominski RM**, Reiter RJ, Schlabritz-Loutsevitch N, Ostrom RS, Slominski AT. Melatonin membrane receptors in peripheral tissues: distribution and functions. *Mol Cell Endocrinol* 2012; **351**: 152-166 [PMID: 22245784 DOI: 10.1016/j.mce.2012.01.004]

3 **Potter GD**, Cade JE, Grant PJ, Hardie LJ. Nutrition and the circadian system. *Br J Nutr* 2016; **116**: 434-442 [PMID: 27221157 DOI: 10.1017/S0007114516002117]

4 **Garaulet M**, Ortega FB, Ruiz JR, Rey-López JP, Béghin L, Manios Y, Cuenca-García M, Plada M, Diethelm K, Kafatos A, Molnár D, Al-Tahan J, Moreno LA. Short sleep duration is associated with increased obesity markers in European adolescents: effect of physical activity and dietary habits. The HELENA study. *Int J Obes* (Lond) 2011; **35**: 1308-1317 [PMID: 21792170 DOI: 10.1038/ijo.2011.149]

5 **Rubio-Sastre P**, Gómez-Abellán P, Martinez-Nicolas A, Ordovás JM, Madrid JA, Garaulet M. Evening physical activity alters wrist temperature circadian rhythmicity. *Chronobiol Int* 2014; **31**: 276-282 [PMID: 24517176 DOI: 10.3109/07420528.2013.833215]

6 **Bonmati-Carrion MA**, Arguelles-Prieto R, Martinez-Madrid MJ, Reiter R, Hardeland R, Rol MA, Madrid JA. Protecting the melatonin rhythm through circadian healthy light exposure. *Int J Mol Sci* 2014; **15**: 23448-23500 [PMID: 25526564 DOI: 10.3390/ijms151223448]

7 **Dawes C**. Circadian rhythms in human salivary flow rate and composition. *J Physiol* 1972; **220**: 529-545 [PMID: 5016036 DOI: 10.1113/jphysiol.1972.sp009721]

8 **Collado MC**, Engen PA, Bandín C, Cabrera-Rubio R, Voigt RM, Green SJ, Naqib A, Keshavarzian A, Scheer FAJL, Garaulet M. Timing of food intake impacts daily rhythms of human salivary microbiota: a randomized, crossover study. *FASEB J* 2018; **32**: 2060-2072 [PMID: 29233857 DOI: 10.1096/fj.201700697RR]

9 **Hoogerwerf WA**. Role of clock genes in gastrointestinal motility. *Am J Physiol Gastrointest Liver Physiol* 2010; **299**: G549-G555 [PMID: 20558764 DOI: 10.1152/ajpgi.00147.2010]

10 **Paulose JK**, Wright JM, Patel AG, Cassone VM. Human Gut Bacteria Are Sensitive to Melatonin and Express Endogenous Circadian Rhythmicity. *PLoS One* 2016; **11**: e0146643 [PMID: 26751389 DOI: 10.1371/journal.pone.0146643]

11 **Forsyth CB**, Voigt RM, Burgess HJ, Swanson GR, Keshavarzian A. Circadian rhythms, alcohol and gut interactions. *Alcohol* 2015; **49**: 389-398 [PMID: 25499101 DOI: 10.1016/j.alcohol.2014.07.021]

12 **Pácha J**, Sumová A. Circadian regulation of epithelial functions in the intestine. *Acta Physiol* (Oxf) 2013; **208**: 11-24 [PMID: 23461998 DOI: 10.1111/apha.12090]

13 **Bando JK**, Colonna M. Innate lymphoid cell function in the context of adaptive immunity. *Nat Immunol* 2016; **17**: 783-789 [PMID: 27328008 DOI: 10.1038/ni.3484]

14 **Voigt RM**, Forsyth CB, Green SJ, Engen PA, Keshavarzian A. Circadian Rhythm and the Gut Microbiome. *Int Rev Neurobiol* 2016; **131**: 193-205 [PMID: 27793218 DOI: 10.1016/bs.irn.2016.07.002]

15 **Liang X**, Bushman FD, FitzGerald GA. Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. *Proc Natl Acad Sci USA* 2015; **112**: 10479-10484 [PMID: 26240359 DOI: 10.1073/pnas.1501305112]

16 **Murakami M**, Tognini P, Liu Y, Eckel-Mahan KL, Baldi P, Sassone-Corsi P. Gut microbiota directs PPARγ-driven reprogramming of the liver circadian clock by nutritional challenge. *EMBO Rep* 2016; **17**: 1292-1303 [PMID: 27418314 DOI: 10.15252/embr.201642463]

17 **Tognini P**, Murakami M, Sassone-Corsi P. Interplay between Microbes and the Circadian Clock. *Cold Spring Harb Perspect Biol* 2018; **10** [PMID: 29038112 DOI: 10.1101/cshperspect.a028365]

18 **Ferrell JM**, Chiang JY. Circadian rhythms in liver metabolism and disease. *Acta Pharm Sin B* 2015; **5**: 113-122 [PMID: 26579436 DOI: 10.1016/j.apsb.2015.01.003]

19 **Tognini P**, Murakami M, Liu Y, Eckel-Mahan KL, Newman JC, Verdin E, Baldi P, Sassone-Corsi P. Distinct Circadian Signatures in Liver and Gut Clocks Revealed by Ketogenic Diet. *Cell Metab* 2017; **26**: 523-538.e5 [PMID: 28877456 DOI: 10.1016/j.cmet.2017.08.015]

20 **Canavan C**, West J, Card T. The epidemiology of irritable bowel syndrome. *Clin Epidemiol* 2014; **6**: 71-80 [PMID: 24523597 DOI: 10.2147/CLEP.S40245]

21 **Morito Y**, Aimi M, Ishimura N, Shimura S, Mikami H, Okimoto E, Sato S, Ishihara S, Kushiyama Y, Katsube T, Adachi K, Kinoshita Y. Association between sleep disturbances and abdominal symptoms. *Intern Med* 2014; **53**: 2179-2183 [PMID: 25274228 DOI: 10.2169/internalmedicine.53.2591]

22 **Kim HI**, Jung SA, Choi JY, Kim SE, Jung HK, Shim KN, Yoo K. Impact of shiftwork on irritable bowel syndrome and functional dyspepsia. *J Korean Med Sci* 2013; **28**: 431-437 [PMID: 23487413 DOI: 10.3346/jkms.2013.28.3.431]

23 **Wells MM**, Roth L, Chande N. Sleep disruption secondary to overnight call shifts is associated with irritable bowel syndrome in residents: a cross-sectional study. *Am J Gastroenterol* 2012; **107**: 1151-1156 [PMID: 22858995 DOI: 10.1038/ajg.2011.486]

24 **Nojkov B**, Rubenstein JH, Chey WD, Hoogerwerf WA. The impact of rotating shift work on the prevalence of irritable bowel syndrome in nurses. *Am J Gastroenterol* 2010; **105**: 842-847 [PMID: 20160712 DOI: 10.1038/ajg.2010.48]

25 **Wisniewska-Jarosinska M**, Chojnacki J, Konturek S, Brzozowski T, Smigielski J, Chojnacki C. Evaluation of urinary 6-hydroxymelatonin sulphate excretion in women at different age with irritable bowel syndrome. *J Physiol Pharmacol* 2010; **61**: 295-300 [PMID: 20610859]

26 **Danilov A**, Kurganova J. Melatonin in Chronic Pain Syndromes. *Pain Ther* 2016; **5**: 1-17 [PMID: 26984272 DOI: 10.1007/s40122-016-0049-y]

27 **Wong RK**, Yang C, Song GH, Wong J, Ho KY. Melatonin regulation as a possible mechanism for probiotic (VSL#3) in irritable bowel syndrome: a randomized double-blinded placebo study. *Dig Dis Sci* 2015; **60**: 186-194 [PMID: 25092036 DOI: 10.1007/s10620-014-3299-8]

28 **Wasiluk D**, Ostrowska L, Stefańska E, Janke A, Jurkowska G. Diet for women with irritable bowel syndrome - a preliminary study *Rocz Panstw Zakl Hig* 2017; **68**: 151-160 [PMID: 28646832]

29 **Guo YB**, Zhuang KM, Kuang L, Zhan Q, Wang XF, Liu SD. Association between Diet and Lifestyle Habits and Irritable Bowel Syndrome: A Case-Control Study. *Gut Liver* 2015; **9**: 649-656 [PMID: 25266811 DOI: 10.5009/gnl13437]

30 **Geremia A**, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun Rev* 2014; **13**: 3-10 [PMID: 23774107 DOI: 10.1016/j.autrev.2013.06.004]

31 **Irwin MR**, Wang M, Ribeiro D, Cho HJ, Olmstead R, Breen EC, Martinez-Maza O, Cole S. Sleep loss activates cellular inflammatory signaling. *Biol Psychiatry* 2008; **64**: 538-540 [PMID: 18561896 DOI: 10.1016/j.biopsych.2008.05.004]

32 **Pagel R**, Bär F, Schröder T, Sünderhauf A, Künstner A, Ibrahim SM, Autenrieth SE, Kalies K, König P, Tsang AH, Bettenworth D, Divanovic S, Lehnert H, Fellermann K, Oster H, Derer S, Sina C. Circadian rhythm disruption impairs tissue homeostasis and exacerbates chronic inflammation in the intestine. *FASEB J* 2017; **31**: 4707-4719 [PMID: 28710114 DOI: 10.1096/fj.201700141RR]

33 **Liu X**, Yu R, Zhu L, Hou X, Zou K. Bidirectional Regulation of Circadian Disturbance and Inflammation in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2017; **23**: 1741-1751 [PMID: 28902123 DOI: 10.1097/MIB.0000000000001265]

34 **Voigt RM**, Forsyth CB, Green SJ, Mutlu E, Engen P, Vitaterna MH, Turek FW, Keshavarzian A. Circadian disorganization alters intestinal microbiota. *PLoS One* 2014; **9**: e97500 [PMID: 24848969 DOI: 10.1371/journal.pone.0097500]

35 **Ananthakrishnan AN**, Khalili H, Konijeti GG, Higuchi LM, de Silva P, Fuchs CS, Richter JM, Schernhammer ES, Chan AT. Sleep duration affects risk for ulcerative colitis: a prospective cohort study. *Clin Gastroenterol Hepatol* 2014; **12**: 1879-1886 [PMID: 24780288 DOI: 10.1016/j.cgh.2014.04.021]

36 **Mazzoccoli G**, Palmieri O, Corritore G, Latiano T, Bossa F, Scimeca D, Biscaglia G, Valvano MR, D'Incà R, Cucchiara S, Stronati L, Annese V, Andriulli A, Latiano A. Association study of a polymorphism in clock gene PERIOD3 and risk of inflammatory bowel disease. *Chronobiol Int* 2012; **29**: 994-1003 [PMID: 22881285 DOI: 10.3109/07420528.2012.705935]

37 **Palmieri O**, Mazzoccoli G, Bossa F, Maglietta R, Palumbo O, Ancona N, Corritore G, Latiano T, Martino G, Rubino R, Biscaglia G, Scimeca D, Carella M, Annese V, Andriulli A, Latiano A. Systematic analysis of circadian genes using genome-wide cDNA microarrays in the inflammatory bowel disease transcriptome. *Chronobiol Int* 2015; **32**: 903-916 [PMID: 26172092 DOI: 10.3109/07420528.2015.1050726]

38 **Schernhammer ES**, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Fuchs CS, Colditz GA. Night-shift work and risk of colorectal cancer in the nurses' health study. *J Natl Cancer Inst* 2003; **95**: 825-828 [PMID: 12783938 DOI: 10.1093/jnci/95.11.825]

39 **Gyarmati G**, Turner MC, Castaño-Vinyals G, Espinosa A, Papantoniou K, Alguacil J, Costas L, Pérez-Gómez B, Martin Sanchez V, Ardanaz E, Moreno V, Gómez-Acebo I, Fernández-Tardon G, Villanueva Ballester V, Capelo R, Chirlaque MD, Santibáñez M, Pollán M, Aragonés N, Kogevinas M. Night shift work and stomach cancer risk in the MCC-Spain study. *Occup Environ Med* 2016; **73**: 520-527 [PMID: 27312400 DOI: 10.1136/oemed-2016-103597]

40 **Devore EE**, Massa J, Papantoniou K, Schernhammer ES, Wu K, Zhang X, Willett WC, Fuchs CS, Chan AT, Ogino S, Giovannucci E, Wei EK. Rotating night shift work, sleep, and colorectal adenoma in women. *Int J Colorectal Dis* 2017; **32**: 1013-1018 [PMID: 28097381 DOI: 10.1007/s00384-017-2758-z]

41 **Lévi F**, Dugué PA, Innominato P, Karaboué A, Dispersyn G, Parganiha A, Giacchetti S, Moreau T, Focan C, Waterhouse J, Spiegel

D; ARTBC Chronotherapy Group. Wrist actimetry circadian rhythm as a robust predictor of colorectal cancer patients survival. *Chronobiol Int* 2014; **31**: 891-900 [PMID: 24927369 DOI: 10.3109/07420528.2014.924523]

42 **Zackular JP**, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, Schloss PD. The gut microbiome modulates colon tumorigenesis. *MBio* 2013; **4**: e00692-e00613 [PMID: 24194538 DOI: 10.1128/mBio.00692-13]

43 **Siffroi-Fernandez S**, Dulong S, Li XM, Filipski E, Gréchez-Cassiau A, Peteri-Brünback B, Meijer L, Lévi F, Teboul M, Delaunay F. Functional genomics identify Birc5/survivin as a candidate gene involved in the chronotoxicity of cyclin-dependent kinase inhibitors. *Cell Cycle* 2014; **13**: 984-991 [PMID: 24552823 DOI: 10.4161/cc.27868]

44 **Wang Y**, Sun N, Lu C, Bei Y, Qian R, Hua L. Upregulation of circadian gene 'hClock' contribution to metastasis of colorectal cancer. *Int J Oncol* 2017; **50**: 2191-2199 [PMID: 28498393 DOI: 10.3892/ijo.2017.3987]

45 **Momma T**, Okayama H, Saitou M, Sugeno H, Yoshimoto N, Takebayashi Y, Ohki S, Takenoshita S. Expression of circadian clock genes in human colorectal adenoma and carcinoma. *Oncol Lett* 2017; **14**: 5319-5325 [PMID: 29113166 DOI: 10.3892/ol.2017.6876]

46 **Fonnes S**, Donatsky AM, Gögenur I. Expression of core clock genes in colorectal tumour cells compared with normal mucosa: a systematic review of clinical trials. *Colorectal Dis* 2015; **17**: 290-297 [PMID: 25418520 DOI: 10.1111/codi.12847]

47 **Sakamoto W**, Takenoshita S. OVEREXPRESSION OF BOTH CLOCK AND BMAL1 INHIBITS ENTRY TO S PHASE IN HUMAN COLON CANCER CELLS. *Fukushima J Med Sci* 2015; **61**: 111-124 [PMID: 26370682 DOI: 10.5387/fms.2015-11]

48 **El-Athman R**, Genov NN, Mazuch J, Zhang K, Yu Y, Fuhr L, Abreu M, Li Y, Wallach T, Kramer A, Schmitt CA, Relógio A. The Ink4a/Arf locus operates as a regulator of the circadian clock modulating RAS activity. *PLoS Biol* 2017; **15**: e2002940 [PMID: 29216180 DOI: 10.1371/journal.pbio.2002940]

49 **Tóth K**, Patai ÁV, Kalmár A, Barták BK, Nagy ZB, Galamb O, Wichmann B, Tulassay Z, Molnár B. Circadian Rhythm of Methylated Septin 9, Cell-Free DNA Amount and Tumor Markers in Colorectal Cancer Patients. *Pathol Oncol Res* 2017; **23**: 699-706 [PMID: 28035516 DOI: 10.1007/s12253-016-0174-2]

50 **Huisman SA**, Ahmadi AR, IJzermans JN, Verhoef C, van der Horst GT, de Bruin RW. Disruption of clock gene expression in human colorectal liver metastases. *Tumour Biol* 2016; **37**: 13973-13981 [PMID: 27492458 DOI: 10.1007/s13277-016-5231-7]

51 **Huisman SA**, Oklejewicz M, Ahmadi AR, Tamanini F, Ijzermans JN, van der Horst GT, de Bruin RW. Colorectal liver metastases with a disrupted circadian rhythm phase shift the peripheral clock in liver and kidney. *Int J Cancer* 2015; **136**: 1024-1032 [PMID: 25045881 DOI: 10.1002/ijc.29089]

52 **Qu F**, Qiao Q, Wang N, Ji G, Zhao H, He L, Wang H, Bao G. Genetic polymorphisms in circadian negative feedback regulation genes predict overall survival and response to chemotherapy in gastric cancer patients. *Sci Rep* 2016; **6**: 22424 [PMID: 26927666 DOI: 10.1038/srep22424]

53 **Rajendran S**, Benna C, Monticelli H, Spiro G, Menin C, Mocellin S. Germline variation of circadian pathway genes and prognosis of gastric cancer patients. *Gut* 2018; **67**: 779-780 [PMID: 28790161 DOI: 10.1136/gutjnl-2017-314740]

54 **Lu H**, Chu Q, Xie G, Han H, Chen Z, Xu B, Yue Z. Circadian gene expression predicts patient response to neoadjuvant chemoradiation therapy for rectal cancer. *Int J Clin Exp Pathol* 2015; **8**: 10985-10994 [PMID: 26617816]

P- Reviewer: Camara-Lemarroy CR, Servillo G **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4304-4310

DOI: 10.3748/wjg.v24.i38.4304

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

EDITORIAL

Challenge to overcome: Nonstructural protein 5A-P32 deletion in direct-acting antiviral-based therapy for hepatitis C virus

Ken Sato, Toshio Uraoka

Ken Sato, Toshio Uraoka, Department of Gastroenterology and Hepatology, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

ORCID number: Ken Sato (0000-0002-3202-7983); Toshio Uraoka (0000-0002-4425-4331).

Author contributions: Sato K reviewed the literature and wrote the first draft of the paper; Uraoka T approved the final version of the article.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Ken Sato, MD, PhD, Associate Professor, Doctor, Department of Gastroenterology and Hepatology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. satoken@gunma-u.ac.jp
Telephone: +81-272-208127
Fax: +81-272-208127

Received: July 17, 2018
Peer-review started: July 17, 2018
First decision: July 25, 2018
Revised: August 2, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 14, 2018

Abstract

Interferon (IFN)-based therapy for hepatitis C virus (HCV) infection has recently been replaced by IFN-free direct-acting antiviral (DAA)-based therapy, which has been established as a 1st line therapy with high efficacy and tolerability due to its reasonable safety profile. Resistance-associated substitutions (RASs) have been a weakness of DAA-based therapy. For example, combination therapy with daclatasvir and asunaprevir (DCV/ASV) is less effective for HCV genotype 1-infected patients with Y93H as a nonstructural protein 5A RAS. However, the problem regarding RASs has been gradually overcome with the advent of recently developed DAAs, such as sofosbuvir-based regimens or combination therapy with glecaprevir and pibrentasvir. Despite the high efficiency of DAA-based therapy, some cases fail to achieve viral eradication. P32 deletion, an NS5A RAS, has been gradually noticed in patients with DCV/ASV failure. P32 deletion has been sporadically reported and the prevalence of this RAS has been considered to be low in patients with DCV/ASV failure. Thus, the picture of P32 deletion has not been fully evaluated. Importantly, currently-commercialized DAA-based combination therapy was not likely to be effective for patients with P32 deletion. Exploring and overcoming this RAS is essential for antiviral therapy for chronic hepatitis C.

Key words: Chronic hepatitis C; Direct-acting antivirals; Resistant-associated substitution; P32 deletion; Non-structural protein 5A

Core tip: P32 deletion, as a nonstructural protein 5A resistance-associated substitution (RAS), has been

gradually noticed in patients who experience treatment failure associated with daclatasvir and asunaprevir as an antiviral therapy for chronic hepatitis C. Information regarding P32 deletion is very limited at the present. Although the prevalence of this RAS is assumed to be low, it was found to be a new threat to current direct-acting antiviral-based combination therapy. There is an urgent need for further basic and clinical studies on this RAS. Exploring and overcoming this RAS is essential for antiviral therapy for chronic hepatitis C.

Sato K, Uraoka T. Challenge to overcome: Nonstructural protein 5A-P32 deletion in direct-acting antiviral-based therapy for hepatitis C virus. *World J Gastroenterol* 2018; 24(38): 4304-4310 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4304.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4304

INTRODUCTION

Interferon (IFN)-based therapy for hepatitis C virus (HCV) infection has recently been replaced with IFN-free direct-acting antiviral (DAA)-based therapy, which has been established as a first-line therapy due to its high efficacy and tolerability due to its reasonable safety profile. Recent DAA-based therapies, such as combination therapy with sofosbuvir [SOF; a nonstructural protein (NS) 5B inhibitor] and ledipasvir (LDV; an NS5A inhibitor) and new DAA-based therapies, such as the combination of glecaprevir [GLE; an NS3/4A protease inhibitor (PI)] and pibrentasvir (PIB; an NS5A inhibitor) have achieved a sustained virological response (SVR) rate of > 95% in clinical trials. Resistance-associated substitutions (RASs), which are baseline polymorphisms at specific amino acid positions, are critical for the efficacy of DAA-based therapy. In particular, Y93H in NS5A is the RAS that is most frequently associated with NS5A inhibitor [*e.g.*, daclatasvir (DCV), LDV, or ombitasvir (OBV)] treatment failure in patients with genotype 1b HCV infection[1]. Several studies[2-6] have also shown resistance to NS5A inhibitors based on Y93H [LDV, 1807-fold; OBV, 77-fold; DCV, 24-fold; and elbasvir (EBR), 17-fold]. PIB, a newly developed NS5A inhibitor has overcome this weakness of prior NS5A inhibitors; Y93H-based NS5A inhibitor-resistance to PIB is only 0.6-fold half maximal effective concentration (EC50) in comparison to the respective wild-type replicon. In fact, the efficacy of GLE/PIB combination therapy was not affected by the existence of Y93H[7,8], which showed a high barrier to Y93H resistance *in vitro*. However, combination therapy with GLE/PIB resulted in treatment failure in two cases with an in-frame deletion of NS5A codon 32 (P32 deletion) at baseline; both cases involved patients with genotype 1b HCV infection who had experienced treatment failure with daclatasvir (DCV) and asunaprevir (ASV; a PI)[9,10]. Thus, this RAS in NS5A has become a focus of attention, as it may represent a new threat to current DAA-based combination therapy for chronic hepatitis C. The impact of P32 deletion on the efficacy of DAA-based therapy has not been fully elucidated.

STUDY ANALYSIS

Integration analysis of RASs based on the Certain-1 and Certain-2 studies, in which combination therapy with GLE/PIB was given to patients with chronic hepatitis C as DAA-based therapy in Japan[10]. Four patients who had genotype 1b or 3 experienced virologic failure. Two of the patients were DCV/ASV-experienced genotype 1b-infected patients, as mentioned above; the others had genotype 3. The two genotype 1b-infected patients with treatment failure both had P32 deletion at baseline and at the time of virologic failure. The prevalence of P32 deletion at baseline was only 6.3% in the DAA-experienced genotype 1b patients (n = 32). Thirty of these patients had experienced DCV/ASV failure; 2 had received a PI-containing therapy without an NS5A inhibitor. A transient-replicon assay showed that P32 deletion was associated with a > 1000-fold EC50 in the replicon with P32 deletion, which was treated with PIB relative to that in the wild-type replicon. Moreover, in the HCV genotype 1b-Con1 replicon treated with currently available NS5A inhibitors [specifically, OBV, DCV, EBR, LDV, and velpatasvir (VEL)], P32 deletion was associated with a > 10000-fold EC50. Thus, P32 deletion is considered to be a new substantial threat to all of the currently available NS5A inhibitors and NS5A inhibitor-containing regimens. Fortunately, the prevalence of P32 deletion suggested to be relatively low.

In Europe, an analysis of RASs was performed based on the MAGELLAN-1 Part 2 study[7], in which combination therapy with GLE/PIB was administered to chronic hepatitis C patients with genotype 1 or 2. One of the 19 genotype-1b patients showed relapse at post-treatment week 8, had a P32 deletion and L28M as an NS5A RAS without any NS3 substitutions at baseline and at virologic failure. The patient also experienced DCV/ASV failure. The MAGELLAN-1 Part 2[7], Certain-1, and Certain-2 studies[10] suggest that P32 deletion exists independently of race and might be associated with DCV/ASV failure.

The analysis of RASs in 14 DAA-naïve genotype 1b patients experienced DCV/ASV failure showed one patient who developed P32 deletion without Y93H or L31M/V after viral breakthrough, which was not observed at baseline[11]. Besides, the prevalence of P32 deletion was 3 (4.8%) patients in 63 patients who experienced ASV/DCV failure, in whom NS5A RASs were successfully analyzed. The prevalence of P32 deletion was not so high in this study. This study also supports the association between the development of P32 deletion and DCV/ASV failure. Interestingly, 10 other analyzable patients who experienced DCV/ASV failure developed Y93H with L31M or L31V. This study suggested the possibility that "P32 deletion" could not coexist with certain RASs.

On the other hand, when combination therapy with

SOF/LDV was administered to genotype 1b-infected patients who had experienced DCV/ASV failure, four patients with P32 deletion at baseline experienced treatment failure with SOF/LDV, and P32 deletion was found to coexist with Y93H in one patient[12]. However, ultra-deep sequencing revealed that the rate of P32 deletion among the total coverage of NS5A codon 32 was only 0.4% in this case, while it was more than 90% in the other three cases without Y93H at baseline. In this case, P32 deletion disappeared and the rate of Y93H increased from 90.0% to 99.6% among the total coverage of NS5A codon 93 at the re-elevation of the viral load. On the other hand, L31F coexisted with P32 deletion in two cases and the rates of L31F and P32 deletion among the total coverage of NS5A codons 31 and 32, respectively were more than 90%. Although L31A or L31M coexisted with P32 deletion in one case, the rate of L31A or L31M among the total coverage of NS5A codon 31 was only 0.9% at baseline or 0.1% at the re-elevation of viral loads, respectively, while the rate of P32 deletion was more than 95% among the total coverage of NS5A codon 32 at both time points. Although L31V coexisted with P32 deletion in one case, the rate of L31V among the total coverage of NS5A codon 31 was 96.3% at baseline and increased to 99.7% at the re-elevation of viral loads, while the rate of P32 deletion was only 0.4% among the total coverage of NS5A codon 32 at baseline and P32 deletion disappeared at the re-elevation of viral loads. This study also suggested the possibility that "P32 deletion" and certain RASs were mutually exclusive. Although the study population was relatively small, given the fact that P32 deletion conferred a > 10000-fold EC50 for LDV[10], this study supports that P32 deletion might be refractory to the combination of SOF/LDV.

A total 24 subjects were given DCV in a 14-d double-blind, placebo-controlled, sequential-panel, multiple-ascending-dose DCV monotherapy study[13]. Among these patients, P32 deletion was detected in one patient with genotype 1b who was treated with DCV (30 mg, twice daily) on days 42, 98, and 182. An *in vitro* analysis of replicon variants revealed that in a replicon with P32 deletion, the replication level was reduced to 29.1% in comparison to the wild-type and that P32 deletion conferred > 390000-fold resistance to DCV in the *in vitro* replicon system. This study supports the finding that the appearance of P32 deletion was related to combination therapy with DCV/ASV as a prior therapy. Importantly, clones with P32 deletion survived for a relatively long period, despite its low replication ability.

A basic study using infectious culture systems[14] showed that HCV variants with P32 deletion conferred more than 1000-fold resistance to the NS5A inhibitors DCV, LDV, OBV, EBR, Ruzasvir, VEL, and PIB, in comparison to the original viruses for both genotype 1a and 1b, which supported the data from the replicon system. Theoretically, this study suggested that - like genotype 1b HCV - genotype 1a HCV with P32 deletion is resistant to NS5A inhibitor treatment. Infectious culture systems mimic the entire viral life cycle[15], but differ from replicons in that they lack the assembly and release processes of infectious viruses[16]. In addition to viral replication, NA5A inhibitors are involved in these steps of the HCV life cycle[16]. The study of infectious culture systems supports P32 deletion is associated with intractability to currently available DAA-based therapies.

A single-institution study in Japan evaluated NS5A RAS in 1516 genotype 1b-infected patients receiving combination therapy with DCV/ASV, SOF/LDV, or OBV/paritaprevir/ritonavir[17]. The frequency of P32 deletion was 0/1516 (0%) at the beginning of DAA therapy. However, P32 deletion was detected at the time of treatment failure in 6/110 (5.45%) patients who exclusively developed ASV/DCV failure. All 6 patients with P32 deletion showed a Fibrosis-4 index of > 3.25, and Interleukin 28B rs8099917 TG as host factors associated with treatment resistance, and a non-response to prior IFN-based therapy. DAA-retreatment was performed in four out of six patients. Three cases re-treated with SOF/LDV combination therapy showed relapse and one case re-treated with GLE/PBV combination therapy showed a non-response. P32 deletion was also detected in the endpoint of re-treatment in these four cases. Besides, the emergence of P32 deletion was observed in patients with several factors associated with treatment resistance. This study suggests that the emergence of P32 deletion was specific to ASV/DCV failure, and that while the rate of emergence was not so high, it might confer resistance to other DAA-based therapies. P32 deletion might develop in the presence of both viral and host factors associated with treatment resistance.

A recent study[18] reported the impact of prior DAA-based therapy on P32 deletion. Among 10 genotype 1-infected patients with SOF/LDV treatment failure, in whom NS5A RASs were evaluated by deep sequencing, one patient had P32 deletion at the time of virologic relapse in post-treatment week 4. P32 deletion was not detected at baseline, and importantly, it was continuously detected until post-treatment week 52. This study suggests that P32 deletion developed after treatment with a combination therapy other than DCV/ASV and that the substitution was maintained for a relatively long period. Thus, it increased the extent of the threat of P32 deletion, although the prevalence in patients experiencing SOF/LDV failure was unclear. This study suggests the need for large-scale cohort studies to evaluate P32 deletion because the previous study[17] showed that this RAS might develop exclusively in patients with DCV/ASV failure.

RASs were evaluated in total 74 patients who experienced DCV/ASV failure in a multi-institutional study in Japan. NS5A deletions were found in seven patients (9.5%)[19]. Six of these were P32 deletions. Four of the 7 NS5A-deletion cases experienced combination therapy with pegylated-interferon (PegIFN)/ribavirin (RBV)/simeprevir and 1 case had D168E as an NS3

RAS that was relatively resistant to combination therapy with DCV/ASV. The change of NS5A quasispecies was evaluated at 2 time points (1-3 mo and 6-28 mo after treatment failure) in four patients with DCV/ASV failure by population sequencing. P32 deletion was persistently detected for more than 2 years after treatment failure in 2 cases and for more than one year after treatment failure in one case. P32 deletion was not detected at 1 mo after treatment failure, but developed at 6 mo after treatment failure in one case. The rate of P32 deletion in NS5A quasispecies was increased in 2 cases, decreased in one case, and unchanged in one case. Given that the study population was relatively large and the survival of the P32 deletion was reported to be relatively long, the study is of value for improving the understanding of this RAS.

A pooled analysis of 5 Phase 2 and 3 global and Japanese studies was performed to investigate emergent RASs in NS5A and NS3 in genotype 1b-infected patients receiving DCV/ASV combination therapy[20]. P32 substitutions were found in 4 of 152 (3%) patients who experienced virologic failure. Although it is not clear whether the P32 substitutions included P32 deletion, the prevalence of P32 deletion is assumed to be low. Besides, NS5A RASs persisted beyond post-treatment week 96, while NS3 RASs were generally no longer detected in the patients who experienced DCV/ASV failure. This study is important for estimating the prevalence of P32 deletion due to the relatively large sample size.

An open-label, phase 2a study in the United States[21] showed that 6 genotype 1a-infected patients who showed no response to previous HCV therapy and who experienced DCV/ASV failure, had no P32 deletions at baseline or the time of virologic failure. This study supports the hypothesis that the prevalence of P32 deletion is low, although the HCV genotype was 1a.

Regarding counterplots against P32 deletion, two studies have examined the effects of 12 wk of combination therapy with SOF/LDV plus RBV in patients with DCV/ASV failure. In one study[22], one patient who had P32 deletion simultaneously with L31F and Q54H (NS5A RASs) without Y93H achieved a SVR at 12 wk post-treatment. In the other study[23], one patient who had P32 deletion simultaneously with L31M (an NS5A RAS) without Y93H and with D168A (as an NS3 RAS) achieved an SVR at 12 wk post-treatment. As the rate of L31M among the total coverage of NS5A codon 31 was unknown in this case, it was difficult to evaluate the association between L31M and P32 deletion. The previous studies[11,12] and this study support "P32 deletion" and at least "Y93H" might be mutually exclusive. Importantly, SOF/LDV plus RBV combination therapy might be one solution for antiviral treatment for hepatitis C patients with P32 deletion although the number of samples was very small.

A recent study[24] reported the prevalence of NS5A RASs in patients who experienced treatment failure with DAA-based therapy that included an NS5A inhibitor and explored a promising therapy for P32 deletion in humanized mice. P32 deletion was detected in 1 of 23 (4.3%) patients with treatment failure under DCV/ASV, DCV/ASV/beclabuvir, or SOF/LDV. In this case, virologic relapse was observed at post-treatment week 24 after DCV/ASV treatment and P32 deletion was detected 25 mo after DCV/ASV treatment. This study supports that the prevalence of P32 deletion was relatively low. Humanized mice can mimic the dynamics of HCV in humans. The wild-type HCV and mutated-type HCV, which were passed on to the mice, were derived from a DAA-naïve patient and a patient with DCV/ASV and SOF/LDV failure, respectively. The efficacy of the combination therapy with 4-wk GLE/PIB in NS3-D168V-infected mice with P32 deletion was low in comparison to wild-type HCV-infected mice. The HCV RNA levels in the mutated-type HCV-infected mice decreased by approximately 2 log copies/mL and reversed to the basal level after the cessation of GLE/PIB combination therapy. In contrast, the HCV RNA levels in the wild-type HCV-infected mice decreased to the lower limit of detection after one week of therapy and were not detected until two weeks after the cessation of the therapy. They tried 4-wk GLE/PIB plus SOF combination therapy in the mutated-type HCV-infected mice. The HCV RNA levels decreased to the lower limit of detection after two weeks of therapy and remained below the limit of detection, although the HCV RNA levels relapsed after the cessation of the therapy in one of four mice. No additional RASs had developed at the time of relapse in mice receiving either GLE/PIB or GLE/PIB plus SOF combination therapy. This study was very useful for providing information about the potential effectiveness of GLE/PIB plus SOF combination therapy in patients with P32 deletion.

In conclusion, P32 deletion in NS5A is a new substantial threat to DAA-based therapy for chronic hepatitis C and has started to garner attention. This RAS is likely to develop after DCV/ASV combination therapy but patients receiving other DAA regimens also have the potential to develop this RAS. The prevalence was estimated - based on the previous studies - to be < 10% in patients among patients with DCV/ASV treatment failure[10,11,17,20,24]. P32 deletion might have a higher relative fitness in comparison to NS3 variants, similarly to other NS5A variants[21]. Regarding antiviral treatment, HCV with P32 deletion might be resistant to GLE/PIB or LDV/SOF combination therapy, while it might be sensitive to "GLE/PIB plus SOF" or "SOF/LDV plus RBV" combination therapy. Interestingly, P32 deletion and Y93H, which are both NS5A RASs and which are critical for DAA-based therapy, might be mutually exclusive. However, the evidence level to support these hypotheses is not so high because the populations of the studies, which included single-institutional studies and preclinical trials, were relatively small.

CONCLUSION

We propose the following strategy for overcoming P32 deletion. (1) Various combinations of DAAs and/or RBV

Table 1 Characteristics of P32 deletion

Characteristic	Description
Position	Nonstructural protein 5A
Frequency in patients who experienced DCV/ASV failure	< 10% (4.3% to 9.5%)
Extent of resistance in the HCV GT1b Con1 replicon	> 1000-fold resistance to PIB and > 10000-fold resistance to DCV, LDV, VEL, EBR, or OBV
Extent of resistance in the infectious culture systems	> 1000-fold resistance to PIB, DCV, LDV, VEL, EBR, OBV, or RZR
Prior DAA therapy to develop P32del	DCV/ASV or SOF/LDV
RAS that is unlikely to be coexistent with	Y93H
Therapy that is unlikely to be effective	GLE/PIB or SOF/LDV
Therapy that is expected to be effective	"GLE/PIB plus SOF" or "SOF/LDV plus RBV"
Therapy that might to be effective	"SOF/VEL plus VOX" or "SOF/VEL"

ASV: Asunaprevir; DAA: Direct-acting antiviral; DCV: Daclatasvir; EBR: Elbasvir; GLE: Glecaprevir: GT: Genotype; HCV: Hepatitis C virus; LDV: Ledipasvir; OBV: Ombitasvir; P32del: P32 deletion; PIB: Pibrentasvir; RAS: Resistance-associated substitution; RBV: Ribavirin; RZR: Ruzasvir; SOF: Sofosbuvir; VEL: Velpatasvir; VOX: Voxilaprevir.

and/or PegIFN should be tried to evaluate the potency of the antiviral effect against HCV with P32 deletion in replicon cells, infectious culture systems, and humanized mice. As mentioned above, several combinations of DAAs have been tried but the number of the combinations is insufficient. A regimen including SOF or RBV is contraindicated for patients with severe renal dysfunction and/or dialysis. Thus, add-on SOF or add-on RBV to combination therapy with an NS5A inhibitor and PI cannot be administered to this special population of patients with P32 deletion. PegIFN can be added to the combination therapy for such cases. However, IFN intolerant/ineligible patients evidently exist due to various reasons, including adverse events, and the addition of PegIFN to combination therapy is not feasible for these patients. In addition to the combination, the order of treatment is important. For example, 'lead-in' therapy with PegIFN with or without RBV prior to the addition of DAAs should be tried. The evaluation of the sensitivity of P32 deletion to PegIFN is especially critical to the design of these strategies. (2) The prevalence of P32 deletion should be clarified using nationwide clinical studies or multinational studies. The prevalence should be determined in patients who are both IFN and DAA-naïve, patients with IFN-experienced and DAA-naïve, patients with IFN-based therapy failure, and patients with DAA-based therapy failure at baseline and at the time of virologic failure. This will help to understand the pathological characteristics of HCV with P32 deletion (Table 1) and will provide useful hints for methods of overcoming P32 deletion. For example, DCV monotherapy[13], DCV/ASV[7,9-12,17,19,22-24], and SOF/LDV were administered as DAA-based therapies before the advent of P32 deletion[18]. However, it is not clear yet whether other patients receiving other DAA-based therapies such as GLE/PIB develop P32 deletion at the time of virologic failure or later. The current data were insufficient due to the small populations of each study. (3) Based on this basic research, large-scale multi-institutional clinical studies of promising combination therapies should be performed and the efficacy against P32 deletion should be evaluated. However, in practice, it may be difficult to collect a sufficient number of patients with P32 deletion because the efficacy of DAA-based therapy is very high and because the prevalence of P32 deletion is assumed to be low. PegIFN-containing regimens can be tried depending on the effects on HCV with P32 deletion in preclinical studies. Two studies[22,23] reported that SOF/LDV plus RBV combination therapy might be effective for P32 deletion, as mentioned above. However, given that the study populations were relatively small, this regimen should be evaluated using in a large cohort. Another problem is that, in some countries - such as Japan and the United Kingdom - SOF/LDV plus RBV combination therapy is not covered by insurance; thus, this regimen cannot always be used in clinical practice. A basic study[24] using humanized mice showed that GLE/PBV plus SOF combination therapy was more effective than GLE/PBV combination therapy for P32 deletion. However, one of the four mice showed virologic relapse. GLE/PBV plus SOF combination therapy should be evaluated in patients with P32 deletion in clinical trials using large number of the subjects. As one mouse case with virologic relapse developed no additional RASs, the optimal treatment period should also be evaluated to avoid the advent of virologic relapse. However, GLE/PBV plus SOF combination therapy is not covered by insurance in all countries. And (4) Recent studies[25,26] showed the promising potential of new SOF-including regimens for genotype 1 patients with failure under DAA-based therapy. These regimens included SOF/VEL plus voxilaprevir, a PI and SOF/VEL. However, P32 deletion was not found at baseline or virologic failure in these studies and thus, the effectiveness of these regimens in the treatment of patients with P32 deletion is not currently available. SOF/VEL plus voxilaprevir or SOF/VEL combination therapy may be tried depending on the impact of these regimens on HCV with P32 deletion in future studies.

REFERENCES

1 **Dietz J**, Susser S, Vermehren J, Peiffer KH, Grammatikos G, Berger A, Ferenci P, Buti M, Müllhaupt B, Hunyady B, Hinrichsen H, Mauss S, Petersen J, Buggisch P, Felten G, Hüppe D, Knecht G, Lutz T, Schott E, Berg C, Spengler U, von Hahn T, Berg T, Zeuzem S, Sarrazin C; European HCV Resistance Study Group. Patterns of Resistance-Associated Substitutions in Patients With Chronic HCV Infection Following Treatment With Direct-Acting Antivirals. *Gastroenterology* 2018; **154**: 976-988.e4 [PMID: 29146520 DOI: 10.1053/j.gastro.2017.11.007]

2 **Krishnan P**, Beyer J, Mistry N, Koev G, Reisch T, DeGoey D, Kati W, Campbell A, Williams L, Xie W, Setze C, Molla A, Collins C, Pilot-Matias T. In vitro and in vivo antiviral activity and resistance

profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. *Antimicrob Agents Chemother* 2015; **59**: 979-987 [PMID: 25451055 DOI: 10.1128/AAC.04226-14]

3 **Wang C**, Jia L, O'Boyle DR 2nd, Sun JH, Rigat K, Valera L, Nower P, Huang X, Kienzle B, Roberts S, Gao M, Fridell RA. Comparison of daclatasvir resistance barriers on NS5A from hepatitis C virus genotypes 1 to 6: implications for cross-genotype activity. *Antimicrob Agents Chemother* 2014; **58**: 5155-5163 [PMID: 24936600 DOI: 10.1128/AAC.02788-14]

4 **Liu R**, Curry S, McMonagle P, Yeh WW, Ludmerer SW, Jumes PA, Marshall WL, Kong S, Ingravallo P, Black S, Pak I, DiNubile MJ, Howe AY. Susceptibilities of genotype 1a, 1b, and 3 hepatitis C virus variants to the NS5A inhibitor elbasvir. *Antimicrob Agents Chemother* 2015; **59**: 6922-6929 [PMID: 26303801 DOI: 10.1128/AAC.01390-15]

5 **Cheng G**, Tian Y, Doehle B, Peng B, Corsa A, Lee YJ, Gong R, Yu M, Han B, Xu S, Dvory-Sobol H, Perron M, Xu Y, Mo H, Pagratis N, Link JO, Delaney W. In Vitro Antiviral Activity and Resistance Profile Characterization of the Hepatitis C Virus NS5A Inhibitor Ledipasvir. *Antimicrob Agents Chemother* 2016; **60**: 1847-1853 [PMID: 26824950 DOI: 10.1128/AAC.02524-15]

6 **Wyles D**, Mangia A, Cheng W, Shafran S, Schwabe C, Ouyang W, Hedskog C, McNally J, Brainard DM, Doehle BP, Svarovskaia E, Miller MD, Mo H, Dvory-Sobol H. Long-term persistence of HCV NS5A resistance-associated substitutions after treatment with the HCV NS5A inhibitor, ledipasvir, without sofosbuvir. *Antivir Ther* 2018; **23**: 229-238 [PMID: 28650844 DOI: 10.3851/IMP3181]

7 **Poordad F**, Pol S, Asatryan A, Buti M, Shaw D, Hézode C, Felizarta F, Reindollar RW, Gordon SC, Pianko S, Fried MW, Bernstein DE, Gallant J, Lin CW, Lei Y, Ng TI, Krishnan P, Kopecky-Bromberg S, Kort J, Mensa FJ. Glecaprevir/Pibrentasvir in patients with hepatitis C virus genotype 1 or 4 and past direct-acting antiviral treatment failure. *Hepatology* 2018; **67**: 1253-1260 [PMID: 29152781 DOI: 10.1002/hep.29671]

8 **Chayama K**, Suzuki F, Karino Y, Kawakami Y, Sato K, Atarashi T, Naganuma A, Watanabe T, Eguchi Y, Yoshiji H, Seike M, Takei Y, Kato K, Alves K, Burroughs M, Redman R, Pugatch DL, Pilot-Matias TJ, Krishnan P, Oberoi RK, Xie W, Kumada H. Efficacy and safety of glecaprevir/pibrentasvir in Japanese patients with chronic genotype 1 hepatitis C virus infection with and without cirrhosis. *J Gastroenterol* 2018; **53**: 557-565 [PMID: 28948366 DOI: 10.1007/s00535-017-1391-5]

9 **Kumada H**, Watanabe T, Suzuki F, Ikeda K, Sato K, Toyoda H, Atsukawa M, Ido A, Takaki A, Enomoto N, Kato K, Alves K, Burroughs M, Redman R, Pugatch D, Pilot-Matias TJ, Krishnan P, Oberoi RK, Xie W, Chayama K. Efficacy and safety of glecaprevir/pibrentasvir in HCV-infected Japanese patients with prior DAA experience, severe renal impairment, or genotype 3 infection. *J Gastroenterol* 2018; **53**: 566-575 [PMID: 29052790 DOI: 10.1007/s00535-017-1396-0]

10 **Krishnan P**, Schnell G, Tripathi R, Beyer J, Reisch T, Dekhtyar T, Irvin M, Xie W, Fu B, Burroughs M, Redman R, Kumada H, Chayama K, Collins C, Pilot-Matias T. Integrated Resistance Analysis of CERTAIN-1 and CERTAIN-2 Studies in Hepatitis C Virus-Infected Patients Receiving Glecaprevir and Pibrentasvir in Japan. *Antimicrob Agents Chemother* 2018; **62**: [PMID: 29180522 DOI: 10.1128/AAC.02217-17]

11 **Kai Y**, Hikita H, Morishita N, Murai K, Nakabori T, Iio S, Hagiwara H, Imai Y, Tamura S, Tsutsui S, Naito M, Nishiuchi M, Kondo Y, Kato T, Suemizu H, Yamada R, Oze T, Yakushijin T, Hiramatsu N, Sakamori R, Tatsumi T, Takehara T. Baseline quasispecies selection and novel mutations contribute to emerging resistance-associated substitutions in hepatitis C virus after direct-acting antiviral treatment. *Sci Rep* 2017; **7**: 41660 [PMID: 28134353 DOI: 10.1038/srep41660]

12 **Akuta N**, Sezaki H, Suzuki F, Fujiyama S, Kawamura Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Suzuki Y, Arase Y, Ikeda K, Kumada H. Ledipasvir plus sofosbuvir as salvage therapy for HCV genotype 1 failures to prior NS5A inhibitors regimens. *J Med Virol* 2017; **89**: 1248-1254 [PMID: 28079269 DOI: 10.1002/jmv.24767]

13 **Wang C**, Sun JH, O'Boyle DR 2nd, Nower P, Valera L, Roberts S, Fridell RA, Gao M. Persistence of resistant variants in hepatitis C virus-infected patients treated with the NS5A replication complex inhibitor daclatasvir. *Antimicrob Agents Chemother* 2013; **57**: 2054-2065 [PMID: 23403428 DOI: 10.1128/AAC.02494-12]

14 **Gottwein JM**, Pham LV, Mikkelsen LS, Ghanem L, Ramirez S, Scheel TKH, Carlsen THR, Bukh J. Efficacy of NS5A Inhibitors Against Hepatitis C Virus Genotypes 1-7 and Escape Variants. *Gastroenterology* 2018; **154**: 1435-1448 [PMID: 29274866 DOI: 10.1053/j.gastro.2017.12.015]

15 **Thomas E**, Liang TJ. Experimental models of hepatitis B and C - new insights and progress. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 362-374 [PMID: 27075261 DOI: 10.1038/nrgastro.2016.37]

16 **McGivern DR**, Masaki T, Williford S, Ingravallo P, Feng Z, Lahser F, Asante-Appiah E, Neddermann P, De Francesco R, Howe AY, Lemon SM. Kinetic analyses reveal potent and early blockade of hepatitis C virus assembly by NS5A inhibitors. *Gastroenterology* 2014; **147**: 453-462.e7 [PMID: 24768676 DOI: 10.1053/j.gastro.2014.04.021]

17 **Kobayashi M**, Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Fujiyama S, Kobayashi M, Saito S, Kawamura Y, Arase Y, Ikeda K, Kumada H. Treatment efficacy of all-oral combination of direct-acting antivirals in chronic hepatitis C patients with NS5A-P32 deletion. *Kanzo* 2017; **58**: 671-673 [DOI: 10.2957/kanzo.58.671]

18 **Doi A**, Hikita H, Sakamori R, Tahata Y, Kai Y, Yamada R, Yakushijin T, Mita E, Ohkawa K, Imai Y, Furuta K, Kodama T, Tatsumi T, Takehara T. Nonstructural protein 5A/P32 deletion after failure of ledipasvir/sofosbuvir in hepatitis C virus genotype 1b infection. *Hepatology* 2018; **68**: 380-383 [PMID: 29425404 DOI: 10.1002/hep.29836]

19 **Iio E**, Ogawa S, Shimada N, Eguchi Y, Hirashima N, Matsuura K, Tanaka Y. Serial changes of NS5A P32 deletion mutant in HCV genotype 1b patients after Daclatasvir/Asunaprevir failure. *Kanzo* 2018; **59**: 230-233 [DOI: 10.2957/kanzo.59.230]

20 **McPhee F**, Hernandez D, Zhou N, Yu F, Kienzle B, Zhao Y, Linaberry M, Noviello S, Yu ML, Ahn SH, Karino Y, Chayama K, Kumada H. Emergence and persistence of NS5A and NS3 resistanceassociated substitutions in patients infected with HCV genotype 1b and treated with daclatasvir and asunaprevir. *Hepatol Int* 2017; **11** Suppl 1: S26

21 **McPhee F**, Hernandez D, Yu F, Ueland J, Monikowski A, Carifa A, Falk P, Wang C, Fridell R, Eley T, Zhou N, Gardiner D. Resistance analysis of hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. *Hepatology* 2013; **58**: 902-911 [PMID: 23504694 DOI: 10.1002/hep.26388]

22 **Ikeda H**, Watanabe T, Shimizu H, Hiraishi T, Kaneko R, Baba T, Takahashi H, Matsunaga K, Matsumoto N, Yasuda H, Okuse C, Iwabuchi S, Suzuki M, Itoh F. Efficacy of ledipasvir/sofosbuvir with or without ribavirin for 12 weeks in genotype 1b HCV patients previously treated with a nonstructural protein 5A inhibitor-containing regimen. *Hepatol Res* 2018; **48**: 802-809 [PMID: 29504692 DOI: 10.1111/hepr.13074]

23 **Suda G**, Ogawa K, Yamamoto Y, Katagiri M, Furuya K, Kumagai K, Konno J, Kimura M, Kawagishi N, Ohara M, Umemura M, Ito J, Izumi T, Nakai M, Sho T, Natsuizaka M, Morikawa K, Tsubota A, Shimada N, Iio E, Tanaka Y, Sakamoto N; NORTE Study Group. Retreatment with sofosbuvir, ledipasvir, and add-on ribavirin for patients who failed daclatasvir and asunaprevir combination therapy. *J Gastroenterol* 2017; **52**: 1122-1129 [PMID: 28315983 DOI: 10.1007/s00535-017-1328-z]

24 **Teraoka Y**, Uchida T, Imamura M, Osawa M, Tsuge M, Abe-Chayama H, Hayes CN, Makokha GN, Aikata H, Miki D, Ochi H, Ishida Y, Tateno C, Chayama K; Hiroshima Liver Study Group. Prevalence of NS5A resistance associated variants in NS5A inhibitor treatment failures and an effective treatment for NS5A-P32 deleted hepatitis C virus in humanized mice. *Biochem Biophys Res Commun* 2018; **500**: 152-157 [PMID: 29621544 DOI: 10.1016/j.bbrc.2018.04.005]

25 **Gane EJ**, Shiffman ML, Etzkorn K, Morelli G, Stedman CAM, Davis MN, Hinestrosa F, Dvory-Sobol H, Huang KC, Osinusi A, McNally J, Brainard DM, McHutchison JG, Thompson AJ,

Sulkowski MS; GS-US-342-1553 Investigators. Sofosbuvir-velpatasvir with ribavirin for 24 weeks in hepatitis C virus patients previously treated with a direct-acting antiviral regimen. *Hepatology* 2017; **66**: 1083-1089 [PMID: 28498551 DOI: 10.1002/hep.29256]

26 **Lawitz E**, Poordad F, Wells J, Hyland RH, Yang Y, Dvory-Sobol H, Stamm LM, Brainard DM, McHutchison JG, Landaverde C, Gutierrez J. Sofosbuvir-velpatasvir-voxilaprevir with or without ribavirin in direct-acting antiviral-experienced patients with genotype 1 hepatitis C virus. *Hepatology* 2017; **65**: 1803-1809 [PMID: 28220512 DOI: 10.1002/hep.29130]

P- Reviewer: Maekawa S, Matsui K **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4311-4329

DOI: 10.3748/wjg.v24.i38.4311

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

REVIEW

Management of bacterial and fungal infections in end stage liver disease and liver transplantation: Current options and future directions

Elda Righi

Elda Righi, Department of Infectious Diseases, Santa Maria della Misericordia University Hospital, Udine 33100, Italy

ORCID number: Elda Righi (0000-0001-8718-1200).

Author contributions: Righi E prepared the manuscript.

Conflict-of-interest statement: No conflict of interest exists.

Manuscript source: Invited manuscript

Correspondence to: Elda Righi, MD, PhD, Doctor, Department of Infectious Diseases, Santa Maria della Misericordia University Hospital, 50, Colugna Street, Udine 33100, Italy. elda.righi@libero.it
Telephone: +39-432-559355
Fax: +39-432-559360

Received: July 14, 2018
Peer-review started: July 16, 2018
First decision: August 27, 2018
Revised: September 11, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 14, 2018

Abstract

Patients with liver cirrhosis are susceptible to infections due to various mechanisms, including abnormalities of humoral and cell-mediated immunity and occurrence of bacterial translocation from the intestine. Bacterial infections are common and represent a reason for progression to liver failure and increased mortality. Fungal infections, mainly caused by *Candida* spp., are often associated to delayed diagnosis and high mortality rates. High level of suspicion along with prompt diagnosis and treatment of infections are warranted. Bacterial and fungal infections negatively affect the outcomes of liver transplant candidates and recipients, causing disease progression among patients on the waiting list and increasing mortality, especially in the early post-transplant period. Abdominal, biliary tract, and bloodstream infections caused by Gram-negative bacteria [*e.g.*, *Enterobacteriaceae* and *Pseudomonas aeruginosa* (*P. aeruginosa*)] and *Staphylococcus* spp. are commonly encountered in liver transplant recipients. Due to frequent exposure to broad-spectrum antibiotics, invasive procedures, and prolonged hospitalizations, these patients are especially at risk of developing infections caused by multidrug resistant bacteria. The increase in antimicrobial resistance hampers the choice of an adequate empiric therapy and warrants the knowledge of the local microbial epidemiology and the implementation of infection control measures. The main characteristics and the management of bacterial and fungal infections in patients with liver cirrhosis and liver transplant recipients are presented.

Key words: Liver cirrhosis; Liver transplant recipients; Bacterial infections; Fungal infections; Multidrug resistant organisms; Management

Core tip: Infections are frequent in patients with liver cirrhosis, liver transplant candidates, and liver transplant recipients and are associated with increased morbidity and mortality. Knowledge of the risk factors, etiology, and type of infections is paramount for the management of severe bacterial and fungal infections in these pa-

tient populations. Increasing rates of infections due to multidrug-resistant pathogens have been reported worldwide and particularly affect liver transplant recipients. The type of bacterial and fungal infections along with their risk factors, management, and future research in patients with liver cirrhosis and liver transplant recipients are presented in the review.

Righi E. Management of bacterial and fungal infections in end stage liver disease and liver transplantation: Current options and future directions. *World J Gastroenterol* 2018; 24(38): 4311-4329 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4311.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4311

INTRODUCTION

Liver cirrhosis (LC) represents a dynamic clinical entity characterized by various stages of progression[1]. From a clinical perspective, LC includes compensated and decompensated stages of disease characterized by different features, prognoses, and predictors of death. Specifically, decompensated cirrhosis is associated with portal hypertension or liver insufficiency and their related complications, including recurrent variceal hemorrhage, refractory ascites, hyponatremia, and/or hepatorenal syndrome. All these clinically evident complications can be further aggravated by the occurrence of infections[1].

Various mechanisms predispose patients with LC to infections. Impairment of immune function has been well documented and is characterized by multiple immune deficiencies, involving not only local liver damage but also deficiencies in systemic innate and acquired immunity[2]. Increased gastrointestinal (GI) permeability and pathological bacterial translocation are considered key factors leading to increased infection susceptibility in LC (Figure 1)[3,4]. Although a clear correlation between bacterial translocation and spontaneous bacterial peritonitis (SBP, one of the most frequent infections in cirrhosis) has not been universally reported[5,6], various factors support this relationship. A GI pathogen such as *Escherichia coli* (*E. coli*), for example, represents a commonly isolated pathogen in cirrhosis and also a major cause of SBP (Table 1)[7,8]. Furthermore, a higher number of pathogenic bacteria, especially Enterobacteriaceae, have been found in the mucosal microbiota composition of the sigmoid in cirrhotic patients compared to healthy controls[9]. Host-related, hospital-related, and drug-related factors also contribute to increased susceptibility to infections in this population (Figure 1). The presence of concomitant comorbidities associated with liver disease, including obesity, alcohol consumption, malnutrition, viral hepatitis and/or HIV infection predisposes to bacterial and fungal infections[10]. Frequent and prolonged hospitalizations along with the use of invasive devices (*e.g.*, urinary and central venous catheters, CVC) pose patients at risk of nosocomial infections such as pneumonia, CVC-related bacteremia, and urinary tract infections. Furthermore, the use of immunosuppressive agents remains frequent in this population[11].

Infections remain one of the principal causes of morbidity and mortality also among liver transplant recipients (LTR)[12]. Bacterial and fungal infections following LT are frequent, occurring in more than 50% of patients mainly due to the type of surgical procedures that, compared to other solid organ transplants, are more complex and may presents complications such as abdominal abscess, bile leaks, and hepatic artery stenosis[13]. Bacterial infections account for up to 70% of all infections in LTR, followed by fungal and viral infections[14]. The interplay among key factors such as patients' net state of immunosuppression, environmental exposure to specific organisms (*e.g.*, nosocomial pathogens), and development of surgical complications affects the timing of specific post-LT infections[14]. The organism's virulence, along with intensity and timing of the exposure, can also impact infections' severity and outcome. Factors known to increase the risk of infections after LT include a Model for End-Stage Liver Disease (MELD) score greater than 30, reoperation (including retransplantation), renal replacement therapy, prolonged intensive care unit (ICU) stay, and older age[15].

An appropriate management of infections in patients with cirrhosis and following liver transplantation implies the knowledge of predisposing risk factors for infections in order to identify high-risk patients, the prompt use of correct diagnostic tools to recognize atypical disease presentations, and early adequate antimicrobial treatment and source control.

The possible etiologies of infections among patients with LC and LTR are diverse and may range from common bacterial and viral pathogens to opportunistic pathogens that are clinically relevant only for immunocompromised patients. In this review, the most common challenges and main principles for the management of bacterial and fungal infections are discussed. The review focuses mainly on nosocomial infections, including those caused by multidrug resistant organisms (MDRO), while other opportunistic infections are not presented in details.

BACTERIAL INFECTIONS IN PATIENTS WITH LIVER CIRRHOSIS

Epidemiology

Bacterial infections are common in patients with LC and can occur at various stages of liver disease, representing the primary cause of admission to emergency departments in this patient population[16]. Among patients admitted to emergency departments, increasing rates of SBP and hepatorenal syndrome have been documented[16]. Patients with LC present a high prevalence of bacterial infections, with 10% reporting more than one episode of infection within the same hospitalization[17,18]. Second infections were reported as independent predictors of mortality in hospitalized patients

Table 1 Main studies reporting the characteristics and mortality rates associated with infections in patients with liver cirrhosis

Year	Prevalence of infections (%); patient *n*	Main infection sites (%)	Most common pathogens (%)	Mortality rates (%)	Ref.
2018	NR; *n* = 312	Only BSI included; primary (32), SBP (16), UTI (11)	GNB (53)	25	[28]
2017	61; *n* = 852	Only BSI included; primary (60), abdominal (33), UTI (7), pneumonia (6)	GNB (60)	23	[22]
2015	38; *n* = 401	Pneumonia (22), UTI (21), SBP (19)	*E. coli* (72)	31	[23]
2012	51; *n* = 207	UTI (52), SBP (23), BSI (21)	GPB (56)	24	[19]
2010	33; *n* = 150	UTI (37), pneumonia (22), BSI (13)	GNB (62)	37	[18]
2007	45; *n* = 233	UTI (43), pneumonia (25), SBP (16)	GNB (65)	18	[20]
2003	25; *n* = 135	UTI (31), SBP (26), pneumonia (25)	NR	9	[25]
2002	22; *n* = 70	BSI (16), CVC-BSI (9), liver abscess (3)	GPB (67)	29	[24]
2002	32; *n* = 507	SBP (24), UTI (19), pneumonia (14), CVC-BSI (8)	GPB (47)	22	[17]
2001	34; *n* = 361	UTI (41), SBP (23), BSI (21), pneumonia (17)	*E. coli* (25)	15	[8]
1994	39; *n* = 132	SBP (44), UTI (26), pneumonia (16)	GNB (65), *E. coli* (62)	29	[27]
1993	47; *n* = 170	SBP (31), UTI (25), pneumonia (21)	GNB (72)	17	[26]

NR: Not reported; BSI: Bloodstream infections; SBP: Spontaneous bacterial peritonitis; UTI: Urinary tract infections; CVC: Central venous catheter; GNB: Gram-negative bacteria; GPB: Gram-positive bacteria.

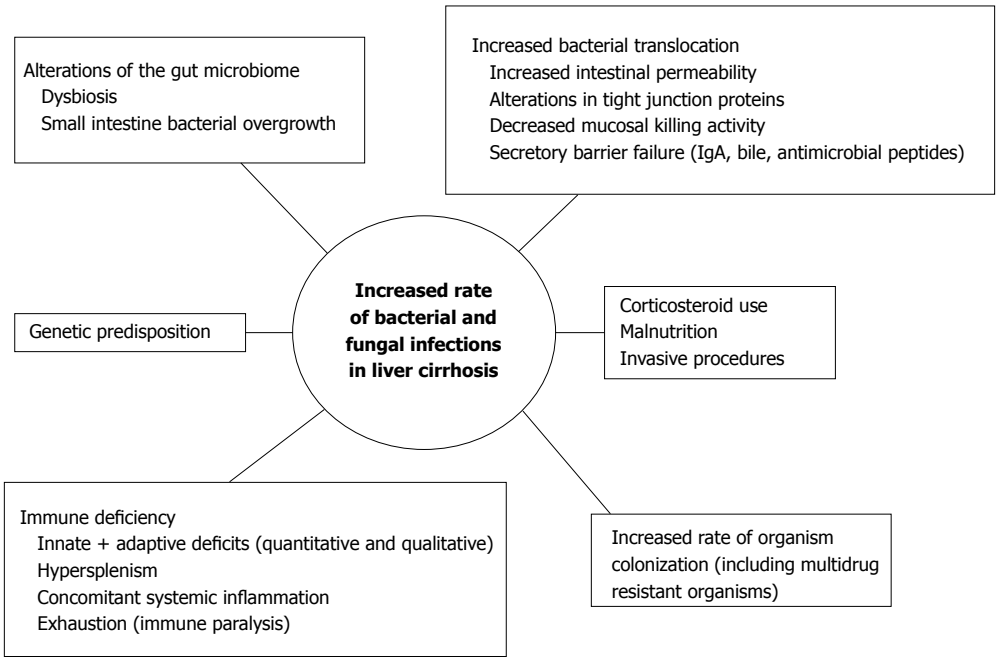


Figure 1 Factors leading to increased susceptibility to infections in patients with liver cirrhosis.

with LC and appeared preventable in the majority of cases[19].

According to reports from different countries, the overall prevalence of bacterial infections in hospitalized patients with LC varies from 22% to 51% (Table 1)[17-28]. Multiple factors have been associated with the occurrence of infections, including increased MELD scores, alcoholic liver disease, protein malnutrition, and GI bleeding[7,8,19,21,29]. Infections often represent the trigger for clinical deterioration or progression to liver decompensation[18,20]. For example, bacterial infections remain a leading cause of acute on chronic liver failure[19]. In a study including 50 cirrhotic patients, the presence of an infectious episode worsened liver function in 62% of cases[18]. Patients with infections were more likely to develop ascites, hepatic encephalopathy, hyponatremia, hepatorenal syndrome, or septic shock compared to noninfected ones. Furthermore, SBP can lead to severe renal failure, which is also associated with poor clinical outcomes[20,30]. In a report encompassing 104 cirrhotic patients with bacterial infections, 34% presented infection-induced renal failure associated with GI infections and SBP. Multivariate analysis confirmed that lack of infection resolution was an independent factor for renal failure ($P = 0.03$)[20].

On the other hand, clinical complications of LC can represent a risk factor for development of infections. Examples are GI bleeding, associated with bacterial infections in up to 45% of patients[7] and SBP, usually reported in patients with ascites, showing prevalence rates that vary between 15% and 25%[8]. Besides SBP, other frequent infections in cirrhotic patients include

urinary tract infections, pneumonia, and skin and soft tissue infections (SSTI) (Table 1)[18,20].

Few reports have also documented high rates of bacterial meningitis among patients with cirrhosis compared to those without liver disease, including pneumococcal infections[31]. Increased creatinine serum levels were associated with mortality in cirrhotic patients with meningitis[32]. The overall incidence of bacteremia, urinary tract infections, pneumonia, meningitis, tuberculosis, and liver abscess appeared increased more than tenfold in LC, and mortality rates of each episode were 3 to 10 times higher than in non-cirrhotic patients[33]. A 10-fold higher risk of bloodstream infections (BSI) in LC compared to the general population was identified in a Danish study showing 47%, 45%, and 8% of Gram-negative, Gram-positive, and polymicrobial BSI, respectively, with overall 30-d case-fatality rate of 0.53[34]. BSI in cirrhotic patients can lead to complications such as deep-seated metastatic infections, including endotipsitis (in patients with transjugular intrahepatic portosystemic shunt) and infective endocarditis[35]. *Streptococcus bovis* endocarditis, in particular, has been associated with advanced liver disease[36]. Types, etiology, and mortality of bacterial infections in LC are reported in Table 1.

Diagnosis and treatment

Due to the increased risk of sepsis and multiorgan failure among cirrhotic patients, prompt identification of symptoms and signs of septic shock and assessment of organ function is paramount[37]. The diagnostic workup should also aim at identifying the source of infection by blood cultures, urine culture, chest X-ray, and lung or abdominal CT scan according to patients' medical history and clinical presentation. Performance of paracentesis with neutrophil count and microbiological culture of ascitic fluid is recommended in all cirrhotic patients hospitalized with ascites to role out SBP[38,39]. Other microbiological tests include sputum and/or bronchoalveolar lavage cultures if pneumonia is suspected, stool cultures (including assays for *Clostridium difficile* diagnosis) in case of GI symptoms, and wound - or intra-abdominal - cultures, when indicated[37].

Data on antimicrobial treatment options in LC are limited, thus recommendations are often based on expert opinion or inferred from studies in non-cirrhotic populations[37]. A study analyzing bloodstream infections in patients with cirrhosis found that timely initiation of an appropriate antimicrobial therapy had a major impact on patients' outcome[40]. Multidrug resistance is a major predictor of inappropriate therapy in LC. For these reasons, antimicrobial treatment should be promptly initiated and, when possible, adjusted according to the microbiological results. Empirical treatment should take into consideration the local epidemiology, including the rates of antimicrobial resistance, the site of infection, and patients' clinical presentation (*e.g.*, septic shock).

Patients with SBP usually have infections caused by enteric pathogens, such as Enterobacteriaceae and *Enterococcus* spp.; infections due to *P. aeruginosa* are also possible, especially among hospitalized patients[11]. Third generation cephalosporins (*e.g.*, ceftriaxone, cefotaxime, or the antipseudomonal cephalosporin ceftazidime) or beta-lactam/beta-lactamase inhibitor combinations such as piperacillin/tazobactam (active also against *Enterococcus* spp.) are frequently used to treat SBP[11]. Although ciprofloxacin represents a potential option for SBP treatment, rates of resistances associated to quinolone use remains high at various centers and among patients receiving long-term norfloxacin prophylaxis[37]. Tigecycline presents good intra-abdominal penetration and is active against *Enterococci* [including *Enterococcus faecium* (*E. faecium*)], *Staphylococcus aureus* (*S. aureus*) and Enterobacteriaceae; nevertheless, its bacteriostatic activity and reduced serum concentrations limit tigecycline use in patients with sepsis. Other broad-spectrum antibiotics, such as carbapenems, should be reserved to the treatment of severe infections or in areas with a high prevalence of ESBL-producing strains[11,37]. Enterobacteriaceae and *Enterococcus* spp. are also common causes of urinary tract infections and can be treated with a third generation cephalosporin or beta-lactam/beta-lactamase inhibitor combinations[11]. In uncomplicated, non-bacteremic infections, oral options such as cotrimoxazole or nitrofurantoin can be used, according to the pathogen's susceptibility. The use of quinolones, however, should be limited due to their high potential for antimicrobial resistance selection.

Nosocomial pneumonia represents a frequent life-threatening infection in LC. Antimicrobial options include a beta-lactam (*e.g.*, ceftazidime, a beta-lactam/beta-lactamase inhibitor combination, or a carbapenem) with or without a quinolone such as ciprofloxacin[11]. If risk factors for MRSA are documented (*e.g.*, MRSA colonization or previous infection), treatment with vancomycin or linezolid can be considered. SSTI, most frequently cellulitis, can be caused by both Gram-negative and Gram-positive pathogens in cirrhotic patients[41].

In severe nosocomial infections, the association of a broad-spectrum antibiotic active against Gram-negative bacilli (*e.g.*, piperacillin/tazobactam or meropenem) with an anti-MRSA drug (*e.g.*, vancomycin, daptomicin) is recommended[11].

Outcome

Although the diagnosis and treatment of infections have improved over the decades, their occurrence still significantly impact on the mortality of patients with liver cirrhosis[29]. In these patients, GI bacterial overgrowth and translocation favor the occurrence of various infections, while the increase of endotoxins levels and cytokines can induce systemic inflammatory responses leading to septic shock, multiorgan dysfunction, and death[29].

A systematic review including 178 studies showed better outcomes in noninfected compared to infected patients with cirrhosis (OR = 3.75, 95%CI: 2.12-4.23)[42].

Mortality of infected patients was 30% at 1 mo and increased to 63% after 12 mo following an infection. Infections and mortality appeared more frequent in patients with Child-Pugh C compared to A or B stage (P = 0.003 and P = 0.0002, respectively)[21]. A prospective study including 312 BSI (53% due to Gram-negative and 47% due to Gram-positive bacteria) showed 30-d mortality rates of 25%[40]. Risk factors associated with mortality included delayed (> 24 h) antibiotic treatment (P < 0.001), inadequate empirical therapy (P < 0.001), and increased Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA) score (P < 0.001). A study analyzing the characteristics of infections in cirrhotic compared to non-cirrhotic patients hospitalized in ICU identified higher prevalence of infections (59% *vs* 51%, P < 0.01), increased rates of abdominal infections, and higher number of Gram-positive infections (including methicillin-resistant *Staphylococcus aureus*, MRSA) in the LC group[43]. In a study showing mortality rates of 37% among LC patients with hospital-acquired infections, sepsis was an independent factor for hospital death (P = 0.005; 95%CI: 1.7-21.4)[18]. Another study identified higher in-hospital mortality in cirrhotic compared to non-cirrhotic patients (42% *vs* 24% respectively, P < 0.001) and among those with septic shock (30% *vs* 49%, P < 0.05)[43]. Based on these data, a novel prognostic stage in the course of cirrhosis was proposed, defining the group of "critically ill decompensated patients", characterized by a risk of progression to death or liver transplant of 60%[42].

FUNGAL INFECTIONS IN LC

Fungal infections in patients with liver cirrhosis are mainly caused by *Candida* spp. and could represent, if not promptly recognized, a cause of treatment failure[44,45]. Studies on fungal infections in LC often refer only to *Candida* spp., are usually limited to retrospective cohorts, and can be biased by underreporting and under diagnosis. Nevertheless, data shown in the few available reports appear worrisome in terms of unfavorable outcomes.

Candidemia may arise both endogenously and exogenously in patients with LC, but nearly always occurs when prolonged antibiotic exposure is documented[40,44]. The use of antibiotics for prevention of SBP, in particular, is frequent and may favor an excessive growth of fungi in the intestinal flora, potentially causing fungal translocation in the peritoneal cavity and development of spontaneous fungal peritonitis (SFP)[46]. SFP is characterized by PMN counts of $\geqslant$ 250 cells/mm^3 in the ascitic fluid along with positive fungal cultures (with or without concomitant SBP) and no apparent intraabdominal sources of infection, while fungal ascites is defined by lower PMN counts[47]. Culture positivity ranged from 0 to 11% in various studies[47]. SFP is mainly caused by *C. albicans*, is frequently nosocomial, and appears associated to higher mortality compared to PBS[47]. Advanced liver disease and GI bleeding have been advocated as risk factors for SFP, since higher GI permeability may be required to favor the translocation of large pathogens such as fungi[47]. SFP should also be considered in the diagnostic workup of hospitalized cirrhotic patients with impaired renal function, such as those with refractory ascites[45]. Other risk factors for SFP include prolonged hospitalizations and performance of invasive procedures[48]. Fungal infections characterized by high mortality have been described in LC patients hospitalized in ICU and in those with alcoholic hepatitis[44,45,49,50]. A retrospective analysis of 120 cirrhotic patients identified the presence of fungal colonization as an independent factor for mortality (P = 0.047)[49]. A study including 185 patients with culture-positive infections documented *Candida* spp. in 19 (10%) cases. Of these, 58% were SFP and 42% candidemia. Only 47% of fungal infections were diagnosed and treated with antifungal agents, while the remaining patients died. Mortality rates at one month were 58% and 29% in patients with fungal infections compared to those with bacterial infection, respectively (P = 0.001)[44]. Similarly, another study encompassing 126 cirrhotic patients with culture-positive ascites identified SFP in 14/126 (11%) patients. Only 43% of patients with cultures positive for fungi received antifungal treatment[45]. In a prospective multicenter study, *Candida* spp. represented 7% of all BSI and was associated with prolonged hospitalizations, prior surgery, CVC placement, neutropenia, and prior antimicrobial use[40]. Compared to other infections, *Candida* BSI had the strongest association with inappropriate empirical therapy[28]. *Bassetti et al*[51] previously analyzed 169 episodes of candidemia and 72 intra-abdominal candidiasis in cirrhotic patients, showing high rates of ICU admission (50%), non-albicans *Candida* infections (46%), and occurrence of septic shock (35%). Thirty-day mortality was 35.3% and was independently associated with candidemia (OR = 2.2, 95%CI: 1.2-4.5), septic shock (OR = 3.2, 95%CI: 1.7-6), and absence of adequate antifungal treatment (OR = 0.4, 95%CI: 0.3-0.9)[51].

These data emphasize the importance of performing fungal cultures and maintaining a high level of suspicion in patients with LC, especially those with impaired renal function and/or receiving antimicrobial treatment with limited clinical response, to ensure early treatment and ultimately reduce mortality (Table 2)[52-54].

Early administration of antifungal treatment has been associated with improved outcomes, especially in patients with severe infections[55,56].

Novel molecules (*e.g.*, azoles such as isavuconazole) and new antifungal classes (*e.g.* echinocandines) have become available for the treatment of invasive fungal infections in the last decades. In patients with LC concerns in the efficacy and safety of antifungals appear linked to resistance to antifungals, patients' reduced tolerance, and altered drug pharmacokinetics caused by advanced liver disease.

Although fluconazole is still widely used due to its favorable pharmacokinetics and tolerability, a shift to non-albicans strains showing lower fluconazole sus-

Table 2 Management of fungal infections in patients with liver cirrhosis

Type	Characteristics	Management	Ref.
SFP, fungemia, disseminated fungal infection (mainly *Candida* spp.)	Delayed diagnosis and therapy. Lack of clinical signs and suspicion. Frequent concomitant SBP. High mortality.	Suspect if peritonitis is not improved after 48 h of empirical antibiotic treatment. Perform fungal cultures (ascites and blood).	[44,45,52,53]
Antifungal prophylaxis	Factors influencing mortality less known. Mortality higher than SBP due to delayed diagnosis.	Indicated for SBP (high risk, previous episode, GI bleeding). No clear indication for fungal infections. Consider in: ICU patients without improvement > 48 h, high prevalence (> 5%) regions, risk factors (corticosteroids, prolonged microbial use, CVC, TPN, high APACHE score, dialysis).	[48,54]
Antifungal treatment	Recommendations for fungal infections in LC.	Prompt initiation. Echinocandins as first-line treatment (*e.g.*, fungemia, nosocomial SFP or critically ill with CA-SFP). Fluconazole indicated if less severe infections. De-escalation if patient is stable and sensitivity tests available.	[52-54]

SFP: Spontaneous fungal peritonitis; SBP: Spontaneous bacterial peritonitis; GI: Gastrointestinal; CVC: Central venous catheter; TPN: Total parenteral nutrition; APACHE: Acute Physiology and Chronic Health Evaluation; LC: Liver cirrhosis; CA: Community-acquired.

ceptibility has been reported[57]. Echinocandins are currently recommended as first line treatment in critically ill patients and in case of reduced susceptibility to fluconazole[58]. Despite evidence of resistance has emerged especially in *C. glabrata*, overall resistance rates to echinocandines remains low[59]. Compared to azoles such as voriconazole, echinocandins present reduced liver toxicity and better tolerability[60]. While dose adjustments are not recommended for any severity of liver disease for micafungin and andulafungin, reduction of caspofungin maintenance dose from 50 to 35 mg/d is suggested[61]. This dose reduction, however, may not be appropriate in critically ill patients who may have sub-therapeutic exposure and efficacy. In patients with liver cirrhosis receiving voriconazole, therapeutic drug monitoring is recommended due to the correlation between trough plasma concentration and occurrence of adverse effects[62].

MANAGEMENT OF INFECTIONS IN CIRRHOTIC PATIENTS

As a general rule, an infection should be suspected in all cirrhotic patients with unexpected clinical deterioration (*e.g.*, new onset of porto-systemic encephalopathy, worsening of renal or liver function tests) due to the known impact of infections on liver disease progression[37].

A prompt diagnosis of infectious processes in patients with liver disease can be hampered by various factors that may act as confounders or mask bacterial and/or fungal infections, thus potentially delaying an effective treatment. Due to the immune impairment that accompanies LC, systemic responses and classical symptoms of infections may be reduced and difficult to diagnose. Furthermore, LC itself may be a cause of low-grade fever in up to 20% of patients[63]. Opportunistic infections can also occur and their recognition may be less immediate, or require longer times to obtain culture positivity. Targeted microbiological cultures (blood, urine and ascites cultures) before administration of antimicrobials are recommended, and the use of markers (*e.g.*, galactomannan, beta-D-glucan) could be considered if fungal infections are suspected[64]. Similarly to other immunocompromised patients, high-resolution chest CT should be preferred to X-rays for pulmonary infections[37]. Besides prompt diagnosis, early appropriate antimicrobial and/or antifungal treatment remain key factors in the management of LC patients with severe infections.

Infections caused by multidrug resistant organisms (MDRO) may represent a cause of treatment failure favoring poor outcomes. Prevalence of MDRO in LC patients reflects the global resistance burden of different countries, thus knowledge of the local patterns of susceptibility is paramount to optimize empirical and targeted therapy in severe infections. Two studies in Italy and Greece identified prevalence rates of MDR infections of 27% and 19%, respectively, mainly caused by ESBL-producing *E. coli* and carbapenem-resistant *K. pneumoniae*[65,66]. MDRO accounted for nearly one-third of BSI in cirrhotic patients in a European multicenter study that identified inadequate empirical therapy as an independent cause of 30-d mortality[40]. Infections were associated with previous antimicrobial exposure and invasive procedures. Most common MDRO were ESBL-producing Enterobacteriaceae (14%), while the highest mortality rates (> 40%) were associated with carbapenem-resistant Enterobacteriaceae, *Candida* spp., and *E. faecium*.

INFECTIONS IN PATIENTS ON THE TRANSPLANT WAITING LIST

Patients on the waiting list are frail and often require multiple hospitalizations, which in turn can favor in-

Table 3 Management of infections in liver transplant recipients

Population/ infection	Risk factor and type of infection	Management	Ref.
Liver transplant candidates/ all infections	Donor-derived. Active/latent infections. Vaccine-preventable infection.	Donor screening. Careful patient history and physical examination. Identification of infections requiring therapy. Immunization.	[160-165]
Liver transplant recipients/ bacterial	Nosocomial infections (ICU, invasive devices). Recurrent infections (anatomical defects). Immunosuppression.	Peri-transplant antibiotic prophylaxis (< 48 h). Prompt diagnostic workup (uncommon presentations, opportunisms). Source control when needed.	[76,83,160]
Liver transplant candidates and recipients/MDRO	Colonization (MRSA, VRE, CRE) linked to increased risk of infections. Risk of transmission between patients and across wards.	Surveillance cultures (CRE, VRE, MRSA) and decolonization (MRSA). Infection control (hand hygiene, isolation, contact precautions).	[102,112,164]

LT: Liver transplantation; ICU: Intensive care unit; MDRO: Multidrug-resistant organisms; MRSA: Methicillin-resistant *Staphylococcus aureus*; VRE: Vancomycin-resistant *enterococci*; CRE: Carbapenem-resistant Enterobacteriaceae.

fections and deteriorate liver function or lead to multi-organ failure. Occurrence of severe infections put patients at risk of dropout from transplant waiting lists, potentially reducing the possibility to undergo LT and causing a destructive impact on the natural progression of cirrhosis. A prospective study evaluating 136 LT candidates developing bacterial infections showed that the majority were delisted or died (42%), while 35% underwent LT[67]. Similarly, occurrence of SBP was documented as a cause of death of removal from the waiting list in 38% of patients with advanced cirrosis[68]. Although higher post-transplant mortality has not been clearly correlated with occurrence of pre-transplant infections, other factors such as increased MELD score, prolonged post-LT intubation and hospitalization were documented among infected LT candidate compared with noninfected ones[69]. Various studies investigating the outcome of LTR after recovery from an infection prior to LT showed an increase length of hospital stay, higher rates of postoperative infections and increased isolation of MDRO compared to patients without infection, although similar survival rates were reported[70]. Careful management of these patients, especially in case of repeated hospitalization, is warranted.

INFECTIONS IN LIVER TRANSPLANT RECIPIENTS

Bacterial infections: Timing

Bacterial infections, especially those caused by nosocomial pathogens, are more common during the early post-transplant period (0-1 mo). Surgical complications can lead to wound infections, peritonitis, hepatic artery thrombosis, and biliary tract ischemia that can cause biloma or strictures, increasing the risk of recurrent cholangitis[12,71,72]. Other factors contributing to bacterial infections in the early postoperative period include mechanical ventilation, prolonged ICU stay, alteration of the mucocutaneous barrier, vascular and urinary catheterization, and profound immunosuppression[73]. A retrospective study including 463 LTR over a 3-year period identified at least one infection in 41% of cases, with biliary tract infections and infections due to staphylococci representing the most common types[72].

Complications occurring during transplantation that imply a more complex and prolonged surgical procedure, such as development of ischemia-reperfusion injury and high amount of blood transfused intraoperatively, may favor surgical site infections[74,75]. A prospective study including LTR with BSI identified CVC-BSI (31%), pneumonia (24%), and abdominal and/or biliary infections (14%) as most common sources of bacteremia. Diabetes mellitus (P = 0.03) and serum albumin level less than 3.0 mg/dL (P = 0.02) were predictors of bacteremia. Mortality at 14 d was higher in patients with BSI compared with nonbacteremic infections (28% *vs* 4%, P = 0.03)[75]. Risk factors for mortality among patients with BSI after LT include ICU stay, abnormal laboratory findings (*e.g.*, greater serum bilirubin level and prothrombin time) and lack of febrile response[75].

Infections in the donors, if controlled, are not considered a contraindication for transplant. However, since they may represent a source of post-transplant bacterial infections, an accurate screening of donors is recommended (Table 3)[76,77]. Opportunistic infections (*e.g.*, herpesvirus infections, nocaridosis, tuberculosis, *etc.*) are considered more common between 1 and 6 mo post-transplant, although pneumonia and intra-abdominal infections can still occur during this period. Risk factors that may favor bacterial infections during the intermediate post-transplantation period include over-immunosuppression, allograft rejection, biliary tract complications, and re-transplantation[73]. In a study analyzing early (< 6 mo) *vs* late post-transplant infections (> 6 mo), the incidence decreased from 11.5 episodes/1000 transplant-days in the first month to 1.9 and 0.3 between 1 mo and 6 mo and after 6 mo, respectively[78]. Gram-positive and Gram-negative bacteria-related infections were equally distributed (14.8% of all infections). A specific risk factor for late infections was the performance of a biliary derivation to jejune, favoring cholangitis and secondary peritonitis in

LTR. Risk for late bacterial infections varies according to the recipient's graft and immune status, with high-risk patients characterized by recurrent rejection and allograft dysfunction requiring intense immunosuppression[73]. Community-acquired infections, however, remain common following LT even among low-risk patients. A high level of suspicion for late bacterial infections should be maintained due to potentially atypical or less expected infection presentations.

Management of infections in LTR includes prompt initiation of antimicrobial treatment and adequate source control (*e.g.*, CVC removal, surgical debridement).

Type of post-transplant bacterial infections

Bloodstream infections: BSI represent an important cause of mortality in LTR[79]. BSI mainly occur during the first post-operative month and appear to be predictors of long-term survival in transplant recipients. A study encompassing 704 LTR at a single center over a 10-year period showed an incidence of BSI of 37% with an overall mortality of 16%[79]. The majority of BSI (39%) occurred within 10 d after LT. Most frequently isolated pathogens were Enterobacteriaceae (41%), *S. aureus* (19.8%), *Enterococci* (13.1%), *P. aeruginosa* (8.8%), and yeasts (7.1%). A similar study including only Gram-negative bloodstream infections identified an incidence of 210/1000 person-years within the first month following transplantation. Compared to kidney transplant recipients, LTR were more likely to develop early infections and had higher BSI-associated mortality[80]. Potential sources of BSI include intra-abdominal infections (IAI), CVC-BSI, pneumonia, and, less frequently, urinary tract infections. Need for re-operation, prolonged use of indwelling vascular catheters, and acute graft rejection represent predisposing factors for BSI[79]. Gram-negative bacilli such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa* are often the most commonly isolated pathogens, although enterococci, viridans streptococci, and polymicrobial infections are frequently reported among LTR[79-81]. Blood cultures from CVC and peripheral vein represent the gold standard for the diagnosis of BSI and CVC-BSI. If pneumonia or urinary tract infections are suspected, additional cultures (*e.g.*, sputum, bronchoalveolar lavage, or urine cultures) and imaging (*e.g.*, chest CT scan or kidney imaging) should be performed. Management of persistent BSI also warrants the investigation of deep-seated infections (*e.g.*, endocarditis, intra-abdominal abscesses, *etc.*) and, when possible, prompt source control measures such as removal of vascular catheters and drainage of collections[37].

Surgical site infections: Surgical site infections (SSI) can occur in up to 10% of patients undergoing LT. SSI are more frequently associated to the early post-transplant period and are mainly caused by *Enterococcus* spp., *E. coli*, and *S. aureus*[82,83]. Although they carry a relatively low mortality risk, SSI are associated with increased morbidity and length of hospital stay. In patients with suspected SSI, obtaining purulent discharge cultures and appropriate imaging (*e.g.*, ultrasounds or CT scan) of a collection is important to achieve a timely diagnosis. Management of SSI is usually based on a combined approach, including surgical debridement and targeted antimicrobial therapy. A prospective study including 107 (9%) patients developing SSI identified as independent risk factors choledochojejunal or hepaticojejunal reconstruction, previous liver or kidney transplant, and transfusion of more than 4 red blood cell units[83].

Intra-abdominal infections: IAI represent common infections, accounting for up to 50% of early bacterial infections following LT, and include intraabdominal abscesses, peritonitis, and cholangitis[84-86]. IAI can be polymicrobial and are mainly caused by *Enterococci*, staphylococci, *Pseudomonas* spp., Enterobacteriaceae, and anaerobes[84]. Risk factors for IAI are often related to complications during transplantation and their severity is increased by hepatic artery thrombosis and arterial stenosis[84,87]. Compared with SSI, IAI can have a major impact on patients' outcome. A study encompassing 57 LTR with biloma showed higher rates of mortality, graft loss and need for re-transplantation compared to patients without IAI[84,87]. Predictors of mortality were renal insufficiency ($P = 0.02$) and infections due to *Candida* spp. or Gram-negative bacteria.

Adequate imaging, such as ultrasounds, CT scan, or MRI, along with prompt source control are often essential to assure an appropriate management of IAI. Surgical approaches include percutaneous drainage of infected foci and control of peritoneal contamination by diversion or resection (*e.g.*, biliary strictures or stones). Patients with diffuse peritonitis from a perforated viscus should undergo prompt emergency surgery. Intraoperative samples and cultures from recently (< 48 h) inserted drains or ascitic fluid collected in blood culture vials should always be performed to achieve a microbiological diagnosis.

Difficult-to-treat bacteria and MDRO

Although any bacteria can potentially be isolated after LT, infections are mainly caused by Enterobacteriaceae, *P. aeruginosa*, enterococci (including *E. faecium*), viridans streptococci, and *S. aureus*[88-90]. Even if an increase in Gram-negative pathogens responsible for infections in LTR has been documented, Gram-positive bacteria remain the most frequent agents of CVC-BSI[91].

Similar to liver cirrhosis, an increasing number of antimicrobial resistant bacteria has been documented among LTR, with prevalence rates varying significantly according to the geographic areas and among different centers[92]. Most case series reporting rates of MDR Gram-negative in solid organ transplant recipients, however, were from endemic areas, resulting in relatively high percentages ranging from 18% to 50%[93,94]. Very few reports specifically documented the rates of resistance

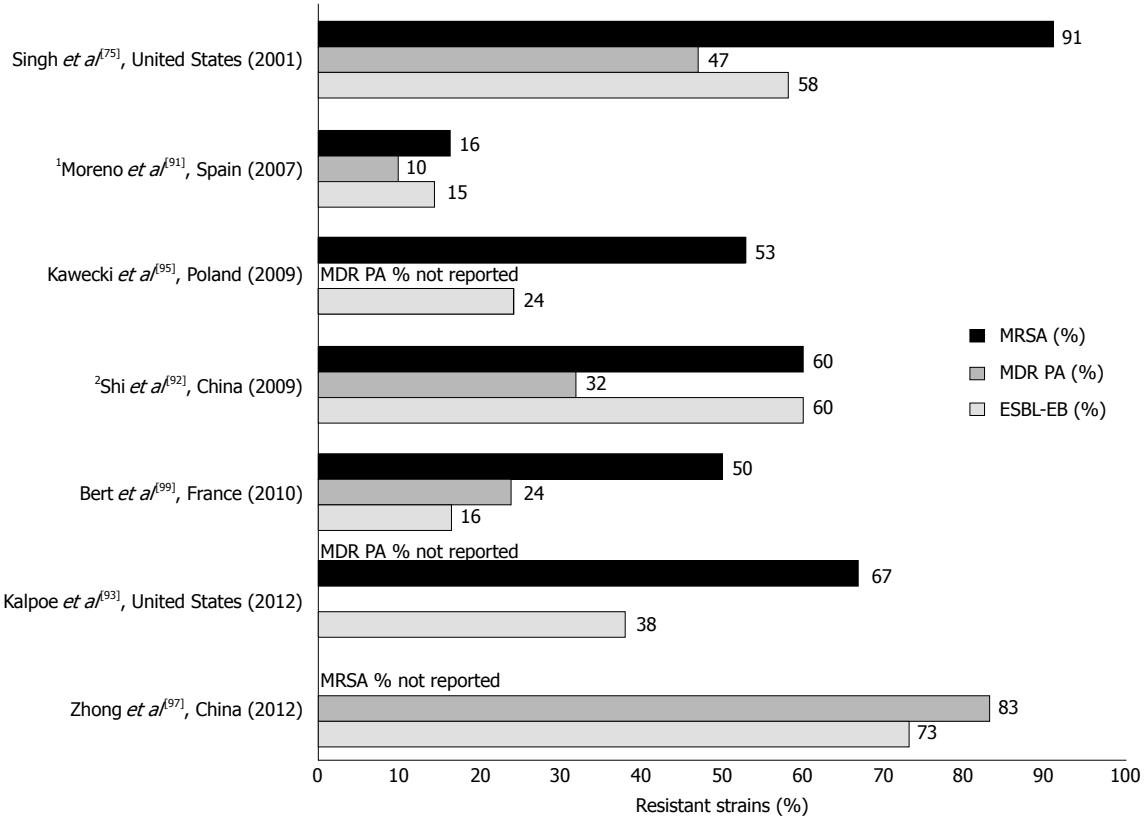


Figure 2 Studies reporting the percentage of infections caused by methicillin-resistant *Staphylococcus aureus*, MDR *Pseudomonas aeruginosa*, and extended-spectrum beta-lactamase-producing Enterobacteriaceae following liver transplantation[79,91-93, 95-97]. [1]Data reported for all solid organ transplants; [2]MRSA data obtained from reference 98[98]. MRSA: Methicillin-resistant *Staphylococcus aureus*; MDR-PA: Multidrug-resistant *Pseudomonas aeruginosa*; ESBL-EB: Extended-spectrum-beta-lactamase Enterobacteriaceae.

among the most frequently isolated pathogens in LTR (Figure 2)[79,91-93,95-98], and larger prospective studies are necessary to understand the global impact of these infections. Table 4 summarizes the current antimicrobial options to treat MDRO.

Methicillin-resistant *S. aureus*: *S. aureus* is an important cause of BSI, pneumonia, wound infections and IAI in LTR, especially within the first 3 post-transplant months[88]. Isolation of methicillin-resistant *S. aureus* (MRSA) in LTR varies across centers and may cause up to 50% of BSI, with important implications for empirical therapy that may result inadequate[99]. MRSA isolation has been linked to several risk factors, including recent surgery (< 2 wk), cytomegalovirus primary infection, extended ICU stay, concomitant major post-transplant infections, peritonitis, and increased prothrombin time[99-101]. *S. aureus* carriers who are transplant candidates have a higher risk (24% to 87%) of post-LT infections and may benefit from decolonization prior to transplantation[102-104]. Pre-transplant identification of colonized patients and subsequent eradication of MRSA may be a valuable strategy for limiting *S. aureus* infections. Decolonization, however, is not permanent; hence it is difficult to determine the optimal timing to decolonize a patient. MRSA colonization is also possible following LT, according to local MRSA prevalence rates, infection control policies, and recipients' general state of illness[105]. Infection control strategies aiming to reduce the transmission of MRSA through multifaceted interventions such as active surveillance, contact isolation, hand hygiene, environmental cleaning, decolonization of carriers, and antimicrobial stewardship are mandatory (Table 3)[106]. Each transplant program, however, should consider the local epidemiology as a key parameter to implement the infection control practices.

Although vancomycin remains the mainstay for treatment of MRSA, various limitations have been associated with its use, including lower efficacy for strains with MIC > 1.0 mg/L and MSSA-mediated infections, reduced tissue penetration, and increased renal toxicity compared to other available options[88,107,108]. Valid alternatives to vancomycin include linezolid, especially in the treatment of MRSA-related pneumonia, and daptomycin (Table 4)[108-111]. Furthermore, novel anti-MRSA options have recently become available for the treatment of MRSA, although most of them have only been approved for SSTI and real-world data, especially in the field of organ transplantation, are still limited[109].

Vancomycin-resistant *Enterococci*: Enterococcal infections are usually associated to CVC-BSI, catheter-associated urinary tract infections, and SSI[112]. Vancomycin resistance among *Enterococci* (VRE), especially

Table 4 Treatment options for multidrug resistant organisms in liver transplant recipients

Pathogens	Recommendation	Antimicrobial regimens	Ref.
MDR Gram-positives			
MRSA	Nasal decolonization with mupirocin. Daptomycin highly bactericidal in BSI; non effective in pulmonary infections. Linezolid and tigecycline bacteriostatic.	Vancomycin[1]/linezolid OR Daptomycin OR Tigecycline OR Novel anti-MRSA cephalosporins (ceftaroline, ceftobiprole)[2].	[107-111]
VRE	Daptomycin highly bactericidal in BSI; non effective in pulmonary infections. Linezolid and tigecycline bacteriostatic.	Linezolid OR Daptomycin OR Tigecycline.	[113,121,122]
MDR Gram-negatives			
ESBL-producing Enterobacteriaceae	Conflicting data on carbapenem superiority *vs* BLBLI. Meropenem recommended for high inoculum infections and unstable patients.	Carbapenems OR Piperacillin/tazobactam.	[175-177]
Carbapenem-resistant Enterobacteriaceae	Test antimicrobial susceptibility (also on colonizing strains). Some evidence of better outcomes with combination therapy *vs* monotherapy. New molecules promising but scarce data in LT.	Ceftazidime/avibactam, OR Combination regimen (at least two active drugs) including colistin/polymixin B, tigecycline, aminoglycosides[1] (gentamycin, amikacin), IV fosfomycin, high-dose prolonged infusion carbapenems. For uncomplicated UTI, consider monotherapy (aminoglycosides, fosfomycin).	[127,137,138,175,178]
MDR *P. aeruginosa*	Test antimicrobial susceptibility. New molecules promising but scarce data in LT.	Combination regimen (at least two active drugs) including colistin, an anti-pseudomonal beta-lactam (if susceptible), aminoglycosides[1], fosfomycin OR Ceftolozane/tazobactam, ceftazidime/avibactam	[175,179,180]

[1]Therapeutic drug monitoring recommended; [2]Approved for skin and soft tissue infections and community-acquired pneumonia (ceftaroline), community-acquired and hospital-acquired pneumonia excluding ventilator-associated pneumonia (ceftobiprole). MDR: Multidrug resistant; BLBLI: Beta-lactam/beta-lactamase inhibitor combination; BSI: Bloodstream infections; LT: Liver transplantation; MDRO: Multidrug-resistant organisms; MRSA: Methicillin-resistant *Staphylococcus aureus*; VRE: Vancomycin-resistant *enterococci*; CRE: Carbapenem-resistant Enterobacteriaceae.

E. faecium, currently represents a concern in various transplant centers[113]. VRE-colonized transplant recipients act as reservoirs for VRE transmission and carry an increased risk of infection, ICU stay, and death[114,115]. GI colonization with VRE among LTR is reported between 3% and 55%[116,117], while reported rates of VRE infections among colonized LTR range between 12% and 32%[114,116-118]. A study including LTR who developed bilomas in the early post-transplant period showed that the most common responsible pathogens were *Enterococci* (37%); of these, 50% were VRE[87]. Common risk factors for VRE infections include antimicrobial use, biliary leaks and strictures, and surgical re-exploration or percutaneous drainage[114-118]. Contact isolation in patients with VRE colonization and infection is recommended (Table 3). Daptomycin and linezolid are commonly used for VRE infections in solid organ transplant recipients, although reduced susceptibility to these antimicrobials has already been reported even in patients without previous exposure to these molecules[119-122].

MDR Gram-negative bacteria: MDR among Gram-negative bacteria is particularly relevant in LTR due to the documented shift from Gram-positive to Gram-negative bacteria infections in the last decade[71,81]. *E. coli*, *K. pneumoniae*, and *P. aeruginosa* currently represent commonly isolated bacteria in BSI after LT[81,92]. Rates of MDRO causing infections in LTR have exponentially increased worldwide, reaching up to 50% in some centers[92,96]. Various risk factors have been associated with antimicrobial resistance, including reoperation, graft rejection, and abdominal infections[96]. Unfortunately, MDRO infections are recognized to cause increased mortality compared to non-resistant infections[79,92,96].

P. aeruginosa is an early nosocomial pathogen and represents a major cause of infection in LTR, accounting for about 6.5% of all BSI[81]. BSI caused by MDR *P. aeruginosa* compared to susceptible strains appeared significantly more frequent in transplant recipients compared to non-transplanted patients. *P. aeruginosa* infections caused by MDR strains reached 43% in the United States and up to 52% in China[92,123]. MDR *P. aeruginosa* causing nosocomial pneumonia in LTR has been reported between 50% and 65%[124].

Rates of extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae in LTR vary between 6% to 13% according to the area, reaching up to 40% in endemic countries[125,126]. Risk factors for ESBL-associated infections include pre-transplant colonization, advanced liver disease, and reoperation. In a study encompassing 317 LTR, independent factors associated with preoperative fecal carriage of ESBL-producing Enterobacteriaceae included exposure to a beta-lactam agent in the month preceding transplantation

($P < 0.001$) and a history of SBP ($P = 0.02$)[125,126]. The occurrence of ESBL-associated infections has increased the use of carbapenems, that usually represent the last resort for antimicrobial therapy in immunocompromised patients. Carbapenem-resistance has subsequently developed, further complicating the management of LTR since the optimal treatment for carbapenem-resistance Enterobacteriaceae is not yet established[127]. Carbapenem-resistant *K. pneumoniae* (CRKP), in particular, has emerged as a major threat for immunocompromised and hospitalized patients worldwide and is associated with significant mortality[128,129]. A retrospective study evaluating 14 CRKP infection episodes after LT showed early onset of infection (median time from LT of 12 d) and mortality rates of 71%. Survival rates were significantly lower for patients with CRKP infections compared to those without (29% *vs* 86%, $P < 0.001$), and represented an independent risk factor for mortality along with MELD scores $\geqslant 30$[93]. Isolation of CRKP in LTR represents a public health threat in endemic countries[94,130]. In one Unites States transplant center, CRKP accounted for 23% of all bacterial infections in LTR[93,82]. Risk for acquisitions of CRKP in the general population that are also commonly encountered in LTR include exposure to broad-spectrum antibiotics, need for invasive devices, and ICU stay[131]. Only recently, some studies have analyzed the presence of specific factors that can be predictive of CRKP infections in LTR. CRKP pre- and post-transplant colonization appeared as an important factor associated with CRKP infections. In a cohort of 41 CRKP rectal carriers (11 at LT and 30 post-LT), 20 patients developed CRKP infections[132]. Compared with 2% of non-colonized patients, rates of infections were 18% and 47% among carriers before and after LT, respectively. Besides carrier status, renal replacement therapy, mechanical ventilation for > 48 h, and HCV recurrence appeared correlated with CRKP infections[132]. Another recent study involving 54 patients with CRKP infections identified as independent risk factors for post-transplant infections the presence of CRKP colonization, reoperation, combined transplantation, MELD > 32, and dialysis[133].

There are currently no guidelines specifically addressing the management of MDRO infections in LTR. Recent recommendations from the Spanish group for the study of infection in transplant recipients (GESITRA) identified as a key point the characterization of the isolate's phenotypic and genotypic resistance profile in order to select a targeted therapy that can be adjusted according to susceptibility results[134]. A specific surgical prophylaxis regimen is currently not recommended for patients colonized with carbapenem-resistant strains. Carrier status in LTR recipients, however, should be timely detected, and empirical therapy in case of infection should include active antibiotics based on available microbiological results. Even if donor and/or recipient colonization are associated with an increased risk of infection, carrier status currently do not represent a contraindication to transplantation, but warrants contact isolation precautions and strict hand hygiene compliance. Due to the high-mortality of these infections and while awaiting real-world data on new antibiotic options, preventive strategies and antimicrobial stewardship programs remain key steps to curtail the impact of carbapenem-resistant infections in this cohort.

Novel molecules targeting MDR Gram-negative are currently available, although data on their use in immunocompromised patients remain scarce[135]. Among new antimicrobial options, ceftazidime-avibactam has demonstrated promising activity against CRKP in preliminary studies[136-138], and meropenem-vaborbactam has recently been approved for the treatment of complicated urinary tract infections caused by carbapenem-resistant Enterobacteriaceae. Both compounds, however, are not active against strains harboring metallo-beta-lactamases (MBLs) that are common in certain geographic areas[139]. Results from observational studies including old antibiotics have shown better outcomes for combination therapy (Table 4), but these results remain conflicting and were not confirmed by all studies[140-142]. New compounds targeting carbapenem-resistant strains, including MBLs (*e.g.*, cefiderocol, aztreonam-avibactam) are currently under investigation[143]. Regarding *Pseudomonas* spp., the novel beta-lactam/beta-lactamase inhibitor ceftolozane-tazobactam has shown good activity against MDR strains, including carbapenem-resistant isolates, with the exception of MBL producers[144].

Fungal infections in liver transplant patients

Although better outcomes have been reported after the introduction of novel antifungals, invasive fungal infections remain an important cause of mortality in LTR, with reported rates between 25% and 81%[145,146]. Fungal infections are frequent in absence of antifungal prophylaxis and can occur in up to 42% of LTR[147,148]. Factors having an impact on the distribution and frequency of fungal infections include changes in surgical techniques, patient and donor organ characteristics, local fungal ecology and resistance, and the use of antifungal prophylaxis. The most common cause of invasive fungal infections in LTR is *Candida* spp. Candida infections, including candidemia, abdominal infections, and biliary infections, are mostly nosocomial and occur early after LT[149]. A retrospective study in LTR identified an overall incidence of fungal infections of 12%, with non-albicans *Candida* accounting for 55% of the infections; of these, half were caused by fluconazole-resistant *C. parapsilosis*[150]. One-year patient survival rates were significantly reduced among patients with fungal infections compared to those without (41% and 80%, respectively). Multivariate analysis showed that pre-transplant fungal colonization was associated with subsequent infections. Various other risk factors have been reported among patients developing fungal infections after LT, including high

blood product volumes during surgery, early surgical re-exploration, choledochojejunostomy, retransplantation, fulminant hepatic failure, and severe renal impairment[145,147,148,151-153].

There is currently no consensus on pre-LT fungal prophylaxis regarding clinical indication, best regimens, and duration. Outcome benefits correlated with the use of antifungal prophylaxis among solid organ transplant recipients appear conflicting, and universal prophylaxis is currently not recommended[154-157]. Pre-transplant risk assessment, however, appears useful to identify patients who are at high-risk for the development of fungal infections[57,158].

Similar to liver cirrhosis, echinocandins and azoles are the most commonly used antifungals for the treatment of *Candida* spp. infections in LTR. Compared to the azoles, very few drug interactions have been reported with echinocandins. Only caspofungin use has been associated with relevant changes in the C_{max} of tacrolimus (up to 20% reduction) and cyclosporine (up to 35% increase in plasma concentration of caspofungin)[159].

Lipid formulations of amphotericin B provide wide-spectrum options for patients that may be at risk of non-Candida infections. Voriconazole (and most recently, isavuconazole) remain the drug of choice for invasive aspergillosis, although limitations in its use in LTR and patients with compromised liver function are represented by significant drug interactions with immunosuppressants and occurrence of liver toxicity[159].

Management of infections in LTR

Bacterial and fungal infections in LTR are often associated to surgical complications, involving pathogens that are typically encountered in nosocomial infections and, more recently, MDRO. Clinical presentations of common bacterial infections may be atypical, and clinical and/or radiological findings may not be evident due to an impairment of the inflammatory responses caused by immunosuppressive therapies. Graft rejection can also be confused with infections. Early diagnoses can therefore be challenging and often require invasive diagnostic procedures that remain key to identify the correct cause of infection and promote a potentially successful therapy. The choice of an appropriate antimicrobial regimen for LTR can be particularly challenging due to the need of an urgent empiric therapy in severe infections, the increased rates of antimicrobial resistance, and the risk of drug toxicity and drug-drug interactions. Knowledge of the local epidemiology is particularly important since the empiric antimicrobial treatment should take into account the coverage of resistant pathogens that colonize or have been previously isolated in LTR, especially in areas that are endemic for MDRO or during outbreaks. Table 3 summarizes the main principles for the management of infections in LTR[160-164].

CONCLUSION

The impact of bacterial and fungal infections on the outcome of patients with liver cirrhosis, liver transplant candidates, and liver transplant recipients remain dramatic despite the advances in antimicrobial therapy and surgical techniques. The mechanisms associated with the increased risk of infections in LC are complex and include genetic predisposition, fecal dysbiosis, disruption of the intestinal barrier causing intestinal hyperpermeability, and multiple immunological deficits (Figure 1). In LTR, immunosuppression and risk factors associated with surgery and prolonged hospital stay represent the main causes for bacterial and fungal infections.

Research into the mechanisms favoring infections in cirrhosis is key to find potential areas of intervention. New strategies to modulate the gut-liver interaction are urgently needed since factors such as systemic inflammation and endotoxemia, that may cause life-threatening complications in LC (*e.g.*, SBP and hepatic encephalopathy), are related to the gut environment[165,166]. Studies on the preservation of microbiome composition and function appeared promising in hematological patients undergoing hematopoietic stem cell transplantation[167]. Preliminary studies on gut microbiota-based therapeutics (*e.g.*, probiotics, prebiotics, rifaximin, *etc.*) in cirrhosis are ongoing[166], but the impact of targeted intervention on gut dysbiosis, bacterial function, or metabolic state is still unclear.

Studies on cirrhosis-associated immune dysfunction appear also important to understand the patterns of pro-inflammatory or immunodeficient phenotypes that can lead to infection susceptibility and/or organ failure in different phases of liver disease, in order to identify potential markers or specific targets of disease progression[168,169].

Bacterial and fungal infections in LT candidates and recipients are often peculiar and may be characterized by confounding factors that favor delayed diagnosis and poor outcomes. For this reason, research dedicated to the development of rapid and accurate diagnostic tools is urgently needed. This is particularly relevant, considering the exponential increase in MDRO, in order to promote the correct use of antibiotics based on the results of microbiological cultures. Specifically, novel instruments of rapid diagnostics (*e.g.*, matrix assisted laser desorption/ionization time-of-flight mass spectrometry, multiplex polymerase chain reaction platforms, *etc.*) may allow for prompt identification of pathogens, giving direction to clinicians dealing with severe infections in terms of broadening, discontinuation, and de-escalation of empiric regimens[170,171].

Furthermore, despite the availability of novel antibiotics in recent years, there is currently no consensus on optimal antimicrobial regimens that can safely and effectively treat infections caused by MDR Gram-negative bacteria in immunocompromised hosts. Results from pathogen-directed clinical trials employing novel antibiotics with broad activity are largely awaited. Limitations in the use of new molecules, however, include the scarce clinical experience from real-world studies, the lack of knowledge of their pharmacokinetics principles in

immunocompromised patients, and an increased risk of toxicity, especially for combination therapies[172].

Finally, the optimization of current strategies directed towards the prevention and treatment of infections in patients with liver disease and LTR remain a key point for their successful management. Examples are represented by the implementation of dedicated antimicrobial stewardship programs and specific bundled interventions based on transplant centers' local needs and epidemiology[171,172]. Studies evaluating antimicrobial stewardship programs in transplant recipients, for example, are limited but have shown promising results[173,174]. All these efforts, however, cannot be successful without the constant involvement of multidisciplinary teams, including transplant surgeons, hepatologists, specialists in infectious diseases and infection control, microbiologists, and pharmacologists[175-180].

REFERENCES

1 **Garcia-Tsao G**, Friedman S, Iredale J, Pinzani M. Now there are many (stages) where before there was one: In search of a pathophysiological classification of cirrhosis. *Hepatology* 2010; **51**: 1445-1449 [PMID: 20077563 DOI: 10.1002/hep.23478]

2 **Albillos A**, Lario M, Álvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. *J Hepatol* 2014; **61**: 1385-1396 [PMID: 25135860 DOI: 10.1016/j.jhep.2014.08.010]

3 **Bellot P**, Francés R, Such J. Pathological bacterial translocation in cirrhosis: pathophysiology, diagnosis and clinical implications. *Liver Int* 2013; **33**: 31-39 [PMID: 23121656 DOI: 10.1111/liv.12021]

4 **Pijls KE**, Jonkers DM, Elamin EE, Masclee AA, Koek GH. Intestinal epithelial barrier function in liver cirrhosis: an extensive review of the literature. *Liver Int* 2013; **33**: 1457-1469 [PMID: 23879434 DOI: 10.1111/liv.12271]

5 **Benjamin J**, Singla V, Arora I, Sood S, Joshi YK. Intestinal permeability and complications in liver cirrhosis: A prospective cohort study. *Hepatol Res* 2013; **43**: 200-207 [PMID: 22726344 DOI: 10.1111/j.1872-034X.2012.01054.x]

6 **Kim BI**, Kim HJ, Park JH, Park DI, Cho YK, Sohn CI, Jeon WK, Kim HS, Kim DJ. Increased intestinal permeability as a predictor of bacterial infections in patients with decompensated liver cirrhosis and hemorrhage. *J Gastroenterol Hepatol* 2011; **26**: 550-557 [PMID: 21332551 DOI: 10.1111/j.1440-1746.2010.06490.x]

7 **Tandon P**, Garcia-Tsao G. Bacterial infections, sepsis, and multiorgan failure in cirrhosis. *Semin Liver Dis* 2008; **28**: 26-42 [PMID: 18293275 DOI: 10.1055/s-2008-1040319]

8 **Borzio M**, Salerno F, Piantoni L, Cazzaniga M, Angeli P, Bissoli F, Boccia S, Colloredo-Mels G, Corigliano P, Fornaciari G, Marenco G, Pistarà R, Salvagnini M, Sangiovanni A. Bacterial infection in patients with advanced cirrhosis: a multicentre prospective study. *Dig Liver Dis* 2001; **33**: 41-48 [PMID: 11303974 DOI: 10.1016/S1590-8658(01)80134-1]

9 **Bajaj JS**, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M, Gillevet PM. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol* 2014; **60**: 940-947 [PMID: 24374295 DOI: 10.1016/j.jhep.2013.12.019]

10 **Bunchorntavakul C**, Chavalitdhamrong D. Bacterial infections other than spontaneous bacterial peritonitis in cirrhosis. *World J Hepatol* 2012; **4**: 158-168 [PMID: 22662285 DOI: 10.4254/wjh.v4.i5.158]

11 **Bunchorntavakul C**, Chamroonkul N, Chavalitdhamrong D. Bacterial infections in cirrhosis: A critical review and practical guidance. *World J Hepatol* 2016; **8**: 307-321 [PMID: 26962397 DOI: 10.4254/wjh.v8.i6.307]

12 **Fishman JA**. Infection in solid-organ transplant recipients. *N Engl J Med* 2007; **357**: 2601-2614 [PMID: 18094380 DOI: 10.1056/NEJMra064928]

13 **Hernandez Mdel P**, Martin P, Simkins J. Infectious Complications After Liver Transplantation. *Gastroenterol Hepatol* (NY) 2015; **11**: 741-753 [PMID: 27134589]

14 **Fishman JA**. Infections in immunocompromised hosts and organ transplant recipients: essentials. *Liver Transpl* 2011; **17** Suppl 3: S34-S37 [PMID: 21748845 DOI: 10.1002/lt.22378]

15 **Sun HY**, Cacciarelli TV, Singh N. Identifying a targeted population at high risk for infections after liver transplantation in the MELD era. *Clin Transplant* 2011; **25**: 420-425 [PMID: 20482564 DOI: 10.1111/j.1399-0012.2010.01262.x]

16 **Pant C**, Olyaee M, Gilroy R, Pandya PK, Olson JC, Oropeza-Vail M, Rai T, Deshpande A. Emergency department visits related to cirrhosis: a retrospective study of the nationwide emergency department sample 2006 to 2011. *Medicine (Baltimore)* 2015; **94**: e308 [PMID: 25569646 DOI: 10.1097/MD.0000000000000308]

17 **Fernández J**, Navasa M, Gómez J, Colmenero J, Vila J, Arroyo V, Rodés J. Bacterial infections in cirrhosis: epidemiological changes with invasive procedures and norfloxacin prophylaxis. *Hepatology* 2002; **35**: 140-148 [PMID: 11786970 DOI: 10.1053/jhep.2002.30082]

18 **Merli M**, Lucidi C, Giannelli V, Giusto M, Riggio O, Falcone M, Ridola L, Attili AF, Venditti M. Cirrhotic patients are at risk for health care-associated bacterial infections. *Clin Gastroenterol Hepatol* 2010; **8**: 979-985 [PMID: 20621200 DOI: 10.1016/j.cgh.2010.06.024]

19 **Bajaj JS**, O'Leary JG, Reddy KR, Wong F, Olson JC, Subramanian RM, Brown G, Noble NA, Thacker LR, Kamath PS; NACSELD. Second infections independently increase mortality in hospitalized patients with cirrhosis: the North American consortium for the study of end-stage liver disease (NACSELD) experience. *Hepatology* 2012; **56**: 2328-2335 [PMID: 22806618 DOI: 10.1002/hep.25947]

20 **Fasolato S**, Angeli P, Dallagnese L, Maresio G, Zola E, Mazza E, Salinas F, Donà S, Fagiuoli S, Sticca A, Zanus G, Cillo U, Frasson I, Destro C, Gatta A. Renal failure and bacterial infections in patients with cirrhosis: epidemiology and clinical features. *Hepatology* 2007; **45**: 223-229 [PMID: 17187409 DOI: 10.1002/hep.21443]

21 **Rosa H**, Silvério AO, Perini RF, Arruda CB. Bacterial infection in cirrhotic patients and its relationship with alcohol. *Am J Gastroenterol* 2000; **95**: 1290-1293 [PMID: 10811341 DOI: 10.1111/j.1572-0241.2000.02026.x]

22 **Xie Y**, Tu B, Xu Z, Zhang X, Bi J, Zhao M, Chen W, Shi L, Zhao P, Bao C, Qin E, Xu D. Bacterial distributions and prognosis of bloodstream infections in patients with liver cirrhosis. *Sci Rep* 2017; **7**: 11482 [PMID: 28904387 DOI: 10.1038/s41598-017-11587-1]

23 **Preveden T**. Bacterial infections in patients with liver cirrhosis. *Med Pregl* 2015; **68**: 187-191 [PMID: 26234027 DOI: 10.2298/MPNS1506187P]

24 **Yoneyama K**, Miyagishi K, Kiuchi Y, Shibata M, Mitamura K. Risk factors for infections in cirrhotic patients with and without hepatocellular carcinoma. *J Gastroenterol* 2002; **37**: 1028-1034 [PMID: 12522534 DOI: 10.1007/s005350200173]

25 **de Mattos AA**, Coral GP, Menti E, Valiatti F, Kramer C. [Bacterial infection in cirrhotic patient]. *Arq Gastroenterol* 2003; **40**: 11-15 [PMID: 14534658 DOI: 10.1590/S0004-28032003000100003]

26 **Caly WR**, Strauss E. A prospective study of bacterial infections in patients with cirrhosis. *J Hepatol* 1993; **18**: 353-358 [PMID: 8228129 DOI: 10.1016/S0168-8278(05)80280-6]

27 **Toledo C**, Flores C, Sáenz M, Jiménez P, Tejero A, Ibarra H, León J, Arce M. [Bacterial infections in hepatic cirrhosis]. *Rev Med Chil* 1994; **122**: 788-794 [PMID: 7732229]

28 **Bartoletti M**, Giannella M, Caraceni P, Domenicali M, Ambretti S, Tedeschi S, Verucchi G, Badia L, Lewis RE, Bernardi M, Viale P. Epidemiology and outcomes of bloodstream infection in patients with cirrhosis. *J Hepatol* 2014; **61**: 51-58 [PMID: 24681345 DOI: 10.1016/j.jhep.2014.03.021]

29 **Bonnel AR**, Bunchorntavakul C, Reddy KR. Immune dysfunction and infections in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2011; **9**: 727-738 [PMID: 21397731 DOI: 10.1016/j.cgh.2011.02.031]

30 **Perdomo Coral G**, Alves de Mattos A. Renal impairment after spontaneous bacterial peritonitis: incidence and prognosis. *Can*

J Gastroenterol 2003; **17**: 187-190 [PMID: 12677269 DOI: 10.1155/2003/370257]
31 **Mølle I**, Thulstrup AM, Svendsen N, Schønheyder HC, Sørensen HT. Risk and case fatality rate of meningitis in patients with liver cirrhosis. *Scand J Infect Dis* 2000; **32**: 407-410 [PMID: 10959650 DOI: 10.1080/003655400750044999]
32 **Barahona-Garrido J**, Hernández-Calleros J, Téllez-Avila FI, Chávez-Tapia NC, Remes-Troche JM, Torre A. Bacterial meningitis in cirrhotic patients: case series and description of the prognostic role of acute renal failure. *J Clin Gastroenterol* 2010; **44**: e218-e223 [PMID: 20453662 DOI: 10.1097/MCG.0b013e3181d88d53]
33 **Vilstrup H**. Cirrhosis and bacterial infections. *Rom J Gastroenterol* 2003; **12**: 297-302 [PMID: 14726975]
34 **Thulstrup AM**, Sørensen HT, Schønheyder HC, Møller JK, Tage-Jensen U. Population-based study of the risk and short-term prognosis for bacteremia in patients with liver cirrhosis. *Clin Infect Dis* 2000; **31**: 1357-1361 [PMID: 11096002 DOI: 10.1086/317494]
35 **Bartoletti M**, Giannella M, Lewis RE, Viale P. Bloodstream infections in patients with liver cirrhosis. *Virulence* 2016; **7**: 309-319 [PMID: 26864729 DOI: 10.1080/21505594.2016.1141162]
36 **Tripodi MF**, Adinolfi LE, Ragone E, Durante Mangoni E, Fortunato R, Iarussi D, Ruggiero G, Utili R. Streptococcus bovis endocarditis and its association with chronic liver disease: an underestimated risk factor. *Clin Infect Dis* 2004; **38**: 1394-1400 [PMID: 15156477 DOI: 10.1086/392503]
37 **Fagiuoli S**, Colli A, Bruno R, Burra P, Craxì A, Gaeta GB, Grossi P, Mondelli MU, Puoti M, Sagnelli E, Stefani S, Toniutto P. Management of infections in cirrhotic patients: report of a consensus conference. *Dig Liver Dis* 2014; **46**: 204-212 [PMID: 24021271 DOI: 10.1016/j.dld.2013.07.015]
38 **Runyon BA**, Canawati HN, Akriviadis EA. Optimization of ascitic fluid culture technique. *Gastroenterology* 1988; **95**: 1351-1355 [PMID: 3049220 DOI: 10.1016/0016-5085(88)90372-1]
39 **Mendler MH**, Agarwal A, Trimzi M, Madrigal E, Tsushima M, Joo E, Santiago M, Flores E, David G, Workman A, Runyon B. A new highly sensitive point of care screen for spontaneous bacterial peritonitis using the leukocyte esterase method. *J Hepatol* 2010; **53**: 477-483 [PMID: 20646775 DOI: 10.1016/j.jhep.2010.04.011]
40 **Bartoletti M**, Giannella M, Lewis R, Caraceni P, Tedeschi S, Paul M, Schramm C, Bruns T, Merli M, Cobos-Trigueros N, Seminari E, Retamar P, Muñoz P, Tumbarello M, Burra P, Torrani Cerenzia M, Barsic B, Calbo E, Maraolo AE, Petrosillo N, Galan-Ladero MA, D'Offizi G, Bar Sinai N, Rodríguez-Baño J, Verucchi G, Bernardi M, Viale P; ESGBIS/BICHROME Study Group. A prospective multicentre study of the epidemiology and outcomes of bloodstream infection in cirrhotic patients. *Clin Microbiol Infect* 2018; **24**: 546.e1-546.e8 [PMID: 28818628 DOI: 10.1016/j.cmi.2017.08.001]
41 **Hamza RE**, Villyoth MP, Peter G, Joseph D, Govindaraju C, Tank DC, Sreesh S, Narayanan P, Vinayakumar KR. Risk factors of cellulitis in cirrhosis and antibiotic prophylaxis in preventing recurrence. *Ann Gastroenterol* 2014; **27**: 374-379 [PMID: 25331211]
42 **Arvaniti V**, D'Amico G, Fede G, Manousou P, Tsochatzis E, Pleguezuelo M, Burroughs AK. Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis. *Gastroenterology* 2010; **139**: 1246-1256, 1256.e1-1256.e5 [PMID: 20558165 DOI: 10.1053/j.gastro.2010.06.019]
43 **Gustot T**, Felleiter P, Pickkers P, Sakr Y, Rello J, Velissaris D, Pierrakos C, Taccone FS, Sevcik P, Moreno C, Vincent JL; EPIC II Group of Investigators. Impact of infection on the prognosis of critically ill cirrhotic patients: results from a large worldwide study. *Liver Int* 2014; **34**: 1496-1503 [PMID: 24606193 DOI: 10.1111/liv.12520]
44 **Alexopoulou A**, Vasilieva L, Agiasotelli D, Dourakis SP. Fungal infections in patients with cirrhosis. *J Hepatol* 2015; **63**: 1043-1045 [PMID: 26095180 DOI: 10.1016/j.jhep.2015.05.032]
45 **Bucsics T**, Schwabl P, Mandorfer M, Peck-Radosavljevic M. Prognosis of cirrhotic patients with fungiascites and spontaneous fungal peritonitis (SFP). *J Hepatol* 2016; **64**: 1452-1454 [PMID: 26916528 DOI: 10.1016/j.jhep.2016.01.039]
46 **Fiore M**, Leone S. Spontaneous fungal peritonitis: Epidemiology, current evidence and future prospective. *World J Gastroenterol* 2016; **22**: 7742-7747 [PMID: 27678356 DOI: 10.3748/wjg.v22.i34.7742]
47 **Hwang SY**, Yu SJ, Lee JH, Kim JS, Yoon JW, Kim YJ, Yoon JH, Kim EC, Lee HS. Spontaneous fungal peritonitis: a severe complication in patients with advanced liver cirrhosis. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 259-264 [PMID: 23996048 DOI: 10.1007/s10096-013-1953-2]
48 **Shizuma T**. Spontaneous bacterial and fungal peritonitis in patients with liver cirrhosis: A literature review. *World J Hepatol* 2018; **10**: 254-266 [PMID: 29527261 DOI: 10.4254/wjh.v10.i2.254]
49 **Lahmer T**, Messer M, Mayr U, Saugel B, Noe S, Schultheiss C, Thies P, Spinner C, Nennstiel S, Schwerdtfeger C, Phillip V, Schmid RM, Huber W. Fungal "colonisation" is associated with increased mortality in medical intensive care unit patients with liver cirrhosis. *Mycopathologia* 2015; **179**: 63-71 [PMID: 25348847 DOI: 10.1007/s11046-014-9825-6]
50 **Lahmer T**, Messer M, Schwerdtfeger C, Rasch S, Lee M, Saugel B, Schmid RM, Huber W. Invasive mycosis in medical intensive care unit patients with severe alcoholic hepatitis. *Mycopathologia* 2014; **177**: 193-197 [PMID: 24710759 DOI: 10.1007/s11046-014-9740-x]
51 **Bassetti M**, Peghin M, Carnelutti A, Righi E, Merelli M, Ansaldi F, Trucchi C, Alicino C, Sartor A, Toniutto P, Wauters J, Laleman W, Tascini C, Menichetti F, Luzzati R, Brugnaro P, Mesini A, Raviolo S, De Rosa FG, Lagunes L, Rello J, Dimopoulos G, Colombo AL, Nucci M, Vena A, Bouza E, Muñoz P, Tumbarello M, Losito R, Martin-Loeches I, Viscoli C. Clinical characteristics and predictors of mortality in cirrhotic patients with candidemia and intra-abdominal candidiasis: a multicenter study. *Intensive Care Med* 2017; **43**: 509-518 [PMID: 28271321 DOI: 10.1007/s00134-017-4717-0]
52 **Gravito-Soares M**, Gravito-Soares E, Lopes S, Ribeiro G, Figueiredo P. Spontaneous fungal peritonitis: a rare but severe complication of liver cirrhosis. *Eur J Gastroenterol Hepatol* 2017; **29**: 1010-1016 [PMID: 28614081 DOI: 10.1097/MEG.0000000000000927]
53 **Fiore M**, Maraolo AE, Leone S, Gentile I, Cuomo A, Schiavone V, Bimonte S, Pace MC, Cascella M. Spontaneous peritonitis in critically ill cirrhotic patients: a diagnostic algorithm for clinicians and future perspectives. *Ther Clin Risk Manag* 2017; **13**: 1409-1414 [PMID: 29081656 DOI: 10.2147/TCRM.S144262]
54 **Nadim MK**, Durand F, Kellum JA, Levitsky J, O'Leary JG, Karvellas CJ, Bajaj JS, Davenport A, Jalan R, Angeli P, Caldwell SH, Fernández J, Francoz C, Garcia-Tsao G, Ginès P, Ison MG, Kramer DJ, Mehta RL, Moreau R, Mulligan D, Olson JC, Pomfret EA, Senzolo M, Steadman RH, Subramanian RM, Vincent JL, Genyk YS. Management of the critically ill patient with cirrhosis: A multidisciplinary perspective. *J Hepatol* 2016; **64**: 717-735 [PMID: 26519602 DOI: 10.1016/j.jhep.2015.10.019]
55 **Morrell M**, Fraser VJ, Kollef MH. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob Agents Chemother* 2005; **49**: 3640-3645 [PMID: 16127033 DOI: 10.1128/AAC.49.9.3640-3645.2005]
56 **Kollef M**, Micek S, Hampton N, Doherty JA, Kumar A. Septic shock attributed to Candida infection: importance of empiric therapy and source control. *Clin Infect Dis* 2012; **54**: 1739-1746 [PMID: 22423135 DOI: 10.1093/cid/cis305]
57 **Grossi PA**, Gasperina DD, Barchiesi F, Biancofiore G, Carafiello G, De Gasperi A, Sganga G, Menichetti F, Montagna MT, Pea F, Venditti M, Viale P, Viscoli C, Nanni Costa A. Italian guidelines for diagnosis, prevention, and treatment of invasive fungal infections in solid organ transplant recipients. *Transplant Proc* 2011; **43**: 2463-2471 [PMID: 21839295 DOI: 10.1016/j.transproceed.2011.06.020]
58 **Cornely OA**, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Flörl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ; ESCMID Fungal Infection Study Group. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. *Clin Microbiol Infect* 2012; **18** Suppl 7: 19-37 [PMID: 23137135 DOI: 10.1111/1469-0691.12039]

59 **Shields RK**, Nguyen MH, Clancy CJ. Clinical perspectives on echinocandin resistance among Candida species. *Curr Opin Infect Dis* 2015; **28**: 514-522 [PMID: 26524326 DOI: 10.1097/QCO.0000000000000215]

60 **Yeoh SF**, Lee TJ, Chew KL, Lin S, Yeo D, Setia S. Echinocandins for management of invasive candidiasis in patients with liver disease and liver transplantation. *Infect Drug Resist* 2018; **11**: 805-819 [PMID: 29881298 DOI: 10.2147/IDR.S165676]

61 **Mistry GC**, Migoya E, Deutsch PJ, Winchell G, Hesney M, Li S, Bi S, Dilzer S, Lasseter KC, Stone JA. Single- and multiple-dose administration of caspofungin in patients with hepatic insufficiency: implications for safety and dosing recommendations. *J Clin Pharmacol* 2007; **47**: 951-961 [PMID: 17660480 DOI: 10.1177/0091270007303764]

62 **Jin H**, Wang T, Falcione BA, Olsen KM, Chen K, Tang H, Hui J, Zhai S. Trough concentration of voriconazole and its relationship with efficacy and safety: a systematic review and meta-analysis. *J Antimicrob Chemother* 2016; **71**: 1772-1785 [PMID: 26968880 DOI: 10.1093/jac/dkw045]

63 **Papp M**, Vitalis Z, Altorjay I, Tornai I, Udvardy M, Harsfalvi J, Vida A, Kappelmayer J, Lakatos PL, Antal-Szalmas P. Acute phase proteins in the diagnosis and prediction of cirrhosis associated bacterial infections. *Liver Int* 2012; **32**: 603-611 [PMID: 22145664 DOI: 10.1111/j.1478-3231.2011.02689.x]

64 **Bassetti M**, Marchetti M, Chakrabarti A, Colizza S, Garnacho-Montero J, Kett DH, Munoz P, Cristini F, Andoniadou A, Viale P, Rocca GD, Roilides E, Sganga G, Walsh TJ, Tascini C, Tumbarello M, Menichetti F, Righi E, Eckmann C, Viscoli C, Shorr AF, Leroy O, Petrikos G, De Rosa FG. A research agenda on the management of intra-abdominal candidiasis: results from a consensus of multinational experts. *Intensive Care Med* 2013; **39**: 2092-2106 [PMID: 24105327 DOI: 10.1007/s00134-013-3109-3]

65 **Salerno F**, Borzio M, Pedicino C, Simonetti R, Rossini A, Boccia S, Cacciola I, Burroughs AK, Manini MA, La Mura V, Angeli P, Bernardi M, Dalla Gasperina D, Dionigi E, Dibenedetto C, Arghittu M; AISF Investigators. The impact of infection by multidrug-resistant agents in patients with cirrhosis. A multicenter prospective study. *Liver Int* 2017; **37**: 71-79 [PMID: 27364035 DOI: 10.1111/liv.13195]

66 **Alexopoulou A**, Papadopoulos N, Eliopoulos DG, Alexaki A, Tsiriga A, Toutouza M, Pectasides D. Increasing frequency of gram-positive cocci and gram-negative multidrug-resistant bacteria in spontaneous bacterial peritonitis. *Liver Int* 2013; **33**: 975-981 [PMID: 23522099 DOI: 10.1111/liv.12152]

67 **Reddy KR**, O'Leary JG, Kamath PS, Fallon MB, Biggins SW, Wong F, Patton HM, Garcia-Tsao G, Subramanian RM, Thacker LR, Bajaj JS; North American Consortium for the Study of End-Stage Liver Disease. High risk of delisting or death in liver transplant candidates following infections: Results from the North American Consortium for the Study of End-Stage Liver Disease. *Liver Transpl* 2015; **21**: 881-888 [PMID: 25845966 DOI: 10.1002/lt.24139]

68 **Mounzer R**, Malik SM, Nasr J, Madani B, Devera ME, Ahmad J. Spontaneous bacterial peritonitis before liver transplantation does not affect patient survival. *Clin Gastroenterol Hepatol* 2010; **8**: 623-628. e1 [PMID: 20417723 DOI: 10.1016/j.cgh.2010.04.013]

69 **Sun HY**, Cacciarelli TV, Singh N. Impact of pretransplant infections on clinical outcomes of liver transplant recipients. *Liver Transpl* 2010; **16**: 222-228 [PMID: 20104499 DOI: 10.1002/lt.21982]

70 **Lin KH**, Liu JW, Chen CL, Wang SH, Lin CC, Liu YW, Yong CC, Lin TL, Li WF, Hu TH, Wang CC. Impacts of pretransplant infections on clinical outcomes of patients with acute-on-chronic liver failure who received living-donor liver transplantation. *PLoS One* 2013; **8**: e72893 [PMID: 24023787 DOI: 10.1371/journal.pone.0072893]

71 **van Delden C**. Bacterial biliary tract infections in liver transplant recipients. *Curr Opin Organ Transplant* 2014; **19**: 223-228 [PMID: 24752064 DOI: 10.1097/MOT.0000000000000083]

72 **Antunes M**, Teixeira A, Fortuna P, Moya B, Martins A, Bagulho L, Pereira JP, Bento L, Perdigoto R, Barroso E, Marcelino P. Infections After Liver Transplantation: A Retrospective, Single-center Study. *Transplant Proc* 2015; **47**: 1019-1024 [PMID: 26036509 DOI: 10.1016/j.transproceed.2015.03.009]

73 **Romero FA**, Razonable RR. Infections in liver transplant recipients. *World J Hepatol* 2011; **3**: 83-92 [PMID: 21603030 DOI: 10.4254/wjh.v3.i4.83]

74 **Martin P**, DiMartini A, Feng S, Brown R Jr, Fallon M. Evaluation for liver transplantation in adults: 2013 practice guideline by the American Association for the Study of Liver Diseases and the American Society of Transplantation. *Hepatology* 2014; **59**: 1144-1165 [PMID: 24716201 DOI: 10.1002/hep.26972]

75 **Singh N**, Paterson DL, Gayowski T, Wagener MM, Marino IR. Predicting bacteremia and bacteremic mortality in liver transplant recipients. *Liver Transpl* 2000; **6**: 54-61 [PMID: 10648578 DOI: 10.1002/lt.500060112]

76 **Fishman JA**, Issa NC. Infection in organ transplantation: risk factors and evolving patterns of infection. *Infect Dis Clin North Am* 2010; **24**: 273-283 [PMID: 20466270 DOI: 10.1016/j.idc.2010.01.005]

77 **Delmonico FL**. Cadaver donor screening for infectious agents in solid organ transplantation. *Clin Infect Dis* 2000; **31**: 781-786 [PMID: 11017830 DOI: 10.1086/314000]

78 **San Juan R**, Aguado JM, Lumbreras C, Díaz-Pedroche C, López-Medrano F, Lizasoain M, Gavalda J, Montejo M, Moreno A, Gurguí M, Torre-Cisneros J; RESITRA Network, Spain,. Incidence, clinical characteristics and risk factors of late infection in solid organ transplant recipients: data from the RESITRA study group. *Am J Transplant* 2007; **7**: 964-971 [PMID: 17391136 DOI: 10.1111/j.1600-6143.2006.01694.x]

79 **Bert F**, Larroque B, Paugam-Burtz C, Janny S, Durand F, Dondero F, Valla DC, Belghiti J, Moreau R, Nicolas-Chanoine MH. Microbial epidemiology and outcome of bloodstream infections in liver transplant recipients: an analysis of 259 episodes. *Liver Transpl* 2010; **16**: 393-401 [PMID: 20209598 DOI: 10.1002/lt.21991]

80 **Al-Hasan MN**, Razonable RR, Eckel-Passow JE, Baddour LM. Incidence rate and outcome of Gram-negative bloodstream infection in solid organ transplant recipients. *Am J Transplant* 2009; **9**: 835-843 [PMID: 19344469 DOI: 10.1111/j.1600-6143.2009.02559.x]

81 **Singh N**, Wagener MM, Obman A, Cacciarelli TV, de Vera ME, Gayowski T. Bacteremias in liver transplant recipients: shift toward gram-negative bacteria as predominant pathogens. *Liver Transpl* 2004; **10**: 844-849 [PMID: 15237367 DOI: 10.1002/lt.20214]

82 **Kawecki D**, Chmura A, Pacholczyk M, Lagiewska B, Adadynski L, Wasiak D, Malkowski P, Sawicka-Grzelak A, Rokosz A, Szymanowska A, Swoboda-Kopec E, Wroblewska M, Rowinski W, Durlik M, Luczak M. Surgical site infections in liver recipients in the early posttransplantation period: etiological agents and susceptibility profiles. *Transplant Proc* 2007; **39**: 2800-2806 [PMID: 18021991 DOI: 10.1016/j.transproceed.2007.08.049]

83 **Asensio A**, Ramos A, Cuervas-Mons V, Cordero E, Sánchez-Turrión V, Blanes M, Cervera C, Gavalda J, Aguado JM, Torre-Cisneros J; Red de Estudio de la Infección en el Trasplante - Grupo de Estudio de la Infección en el Trasplante. Effect of antibiotic prophylaxis on the risk of surgical site infection in orthotopic liver transplant. *Liver Transpl* 2008; **14**: 799-805 [PMID: 18508358 DOI: 10.1002/lt.21435]

84 **Safdar N**, Said A, Lucey MR, Knechtle SJ, D'Alessandro A, Musat A, Pirsch J, McDermott J, Kalayoglu M, Maki DG. Infected bilomas in liver transplant recipients: clinical features, optimal management, and risk factors for mortality. *Clin Infect Dis* 2004; **39**: 517-525 [PMID: 15356815 DOI: 10.1086/422644]

85 **Akin K**, Ozturk A, Guvenc Z, Isiklar I, Haberal M. Localized fluid collections after liver transplantation. *Transplant Proc* 2006; **38**: 627-630 [PMID: 16549192 DOI: 10.1016/j.transproceed.2006.02.006]

86 **Kim YJ**, Kim SI, Wie SH, Kim YR, Hur JA, Choi JY, Yoon SK, Moon IS, Kim DG, Lee MD, Kang MW. Infectious complications in living-donor liver transplant recipients: a 9-year single-center experience. *Transpl Infect Dis* 2008; **10**: 316-324 [PMID: 18507752 DOI: 10.1111/j.1399-3062.2008.00315.x]

87 **Said A**, Safdar N, Lucey MR, Knechtle SJ, D'Alessandro A, Musat A, Pirsch J, Kalayoglu M, Maki DG. Infected bilomas in liver transplant recipients, incidence, risk factors and implications for prevention. *Am J Transplant* 2004; **4**: 574-582 [PMID: 15023150 DOI: 10.1111/j.1600-6143.2004.00374.x]

88 **Garzoni C**, AST Infectious Diseases Community of Practice.

Multiply resistant gram-positive bacteria methicillin-resistant, vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus (MRSA, VISA, VRSA) in solid organ transplant recipients. *Am J Transplant* 2009; **9** Suppl 4: S41-S49 [PMID: 20070694 DOI: 10.1111/j.1600-6143.2009.02892.x]

89 **Muñoz P**, AST Infectious Diseases Community of Practice. Multiply resistant gram-positive bacteria: vancomycin-resistant enterococcus in solid organ transplant recipients. *Am J Transplant* 2009; **9** Suppl 4: S50-S56 [PMID: 20070695 DOI: 10.1111/j.1600-6143.2009.02893.x]

90 **van Delden C**, Blumberg EA; AST Infectious Diseases Community of Practice. Multidrug resistant gram-negative bacteria in solid organ transplant recipients. *Am J Transplant* 2009; **9** Suppl 4: S27-S34 [PMID: 20070690 DOI: 10.1111/j.1600-6143.2009.02890.x]

91 **Moreno A**, Cervera C, Gavaldá J, Rovira M, de la Cámara R, Jarque I, Montejo M, de la Torre-Cisneros J, Miguel Cisneros J, Fortún J, López-Medrano F, Gurguí M, Muñoz P, Ramos A, Carratalá J. Bloodstream infections among transplant recipients: results of a nationwide surveillance in Spain. *Am J Transplant* 2007; **7**: 2579-2586 [PMID: 17868067 DOI: 10.1111/j.1600-6143.2007.01964.x]

92 **Shi SH**, Kong HS, Xu J, Zhang WJ, Jia CK, Wang WL, Shen Y, Zhang M, Zheng SS. Multidrug resistant gram-negative bacilli as predominant bacteremic pathogens in liver transplant recipients. *Transpl Infect Dis* 2009; **11**: 405-412 [PMID: 19638006 DOI: 10.1111/j.1399-3062.2009.00421.x]

93 **Kalpoe JS**, Sonnenberg E, Factor SH, del Rio Martin J, Schiano T, Patel G, Huprikar S. Mortality associated with carbapenem-resistant Klebsiella pneumoniae infections in liver transplant recipients. *Liver Transpl* 2012; **18**: 468-474 [PMID: 22467548 DOI: 10.1002/lt.23374]

94 **Bergamasco MD**, Barroso Barbosa M, de Oliveira Garcia D, Cipullo R, Moreira JC, Baia C, Barbosa V, Abboud CS. Infection with Klebsiella pneumoniae carbapenemase (KPC)-producing K. pneumoniae in solid organ transplantation. *Transpl Infect Dis* 2012; **14**: 198-205 [PMID: 22093103 DOI: 10.1111/j.1399-3062.2011.00688.x]

95 **Kawecki D**, Chmura A, Pacholczyk M, Lagiewska B, Adadynski L, Wasiak D, Czerwinski J, Malkowski P, Sawicka-Grzelak A, Kot K, Wroblewska M, Rowinski W, Durlik M, Paczek L, Luczak M. Bacterial infections in the early period after liver transplantation: etiological agents and their susceptibility. *Med Sci Monit* 2009; **15**: CR628-CR637 [PMID: 19946234]

96 **Singh N**, Gayowski T, Rihs JD, Wagener MM, Marino IR. Evolving trends in multiple-antibiotic-resistant bacteria in liver transplant recipients: a longitudinal study of antimicrobial susceptibility patterns. *Liver Transpl* 2001; **7**: 22-26 [PMID: 11150417 DOI: 10.1053/jlts.2001.20769]

97 **Zhong L**, Men TY, Li H, Peng ZH, Gu Y, Ding X, Xing TH, Fan JW. Multidrug-resistant gram-negative bacterial infections after liver transplantation - spectrum and risk factors. *J Infect* 2012; **64**: 299-310 [PMID: 22198738 DOI: 10.1016/j.jinf.2011.12.005]

98 **Shi SH**, Kong HS, Jia CK, Xu J, Zhang WJ, Wang WL, Shen Y, Zhang M, Zheng SS. Coagulase-negative staphylococcus and enterococcus as predominant pathogens in liver transplant recipients with Gram-positive coccal bacteremia. *Chin Med J* (Engl) 2010; **123**: 1983-1988 [PMID: 20819529]

99 **Bert F**, Bellier C, Lassel L, Lefranc V, Durand F, Belghiti J, Mentré F, Fantin B. Risk factors for Staphylococcus aureus infection in liver transplant recipients. *Liver Transpl* 2005; **11**: 1093-1099 [PMID: 16123951 DOI: 10.1002/lt.20491]

100 **Singh N**, Paterson DL, Chang FY, Gayowski T, Squier C, Wagener MM, Marino IR. Methicillin-resistant Staphylococcus aureus: the other emerging resistant gram-positive coccus among liver transplant recipients. *Clin Infect Dis* 2000; **30**: 322-327 [PMID: 10671336 DOI: 10.1086/313658]

101 **Florescu DF**, McCartney AM, Qiu F, Langnas AN, Botha J, Mercer DF, Grant W, Kalil AC. Staphylococcus aureus infections after liver transplantation. *Infection* 2012; **40**: 263-269 [PMID: 22124952 DOI: 10.1007/s15010-011-0224-3]

102 **Bert F**, Galdbart JO, Zarrouk V, Le Mée J, Durand F, Mentré F, Belghiti J, Lambert-Zechovsky N, Fantin B. Association between nasal carriage of Staphylococcus aureus and infection in liver transplant recipients. *Clin Infect Dis* 2000; **31**: 1295-1299 [PMID: 11073769 DOI: 10.1086/317469]

103 **Desai D**, Desai N, Nightingale P, Elliott T, Neuberger J. Carriage of methicillin-resistant Staphylococcus aureus is associated with an increased risk of infection after liver transplantation. *Liver Transpl* 2003; **9**: 754-759 [PMID: 12827565 DOI: 10.1053/jlts.2003.50142]

104 **Russell DL**, Flood A, Zaroda TE, Acosta C, Riley MM, Busuttil RW, Pegues DA. Outcomes of colonization with MRSA and VRE among liver transplant candidates and recipients. *Am J Transplant* 2008; **8**: 1737-1743 [PMID: 18557723 DOI: 10.1111/j.1600-6143.2008.02304.x]

105 **Santoro-Lopes G**, de Gouvêa EF, Monteiro RC, Branco RC, Rocco JR, Halpern M, Ferreira AL, de Araújo EG, Basto ST, Silveira VG, Ribeiro-Filho J. Colonization with methicillin-resistant Staphylococcus aureus after liver transplantation. *Liver Transpl* 2005; **11**: 203-209 [PMID: 15666377 DOI: 10.1002/lt.20338]

106 **Singh N**, Squier C, Wannstedt C, Keyes L, Wagener MM, Cacciarelli TV. Impact of an aggressive infection control strategy on endemic Staphylococcus aureus infection in liver transplant recipients. *Infect Control Hosp Epidemiol* 2006; **27**: 122-126 [PMID: 16465627 DOI: 10.1086/500651]

107 **Wunderink RG**, Niederman MS, Kollef MH, Shorr AF, Kunkel MJ, Baruch A, McGee WT, Reisman A, Chastre J. Linezolid in methicillin-resistant Staphylococcus aureus nosocomial pneumonia: a randomized, controlled study. *Clin Infect Dis* 2012; **54**: 621-629 [PMID: 22247123 DOI: 10.1093/cid/cir895]

108 **Bassetti M**, Righi E. Safety profiles of old and new antimicrobials for the treatment of MRSA infections. *Expert Opin Drug Saf* 2016; **15**: 467-481 [PMID: 26764972 DOI: 10.1517/14740338.2016.1142528]

109 **Bassetti M**, Righi E, Carnelutti A. New therapeutic options for skin and soft tissue infections. *Curr Opin Infect Dis* 2016; **29**: 99-108 [PMID: 26859726 DOI: 10.1097/QCO.0000000000000241]

110 **Len O**, Montejo M, Cervera C, Fariñas MC, Sabé N, Ramos A, Cordero E, Torre-Cisneros J, Martín-Dávila P, Azanza JR, Pahissa A, Gavaldà J; DAPTOSOT (Daptomycin in Solid Organ Transplantation) Study Group. Daptomycin is safe and effective for the treatment of gram-positive cocci infections in solid organ transplantation. *Transpl Infect Dis* 2014; **16**: 532-538 [PMID: 24834833 DOI: 10.1111/tid.12232]

111 **Radunz S**, Juntermanns B, Kaiser GM, Treckmann J, Mathe Z, Paul A, Saner FH. Efficacy and safety of linezolid in liver transplant patients. *Transpl Infect Dis* 2011; **13**: 353-358 [PMID: 21355969 DOI: 10.1111/j.1399-3062.2011.00617.x]

112 **Bucheli E**, Kralidis G, Boggian K, Cusini A, Garzoni C, Manuel O, Meylan PR, Mueller NJ, Khanna N, van Delden C, Berger C, Koller MT, Weisser M; Swiss Transplant Cohort Study. Impact of enterococcal colonization and infection in solid organ transplantation recipients from the Swiss transplant cohort study. *Transpl Infect Dis* 2014; **16**: 26-36 [PMID: 24330137 DOI: 10.1111/tid.12168]

113 **Patel G**, Snydman DR; AST Infectious Diseases Community of Practice. Vancomycin-resistant Enterococcus infections in solid organ transplantation. *Am J Transplant* 2013; **13** Suppl 4: 59-67 [PMID: 23464999 DOI: 10.1111/ajt.12099]

114 **McNeil SA**, Malani PN, Chenoweth CE, Fontana RJ, Magee JC, Punch JD, Mackin ML, Kauffman CA. Vancomycin-resistant enterococcal colonization and infection in liver transplant candidates and recipients: a prospective surveillance study. *Clin Infect Dis* 2006; **42**: 195-203 [PMID: 16355329 DOI: 10.1086/498903]

115 **Gearhart M**, Martin J, Rudich S, Thomas M, Wetzel D, Solomkin J, Hanaway MJ, Aranda-Michel J, Weber F, Trumball L, Bass M, Zavala E, Steve Woodle E, Buell JF. Consequences of vancomycin-resistant Enterococcus in liver transplant recipients: a matched control study. *Clin Transplant* 2005; **19**: 711-716 [PMID: 16313314 DOI: 10.1111/j.1399-0012.2005.00362.x]

116 **Dominguez EA**, Davis JC, Langnas AN, Winfield B, Cavalieri SJ, Rupp ME. An outbreak of vancomycin-resistant Enterococcus faecium in liver transplant recipients. *Liver Transpl Surg* 1997; **3**: 586-590 [PMID: 9404957 DOI: 10.1002/lt.500030605]

117 **Bakir M**, Bova JL, Newell KA, Millis JM, Buell JF, Arnow PM. Epidemiology and clinical consequences of vancomycin-resistant enterococci in liver transplant patients. *Transplantation* 2001; **72**:

1032-1037 [PMID: 11579296 DOI: 10.1097/00007890-200109270-00009]
118 **Hagen EA**, Lautenbach E, Olthoff K, Blumberg EA. Low prevalence of colonization with vancomycin-resistant Enterococcus in patients awaiting liver transplantation. *Am J Transplant* 2003; **3**: 902-905 [PMID: 12814484 DOI: 10.1034/j.1600-6143.2003.00169.x]
119 **Arias CA**, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. *Nat Rev Microbiol* 2012; **10**: 266-278 [PMID: 22421879 DOI: 10.1038/nrmicro2761]
120 **Niebel M**, Perera MT, Shah T, Marudanayagam R, Martin K, Oppenheim BA, David MD. Emergence of linezolid resistance in hepatobiliary infections caused by Enterococcus faecium. *Liver Transpl* 2016; **22**: 201-208 [PMID: 26335577 DOI: 10.1002/lt.24328]
121 **King ST**, Usery JB, Holloway K, Koeth L, Cleveland KO, Gelfand MS. Successful therapy of treatment-emergent, non-clonal daptomycin-non-susceptible Enterococcus faecium infections. *J Antimicrob Chemother* 2011; **66**: 2673-2675 [PMID: 21846674 DOI: 10.1093/jac/dkr343]
122 **Linden PK**, Pasculle AW, Manez R, Kramer DJ, Fung JJ, Pinna AD, Kusne S. Differences in outcomes for patients with bacteremia due to vancomycin-resistant Enterococcus faecium or vancomycin-susceptible E. faecium. *Clin Infect Dis* 1996; **22**: 663-670 [PMID: 8729206 DOI: 10.1093/clinids/22.4.663]
123 **Johnson LE**, D'Agata EM, Paterson DL, Clarke L, Qureshi ZA, Potoski BA, Peleg AY. Pseudomonas aeruginosa bacteremia over a 10-year period: multidrug resistance and outcomes in transplant recipients. *Transpl Infect Dis* 2009; **11**: 227-234 [PMID: 19302282 DOI: 10.1111/j.1399-3062.2009.00380.x]
124 **Shi SH**, Kong HS, Jia CK, Zhang WJ, Xu J, Wang WL, Shen Y, Zhang M, Zheng SS. Risk factors for pneumonia caused by multidrug-resistant Gram-negative bacilli among liver recipients. *Clin Transplant* 2010; **24**: 758-765 [PMID: 20047611 DOI: 10.1111/j.1399-0012.2009.01184.x]
125 **Bert F**, Larroque B, Paugam-Burtz C, Dondero F, Durand F, Marcon E, Belghiti J, Moreau R, Nicolas-Chanoine MH. Pretransplant fecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae and infection after liver transplant, France. *Emerg Infect Dis* 2012; **18**: 908-916 [PMID: 22607885 DOI: 10.3201/eid1806.110139]
126 **Bert F**, Larroque B, Dondero F, Durand F, Paugam-Burtz C, Belghiti J, Moreau R, Nicolas-Chanoine MH. Risk factors associated with preoperative fecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae in liver transplant recipients. *Transpl Infect Dis* 2014; **16**: 84-89 [PMID: 24330161 DOI: 10.1111/tid.12169]
127 **Bassetti M**, Giacobbe DR, Giamarellou H, Viscoli C, Daikos GL, Dimopoulos G, De Rosa FG, Giamarellos-Bourboulis EJ, Rossolini GM, Righi E, Karaiskos I, Tumbarello M, Nicolau DP, Viale PL, Poulakou G; Critically Ill Patients Study Group of the European Society of Clinical Microbiology and Infectious Disease (ESCMID); Hellenic Society of Chemotherapy (HSC) and Società Italiana di Terapia Antinfettiva (SITA). Management of KPC-producing Klebsiella pneumoniae infections. *Clin Microbiol Infect* 2018; **24**: 133-144 [PMID: 28893689 DOI: 10.1016/j.cmi.2017.08.030]
128 **Patel G**, Huprikar S, Factor SH, Jenkins SG, Calfee DP. Outcomes of carbapenem-resistant Klebsiella pneumoniae infection and the impact of antimicrobial and adjunctive therapies. *Infect Control Hosp Epidemiol* 2008; **29**: 1099-1106 [PMID: 18973455 DOI: 10.1086/592412]
129 **Nguyen M**, Eschenauer GA, Bryan M, O'Neil K, Furuya EY, Della-Latta P, Kubin CJ. Carbapenem-resistant Klebsiella pneumoniae bacteremia: factors correlated with clinical and microbiologic outcomes. *Diagn Microbiol Infect Dis* 2010; **67**: 180-184 [PMID: 20356699 DOI: 10.1016/j.diagmicrobio.2010.02.001]
130 **Pereira MR**, Scully BF, Pouch SM, Uhlemann AC, Goudie S, Emond JE, Verna EC. Risk factors and outcomes of carbapenem-resistant Klebsiella pneumoniae infections in liver transplant recipients. *Liver Transpl* 2015; **21**: 1511-1519 [PMID: 26136397 DOI: 10.1002/lt.24207]
131 **Swaminathan M**, Sharma S, Poliansky Blash S, Patel G, Banach DB, Phillips M, LaBombardi V, Anderson KF, Kitchel B, Srinivasan A, Calfee DP. Prevalence and risk factors for acquisition of carbapenem-resistant Enterobacteriaceae in the setting of endemicity. *Infect Control Hosp Epidemiol* 2013; **34**: 809-817 [PMID: 23838221 DOI: 10.1086/671270]
132 **Giannella M**, Bartoletti M, Morelli MC, Tedeschi S, Cristini F, Tumietto F, Pasqualini E, Danese I, Campoli C, Lauria ND, Faenza S, Ercolani G, Lewis R, Pinna AD, Viale P. Risk factors for infection with carbapenem-resistant Klebsiella pneumoniae after liver transplantation: the importance of pre- and posttransplant colonization. *Am J Transplant* 2015; **15**: 1708-1715 [PMID: 25754742 DOI: 10.1111/ajt.13136]
133 **Freire MP**, Oshiro IC, Pierrotti LC, Bonazzi PR, de Oliveira LM, Song AT, Camargo CH, van der Heijden IM, Rossi F, Costa SF, D'Albuquerque LA, Abdala E. Carbapenem-Resistant Enterobacteriaceae Acquired Before Liver Transplantation: Impact on Recipient Outcomes. *Transplantation* 2017; **101**: 811-820 [PMID: 28009779 DOI: 10.1097/TP.0000000000001620]
134 **Aguado JM**, Silva JT, Fernández-Ruiz M, Cordero E, Fortún J, Gudiol C, Martínez-Martínez L, Vidal E, Almenar L, Almirante B, Cantón R, Carratalá J, Caston JJ, Cercenado E, Cervera C, Cisneros JM, Crespo-Leiro MG, Cuervas-Mons V, Elizalde-Fernández J, Fariñas MC, Gavaldà J, Goyanes MJ, Gutiérrez-Gutiérrez B, Hernández D, Len O, López-Andujar R, López-Medrano F, Martín-Dávila P, Montejo M, Moreno A, Oliver A, Pascual A, Pérez-Nadales E, Román-Broto A, San-Juan R, Serón D, Solé-Jover A, Valerio M, Muñoz P, Torre-Cisneros J; Spanish Society of Transplantation (SET); Group for Study of Infection in Transplantation of the Spanish Society of Infectious Diseases and Clinical Microbiology (GESITRA-SEIMC); Spanish Network for Research in Infectious Diseases (REIPI) (RD16/0016). Management of multidrug resistant Gram-negative bacilli infections in solid organ transplant recipients: SET/GESITRA-SEIMC/REIPI recommendations. *Transplant Rev* (Orlando) 2018; **32**: 36-57 [PMID: 28811074 DOI: 10.1016/j.trre.2017.07.001]
135 **Bassetti M**, Righi E. New antibiotics and antimicrobial combination therapy for the treatment of gram-negative bacterial infections. *Curr Opin Crit Care* 2015; **21**: 402-411 [PMID: 26263298 DOI: 10.1097/MCC.0000000000000235]
136 **Tumbarello M**, Trecarichi EM, Corona A, De Rosa FG, Bassetti M, Mussini C, Menichetti F, Viscoli C, Campoli C, Venditti M, De Gasperi A, Mularoni A, Tascini C, Parruti G, Pallotto C, Sica S, Concia E, Cultrera R, De Pascale G, Capone A, Antinori S, Corcione S, Righi E, Losito AR, Digaetano M, Amadori F, Giacobbe DR, Ceccarelli G, Mazza E, Raffaelli F, Spanu T, Cauda R, Viale P. Efficacy of Ceftazidime-avibactam Salvage Therapy in Patients with Infections Caused by KPC-producing Klebsiella pneumoniae. *Clin Infect Dis* 2018 [PMID: 29893802 DOI: 10.1093/cid/ciy492]
137 **Shields RK**, Nguyen MH, Chen L, Press EG, Potoski BA, Marini RV, Doi Y, Kreiswirth BN, Clancy CJ. Ceftazidime-Avibactam Is Superior to Other Treatment Regimens against Carbapenem-Resistant Klebsiella pneumoniae Bacteremia. *Antimicrob Agents Chemother* 2017; **61** [PMID: 28559250 DOI: 10.1128/AAC.00883-17]
138 **van Duin D**, Lok JJ, Earley M, Cober E, Richter SS, Perez F, Salata RA, Kalayjian RC, Watkins RR, Doi Y, Kaye KS, Fowler VG Jr, Paterson DL, Bonomo RA, Evans S; Antibacterial Resistance Leadership Group. Colistin Versus Ceftazidime-Avibactam in the Treatment of Infections Due to Carbapenem-Resistant Enterobacteriaceae. *Clin Infect Dis* 2018; **66**: 163-171 [PMID: 29020404 DOI: 10.1093/cid/cix783]
139 **Cho JC**, Zmarlicka MT, Shaeer KM, Pardo J. Meropenem/Vaborbactam, the First Carbapenem/β-Lactamase Inhibitor Combination. *Ann Pharmacother* 2018; **52**: 769-779 [PMID: 29514462 DOI: 10.1177/1060028018763288]
140 **Tumbarello M**, Viale P, Viscoli C, Trecarichi EM, Tumietto F, Marchese A, Spanu T, Ambretti S, Ginocchio F, Cristini F, Losito AR, Tedeschi S, Cauda R, Bassetti M. Predictors of mortality in bloodstream infections caused by Klebsiella pneumoniae carbapenemase-producing K. pneumoniae: importance of combination therapy. *Clin Infect Dis* 2012; **55**: 943-950 [PMID: 22752516 DOI: 10.1093/cid/cis588]
141 **Qureshi ZA**, Paterson DL, Potoski BA, Kilayko MC, Sandovsky

G, Sordillo E, Polsky B, Adams-Haduch JM, Doi Y. Treatment outcome of bacteremia due to KPC-producing Klebsiella pneumoniae: superiority of combination antimicrobial regimens. *Antimicrob Agents Chemother* 2012; **56**: 2108-2113 [PMID: 22252816 DOI: 10.1128/AAC.06268-11]

142 **Paul M**, Daikos GL, Durante-Mangoni E, Yahav D, Carmeli Y, Benattar YD, Skiada A, Andini R, Eliakim-Raz N, Nutman A, Zusman O, Antoniadou A, Pafundi PC, Adler A, Dickstein Y, Pavleas I, Zampino R, Daitch V, Bitterman R, Zayyad H, Koppel F, Levi I, Babich T, Friberg LE, Mouton JW, Theuretzbacher U, Leibovici L. Colistin alone versus colistin plus meropenem for treatment of severe infections caused by carbapenem-resistant Gram-negative bacteria: an open-label, randomised controlled trial. *Lancet Infect Dis* 2018; **18**: 391-400 [PMID: 29456043 DOI: 10.1016/S1473-3099(18)30099-9]

143 **Bassetti M**, Russo A, Carnelutti A, La Rosa A, Righi E. Antimicrobial resistance and treatment: an unmet clinical safety need. *Expert Opin Drug Saf* 2018; **17**: 669-680 [PMID: 29897796 DOI: 10.1080/14740338.2018.1488962]

144 **Haidar G**, Philips NJ, Shields RK, Snyder D, Cheng S, Potoski BA, Doi Y, Hao B, Press EG, Cooper VS, Clancy CJ, Nguyen MH. Ceftolozane-Tazobactam for the Treatment of Multidrug-Resistant Pseudomonas aeruginosa Infections: Clinical Effectiveness and Evolution of Resistance. *Clin Infect Dis* 2017; **65**: 110-120 [PMID: 29017262 DOI: 10.1093/cid/cix182]

145 **Husain S**, Tollemar J, Dominguez EA, Baumgarten K, Humar A, Paterson DL, Wagener MM, Kusne S, Singh N. Changes in the spectrum and risk factors for invasive candidiasis in liver transplant recipients: prospective, multicenter, case-controlled study. *Transplantation* 2003; **75**: 2023-2029 [PMID: 12829905 DOI: 10.1097/01.TP.0000065178.93741.72]

146 **Rabkin JM**, Oroloff SL, Corless CL, Benner KG, Flora KD, Rosen HR, Olyaei AJ. Association of fungal infection and increased mortality in liver transplant recipients. *Am J Surg* 2000; **179**: 426-430 [PMID: 10930495 DOI: 10.1016/S0002-9610(00)00366-4]

147 **Collins LA**, Samore MH, Roberts MS, Luzzati R, Jenkins RL, Lewis WD, Karchmer AW. Risk factors for invasive fungal infections complicating orthotopic liver transplantation. *J Infect Dis* 1994; **170**: 644-652 [PMID: 8077723 DOI: 10.1093/infdis/170.3.644]

148 **Briegel J**, Forst H, Spill B, Haas A, Grabein B, Haller M, Kilger E, Jauch KW, Maag K, Ruckdeschel G. Risk factors for systemic fungal infections in liver transplant recipients. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 375-382 [PMID: 7556225 DOI: 10.1007/BF02114892]

149 **Bassetti M**, Peghin M, Carnelutti A, Righi E, Merelli M, Ansaldi F, Trucchi C, Alicino C, Sartor A, Wauters J, Lagrou K, Tascini C, Menichetti F, Mesini A, De Rosa FG, Lagunes L, Rello J, Colombo AL, Vena A, Munoz P, Tumbarello M, Sganga G, Martin-Loeches I, Viscoli C. Invasive Candida Infections in Liver Transplant Recipients: Clinical Features and Risk Factors for Mortality. *Transplant Direct* 2017; **3**: e156 [PMID: 28573191 DOI: 10.1097/TXD.0000000000000673]

150 **Raghuram A**, Restrepo A, Safadjou S, Cooley J, Orloff M, Hardy D, Butler S, Koval CE. Invasive fungal infections following liver transplantation: incidence, risk factors, survival, and impact of fluconazole-resistant Candida parapsilosis (2003-2007). *Liver Transpl* 2012; **18**: 1100-1109 [PMID: 22577087 DOI: 10.1002/lt.23467]

151 **Nieto-Rodriguez JA**, Kusne S, Mañez R, Irish W, Linden P, Magnone M, Wing EJ, Fung JJ, Starzl TE. Factors associated with the development of candidemia and candidemia-related death among liver transplant recipients. *Ann Surg* 1996; **223**: 70-76 [PMID: 8554421 DOI: 10.1097/00000658-199601000-00010]

152 **George MJ**, Snydman DR, Werner BG, Griffith J, Falagas ME, Dougherty NN, Rubin RH. The independent role of cytomegalovirus as a risk factor for invasive fungal disease in orthotopic liver transplant recipients. Boston Center for Liver Transplantation CMVIG-Study Group. Cytogam, MedImmune, Inc. Gaithersburg, Maryland. *Am J Med* 1997; **103**: 106-113 [PMID: 9274893 DOI: 10.1016/S0002-9343(97)80021-6]

153 **Patel R**, Portela D, Badley AD, Harmsen WS, Larson-Keller JJ, Ilstrup DM, Keating MR, Wiesner RH, Krom RA, Paya CV. Risk factors of invasive Candida and non-Candida fungal infections after liver transplantation. *Transplantation* 1996; **62**: 926-934 [PMID: 8878386 DOI: 10.1097/00007890-199610150-00010]

154 **Cruciani M**, Mengoli C, Malena M, Bosco O, Serpelloni G, Grossi P. Antifungal prophylaxis in liver transplant patients: a systematic review and meta-analysis. *Liver Transpl* 2006; **12**: 850-858 [PMID: 16628697 DOI: 10.1002/lt.20690]

155 **Winston DJ**, Pakrasi A, Busuttil RW. Prophylactic fluconazole in liver transplant recipients. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 1999; **131**: 729-737 [PMID: 10577295 DOI: 10.7326/0003-4819-131-10-199911160-00003]

156 **Fortún J**, Martín-Davila P, Moreno S, Barcena R, de Vicente E, Honrubia A, García M, Nuño J, Candela A, Uriarte M, Pintado V. Prevention of invasive fungal infections in liver transplant recipients: the role of prophylaxis with lipid formulations of amphotericin B in high-risk patients. *J Antimicrob Chemother* 2003; **52**: 813-819 [PMID: 14563893 DOI: 10.1093/jac/dkg450]

157 **Hadley S**, Huckabee C, Pappas PG, Daly J, Rabkin J, Kauffman CA, Merion RM, Karchmer AW. Outcomes of antifungal prophylaxis in high-risk liver transplant recipients. *Transpl Infect Dis* 2009; **11**: 40-48 [PMID: 19144094 DOI: 10.1111/j.1399-3062.2008.00361.x]

158 **Hashemizadeh Z**, Badiee P, Malekhoseini SA, Raeisi Shahraki H, Geramizadeh B, Montaseri H. Observational Study of Associations between Voriconazole Therapeutic Drug Monitoring, Toxicity, and Outcome in Liver Transplant Patients. *Antimicrob Agents Chemother* 2017; **61** [PMID: 28923870 DOI: 10.1128/AAC.01211-17]

159 **Ullmann AJ**. Review of the safety, tolerability, and drug interactions of the new antifungal agents caspofungin and voriconazole. *Curr Med Res Opin* 2003; **19**: 263-271 [PMID: 12841918 DOI: 10.1185/030079903125001884]

160 **Kim SI**. Bacterial infection after liver transplantation. *World J Gastroenterol* 2014; **20**: 6211-6220 [PMID: 24876741 DOI: 10.3748/wjg.v20.i20.6211]

161 **O'Leary JG**, Reddy KR, Wong F, Kamath PS, Patton HM, Biggins SW, Fallon MB, Garcia-Tsao G, Subramanian RM, Malik R, Thacker LR, Bajaj JS; North American Consortium for the Study of End-Stage Liver Disease. Long-term use of antibiotics and proton pump inhibitors predict development of infections in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2015; **13**: 753-759.e1-2 [PMID: 25130937 DOI: 10.1016/j.cgh.2014.07.060]

162 **Fischer SA**, Lu K; AST Infectious Diseases Community of Practice. Screening of donor and recipient in solid organ transplantation. *Am J Transplant* 2013; **13** Suppl 4: 9-21 [PMID: 23464994 DOI: 10.1111/ajt.12094]

163 **Ferrarese A**, Zanetto A, Becchetti C, Sciarrone SS, Shalaby S, Germani G, Gambato M, Russo FP, Burra P, Senzolo M. Management of bacterial infection in the liver transplant candidate. *World J Hepatol* 2018; **10**: 222-230 [PMID: 29527258 DOI: 10.4254/wjh.v10.i2.222]

164 **Santoro-Lopes G**, de Gouvêa EF. Multidrug-resistant bacterial infections after liver transplantation: an ever-growing challenge. *World J Gastroenterol* 2014; **20**: 6201-6210 [PMID: 24876740 DOI: 10.3748/wjg.v20.i20.6201]

165 **Bajaj JS**, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, Monteith P, Noble NA, Sikaroodi M, Gillevet PM. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G675-G685 [PMID: 22821944 DOI: 10.1152/ajpgi.00152.2012]

166 **Fukui H**. Gut Microbiome-based Therapeutics in Liver Cirrhosis: Basic Consideration for the Next Step. *J Clin Transl Hepatol* 2017; **5**: 249-260 [PMID: 28936406 DOI: 10.14218/JCTH.2017.00008]

167 **Taur Y**. Intestinal microbiome changes and stem cell transplantation: Lessons learned. *Virulence* 2016; **7**: 930-938 [PMID: 27805463 DOI: 10.1080/21505594.2016.1250982]

168 **Dirchwolf M**, Podhorzer A, Marino M, Shulman C, Cartier M, Zunino M, Paz S, Muñoz A, Bocassi A, Gimenez J, Di Pietro L, Romero G, Fainboim H, Fainboim L. Immune dysfunction in cirrhosis: Distinct cytokines phenotypes according to cirrhosis severity. *Cytokine* 2016; **77**: 14-25 [PMID: 26517154 DOI: 10.1016/j.cyto.2015.10.006]

169 **Robinson MW**, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol* 2016; **13**:

267-276 [PMID: 27063467 DOI: 10.1038/cmi.2016.3]
170 **Egli A**, Osthoff M, Goldenberger D, Halter J, Schaub S, Steiger J, Weisser M, Frei R. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) directly from positive blood culture flasks allows rapid identification of bloodstream infections in immunosuppressed hosts. *Transpl Infect Dis* 2015; **17**: 481-487 [PMID: 25704776 DOI: 10.1111/tid.12373]
171 **Sato A**, Kaido T, Iida T, Yagi S, Hata K, Okajima H, Takakura S, Ichiyama S, Uemoto S. Bundled strategies against infection after liver transplantation: Lessons from multidrug-resistant Pseudomonas aeruginosa. *Liver Transpl* 2016; **22**: 436-445 [PMID: 26824429 DOI: 10.1002/lt.24407]
172 **Abbo LM**, Ariza-Heredia EJ. Antimicrobial stewardship in immunocompromised hosts. *Infect Dis Clin North Am* 2014; **28**: 263-279 [PMID: 24857392 DOI: 10.1016/j.idc.2014.01.008]
173 **Aitken SL**, Palmer HR, Topal JE, Gabardi S, Tichy E. Call for antimicrobial stewardship in solid organ transplantation. *Am J Transplant* 2013; **13**: 2499 [PMID: 23865747 DOI: 10.1111/ajt.12364]
174 **So M**, Yang DY, Bell C, Humar A, Morris A, Husain S. Solid organ transplant patients: are there opportunities for antimicrobial stewardship? *Clin Transplant* 2016; **30**: 659-668 [PMID: 26992472 DOI: 10.1111/ctr.12733]
175 **van Duin D**, van Delden C; AST Infectious Diseases Community of Practice. Multidrug-resistant gram-negative bacteria infections in solid organ transplantation. *Am J Transplant* 2013; **13** Suppl 4: 31-41 [PMID: 23464996 DOI: 10.1111/ajt.12096]
176 **Vardakas KZ**, Tansarli GS, Rafailidis PI, Falagas ME. Carbapenems versus alternative antibiotics for the treatment of bacteraemia due to Enterobacteriaceae producing extended-spectrum β-lactamases: a systematic review and meta-analysis. *J Antimicrob Chemother* 2012; **67**: 2793-2803 [PMID: 22915465 DOI: 10.1093/jac/dks301]
177 **Gutiérrez-Gutiérrez B**, Pérez-Galera S, Salamanca E, de Cueto M, Calbo E, Almirante B, Viale P, Oliver A, Pintado V, Gasch O, Martínez-Martínez L, Pitout J, Akova M, Peña C, Molina J, Hernández A, Venditti M, Prim N, Origüen J, Bou G, Tacconelli E, Tumbarello M, Hamprecht A, Giamarellou H, Almela M, Pérez F, Schwaber MJ, Bermejo J, Lowman W, Hsueh PR, Mora-Rillo M, Natera C, Souli M, Bonomo RA, Carmeli Y, Paterson DL, Pascual A, Rodríguez-Baño J. A Multinational, Preregistered Cohort Study of β-Lactam/β-Lactamase Inhibitor Combinations for Treatment of Bloodstream Infections Due to Extended-Spectrum-β-Lactamase-Producing Enterobacteriaceae. *Antimicrob Agents Chemother* 2016; **60**: 4159-4169 [PMID: 27139473 DOI: 10.1128/AAC.00365-16]
178 **Kaye KS**, Bhowmick T, Metallidis S, Bleasdale SC, Sagan OS, Stus V, Vazquez J, Zaitsev V, Bidair M, Chorvat E, Dragoescu PO, Fedosiuk E, Horcajada JP, Murta C, Sarychev Y, Stoev V, Morgan E, Fusaro K, Griffith D, Lomovskaya O, Alexander EL, Loutit J, Dudley MN, Giamarellos-Bourboulis EJ. Effect of Meropenem-Vaborbactam vs Piperacillin-Tazobactam on Clinical Cure or Improvement and Microbial Eradication in Complicated Urinary Tract Infection: The TANGO I Randomized Clinical Trial. *JAMA* 2018; **319**: 788-799 [PMID: 29486041 DOI: 10.1001/jama.2018.0438]
179 **Huntington JA**, Sakoulas G, Umeh O, Cloutier DJ, Steenbergen JN, Bliss C, Goldstein EJ. Efficacy of ceftolozane/tazobactam versus levofloxacin in the treatment of complicated urinary tract infections (cUTIs) caused by levofloxacin-resistant pathogens: results from the ASPECT-cUTI trial. *J Antimicrob Chemother* 2016; **71**: 2014-2021 [PMID: 26994090 DOI: 10.1093/jac/dkw053]
180 **Walkty A**, DeCorby M, Lagacé-Wiens PR, Karlowsky JA, Hoban DJ, Zhanel GG. In vitro activity of ceftazidime combined with NXL104 versus Pseudomonas aeruginosa isolates obtained from patients in Canadian hospitals (CANWARD 2009 study). *Antimicrob Agents Chemother* 2011; **55**: 2992-2994 [PMID: 21422208 DOI: 10.1128/AAC.01696-10]

P- Reviewer: Jha AK, Kim DJ, Zhu X **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4330-4340

DOI: 10.3748/wjg.v24.i38.4330

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

MINIREVIEWS

Towards hepatitis C virus elimination: Egyptian experience, achievements and limitations

Dalia Omran, Mohamed Alboraie, Rania A Zayed, Mohamed-Naguib Wifi, Mervat Naguib, Mohamed Eltabbakh, Mohamed Abdellah, Ahmed Fouad Sherief, Sahar Maklad, Heba Hamdy Eldemellawy, Omar Khalid Saad, Doaa Mohamed Khamiss, Mohamed El Kassas

Dalia Omran, Department of Endemic Medicine and Hepatology, Faculty of Medicine, Cairo University, Cairo 11651, Egypt

Mohamed Alboraie, Mohamed Abdellah, Department of Internal Medicine, Al-Azhar University, Cairo 11651 , Egypt

Rania A Zayed, Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo 11651, Egypt

Mohamed-Naguib Wifi, Mervat Naguib, Department of Internal Medicine, Faculty of Medicine, Cairo University, Cairo 11599, Egypt

Mohamed Eltabbakh, Ahmed Fouad Sherief, Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Cairo 11566, Egypt

Sahar Maklad, National Hepatology and Tropical Medicine Research Institute, Cairo 11599, Egypt

Heba Hamdy Eldemellawy, Department of Internal Medicine, Faculty of Medicine, Beni-Suef University, Beni-Suef 62511, Egypt

Omar Khalid Saad, Faculty of Medicine, Ain Shams University, Cairo 11566, Egypt

Doaa Mohamed Khamiss, Department of Clinical and Chemical Pathology, El-monera hospital, Ministry of Health, Cairo 11562, Egypt

Mohamed El Kassas, Department of Endemic Medicine, Faculty of Medicine, Helwan University, Cairo 11599, Egypt

ORCID number: Dalia Omran (0000-0002-5513-6955); Mohamed Alboraie (0002-8490-9822); Rania A Zayed (0000-0001-7920-7060); Mohamed-Naguib Wifi (0000-0002-3403-7106); Mervat Naguib (0000-0002-9345-1542); Mohamed Eltabbakh (0000-0003-2836-807x); Mohamed Abdellah (0000-0001-9329-6063); Ahmed Fouad Sherief(0000-0003-1828-004x); Sahar Maklad (0000-0002-7411-6108); Heba Hamdy Eldemellawy (0000-0001-6618-6168); Omar Khalid Saad (0000-0002-6298-993x); Doaa Mohamed Khamiss (0000-0002-1693-5308); Mohamed El Kassas (0000-0002-3396-6894).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision, editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Manuscript source: Invited manuscript

Correspondence to: Dalia Omran, MD, Professor, Department of Endemic Medicine and Hepatology, Faculty of Medicine, Cairo University, Egypt. daliaomran@kasralainy.edu.eg
Telephone: +20-10-00087802
Fax: +20-2-23682030

Received: March 27, 2018
Peer-review started: March 27, 2018
First decision: April 3, 2018
Revised: September 13, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 14, 2018

Abstract

Worldwide, more than one million people die each

year from hepatitis C virus (HCV) related diseases, and over 300 million people are chronically infected with hepatitis B or C. Egypt used to be on the top of the countries with heavy HCV burden. Some countries are making advances in elimination of HCV, yet multiple factors preventing progress; remain for the majority. These factors include lack of global funding sources for treatment, late diagnosis, poor data, and inadequate screening. Treatment of HCV in Egypt has become one of the top national priorities since 2007. Egypt started a national treatment program intending to provide cure for Egyptian HCV-infected patients. Mass HCV treatment program had started using Pegylated interferon and ribavirin between 2007 and 2014. Yet, with the development of highly-effective direct acting antivirals (DAAs) for HCV, elimination of viral hepatitis has become a real possibility. The Egyptian National Committee for the Control of Viral Hepatitis did its best to provide Egyptian HCV patients with DAAs. Egypt adopted a strategy that represents a model of care that could help other countries with high HCV prevalence rate in their battle against HCV. This review covers the effects of HCV management in Egyptian real life settings and the outcome of different treatment protocols. Also, it deals with the current and future strategies for HCV prevention and screening as well as the challenges facing HCV elimination and the prospect of future eradication of HCV.

Key words: Hepatitis C virus; Egypt; Direct acting antivirals; Screening; Elimination; Limitations

Core tip: Egypt started a large treatment program intended to provide therapy for Egyptian hepatitis C virus (HCV)-infected patients. The Egyptian National Committee for the Control of Viral Hepatitis provides a model of care that could help other countries with high HCV prevalence rate in their battle against HCV. This review covers the results of HCV management in Egyptian real life settings and the outcome of different treatment protocols. Also, it covers the current and future strategies for HCV prevention and screening. Lastly, we discussed the challenges facing HCV elimination and the prospect of future eradication of HCV.

Omran D, Alboraie M, Zayed RA, Wifi MN, Naguib M, Eltabbakh M, Abdellah M, Sherief AF, Maklad S, Eldemellawy HH, Saad OK, Khamiss DM, El Kassas M. Towards hepatitis C virus elimination: Egyptian experience, achievements and limitaions. *World J Gastroenterol* 2018; 24(38): 4330-4340 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4330.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4330

INTRODUCTION

Hepatitis C virus (HCV) is a major health problem worldwide. In 2015, the global prevalence of HCV infection was 1.0%, with the highest prevalence in the Eastern Mediterranean Region (2.3%) followed by the European one (1.5%). The annual mortality due to HCV-related complications is estimated to be approximately 700000 deaths[1].

Seven HCV genotype strains have been identified and classified according to the phylogenetic and sequence analyses of the whole viral genomes[2]. Genotype strains differ at 30%-35% of the nucleotide sites[3]. HCV genotype 4 is the predominant type among chronically infected Egyptian patients[4].

HCV BURDEN IN EGYPT

The highest prevalence of HCV infection is present in Egypt, with 92.5% of patients infected with genotype 4, 3.6% patients with genotype 1, 3.2% patients with multiple genotypes, and < 1% patients with other genotypes[2,3,5,6] (Figure 1).

In Egypt, widespread HCV infection was caused by a mass-scale treatment campaign of intravenous antischistosomal injections executed between 1950 and 1980[7]. In 1996, the HCV seroprevalence was > 40% among adults, whereas in 2008, the Demographic Health Survey (DHS) showed a seroprevalence of 14.7% and viremic prevalence of 9.7% in 15-59-year-old patients[8,9]. The latest DHS in 2015 reported a seroprevalence of 10% and viremic prevalence of 7%[10]. As per the DHS, the HCV burden significantly decreased approximately 30% between 2008 and 2015 (Figure 2). However, in the 2008 DHS, this apparent decline in HCV seroprevalence was not attributed exclusively to the decrease in the newly acquired infections but to the aging of patients infected 50 years ago in the mass campaigns held for treatment of schistosomiasis[11].

Notably, HCV is mostly prevalent among lower socioeconomic sections of the population[12]. The HCV prevalence rate is higher in rural areas (12%) than in urban areas (7%); HCV prevalence is also related to wealth: 12% in the lowest wealth quintile compared to 7% in the highest quintile. In Egypt, 26% of the population lives below the national poverty line with income of < 1.6 USD per day[9]. Therefore, hepatitis C infection can be considered as a socioeconomic condition.

In addition to economic burden, HCV infection causes life-compromising health burden. It was estimated that the annual rates of liver decompensation, death/transplantation, and hepatocellular carcinoma (HCC) in HCV patients to be 6.37%, 4.58%, and 3.36%, respectively[13].

In 2015, the economic burden of HCV in Egypt was estimated to be $3.81 billion, whereas direct health care costs of HCV-associated diseases exceeded $700 million annually[14]. It is expected that the economic burden will exponentially increase as HCV-infected individuals progress to more advanced disease stages of decompensated cirrhosis, HCC, and liver-related mortality[15]. The cumulative total economic burden of HCV disease was estimated at $89.1 billion between

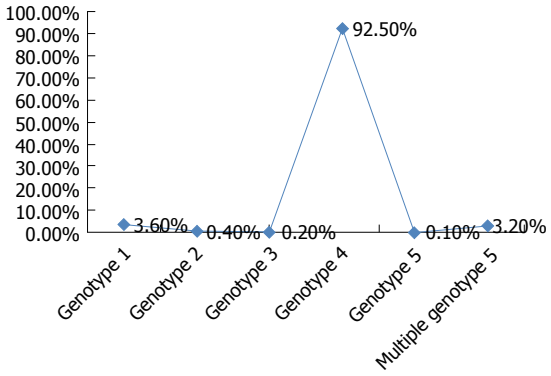


Figure 1 Frequency distribution of different hepatitis C virus genotypes in Egypt.

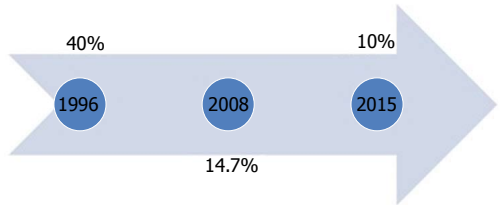


Figure 2 Timeline of hepatitis C virus prevalence in Egypt among adults.

2013 and 2030[16].

NATIONAL TREAMENT PROGRANM FOR MANAGING HCV IN EGYPT

In response to the major problem of HCV in Egypt, the Egyptian Ministry of Health and Population (MOH) launched the National Committee for Control of Viral Hepatitis (NCCVH) in 2006 to be in charge of combating HCV epidemic in the country[17]. The Egyptian NCCVH started its work by issuing the national treatment strategy for controlling HCV infection, which represented the road map for the activity of NCCVH[18]. Many stakeholders were represented in the NCCVH, including experts in the field of HCV from Egypt in addition to some international experts[19]. Based on the national treatment strategy, the main initiative tasks for the NCCVH were to estimate the actual HCV disease burden and develop a reliable infrastructure to function as a base for the national treatment program activities[17,20]. The main challenge at that time was the high cost of antiviral medications, and thus, providing such medications for free (on the expense of the MOH) or at a highly reduced cost was the main pillar for attracting patients to the project. The infrastructure of the program relied mainly on establishing a wide network of specialized centers that provide integrated care for HCV patients. This integrated care includes all required services needed for HCV management, including screening, evaluation of the patients for their eligibility for treatment, administration of therapy, and follow-up during as well as after treatment. A well-trained multidisciplinary team, composed of hepatologists, pathologists, radiologists, clinical pharmacists, and all other specialties participating in assessment and follow-up, in each center is in charge of the management of patients[17]. These centers reached 64 facilities in 2018, covering the country with < 50 km between every two bordering centers, and were interconnected through a special dedicated intranet.

The National Network for Treatment Centers responsible for data transfer and other administrative issues, monitoring work progress, issuing treatment protocols, and overseeing these centers comes under the governance of the NCCVH. After the introduction of new direct-acting antivirals (DAAs), the story continued with obtaining these drugs at very low prices through negotiations between the NCCVH and the manufacturing companies. This allowed the treatment of a large number of patients in the first phase of the treatment program, and the number maximized to approximately 150000 patients in 3 years after the introduction of the locally produced generic DAAs. As expected, such a large nationwide program represented a huge financial challenge to a resource-limited country like Egypt. The main funds for the program were through the governmental support funds issued to patients who are not under the umbrella of health insurance[20]. These funds were utilized for pre-treatment investigations, cost of medications, and follow-up laboratory tests during treatment.

RESULTS OF HCV MANAGEMENT IN EGYPTIAN REAL-LIFE SETTINGS

The mass HCV treatment program started administering pegylated interferon and ribavirin between 2007 and 2014[17]. However, the response to pegylated interferon and ribavirin was not satisfactory and was associated with many adverse events[21].

In October 2014, the introduction of sofosbuvir markedly changed therapeutic outcomes (Table 1). Ruane *et al*[22] treated 60 chronic hepatitis C patients of Egyptian ancestry with sofosbuvir and ribavirin for 12 wk or 24 wk. In their study, sustained virological response (SVR) rates ranged from 68% to 93%, being more in patients who received 24 wk of therapy. Similar results were obtained in Egypt by Doss *et al*[23] who used sofosbuvir and ribavirin for treating treatment-naive and experienced patients. SVR rates were 77% in patients treated for 12 wk and 90% in those treated for 24 wk, with favorable response in non-cirrhotic patients.

Elsharkawy *et al*[24] compared interferon-containing sofosbuvir triple therapy (sofosbuvir, pegylated interferon, and ribavirin) versus dual therapy (sofosbuvir and ribavirin) in 14409 patients and found the SVR rates to be 94% with triple therapy and 78.7% with dual therapy. Predictors of SVR were female gender, being easy to treat patient, and previous treatment with interferon and ribavirin. El kassas *et al*[25] evaluated the efficacy of different DAA combinations in Egyptian patients receiving

Table 1 Highlight on some recent studies exploring the efficacy of different direct acting antivirals combinations in Egypt

Ref.	Year of publication	Number of patients	Regimen used	Sustainted virologic response rate
Ruane *et al*[22]	2015	60	Sofosbuvir and ribavirin for 12 wk or 24 wk	68% for 12 wk or 24 wk and 93% for patients treated for 24 wk
Doss *et al*[23]	2015	103	Sofosbuvir and ribavirin for 12 wk or 24 wk	77% in patients treated for 12 wk and 90% for patients treated for 24 wk
Elsharkawy *et al*[24]	2017	14409	Triple; sofosbuvir, pegylated interferon and ribavirin versus dual; sofosbuvir and ribavirin	94% with triple therapy and 78.7% with dual therapy
El Kassas *et al*[25]	2018	7042	Different combinations used	82.9%-100.0%
Ahmed *et al*[26]	2018	300	Sofosbuvir plus daclatasvir with or without ribavirin for 12-24 wk	96.55% and 84.54%
Abd-Elsalam *et al*[27]	2018	2400	Sofosbuvir and ribavirin	71.20%.
El Kassas *et al*[28]	2018	10083	A 4-wk lead-in phase of sofosbuvir, pegylated interferon and ribavirin followed by 12 wk therapy with sofosbuvir and daclatasvir versus 12 wk therapy with sofosbuvir and daclatasvir	100% in those whoe received the lead in phase treatment regimen and 98.9% in those who received sofosbuvir and daclatasvir only
El-Khayat *et al*[29]	2017	583	Sofosbuvir and simeprevir for 12 wk	95.70%
Eletreby *et al*[30]	2017	6211	Sofosbuvir and simeprevir for 12 wk	94.00%
Abdel-Moneim *et al*[31]	2018	946	Sofosbuvir, daclatasvir versus Sofosbuvir, daclatasvir and ribavirin	95% and 92%
Omar *et al*[34]	2018	18378	Daclatasvir plus sofosbuvir	95.10%.
El-Khayat *et al*[35]	2018	144	Ledipasvir plus sofosbuvir	99.00%
Elsharkawy *et al*[36]	2018	337042	Different combinations used	82.7% to 98.0%

treatment in one of the NCCVH centers and found the SVR rates to be 82.9%-100%.

In the study by Ahmed *et al*[26], sofosbuvir plus daclatasvir with or without ribavirin for 12-24 wk were used to treat 300 HCV genotype 4 Egyptian patients and the SVR12 rates were 96.55% in non-cirrhotic and 84.54% in cirrhotic patients. Further, Abd-Elsalam *et al*[27] assessed the safety of dual therapy (sofosbuvir and ribavirin) in 2400 cirrhotic patents and found the overall SVR rate to be 71.2%.

In another interesting and unique study, El Kassas *et al*[28] evaluated a 4-wk lead-in phase of sofosbuvir, pegylated interferon, and ribavirin, followed by a 12-wk therapy with sofosbuvir and daclatasvir. In their study, the SVR rates were surprisingly very high (100%) compared with that in a group of patients who received the 12-wk therapy with sofosbuvir and daclatasvir without lead-in phase treatment (SVR: 98.9%).

The combination of sofosbuvir plus simeprevir has been tested in two large real-life cohorts in Egypt[29,30]. In a study by El-Khayat *et al*[29], a 12-wk regimen of simeprevir plus sofosbuvir led to an overall SVR rate of 95.7%, which increased to 98.9% in patients with mild fibrosis and to 100% in treatment-experienced patients. Another study using simeprevir plus sofosbuvir achieved overall SVR rates of 94% and SVR rates of 96% and 93% in easy- and difficult-to-treat groups, respectively[30].

Sofosbuvir, daclatasvir, and ribavirin combination has proven to be safe and effective in several studies in Egypt. A cohort of 946 Egyptian patients with chronic HCV was enrolled for treatment with sofosbuvir and daclatasvir with and without ribavirin[31]. An overall SVR12 rate of 94% was achieved; 95% in the easy-to-treat group receiving Sofosbuvir and daclatasvir, and 92% in the difficult-to-treat group receiving Sofosbuvir, daclatasvir, and ribavirin[31]. El-Khayat *et al*[32] reported 92% SVR rates in naïve cirrhotic patients and 87% in previously treated patients. Most reported adverse events included anemia, fatigue, headache, and pruritus. Serious adverse events were HCC and hepatic encephalopathy, and they were present in patients with Child-Turcotte-Pugh score class B. Mohemd *et al*[33] showed that DAA combinations led to improvements in biochemical parameters and clinical outcomes in their study.

A large cohort of patients (18378) who received generic sofosbuvir /daclatasvir with or without ribavirin showed an overall SVR rate of 95.1% and a discontinuation rate of 1.5%, with most discontinuations being due to patient withdrawal and pregnancy[34]. Mortality was reported in five patients[34].

Ledipasvir plus sofosbuvir combination has recently been approved for treating adolescents with chronic hepatitis C. El-Khayat *et al*[35] evaluated the safety and efficacy of this regimen in Egyptian adolescents and found it to be well tolerated, safe, and effective, with SVR12 rates of 99%. Relapses were more in treatment-naïve patients, and no case of serious adverse events, treatment discontinuation, or death was reported. Skin rash, pruritus, and diarrhea were the minor adverse events observed[35].

In the study that evaluated real-life HCV treatment outcomes of DAAs in the largest cohort of 337042 patients with different fibrosis stages (F0-F4) in Egypt, sofosbuvir and ribavirin therapy for 24 wk showed the lowest SVR12 rate (82.7%) compared with other regimens that showed SVR rates between 94% and 98%[36].

Another recent study has evaluated adverse events in

149,816 chronic hepatitis C patients treated with different regimens in the national HCV treatment program in Egypt. In this study, adverse events developed in 1.7% patients, with serious adverse events occurring in 68% of them, mainly in patients treated with sofosbuvir and ribavirin. Anemia and hyperbilirubinemia were the most commonly reported adverse events. HCC and mortality were reported in 0.02% and 0.06% of treated patients, respectively. Adverse events were more common in patients who were males and with cirrhosis, high bilirubin levels, and low hemoglobin, platelet, and albumin levels[37].

PREVENTIVE MEASURES AND AWARENESS CAMPAIGNS

Egypt is supporting a comprehensive approach for tackling hepatitis through "Plan of action for the prevention, care and treatment of viral hepatitis, Egypt 2014-2018"[38,39].

Primary prevention of HCV

Primary prevention of disease requires strict measures to prevent HCV transmission to vulnerable people. This aim can be achieved by

Strengthening surveillance to detect viral hepatitis transmission and disease: Guided by the MOH viral hepatitis centers, surveillance programs to detect viral hepatitis were expanded to many facilities other than hospitals, including, antenatal care units, prisons, dialysis units and patients requiring frequent medical intervention[38].

Promoting infection control practices to reduce viral hepatitis transmission: Viral hepatitis transmission in Egypt is largely related to improper infection control practice during various medical procedures as; dental, obstetric, injection administration and blood transfusion[23,40,41].

In 2002, MOH, NAMRU-3, and WHO developed a plan to establish an organizational infection control (IC) program structure, develop IC guidelines, train health care workers (HCWs), promote occupational safety, and establish a system for monitoring and evaluating IC activities in Egypt[42]. The plan implementation was assessed in 2011, denoting improved infection control practice, HCWs compliance and substantial reduction in iatrogenic HCV transmission[38].

Improving blood safety to reduce viral hepatitis transmission: Blood transfusion services providers should implement strict measures to ensure blood safety[38]. Special precautions should be followed in hemodialysis centers; HCV patients should use certain hemodialysis instruments other than those used for non-infected individuals, healthcare providers should wear protective gloves while dealing with HCV patients and the hemodialysis instruments[43].

HCV vaccine: HCV vaccine is an important research issue. Two promising studies are in progress; one by GlaxoSmithKline and another that was launched in March 2011 as a clinical trial by National Liver Institute, Menofyia University, Egypt[44].

Secondary prevention

Early detection and treatment of HCV patients is the goal of Egypt's treatment program starting in 2014, intending HCV prevalence reduction to < 2% in 10 years, in line with global targets. In addition, Egypt has aimed to treat 250000 people annually up to 2020 in the first phase of their treatment program[45] aiming at reducing the number of viremic patients, thus limiting the ongoing HCV transmission.

With the availability of DAAs, Egypt is struggling to eliminate HCV and HCV-related morbidity by 2030[46].

COMMUNITY-BASED PARTICIPATION IN HCV BATTLE

Considering the high prevalence of HCV infection in Egypt, many interventions are required to limit HCV. The first and the most important step is to stop transmission. Shaving at barbershops is a known risk factor for transmission of both HCV and HBV infections[47]. Targeted educational programs on the importance of equipment sterilization are promoted[48].

As illiteracy was known to be a risk factor for HCV transmission in Egypt, the endorsement of health education about the usage of single-use syringes, screening, and treatment of the diagnosed patients decreased the transmission rate and HCV-related complications[49]. Education program directed to awareness about infection risks due to having sex with multiple partner and IV drug use, and precautions to reduce infection transmission by single needle use and cleaning the injection site[50,51].

Among dialysis patients, the annual HCV incidence rate was 28% in 2001. The introduction of IC practices by the MOH resulted in a major decline in this percentage to 6% in 2012[52].

Recently, Information Education Communication had attempts to increase awareness about viral hepatitis in Egypt through, hotlines for counseling, universities vaccination campaign and World Hepatitis Day celebration. World Hepatitis Day celebration meant to bring all stakeholders together and convey an important message to the community. Stakeholders shared in policy making and implementation of action plan[38].

SCREENING PROGRAMS

Most HCV infected patients are unaware of being infected until they develop hepatic cirrhosis[53,54]. Egypt has high HCV transmission rate with around 416000 new infections each year[55], related to self-sustained spread of infection; each HCV patient can transmit the infection to

3.54 subjects[56].

Screening programs helps to identify asymptomatic HCV patients to benefit from early treatment and counseling programs to maintain their health by avoiding high-risk behaviors and awareness about self-protection and prevention of further HCV spread in the community[55].

Due to the unavailability of HCV vaccine as well as the estimated large number of current and ongoing infections, the preventive measures, namely screening, should be prioritized at the same level as the treatment campaigns[57].

In 2008, the Egyptian Demographic Health Survey reported HCV antibody prevalence of 14.7%. The study sample included 11126 women and men aged 15-59 years. It was the first nationwide representative sample for anti-HCV testing performed in Egypt. The blood samples were tested by a third-generation enzyme-linked immunosorbent assay to detect the anti-HCV antibody. Sera positive for anti-HCV antibodies were tested for HCV RNA[9]. This was followed by another screening in 2015[11].

Similar to other developing countries with a high HCV disease burden, Egypt has limited funds to support large-scale prevention programs. Therefore, prioritizing prevention activities, such as screening programs, through specific high-risk groups are essential[58].

In the past, blood transfusion or transfusion of other blood products was a major risk factor for HCV infection. In some historic cohorts, $\geqslant$ 10% of the patients who received blood transfusions were infected with hepatitis C[59]. However, since the early 1990s, blood donor screening for HCV has nearly eliminated this transmission route. Several studies conducted on Egyptian blood donors have shown higher prevalence of HCV among paid blood donors and family replacement blood donors than among voluntary donors[60,61]. Further, male donors had a higher prevalence of HCV than females[62], and blood donors from rural areas had a higher prevalence than those from urban areas[63].

Some other screening studies were specifically conducted on children, spouses, and family contacts of HCV-infected patients in Egypt[64-66]. In one such study, the HCV prevalence among spouses of infected patients was as high as 35.5%[67]. In another study, the prevalence among family contacts of infected patients was found to be 5.7%[68].

Screening among hospitalized and special clinic populations revealed elevated HCV prevalence among individuals receiving even minor medical care procedures in and outside established health care facilities[69].

Studying the prevalence of HCV exposure among HCWs, would give us data on the priority treatment and prevention of HCV in Egypt. Several studies were conducted among populations in select HCV-relevant professions[70-73]. The anti-HCV prevalence among HCWs worldwide ranges from 0% to 9.7%[71]. This is comparable with that of the general populations of the same country[72,73]. For example, in 2008, a cross-sectional study performed on 1770 HCWs at Cairo found the anti-HCV prevalence to be 8%[74]. This was comparable to the anti-HCV prevalence in the general populations of Cairo governorate in 2008[9]. Moreover, it was approximately similar to that (7.39%) in general populations of rural Lower Egypt governorates[11]. The high anti-HCV prevalence among Egyptian HCWs and their importance as a source for transmitting HCV suggests mass screening of all HCWs dealing with infectious secretions or tissues and those performing exposure-prone procedures.

LIMITATIONS FOR HCV ELIMINATION

Being an economically constrained country, budget limitation is the main challenge for HCV elimination in Egypt. HCV elimination challenge lies not only in the costs of treatment but also in the costs of diagnosis. Most patients remain undiagnosed and are therefore not appropriately managed. Inadequate diagnosis relates to investigation cost and the high illiteracy rates and low HCV awareness among the general population about the transmission modes and high-risk behaviors[75]. Hence, the majority of infected individuals in Egypt are unaware of their infected status. To achieve 2030 disease elimination target, the number of newly diagnosed patients annually must exceed 350000/year together with a decrease in the incidence of new cases by > 20% annually[76].

Coordination of all efforts is needed to train health care personnel, to deliver efficient care, counseling, and treatment to patients with chronic HCV in accordance with the national and international guidelines.

IC is another limitation to HCV elimination in Egypt. The overall IC had been assessed. In 2003, the overall IC score was 19. In response, the Egyptian MOH launched an IC program to promote safe practices in hospitals and health care facilities. This increased the overall IC score to 68.9 in 2011. However, Egypt still lacks formal IC programs in many health care facilities, professional health care workers with training or expertise in IC, and adequate equipment sterilization, reprocessing practices, and waste management. Limiting the ongoing HCV transmission in Egypt necessitates wider application of IC standards to all health and dental care providers beyond MOH facilities as well as increasing the community awareness.

Until recently, only 40% of HCV-infected individuals were enrolled in government programs for HCV eradication, whereas the HCV treatment expenditure for the remaining 60% individuals comes from Health Insurance Organization, insurance companies, and private payments[77]. Currently, the MOH financially sponsors the treatment for all patients who are not covered by health insurance[17]. Local pharmaceutical companies provide generic versions so as to make DAAs more available for individuals who cannot be treated through governmental schemes. In this context, the national health surveys in Egypt need to be expanded to make all infected

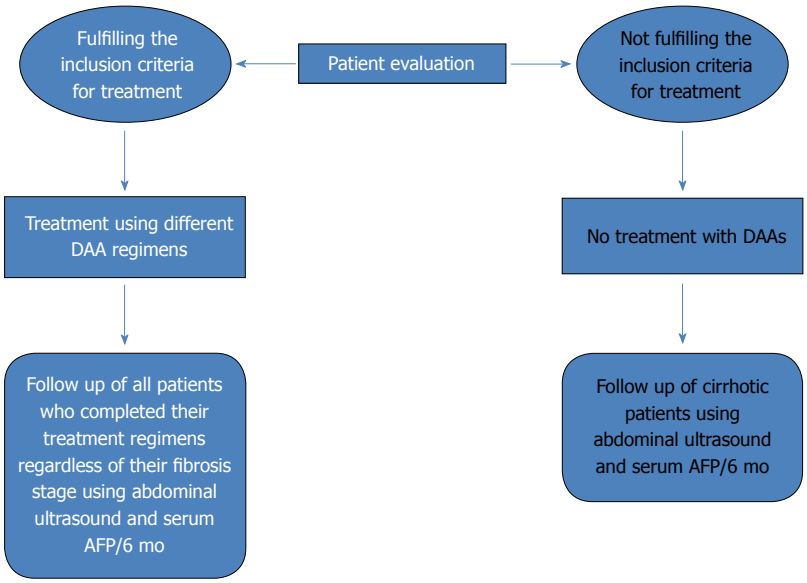


Figure 3 Management of hepatitis C virus patients in Egyptian viral hepatitis treatment centers. DAAs: Direct acting antivirals.

individuals aware of their infection status so that they can seek treatment.

FUTURE PROSPECTIVE FOR HCV ELIMINATION

Elimination of an ongoing nightmare like HCV is a national and global dream because of its burden on all aspects of life. Such a dream comes with its future perspectives. We plan to build centers for controlling and treating HCV as nuclei for integrated multidisciplinary centers for liver disease management and screening of treated patients for HCC and centers of excellence for HCC treatment as well as liver transplantation.

To date, deceased donor liver transplantation has not been implemented in any treatment center program despite law approval by the Egyptian Parliament in 2010. The current practice of living donor liver transplantation (LDLT) is the only choice to save many lives and is implemented in nearly 13 centers, with an increase in the total number of LDLT cases to approximately 2400 with improving results[78]. HCC incidence is increasing worldwide. Globally, it is considered the second cause of cancer-related death[79,80]. To date, in Egypt, HCC is known to be the second most common cancer in men and the sixth most common cancer in women, and unfortunately, no current program has yet been implemented for early detection and management of such cases[81]. However, it does not seem like a national dilemma as a Canadian study stated that most HCC cases referred to tertiary treatment centers are in palliative stages[82]. Therefore, because of the obvious advantages of early intervention in HCC, surveillance measures with early detection seem to be the only plausible option. In our experience, HCC developing after chronic hepatitis C treatment with DAAs showed a more infiltrative pattern of lesions[83]. However, the possible role of DAAs in HCC development needs to be further studied to verify the assumption and to identify the possible associated factors. Currently, we are endorsing a surveillance program for patients who have completed DAA therapies in Egypt. This surveillance program is based on the recall of all patients regardless of their fibrosis stage. Such patients will be subjected to abdominal ultrasound and serum alpha fetoprotein measurement every 6 mo (Figure 3). In Egypt, we aim to implement a long-term follow-up and screening program for our HCV patients so that such treatment centers also function as early detection centers for HCC. Such a program would encourage the government to implement therapeutic options for the early detected cases of HCC with higher success rates simultaneously with the running program for HCV eradication. Such an accomplishment will create centers of excellence targeting all HCV-related complications with radical therapeutic options.

All scientific breakthroughs were originally dreams, followed by future perspective plans and program implementation; we are surely dedicated and insistent on creating such a new breakthrough.

CONCLUSION

The high prevalence of HCV infection in Egypt, which is considered the highest worldwide, prompted the launch of Egypt's pioneering experience against HCV, aiming to eradicate viral hepatitis by 2030. The strategic plan targeted both treatment and prevention of new transmissions. The introduction of DAAs is a milestone in HCV eradication plan, with SVR rate of almost 100% obtained using certain DAA combinations. Preventing new transmissions is a real challenge that requires collaborative efforts to increase population awareness about transmission modes, safe practices,

and importance of screening and early diagnosis. The battle against HCV presented a huge financial burden on Egypt as it is a resource-limited country. It was difficult to provide funding sources to the HCV eradication program as only 40% of the HCV-infected individuals are enrolled in government programs for HCV eradication, whereas the HCV treatment expenditure for the remaining 60% individuals comes from Health Insurance Organization, insurance companies, and private out-of-pocket spending. Fortunately, funds were secured from the governmental support funds issued to individuals who are not under the umbrella of health insurance, and a large number of patients achieved SVR. Egypt provides a role model in planning and implementing the life-long goal of viral hepatitis eradication, which can be implemented in other countries to copy the Egyptian campaign target "For a world free of hepatitis C."

REFERENCES

1 **World Health Organization (WHO)**. Hepatitis C Fact sheet. July 2016
2 **Simmonds P**, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan SW, Chayama K, Chen DS. A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* 1994; **19**: 1321-1324 [PMID: 8175159 DOI: 10.1002/hep.1840190538]
3 **Smith DB**, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, Simmonds P. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology* 2014; **59**: 318-327 [PMID: 24115039 DOI: 10.1002/hep.26744]
4 **Messina JP**, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, Barnes E. Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology* 2015; **61**: 77-87 [PMID: 25069599 DOI: 10.1002/hep.27259]
5 **Polaris Observatory HCV Collaborators**. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol* 2017; **2**: 161-176 [PMID: 28404132 DOI: 10.1016/S2468-1253(16)30181-9]
6 **Kouyoumjian SP**, Chemaitelly H, Abu-Raddad LJ. Characterizing hepatitis C virus epidemiology in Egypt: systematic reviews, meta-analyses, and meta-regressions. *Sci Rep* 2018; **8**: 1661 [PMID: 29374178 DOI: 10.1038/s41598-017-17936-4]
7 **Rao MR**, Naficy AB, Darwish MA, Darwish NM, Schisterman E, Clemens JD, Edelman R. Further evidence for association of hepatitis C infection with parenteral schistosomiasis treatment in Egypt. *BMC Infect Dis* 2002; **2**: 29 [PMID: 12464161 DOI: 10.1186/1471-2334-2-29]
8 **Abdel-Aziz F**, Habib M, Mohamed MK, Abdel-Hamid M, Gamil F, Madkour S, Mikhail NN, Thomas D, Fix AD, Strickland GT, Anwar W, Sallam I. Hepatitis C virus (HCV) infection in a community in the Nile Delta: population description and HCV prevalence. *Hepatology* 2000; **32**: 111-115 [PMID: 10869297 DOI: 10.1053/jhep.2000.8438]
9 **El-Zanaty F**, Way A. Egypt Demographic and Health Survey 2008. Egyptian: Ministry of Health. Cairo: El-Zanaty and Associates and Macro International; 2009: 31 Available from: URL: http://dhsprogram.com/pubs/pdf/FR220/FR220.pdf
10 **Ministry of Health and Population/Egypt**, El-Zanaty and Associates/Egypt, and ICF International. 2015. Egypt Health Issues Survey 2015. Cairo, Egypt: Ministry of Health and Population/EgyptandICFInternational. Available from: URL: http://dhsprogram.com/pubs/pdf/FR313/FR313.pdf
11 **Kandeel A**, Genedy M, El-Refai S, Funk AL, Fontanet A, Talaat M. The prevalence of hepatitis C virus infection in Egypt 2015: implications for future policy on prevention and treatment. *Liver Int* 2017; **37**: 45-53 [PMID: 27275625 DOI: 10.1111/liv.13186]
12 **Esmat G**. Hepatitis C in the Eastern Mediterranean Region. *East Mediterr Health J* 2013; **19**: 587-588 [PMID: 24975301 DOI: 10.26719/2013.19.7.587]
13 **Alazawi W**, Cunningham M, Dearden J, Foster GR. Systematic review: outcome of compensated cirrhosis due to chronic hepatitis C infection. *Aliment Pharmacol Ther* 2010; **32**: 344-355 [PMID: 20497143 DOI: 10.1111/j.1365-2036.2010.04370.x]
14 **World Bank Health Expenditure**, Total (% of GDP) Available from: URL: http://data.worldbank.org/indicator/SH.XPD.TOTL.ZS
15 **Wedemeyer H**, Duberg AS, Buti M, Rosenberg WM, Frankova S, Esmat G, Örmeci N, Van Vlierberghe H, Gschwantler M, Akarca U, Aleman S, Balık I, Berg T, Bihl F, Bilodeau M, Blasco AJ, Brandão Mello CE, Bruggmann P, Calinas F, Calleja JL, Cheinquer H, Christensen PB, Clausen M, Coelho HS, Cornberg M, Cramp ME, Dore GJ, Doss W, El-Sayed MH, Ergör G, Estes C, Falconer K, Félix J, Ferraz ML, Ferreira PR, García-Samaniego J, Gerstoft J, Giria JA, Gonçales FL Jr, Guimarães Pessôa M, Hézode C, Hindman SJ, Hofer H, Husa P, Idilman R, Kåberg M, Kaita KD, Kautz A, Kaymakoglu S, Krajden M, Krarup H, Laleman W, Lavanchy D, Lázaro P, Marinho RT, Marotta P, Mauss S, Mendes Correa MC, Moreno C, Müllhaupt B, Myers RP, Nemecek V, Øvrehus AL, Parkes J, Peltekian KM, Ramji A, Razavi H, Reis N, Roberts SK, Roudot-Thoraval F, Ryder SD, Sarmento-Castro R, Sarrazin C, Semela D, Sherman M, Shiha GE, Sperl J, Stärkel P, Stauber RE, Thompson AJ, Urbanek P, Van Damme P, van Thiel I, Vandijck D, Vogel W, Waked I, Weis N, Wiegand J, Yosry A, Zekry A, Negro F, Sievert W, Gower E. Strategies to manage hepatitis C virus (HCV) disease burden. *J Viral Hepat* 2014; **21** Suppl 1: 60-89 [PMID: 24713006 DOI: 10.1111/jvh.12249]
16 **Estes C**, Abdel-Kareem M, Abdel-Razek W, Abdel-Sameea E, Abuzeid M, Gomaa A, Osman W, Razavi H, Zaghla H, Waked I. Economic burden of hepatitis C in Egypt: the future impact of highly effective therapies. *Aliment Pharmacol Ther* 2015; **42**: 696-706 [PMID: 26202593 DOI: 10.1111/apt.13316]
17 **El-Akel W**, El-Sayed MH, El Kassas M, El-Serafy M, Khairy M, Elsaeed K, Kabil K, Hassany M, Shawky A, Yosry A, Shaker MK, ElShazly Y, Waked I, Esmat G, Doss W. National treatment programme of hepatitis C in Egypt: Hepatitis C virus model of care. *J Viral Hepat* 2017; **24**: 262-267 [PMID: 28145032 DOI: 10.1111/jvh.12668]
18 **Doss W**, Mohamed MK, Esmat G, El Sayed M, Fontanet A, Cooper S, El Sayed N. Egyptian National Control Strategy for Viral Hepatitis 2008-2012. Arab Republic of Egypt, Ministry of Health and Population, National Committee for the Control of Viral Hepatitis; 2008. Available from: URL: http://www.hepnile.org/images/stories/doc/NSP_10_April_2008_final2.pdf
19 **Esmat G**, El Kassas M. The control of HCV in Egypt. Hepatitis B and C Public Policy Association Newsletter. 2017; 4-5 Available from: URL: http://www.hepbcppa.org/wpcontent/uploads/2017/06/HepBC-June17.pdf
20 **Gomaa A**, Allam N, Elsharkawy A, El Kassas M, Waked I. Hepatitis C infection in Egypt: prevalence, impact and management strategies. *Hepat Med* 2017; **9**: 17-25 [PMID: 28553150 DOI: 10.2147/HMER.S113681]
21 **El Raziky M**, Fathalah WF, El-Akel WA, Salama A, Esmat G, Mabrouk M, Salama RM, Khatab HM. The Effect of Peginterferon Alpha-2a vs. Peginterferon Alpha-2b in Treatment of Naive Chronic HCV Genotype-4 Patients: A Single Centre Egyptian Study. *Hepat Mon* 2013; **13**: e10069 [PMID: 23922556 DOI: 10.5812/hepatmon.10069]
22 **Ruane PJ**, Ain D, Stryker R, Meshrekey R, Soliman M, Wolfe PR, Riad J, Mikhail S, Kersey K, Jiang D, Massetto B, Doehle B, Kirby BJ, Knox SJ, McHutchison JG, Symonds WT. Sofosbuvir plus ribavirin for the treatment of chronic genotype 4 hepatitis C virus infection in patients of Egyptian ancestry. *J Hepatol* 2015; **62**: 1040-1046 [PMID: 25450208 DOI: 10.1016/j.jhep.2014.10.044]
23 **Doss W**, Shiha G, Hassany M, Soliman R, Fouad R, Khairy M, Samir

W, Hammad R, Kersey K, Jiang D, Doehle B, Knox SJ, Massetto B, McHutchison JG, Esmat G. Sofosbuvir plus ribavirin for treating Egyptian patients with hepatitis C genotype 4. *J Hepatol* 2015; **63**: 581-585 [PMID: 25937436 DOI: 10.1016/j.jhep.2015.04.023]

24 **Elsharkawy A**, Fouad R, El Akel W, El Raziky M, Hassany M, Shiha G, Said M, Motawea I, El Demerdash T, Seif S, Gaballah A, El Shazly Y, Makhlouf MA, Waked I, Abdelaziz AO, Yosry A, El Serafy M, Thursz M, Doss W, Esmat G. Sofosbuvir-based treatment regimens: real life results of 14 409 chronic HCV genotype 4 patients in Egypt. *Aliment Pharmacol Ther* 2017; **45**: 681-687 [PMID: 28070899 DOI: 10.1111/apt.13923]

25 **El Kassas M**, Alboraie M, Omran D, Salaheldin M, Wifi MN, ElBadry M, El Tahan A, Ezzat S, Moaz E, Farid AM, Omar H, Abouelkhair M, Afify S, Elsaeed K, Shazly Y, Doss W, Esmat G. An account of the real-life hepatitis C management in a single specialized viral hepatitis treatment centre in Egypt: results of treating 7042 patients with 7 different direct acting antiviral regimens. *Expert Rev Gastroenterol Hepatol* 2018; Epub ahead of print [PMID: 29757684 DOI: 10.1080/17474124.2018.1476137]

26 **Ahmed OA**, Elsebaey MA, Fouad MHA, Elashry H, Elshafie AI, Elhadidy AA, Esheba NE, Elnaggar MH, Soliman S, Abd-Elsalam S. Outcomes and predictors of treatment response with sofosbuvir plus daclatasvir with or without ribavirin in Egyptian patients with genotype 4 hepatitis C virus infection. *Infect Drug Resist* 2018; **11**: 441-445 [PMID: 29628768 DOI: 10.2147/IDR.S160593]

27 **Abd-Elsalam S**, Sharaf-Eldin M, Soliman S, Elfert A, Badawi R, Ahmad YK. Efficacy and safety of sofosbuvir plus ribavirin for treatment of cirrhotic patients with genotype 4 hepatitis C virus in real-life clinical practice. *Arch Virol* 2018; **163**: 51-56 [PMID: 28983675 DOI: 10.1007/s00705-017-3573-0]

28 **El Kassas M**, Omran D, Elsaeed K, Alboraie M, Elakel W, El Tahan A, Abd El Latif Y, Nabeel MM, Korany M, Ezzat S, El-Serafy M, ElShazly Y, Doss W, Esmat G. Spur-of-the-Moment Modification in National Treatment Policies Leads to a Surprising HCV Viral Suppression in All Treated Patients: Real-Life Egyptian Experience. *J Interferon Cytokine Res* 2018; **38**: 81-85 [PMID: 29356573 DOI: 10.1089/jir.2017.0121]

29 **El-Khayat HR**, Fouad YM, Maher M, El-Amin H, Muhammed H. Efficacy and safety of sofosbuvir plus simeprevir therapy in Egyptian patients with chronic hepatitis C: a real-world experience. *Gut* 2017; **66**: 2008-2012 [PMID: 27511197 DOI: 10.1136/gutjnl-2016-312012]

30 **Eletreby R**, Elakel W, Said M, El Kassas M, Seif S, Elbaz T, El Raziky M, Abdel Rehim S, Zaky S, Fouad R, Gamal Eldeen H, Abdo M, Korany M, Yosry A, El Serafy M, El-Sayed MH, ElShazly Y, Waked I, Doss W, Esmat G. Real life Egyptian experience of efficacy and safety of Simeprevir/Sofosbuvir therapy in 6211 chronic HCV genotype IV infected patients. *Liver Int* 2017; **37**: 534-541 [PMID: 27712017 DOI: 10.1111/liv.13266]

31 **Abdel-Moneim A**, Aboud A, Abdel-Gabaar M, Zanaty MI, Ramadan M. Efficacy and safety of sofosbuvir plus daclatasvir with or without ribavirin: large real-life results of patients with chronic hepatitis C genotype 4. *Hepatol Int* 2018; Epub ahead of print [PMID: 29754329 DOI: 10.1007/s12072-018-9868-8]

32 **El-Khayat H**, Fouad Y, Mohamed HI, El-Amin H, Kamal EM, Maher M, Risk A. Sofosbuvir plus daclatasvir with or without ribavirin in 551 patients with hepatitis C-related cirrhosis, genotype 4. *Aliment Pharmacol Ther* 2018; **47**: 674-679 [PMID: 29314146 DOI: 10.1111/apt.14482]

33 **Mohamed MS**, Hanafy AS, Bassiony MAA, Hussein S. Sofosbuvir and daclatasvir plus ribavirin treatment improve liver function parameters and clinical outcomes in Egyptian chronic hepatitis C patients. *Eur J Gastroenterol Hepatol* 2017; **29**: 1368-1372 [PMID: 28953002 DOI: 10.1097/MEG.0000000000000963]

34 **Omar H**, El Akel W, Elbaz T, El Kassas M, Elsaeed K, El Shazly H, Said M, Yousif M, Gomaa AA, Nasr A, AbdAllah M, Korany M, Ismail SA, Shaker MK, Doss W, Esmat G, Waked I, El Shazly Y. Generic daclatasvir plus sofosbuvir, with or without ribavirin, in treatment of chronic hepatitis C: real-world results from 18 378 patients in Egypt. *Aliment Pharmacol Ther* 2018; **47**: 421-431 [PMID: 29193226 DOI: 10.1111/apt.14428]

35 **El-Khayat HR**, Kamal EM, El-Sayed MH, El-Shabrawi M, Ayoub H, RizK A, Maher M, El Sheemy RY, Fouad YM, Attia D. The effectiveness and safety of ledipasvir plus sofosbuvir in adolescents with chronic hepatitis C virus genotype 4 infection: a real-world experience. *Aliment Pharmacol Ther* 2018; **47**: 838-844 [PMID: 29349793 DOI: 10.1111/apt.14502]

36 **Elsharkawy A**, El-Raziky M, El-Akel W, El-Saeed K, Eletreby R, Hassany M, El-Sayed MH, Kabil K, Ismail SA, El-Serafy M, Abdelaziz AO, Shaker MK, Yosry A, Doss W, El-Shazly Y, Esmat G, Waked I. Planning and prioritizing direct-acting antivirals treatment for HCV patients in countries with limited resources: Lessons from the Egyptian experience. *J Hepatol* 2017; : [PMID: 29223371 DOI: 10.1016/j.jhep.2017.11.034]

37 **Attia D**, El Saeed K, Elakel W, El Baz T, Omar A, Yosry A, Elsayed MH, Said M, El Raziky M, Anees M, Doss W, El Shazly Y, Wedemeyer H, Esmat G. The adverse effects of interferon-free regimens in 149 816 chronic hepatitis C treated Egyptian patients. *Aliment Pharmacol Ther* 2018; **47**: 1296-1305 [PMID: 29504152 DOI: 10.1111/apt.14538]

38 **Wanis H**, Hussein A, El Shibiny A, et al. HCV treatment in Egypt - why cost remains a challenge Egyptian initiative for personal rights. 2014: 6-18 Available from: URL: http://www.eipr.org/sites/default/files/pressreleases/pdf/hcv_treatment_in_egypt.pdf

39 **Medhat A**, Shehata M, Magder LS, Mikhail N, Abdel-Baki L, Nafeh M, Abdel-Hamid M, Strickland GT, Fix AD. Hepatitis c in a community in Upper Egypt: risk factors for infection. *Am J Trop Med Hyg* 2002; **66**: 633-638 [PMID: 12201604 DOI: 10.4269/ajtmh.2002.66.633]

40 **Mostafa A**, Taylor SM, el-Daly M, el-Hoseiny M, Bakr I, Arafa N, Thiers V, Rimlinger F, Abdel-Hamid M, Fontanet A, Mohamed MK. Is the hepatitis C virus epidemic over in Egypt? Incidence and risk factors of new hepatitis C virus infections. *Liver Int* 2010; **30**: 560-566 [PMID: 20141592 DOI: 10.1111/j.1478-3231.2009.02204.x]

41 **Talaat M**, Radwan E, El-Sayed N, Ismael T, Hajjeh R, Mahoney FJ. Case-control study to evaluate risk factors for acute hepatitis B virus infection in Egypt. *East Mediterr Health J* 2010; **16**: 4-9 [PMID: 20214150 DOI: 10.26719/2010.16.1.4]

42 **Talaat M**, el-Oun S, Kandeel A, Abu-Rabei W, Bodenschatz C, Lohiniva AL, Hallaj Z, Mahoney FJ. Overview of injection practices in two governorates in Egypt. *Trop Med Int Health* 2003; **8**: 234-241 [PMID: 12631314 DOI: 10.1046/j.1365-3156.2003.01015.x]

43 **Hagan H**, Jarlais DC, Friedman SR, Purchase D, Alter MJ. Reduced risk of hepatitis B and hepatitis C among injection drug users in the Tacoma syringe exchange program. *Am J Public Health* 1995; **85**: 1531-1537 [PMID: 7485666 DOI: 10.2105/AJPH.85.11.1531]

44 **Abdel-Ghaffar TY**, Sira MM, El Naghi S. Hepatitis C genotype 4: The past, present, and future. *World J Hepatol* 2015; **7**: 2792-2810 [PMID: 26668691 DOI: 10.4254/wjh.v7.i28.2792]

45 Gamal E. Situation of HCV in Egypt Towards an End to HCV Epidemic.2014. Available from: URL: http://www.liver-eg.org/assets/esmatt-hepatitis-c-situation-in-egypt.pdf

46 **Ayoub HH**, Abu-Raddad LJ. Impact of treatment on hepatitis C virus transmission and incidence in Egypt: A case for treatment as prevention. *J Viral Hepat* 2017; **24**: 486-495 [PMID: 28039923 DOI: 10.1111/jvh.12671]

47 **Paez Jimenez A**, El-Din NS, El-Hoseiny M, El-Daly M, Abdel-Hamid M, El Aidi S, Sultan Y, El-Sayed N, Mohamed MK, Fontanet A. Community transmission of hepatitis B virus in Egypt: results from a case-control study in Greater Cairo. *Int J Epidemiol* 2009; **38**: 757-765 [PMID: 19420088 DOI: 10.1093/ije/dyp194]

48 **Metwally A**, Mohsen A, Saleh R, Foaud W, Ibrahim N, Rabaah T, El-Sayed M. Prioritizing High-Risk Practices and Exploring New Emerging Ones Associated With Hepatitis C Virus Infection in Egypt. *Iran J Public Health* 2014; **43**: 1385-1394 [PMID: 26060701]

49 **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]
50 **Dolan K,** MacDonald M, Silins E, Topp L. Needle and syringe programs: A review of the evidence. Canberra: Australian Government Department of Health and Ageing. 2005 Available from: URL: https://www.health.gov.au/internet/main/publishing.nsf/content/.../$File/evid.pdf
51 **Islam MM**, Topp L, Conigrave KM, Haber PS, White A, Day CA. Sexually transmitted infections, sexual risk behaviours and perceived barriers to safe sex among drug users. *Aust N Z J Public Health* 2013; **37**: 311-315 [PMID: 23895472 DOI: 10.1111/1753-6405.12077]
52 **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]
53 **Calès P**, Boursier J, Bertrais S, Oberti F, Gallois Y, Fouchard-Hubert I, Dib N, Zarski JP, Rousselet MC; multicentric groups (SNIFF 14 & 17, ANRS HC EP 23 Fibrostar). Optimization and robustness of blood tests for liver fibrosis and cirrhosis. *Clin Biochem* 2010; **43**: 1315-1322 [PMID: 20713037 DOI: 10.1016/j.clinbiochem.2010.08.010]
54 **Nguyen LH**, Nguyen MH. Systematic review: Asian patients with chronic hepatitis C infection. *Aliment Pharmacol Ther* 2013; **37**: 921-936 [PMID: 23557103 DOI: 10.1111/apt.12300]
55 **Kandeel AM**, Talaat M, Afifi SA, El-Sayed NM, Abdel Fadeel MA, Hajjeh RA, Mahoney FJ. Case control study to identify risk factors for acute hepatitis C virus infection in Egypt. *BMC Infect Dis* 2012; **12**: 294 [PMID: 23145873 DOI: 10.1186/1471-2334-12-294]
56 **Breban R**, Arafa N, Leroy S, Mostafa A, Bakr I, Tondeur L, Abdel-Hamid M, Doss W, Esmat G, Mohamed MK, Fontanet A. Effect of preventive and curative interventions on hepatitis C virus transmission in Egypt (ANRS 1211): a modelling study. *Lancet Glob Health* 2014; **2**: e541-e549 [PMID: 25304421 DOI: 10.1016/S2214-109X(14)70188-3]
57 U.S. Preventive Services Task Force. Final recommendation statement: Hepatitis C screening. USPSTF. 2013 Available from: URL: http://www.uspreventiveservicestaskforce.org/Page/Document/Recommendation Statement Final/hepatitis-c-screening
58 **Artenie AA**, Bruneau J, Lévesque A, Wansuanganyi JM. Role of primary care providers in hepatitis C prevention and care: one step away from evidence-based practice. *Can Fam Physician* 2014; **60**: 881-882, e468-e470 [PMID: 25316735]
59 **Alter HJ**, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; **321**: 1494-1500 [PMID: 2509915 DOI: 10.1056/NEJM198911303212202]
60 **Elkareh S**. HCV screening of donors in Governmental Blood Transfusion Centers at Menoufia Governorate (from Jan. 2008 to Oct. 2008). *Vox Sang* 2009; **96**: 89-90
61 **Eita N**. Prevalence of HCV and HBV infections among blood donors in Dakahilia, Egypt. *Vox Sang* 2009; **96**: 106-107
62 **Rushdy O**, Moftah F, Zakareya S. Trasmitted transfused viral infections among blood donors during years 2006 and 2007 in suez canal area, Egypt. *Vox Sang* 2009; **96**: 86-87
63 **Arthur RR**, Hassan NF, Abdallah MY, el-Sharkawy MS, Saad MD, Hackbart BG, Imam IZ. Hepatitis C antibody prevalence in blood donors in different governorates in Egypt. *Trans R Soc Trop Med Hyg* 1997; **91**: 271-274 [PMID: 9231192 DOI: 10.1016/S0035-9203(97)90070-5]
64 **El-Sherbini A**, Hassan W, Abdel-Hamid M, Naeim A. Natural history of hepatitis C virus among apparently normal schoolchildren: follow-up after 7 years. *J Trop Pediatr* 2003; **49**: 384-385 [PMID: 14725420 DOI: 10.1093/tropej/49.6.384]
65 **Mohamed MK**, Magder LS, Abdel-Hamid M, El-Daly M, Mikhail NN, Abdel-Aziz F, Medhat A, Thiers V, Strickland GT. Transmission of hepatitis C virus between parents and children. *Am J Trop Med Hyg* 2006; **75**: 16-20 [PMID: 16837701 DOI: 10.4269/ajtmh.2006.75.16]
66 **El Sherbini A**, Mohsen SA, Hasan W, Mostafa S, El Gohary K, Moneib A, Abaza AH. The peak impact of an Egyptain outbreak of hepatitis C virus: has it passed or has not yet occurred? *Liver Int* 2007; **27**: 876-877 [PMID: 17617132 DOI: 10.1111/j.1478-3231.2007.01501.x]
67 **Morad WS**. Transmission of hepatitis C between spouses an epidemiological study at National Liver Institute hospital. *Int J Infect Dis*. 2011; **15**: S81 [DOI: 10.1016/S1201-9712(11)60279-8]
68 **El-Zayadi A**, Khalifa AA, El-Misiery A, Naser AM, Dabbous H, Aboul-Ezz AA. Evaluation of risk factors for intrafamilial transmission of HCV infection in Egypt. *J Egypt Public Health Assoc* 1997; **72**: 33-51 [PMID: 17265624]
69 **Mohamoud YA**, Mumtaz GR, Riome S, Miller D, Abu-Raddad LJ. The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis. *BMC Infect Dis* 2013; **13**: 288 [PMID: 23799878 DOI: 10.1186/1471-2334-13-288]
70 **Shalaby S**, Kabbash IA, El Saleet G, Mansour N, Omar A, El Nawawy A. Hepatitis B and C viral infection: prevalence, knowledge, attitude and practice among barbers and clients in Gharbia governorate, Egypt. *East Mediterr Health J* 2010; **16**: 10-17 [PMID: 20214151 DOI: 10.26719/2010.16.1.10]
71 **Coppola N**, De Pascalis S, Onorato L, Calò F, Sagnelli C, Sagnelli E. Hepatitis B virus and hepatitis C virus infection in healthcare workers. *World J Hepatol* 2016; **8**: 273-281 [PMID: 26925201 DOI: 10.4254/wjh.v8.i5.273]
72 **Arguillas MO**, Domingo EO, Tsuda F, Mayumi M, Suzuki H. Seroepidemiology of hepatitis C virus infection in the Philippines: a preliminary study and comparison with hepatitis B virus infection among blood donors, medical personnel, and patient groups in Davao, Philippines. *Gastroenterol Jpn* 1991; **26** Suppl 3: 170-175 [PMID: 1909261 DOI: 10.1007/BF02779292]
73 **Ozsoy MF**, Oncul O, Cavuslu S, Erdemoglu A, Emekdas G, Pahsa A. Seroprevalences of hepatitis B and C among health care workers in Turkey. *J Viral Hepat* 2003; **10**: 150-156 [PMID: 12614472 DOI: 10.1046/j.1365-2893.2003.00404.x]
74 **Okasha O**, Munier A, Delarocque-Astagneau E, El Houssinie M, Rafik M, Bassim H, Hamid MA, Mohamed MK, Fontanet A. Hepatitis C virus infection and risk factors in health-care workers at Ain Shams University Hospitals, Cairo, Egypt. *East Mediterr Health J* 2015; **21**: 199-212 [PMID: 26074220]
75 **Central Agency for Public Mobilisation and Statistics (CAPMAS)**. Poverty indicators according to income, expenditure and consump¬tion data 2012-2013. 2013 Available from: URL: http://www.capmas.gov.eg/pepo/a.pdf
76 **Waked I**, Doss W, El-Sayed MH, Estes C, Razavi H, Shiha G, Yosry A, Esmat G. The current and future disease burden of chronic hepatitis C virus infection in Egypt. *Arab J Gastroenterol* 2014; **15**: 45-52 [PMID: 25097045 DOI: 10.1016/j.ajg.2014.04.003]
77 **Iskander D**. The Right to Health: a case study on Hepatitis C in Egypt. MS.c. thesis, American University in Cairo. 2013. Available from: URL: https://dar.aucegypt.edu/bitstream/handle/10526/3748/Thesis%20IHRL%20%20Dina%20Iskander%20Dec2013.pdf?sequence=3
78 **Amer KE**, Marwan I. Living donor liver transplantation in Egypt. *Hepatobiliary Surg Nutr* 2016; **5**: 98-106 [PMID: 27115003 DOI: 10.3978/j.issn.2304-3881.2015.10.03]
79 **World Health Organization**. Mortality database. WHO statistical information system. Available from: URL: http://www.who.int/whosis
80 **Venook AP**, Papandreou C, Furuse J, de Guevara LL. The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. *Oncologist* 2010; **15** Suppl 4: 5-13 [PMID: 21115576 DOI: 10.1634/theoncologist.2010-S4-05]
81 Egyptian National Cancer Registry. Available from: URL: http://www.cancerregistry.gov.eg
82 **Khalili K**, Menezes R, Yazdi LK, Jang HJ, Kim TK, Sharma S, Feld J, Sherman M. Hepatocellular carcinoma in a large Canadian urban centre: stage at treatment and its potential determinants. *Can*

J Gastroenterol Hepatol 2014; **28**: 150-154 [PMID: 24619637 DOI: 10.1155/2014/561732]

83 **Abdelaziz AO**, Nabil MM, Abdelmaksoud AH, Shousha HI, Cordie AA, Hassan EM, Omran DA, Leithy R, Elbaz TM. De-novo versus recurrent hepatocellular carcinoma following direct-acting antiviral therapy for hepatitis C virus. *Eur J Gastroenterol Hepatol* 2018; **30**: 39-43 [PMID: 29064851 DOI: 10.1097/MEG.0000000000001004]

P- Reviewer: Inoue K, Sazci A, Shimizu Y **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4341-4355

DOI: 10.3748/wjg.v24.i38.4341

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

ORIGINAL ARTICLE

Basic Study

Temporal clinical, proteomic, histological and cellular immune responses of dextran sulfate sodium-induced acute colitis

Natalia Schneider Nunes, Saejeong Kim, Maggie Sundby, Parwathy Chandran, Scott Robert Burks, Ana Helena Paz, Joseph Alan Frank

Natalia Schneider Nunes, Saejeong Kim, Maggie Sundby, Parwathy Chandran, Scott Robert Burks, Joseph Alan Frank, Frank Laboratory, Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, Bethesda, MD 20892, United States

Natalia Schneider Nunes, Ana Helena Paz, Gastroenterology and Hepatology Sciences Graduate Program, Universidade Federal do Rio Grande do Sul, Porto Alegre 90035-093, Brazil

Joseph Alan Frank, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD 20892, United States

ORCID number: Natalia Schneider Nunes (0000-0003-1950-4391); Saejeong Kim (0000-0003-0979-8162); Maggie Sundby (0000-0002-2046-0181); Parwathy Chandran (0000-0001-7435-730X); Scott Robert Burks (0000-0003-2030-3845); Ana Helena Paz (0000-0003-3668-7054); Joseph Alan Frank (0000-0001-8636-7273).

Author contributions: Nunes NS, Paz AH and Frank JA assisted in research design; Nunes NS carried out the research; Nunes NS and Frank JA analyzed data and wrote manuscript; Sundby M and Paz AH assisted with histological analysis; Kim S and Chandran P assisted with flow cytometry analysis; Burks SR assisted with proteomic analysis; all authors reviewed and approved manuscript.

Institutional animal care and use committee statement: All procedures involving animal subjects were reviewed and approved by the Animal Care and Use Committee of the Clinical Center at the National Institutes of Health (ACUC protocol number: LDRR16-02).

Conflict-of-interest statement: The authors declare that they have no conflict-of-interests.

Data sharing statement: Materials used for the current study are available from the corresponding author on reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Natalia S Nunes, PhD, Frank Laboratory, Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, Bldg 10 Room B1N256, 10 Center Drive, Bethesda, MD 20892,
United States. natalia.schneidernunes@nih.gov
Telephone: +1-301-9063353

Received: June 21, 2018
Peer-review started: June 21, 2018
First decision: August 1, 2018
Revised: August 6, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 14, 2018

Abstract

AIM

To investigate the temporal clinical, proteomic, histological and cellular immune profiles of dextran sulfate sodium (DSS)-induced acute colitis.

METHODS

Acute colitis was induced in C57BL/6 female mice by

administration of 1%, 2% or 3% DSS in drinking water for 7 d. Animals were monitored daily for weight loss, stool consistency and blood in the stool, while spleens and colons were harvested on day 8. A time course analysis was performed in mice ingesting 3% DSS, which included colon proteomics through multiplex assay, colon histological scoring by a blinded investigator, and immune response through flow cytometry or immunohistochemistry of the spleen, mesenteric lymph node and colon.

RESULTS

Progressive worsening of clinical colitis was observed with increasing DSS from 1% to 3%. In mice ingesting 3% DSS, colon shortening and increase in pro-inflammatory factors starting at day 3 was observed, with increased spleen weights at day 6 and day 8. This coincided with cellular infiltration in the colon from day 2 to day 8, with progressive accumulation of macrophages F4/80$^+$, T helper CD4$^+$ (Th), T cytotoxic CD8$^+$ (Tcyt) and T regulatory CD25$^+$ (Treg) cells, and progressive changes in colonic pathology including destruction of crypts, loss of goblet cells and depletion of the epithelial barrier. Starting on day 4, mesenteric lymph node and/or spleen presented with lower levels of Treg, Th and Tcyt cells, suggesting an immune cell tropism to the gut.

CONCLUSION

These results demonstrate that the severity of experimental colitis is dependent on DSS concentration, correlated with clinical, proteomic, histological and cellular immune response on 3% DSS.

Key words: Ulcerative colitis; Dextran sulfate sodium; Proteomics; Inflammatory bowel diseases; Inflammation

Core tip: Our study contributes to a better understanding of the dextran sulfate sodium (DSS) acute colitis model in order to provide a stronger basis for novel therapies. Colonic proteomic temporal analysis reveals an increase in cytokines with a strong influx of immune cells. The highest cytokine levels were observed when animals were no longer drinking DSS, suggesting a rebound response. Secondary lymphoid organs contribute by sending different immune cells to the colon during the acute phase, such as CD4$^+$, CD8$^+$ and CD25$^+$ T cells. Our results demonstrate involvement of the adaptive and innate immune responses during the acute phase of DSS-induced colitis.

Nunes NS, Kim S, Sundby M, Chandran P, Burks SR, Paz AH, Frank JA. Temporal clinical, proteomic, histological and cellular immune responses of dextran sulfate sodium-induced acute colitis. *World J Gastroenterol* 2018; 24(38): 4341-4355 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4341.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4341

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases and consist mainly of ulcerative colitis (UC) and Crohn's disease (CD). UC usually presents with symptoms of diarrhea, weight loss, abdominal pain and blood in the stool and the development of IBD is associated with genetic, environmental and microbial factors[1,2]. Despite the rapid rise of IBD in the United States (US) and Europe, even with the advent of biological therapies, there are no current treatments that will sustain remission. Numerous animal models, including the chemically inducible colitis model of dextran sulfate sodium (DSS), have been developed to understand the pathobiology of IBD and evaluate novel therapies[3-5]. When DSS is added to drinking water, mice develop colitis that can be modulated by altering the DSS concentration, molecular weight and microbiota[6,7]. DSS primarily causes disruption of the intestinal barrier, allowing access of antigens and pro-inflammatory factors from the intestinal contents to the mucosal layer of the large bowel. Moreover, the exact mechanism has not been thoroughly elucidated[6,8,9]. Histological characteristics of DSS colitis includes the depletion of crypts, infiltration of neutrophils, ulceration and inflammation of the mucosal and submucosal layers[10]. Initial studies[11] suggested that human UC was predominantly associated with a Th2 immune response [interleukin (IL)-5], however it has been shown that other factors from Th1 [tumor necrosis factor alpha (TNFα)] and Th17 (IL-17, IL-23) profiles are also implicated in the development of the disease[12-15]. Although the DSS acute and chronic colitis models are not solely dependent on B and T cell responses, a complex interplay between innate and adaptive immune system occurs, in which neutrophils (N), eosinophils (E), macrophages (M), dendritic cells (DC), T cells and B cells participate in the exaggerated presentation of the disease[15-18].

Previous studies have individually investigated the clinical manifestations of DSS induced colitis with temporal proteomic, immune cells infiltration, histological changes in the colon and transcriptional genomics[9,15,16,19-21]. In the current study, the relationship between daily clinical activities along with temporal molecular analysis, histological features and immune cell trafficking were investigated during the acute phase of DSS colitis, to further the understanding of the interaction of these factors in disease development.

MATERIALS AND METHODS

Animals

The protocol was approved by the Animal Care and Use Committee at our institution. C57BL/6 female mice 6-8 wk old from Charles River Laboratories (Wilmington, MA, United States) were used for the experiments. Animals were housed in specific pathogen-free conditions with

Table 1 Disease activity index scoring

Score	Weight loss	Stool consistency	Bleeding
0	None	Normal	No bleeding
1	1%-5%	-	-
2	5%-10%	Loose stools	Slight bleeding
3	10%-15%	-	-
4	More than 15%	Watery diarrhea	Gross bleeding

12h-12h light-dark cycles under controlled humidity and temperature.

DSS colitis model

Experimental acute colitis was induced by administration of 1%, 2% or 3% (wt/vol) DSS (36000-50000 Da - MP Biomedicals, Solon, OH, Unites States) in drinking water *ad libitum* for 7 d and euthanized at day 8. Control animals were allowed sterilized tap water *ad libitum*. For the time course analysis, mice received 3% DSS for 7 d and were euthanized on day 0, day 2, day 3, day 4, day 5, day 6 and day 8. Euthanasia was performed through isoflurane anesthesia followed by cervical dislocation for collection of biological samples.

Clinical activity

Animals (n = 6/DSS group and n = 6/time point) were daily evaluated through disease activity index (DAI), as previously described[7,22]. Table 1 contains the grading criteria used for the DSS colitis model. Briefly, animals were evaluated for weight loss (0-4), stool consistency (0-4) and blood in the stool (0-4), in which DAI reaches a maximum score of 12. After euthanasia at different time points, the entire colon was collected and cleaned with flushing PBS (Phosphate buffered saline) 1 ×. Colon was weighed, and the colon length was measured from the caecum to the anus. Spleen was weighted and further processed for flow cytometry analysis.

Proteomics

Colon samples from animals (n = 6/each time point) receiving 3% DSS were snap frozen and later homogenized for protein extraction. Briefly, frozen colon samples were processed in cell lysis buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris-HCl and 0.05% Tween-20, with addition of protease inhibitor (Thermo Scientific, Waltham, MA, United States) and 1.0 mm Zyrconium Beads. Samples were centrifuged twice at 14000 r/min at 4 ℃ for 20 min and supernatant was collected. Aliquots were kept at -80 ℃ until further analysis. Samples were quantified through bicinchoninic acid assay (BCA - Thermo Scientific, Waltham, MA, United States) and diluted to a final concentration of 1 mg/mL of total protein. Colon homogenates were analyzed by MILLIPLEX Map Mouse Cytokine/Chemokine Panel (EMD Millipore, Billerica, MA, United States) using Bio-Plex 200 (Bio-Rad) according to manufacturer specifications.

Flow cytometry

Spleen and mesenteric lymph nodes (MLN) (n = 6/each time point) were collected and processed for flow cytometry analysis. Tissue samples were smashed between two frosted glass slides in the presence of ammonium-chloride-potassium lysing buffer (Lonza, Walkersville, MD, United States) until they were dissociated. PBS 1 × was added and samples were centrifuged at 1500 r/min at 4 ℃ for 10 min. Cells were re-suspended in PBS 1 ×, filtered through a 70 μm filter and centrifuged at 1500 r/min at 4 ℃ for 10 min. The pellet was incubated in 10% formalin for 35 min at 4 ℃ and washed in PBS 1 ×. Samples were kept at 4 ℃ until flow cytometry analysis. The single cell suspension was incubated with the proper amounts of antibodies in Stain Buffer (BD Pharmingen, San Jose, CA, United States) for 35 min on ice protected from light, following manufacturer instructions. Samples were loaded in a V-bottom 96-well plate and read in Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, United States). Data were analyzed using Accuri C6 Flow Cytometer software. Immune cells were characterized for T helper cells ($CD3^+CD4^+$), T regulatory cells ($CD3^+CD4^+CD25^+$), T cytotoxic cells ($CD3^+CD8^+$), B cells ($B220^+$) and Macrophages ($F4/80^+$). Antibodies used were FITC F4/80 (Rat, 0.5 mg/mL, eBioscience), PE CD25 (Rat, 0.2 mg/mL, BD Pharmingen), Alexa Fluor 488 B220 (Rat, 0.5 mg/mL, Biolegend), APC CD4 (Rat, 0.2 mg/mL, BD Pharmingen), FITC CD3 (Rat, 0.5 mg/mL, BD Pharmingen) and PE CD8a (Rat, 0.2 mg/mL, BD Pharmingen). Enriched $F4/80^+$ and $CD3^+CD4^+CD25^+$ populations were separated prior to flow cytometry analysis using Magnetic Cell Separation MicroBeads (MACS - Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer instructions in $CD4^+CD25^+$ Regulatory T cell isolation kit and with F4/80 MicroBeads Ultrapure. There were collected 20000 events for each sample and results are presented as the mean ± SD percentage of the total number of cells. Isotypes were also analyzed for each antibody and sample. Flow cytometry gating can be found in Supplementary Figures 1 and 2.

Histological and immunohistochemistry evaluation

For bright field microscopy, colon samples (n = 4/DSS group and n = 4/each time point) freshly collected from animals receiving 3% DSS were washed with PBS 1 ×, cut longitudinally and kept in 10% NBF (neutral buffered formalin) as a *Swiss roll* for 24 h at room temperature (RT). Tissue was kept in PBS 1 × until further processed into paraffin blocks. 3 μm sections were stained with Guills II hematoxylin and Eosin-Y (HE) for morphologic analysis. The histological evaluation was performed as previously described[7]. Briefly, the tissue was analyzed for grade of inflammation (0-3), extent within the intestine layers (0-3), regeneration (0-4), crypt damage (0-4) and percentage of involvement (0-4), reaching a maximum score of 56 (Table 2)[23]. Images were obtained on a Leica Aperio ScanScope CS using a 20 × air objective (NA = 0.75, Leica Microsystems, Buffalo Grove, IL, United States) and Aperio ImageScope software. HE staining was done on spleen and MLN using

Table 2 Histological scoring

Feature graded	Grade	Description
Inflammation	0	None
	1	Slight
	2	Moderate
	3	Severe
Extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Percent involvement	1	1%-25%
	2	26%-50%
	3	51%-75%
	4	76%-100%

the same method as colon samples.

For immunohistochemistry studies, FFPE (formalin fixed paraffin embedded) 3 μm colon and MLN samples (n = 4/each time point) were cut using a Leica Manual Microtome, mounted on adhesive slides, left at 20 ℃ overnight and then baked for 1 h at 65 ℃ the next day. Samples were incubated in antigen unmasking solution (citrate-based, pH = 6.0; Vector Laboratories, Burlingame, CA, United States) at 100 ℃ for 40 min and blocked with SuperBlock Blocking Buffer (Thermo Scientific, Waltham, MA, United States) for 20 min at RT. Primary antibodies CD4 (Rabbit, 0.623 mg/mL, Abcam), CD8 (Rabbit, 1 mg/mL, Abcam), F4/80 (Rabbit, 0.23 mg/mL, Novus Biologicals), B220 (Rat, 0.5 mg/mL, Invitrogen) and CD25 (Goat, 0.2 mg/mL, Invitrogen) were incubated at RT for 1 h. Samples were incubated with Peroxidazed 1 (BioCare Medical, Pacheco, CA, United States) for 5 min at RT, followed by incubation with the respective secondary HRP (Horseradish Peroxidase) antibody for 30 min at RT. Samples were incubated with Immpact DAB (3,3-diaminobendizine) Peroxidase HRP substrate (Vector Laboratories, Burlingame, CA, United States) for 5 min at RT. All samples were counterstained for 5 min with warmed 60 ℃ Methyl Green (Vector Laboratories, Burlingame, CA, United States). Respective isotypes were also analyzed. Images were obtained on a Leica Aperio ScanScope CS using a 10 × air objective (NA = 0.75, Leica Microsystems, Buffalo Grove, IL, United States) and Aperio ImageScope software. Photomicrographs were obtained from the whole area of the colon or MLN and analyzed through ImageJ. The quantification was done following the ratio of positive cells by the total area, multiplied by 100 and represented in percentage.

Statistical analysis

Statistical analysis was performed using Prism 7 (Graph Pad Inc., La Jolla, CA, United States). Experiments were evaluated through multiple student's t-test and one-way ANOVA followed by Dunnett post-hoc test. $P < 0.05$ was considered statistically significant. Data are presented as mean ± SD.

RESULTS

This study demonstrates the progressive aggressiveness of colitis with increasing DSS concentration from 1%-3% based on clinical and histological results. That led us to focus on the evaluation of the proteomic profile and immune cell infiltration in the colon of mice ingesting 3% DSS. We observed worsening of colonic pathology with lymphocytic, macrophage and eosinophilic infiltration that was associated with increasing pro and anti-inflammatory cytokines, chemokines and trophic factors (CCTF) expression in the colon over day 2 to day 8.

Comparison between 1%, 2% and 3% DSS

Acute DSS chemically induced colitis was evaluated at three dose levels of 1%, 2% and 3% for 7 d in the drinking water of mice and the clinical course was monitored and scored for the presence of bloody stools, watery diarrhea and weight loss for 8 d. In comparison to the control group, all three percentages of DSS in water resulted in progressive and increased clinical scores (Figure 1). The 1% DSS group exhibited weight loss starting on day 7, while the 2% DSS group showed variability in decreasing weight starting at day 6 (Figure 1). In comparison, mice that ingested 3% DSS showed prominent weight loss from day 5, reaching around -20% by day 8. For all DSS groups, colon lengths significantly decreased ($P < 0.05$) compared to control mice (Figure 1). The mean splenic weights significantly increased ($P < 0.05$) in mice ingesting 2% and 3% DSS compared to the control group, indicative of a robust systemic immune response (Figure 1). Histological scores were significantly higher ($P < 0.05$) in the 2% and 3% DSS groups, with clear evidence of destruction of crypts, loss of goblet cells, depletion of the epithelial barrier and infiltration of neutrophils and eosinophils at time of euthanasia (day 8) on HE staining (Figures 2 and 3). While there was no difference in colon weights amongst the groups compared to control mice, colon shortening was apparent in the 3% DSS cohort starting on day 3 (Figure 3). Splenic weights were significantly increased at day 6 and day 8, representing a systemic response one day before 3% DSS withdrawal and an increased histological inflammation (Figure 3). Overall, mice ingesting 3% DSS had greater clinical scores, weight loss, colon shortening, spleen weights and histological scores that led us to investigate the proteomic and immunological changes over time.

Colon proteomics

Proteomic changes following the introduction of 3% DSS in water were determined based on multiplex ELISA and showed a significant increase ($P < 0.05$, ANOVA compared to control) in expression of pro-

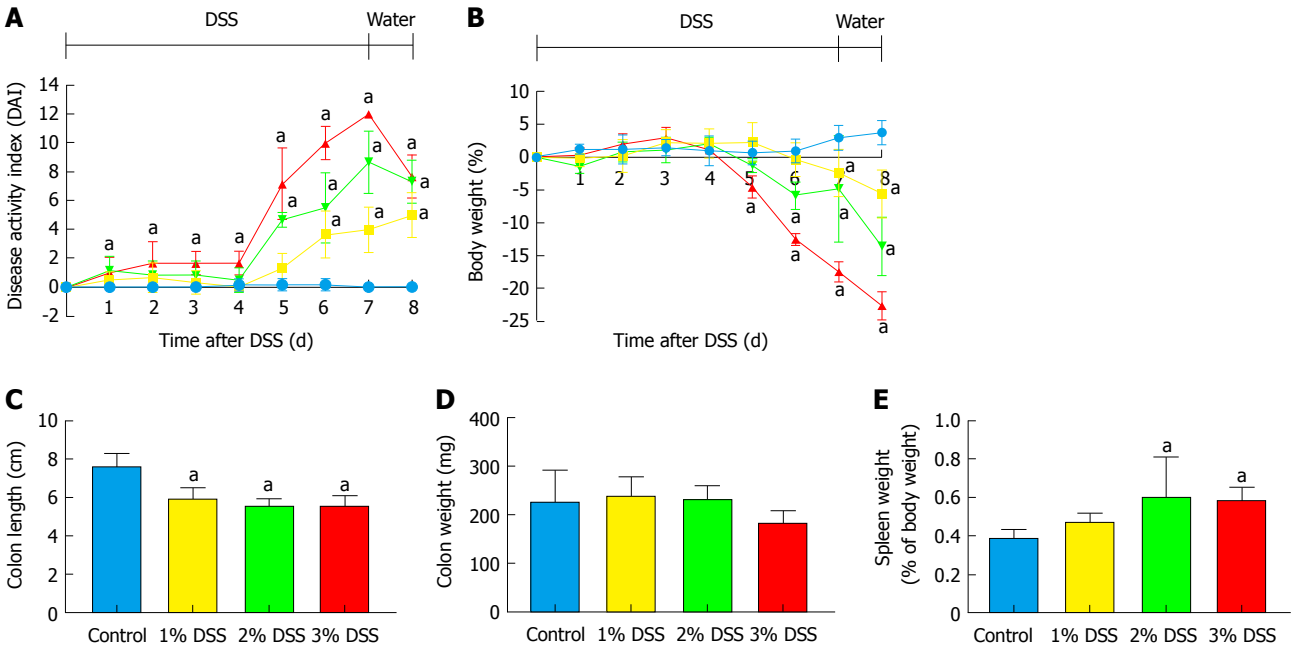


Figure 1 Comparison between 1%, 2% and 3% dextran sulfate sodium. Experimental colitis was induced by the administration of DSS in drinking water for 7 d at three different concentrations. Were performed daily clinical evaluations for (A) disease activity index and (B) weight loss. At the end of 8 d, animals were euthanized and were collected measurements for (C) colon length, (D) colon weight and (E) spleen weight. Multiple student's t test for clinical scores and one-way ANOVA followed by Dunnett post-hoc test for colon length, colon weight and spleen weight. [a]$P < 0.05$ *vs* control. $n = 6$/DSS group. DSS: Dextran sulfate sodium.

inflammatory cytokines and chemokines: IL-1α, IL-1β, IL-5, IL-6, IL-9, IL-17, eotaxin, granulocyte-colony stimulating factor (G-CSF), interferon γ (IFN-γ), keratinocyte chemoattractant (KC), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte chemoattractant protein 1 (MCP-1), monokine induced by gamma interferon (MIG), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, MIP-2, regulated on activation, normal T cell expressed and secreted (RANTES) and TNFα starting at day 3 (Figure 4; Supplementary Figure 1 for raw data). Both pro and anti-inflammatory CCTF were elevated on day 8, when animals were ingesting water. There was a significant ($P < 0.05$) decrease in detection of IL-2, IL-10, IL-15, macrophage-colony stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF) compared to control while animals were administered DSS 3%. We observed no changes in expression of IL-4, IL-7, IL12p40 and IL12p70 while animals ingested 3% DSS (Figure 4; Supplementary Figure 1). These results show that there are progressive inflammatory alterations in the colonic microenvironment that peaks one day after discontinuing DSS.

Immune cells reaction to 3% DSS

Immune cells population trafficking into the colon from the spleen and MLN were characterized from mice receiving 3% DSS by flow cytometry and IHC. The presence of macrophages (F4/80$^+$) progressively increased ($P < 0.05$) in the colon from day 2 to day 8, while the spleen and MLN did not show differences when compared to control, besides a small increase in the MLN at day 4 (Figures 5 and 6). This observation would suggest that monocyte tropism to the colon probably originated from bone marrow instead of the secondary lymphoid organs. Cytotoxic T cells and Th cells were significantly elevated ($P < 0.05$) in the colon starting around day 6 and day 8, whereas Treg started to increase on day 4 as a countermeasure to the inflammatory environment in the colon. In comparison, Th cells were decreased only in the spleen from day 4 and forward, while both spleen and MLN demonstrated lower levels of Tcyt cells around day 6 and day 8. Treg were significantly ($P < 0.05$) decreased in the spleen and MLN starting on day 4 and day 6. B-cells (B220$^+$) were significantly ($P < 0.05$) elevated in the MLN on day 8 after cessation of DSS, otherwise there was no changes compared to the control in the spleen and colon during the experiment. Figure 6 summarizes the fold changes in immune cell populations compared to day 0 (control) in the colon, spleen and MLN over the course of 8 d in this experiment and depict the trafficking of cells from secondary lymphoid tissues into the colon that resulted in an inflammatory response to 3% DSS. Detailed data on flow cytometry analysis can be found in supplementary figures 2 and 3. Images from HE and/or IHC analysis can be found in supplementary figures 4 and 5.

DISCUSSION

DSS is a chemically induced model of colitis characterized by a disruption of the epithelial barrier, resulting in microfloral substances entering the colonic mucosa and activating an innate immune response that produces local inflammatory factors[24]. It closely resembles human UC, which affects over 3.5 million people worldwide[25,26].

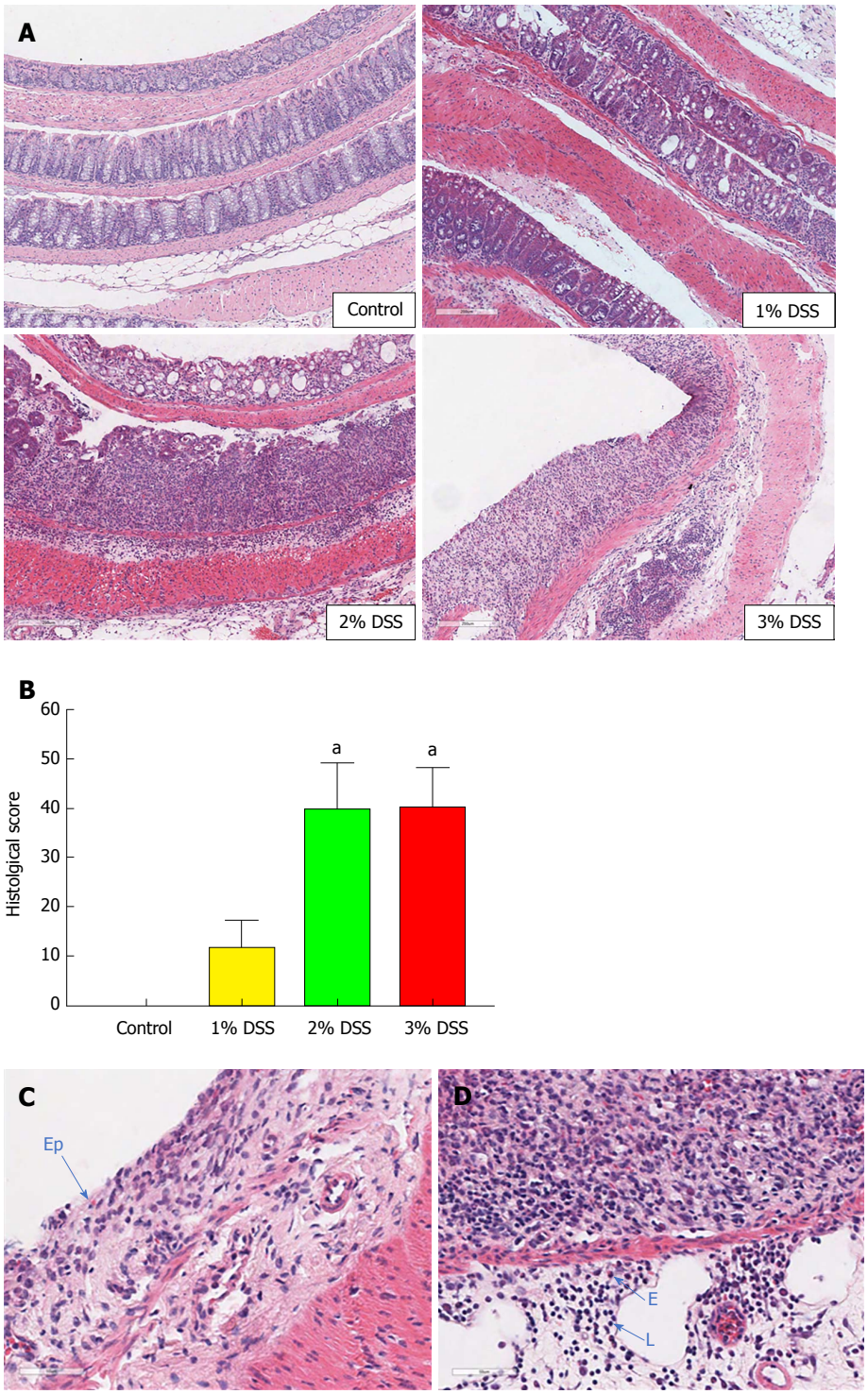


Figure 2 Histological comparisons between 1%, 2% and 3% dextran sulfate sodium. At the end of 8 d, the difference between DSS concentration was visible in (A) colonic damage (crypt depletion, inflammation, loss of epithelial barrier) compared to control. (B) Histological scores were similar between 2% and 3% DSS, where at the end of 8 d it is possible to visualize (C) the loss of the Ep and (D) infiltration of eosinophils (E) and lymphocytes (L). One-way ANOVA followed by Dunnet post-hoc test. $^{a}P < 0.05$ compared to control. n = 4/DSS group. DSS: Dextran sulfate sodium; Ep: Epithelial layer.

The acute tissue damage is characterized by a Th1/Th17 immune cell profile that leads to disease progression[15]. Previous studies have approached the analysis of acute DSS colitis by focusing on individual pathological features of the disease[9,15,16,19-21]. However, in the current study we demonstrate the temporal changes in clinical symptoms, histological features, immune cell population and proteomic response during the acute phase of DSS colitis.

We observed that the severity of experimental colitis was dependent on DSS concentration, and that clinical changes started as early as day 1, following initial ingestion. Increasing DSS concentration correlated with clinical disease severity, although on gross pathological

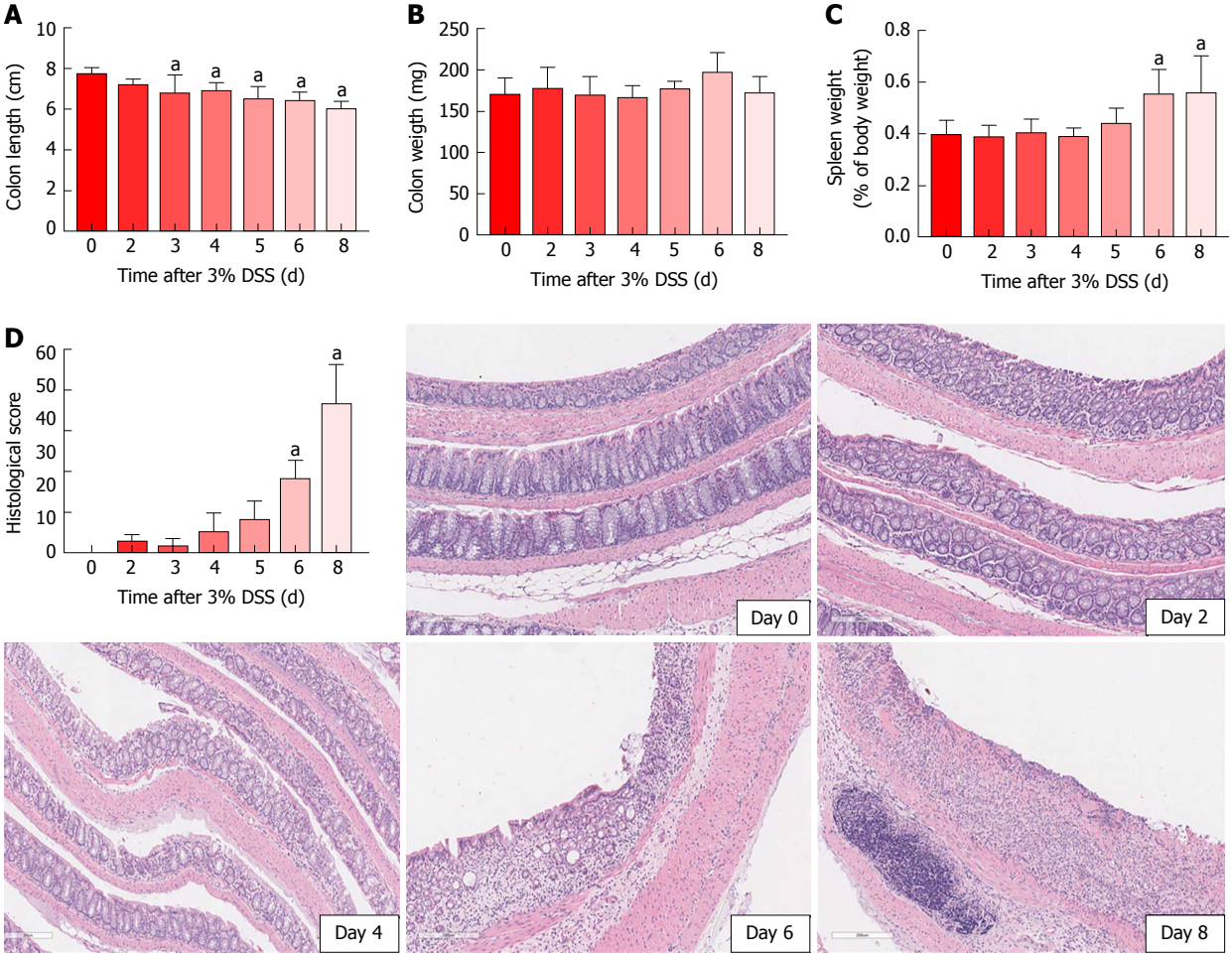


Figure 3 Colon and spleen in 3% dextran sulfate sodium time course. Experimental colitis was induced by the administration of DSS 3% in drinking water for 7 d. Colon shortening started as early as day 3 (A), with no changes in colon weight (B). Spleen weight was increased on day 6 and day 8 (C), at the same time as histological scores were augmented, demonstrating crypt depletion, loss of goblet cells, loss of the epithelial layer and inflammatory cells' infiltration (D). One-way ANOVA followed by Dunnett post-hoc test. $^{a}P < 0.05$ compared to control (day 0). n = 4/each time point for histology and n = 6/each time point for colon and spleen measurements. DSS: Dextran sulfate sodium.

examination all groups presented the same level of colon shortening. The difference between clinical and pathology suggest a mismatch (*i.e.*, clinical disease severity does not correlate with histological scores) in the DSS colitis model. Our data contradicts the previous report[9], in which animals started to improve clinically and histologically after DSS withdrawal. These differences between studies may reflect the influence of the microbiome and/or the animals' age, as previously reported in experimental DSS colitis[27,28]. In addition, it has been reported that UC patients in clinical and endoscopic based remission presenting with active histological inflammation possess a higher risk for clinical relapse[29,30]. In this way, our study may provide an understanding of the pathological and clinical response of severe human UC, with higher chances of relapsing and chronic disease. Since histological improvement could be seen as a new therapeutic approach and predictor of clinical relapse[31,32], the DSS clinical and molecular time course may be useful for evaluating novel therapeutic approaches with the goal of clinical pathological complete remission.

Morphological examination of the colon following 7 d of 3% DSS ingestion revealed that there is colonic shortening starting by day 3 and progressively decreases in size to day 8. In comparison, the weight of the colon does not change during DSS ingestion but splenic weight increases on day 6 and day 8, in agreement to previous studies in which splenic hypertrophy was observed in DSS colitis[33-36]. The increase in splenic size may represent congestion associated with an apparent proliferation of immune cells by HE staining. 3% DSS induces temporal changes in CCTF during the 8 d that can be segregated into four patterns: (pattern A) Progressive decreased expression of CCTF starting around day 4 (*i.e.*, IL-2, IL-10 and IL-15); (pattern B) progressive increased expression of CCTF starting around day 4 (*i.e.*, IL-6, Eotaxin, G-CSF, KC, LIF, MCP-1, MIP-2 and TNFα); (pattern C) increased expression of CCTF after stopping 3% DSS (*i.e.*, day 8) of IL-1α, IL1β, IL-5, IL-17, IFN-γ, LIX, MIG, MIP-1a MIP-1b, and RANTES; and (pattern D) little or no change in CCTF from controls (*i.e.*, IL-4, IL-7, IL-9, IL-12(p40), IL-12(p70), M-CSF and VEGF). The four patterns contain a mixture of pro-inflammatory and anti-inflammatory CCTF as well as chemoattractants associated with the influx of immune cell populations (*i.e.*, neutrophils,

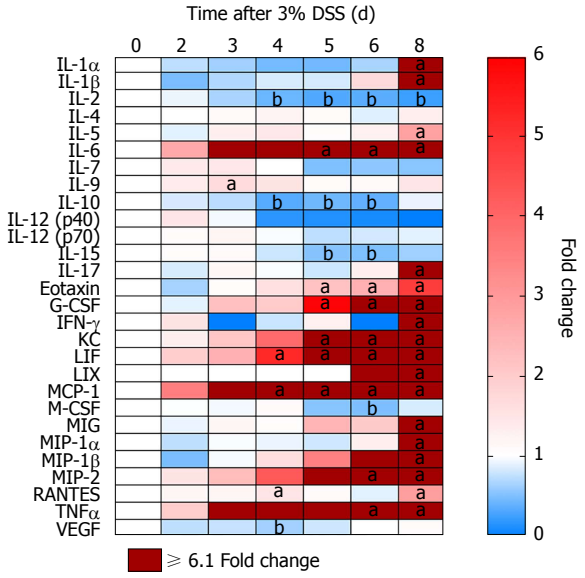


Figure 4 Colonic proteomic analysis from 3% dextran sulfate sodium time course. After induction of ulcerative colitis with 3% DSS, colon samples were collected at different time points from day 0 to 8. Proteomics was analyzed through multiplex ELISA assay and revealed four patterns: (pattern A) Progressive decreased expression of IL-2, IL-10 and IL-15 around day 4; (pattern B) Progressive increased expression IL-6, Eotaxin, G-CSF, KC, LIF, MCP-1, MIP-2 and TNFα around day 4; (pattern C) Increased expression of CCTF after stopping 3% DSS (*i.e.*, day 8) of IL-1α, IL1β, IL-5, IL-17, IFN-γ, LIX, MIG, MIP-1α MIP-1β, and RANTES; and (pattern D) Little or no change in IL-4, IL-7, IL-9, IL-12(p40), IL-12(p70), M-CSF and VEGF. One-way ANOVA followed by Dunnett post-hoc test. [a]$P < 0.05$ fold increases compared to control (day 0). [b]$P < 0.05$ fold decreases compared to control (day 0). n = 5-6/each time point. DSS: Dextran sulfate sodium; G-CSF: Granulocyte-colony stimulating factor; KC: Keratinocyte chemoattractant; LIF: Leukemia inhibitory factor; MCP-1: Monocyte chemoattractant protein 1; TNFα: Tumor necrosis factor alpha; IFN-γ: Interferon γ; LIX: Lipopolysaccharide-induced CXC chemokine; MIP-1α: Macrophage inflammatory protein 1α; RANTES : Regulated on activation, normal T cell expressed and secreted.

eosinophils and macrophages) into the inflamed colon associated with loss of the normal epithelial barrier. For patterns A and B, changes in CCTF expression coincided with clinical worsening of colitis.

The decreased expression of IL-2, IL-10, and IL-15 starting on day 4 corresponds to the inflammatory response and progressive colonic damage. Decreased expression of IL-2 was previously seen in mononuclear cells derived from UC patient's gut mucosa[37,38], as well as the disruption of the IL-2 gene in an animal model that exacerbated activation of lymphocytes, resembling autoimmunity[39,40]. In addition, the decreased expression of IL-10 would result in increased mucosal barrier disruption and increased TNFα and reactive oxygen species in the DSS model[41]. In the current study, there was a decrease in IL-15 starting at day 4, which should have attenuated colitis, based on results from DSS administration in the knockout mouse model[42]. It has been reported that the absence of IL-15 provokes a decrease in Foxp3 (Treg) and an increase in RORγt (Th17) by $CD4^+$ T cells in the colon[43]. Such effect was not observed in our study, possibly due to a significant increase in IL-17 that may have contributed with other CCTFs to disease worsening.

Starting around day 4 of 3% DSS exposure, a progressive increase in IL-6, Eotaxin, G-CSF, KC, LIF, MCP-1, MIP-2 and TNFα was observed. IL-6 and TNFα interfere with epithelial tight junctions, increasing intestinal barrier permeability allowing for water loss and the paracellular influx of molecules including the intrusion of pathogens that perpetuates the inflammatory process[44,45]. Elevation in IL-6 levels has an anti-apoptotic effect on lymphocytes, in addition to the increase of adhesion molecules that facilitate their migration to the gut[46,47]. Eotaxin, G-CSF, KC, LIF, MCP-1 and MIP-2 are chemokines associated with the influx of eosinophils, neutrophils and macrophages in the colon in active IBD[48-50]. Eotaxin is observed in DSS induced eosinophilic inflammation and promotes the recruitment of $F4/80^+CD11b^+CCR2^+Ly6C^{high}$ inflammatory monocytes to the colon that correlates with eosinophilic inflammation[50]. MIP-2 has also been associated with increased inflammatory response in DSS induced colitis with increased myeloperoxidase activity and neutrophils infiltration in the colon and small intestine in a transgenic mouse model[51].

Increased expression of G-CSF, KC, LIF, and MCP-1 in DSS models has been associated with anti-apoptotic, anti-inflammatory phenotype with improvement in clinical scores, regulation of the immune response and morphological changes in the colon. G-CSF has been reported to reduce apoptosis of epithelial cells and along with other cytokines, helps in bacterial clearance through neutrophil recruitment to maintain the mucosal barrier integrity in IBD[52-54]. Treatment with recombinant G-CSF ameliorated DSS colitis by attenuating weight loss, stool score and shortening of the colon. In addition, inflammation, epithelial damage and cell apoptosis were attenuated in the rectum[54]. DSS acute colitis in KC deficient mice results in the increase of weight loss, bloody stools, inflammation and a moribund appearance, presenting higher histological scores but lower neutrophil infiltration compared to wild type (WT) animals[55]. LIF was found to be elevated in IBD patients[56] and has been shown to act in tissue damage by recruiting inflammatory cells to the injury site[57,58]. However, studies have shown that LIF also has an anti-inflammatory effect, stimulating repair and up-regulating Treg cells[58,59]. In our study, LIF expression increased on day 6 and could be responsible for modulating inflammation in the colon. In UC patients, the level of MCP-1 is directly related to disease activity[20,60-66]. It has been reported that intraperitoneal administration of MCP-1 significantly inhibited acute DSS colitis with lower clinical scores, increased survival, reduced weight loss, decreased production of IL-12 and IFN-γ associated with less inflammation[67]. MCP-1 may contribute to inflammation and colon shortening in our study, as well as inducing the elevation of pro-inflammatory CCTF by the end of 8 d. Although several CCTF included in Pattern B could be associated with improvements in clinical and pathological outcomes, in the current study, a predominant inflammatory microenvironment with disruption of the epithelium and infiltration of N and E into the lamina propria was observed in the colon.

Following cessation of DSS on day 7 (*i.e.*, Pattern C), we detected significant increased expression of IL-

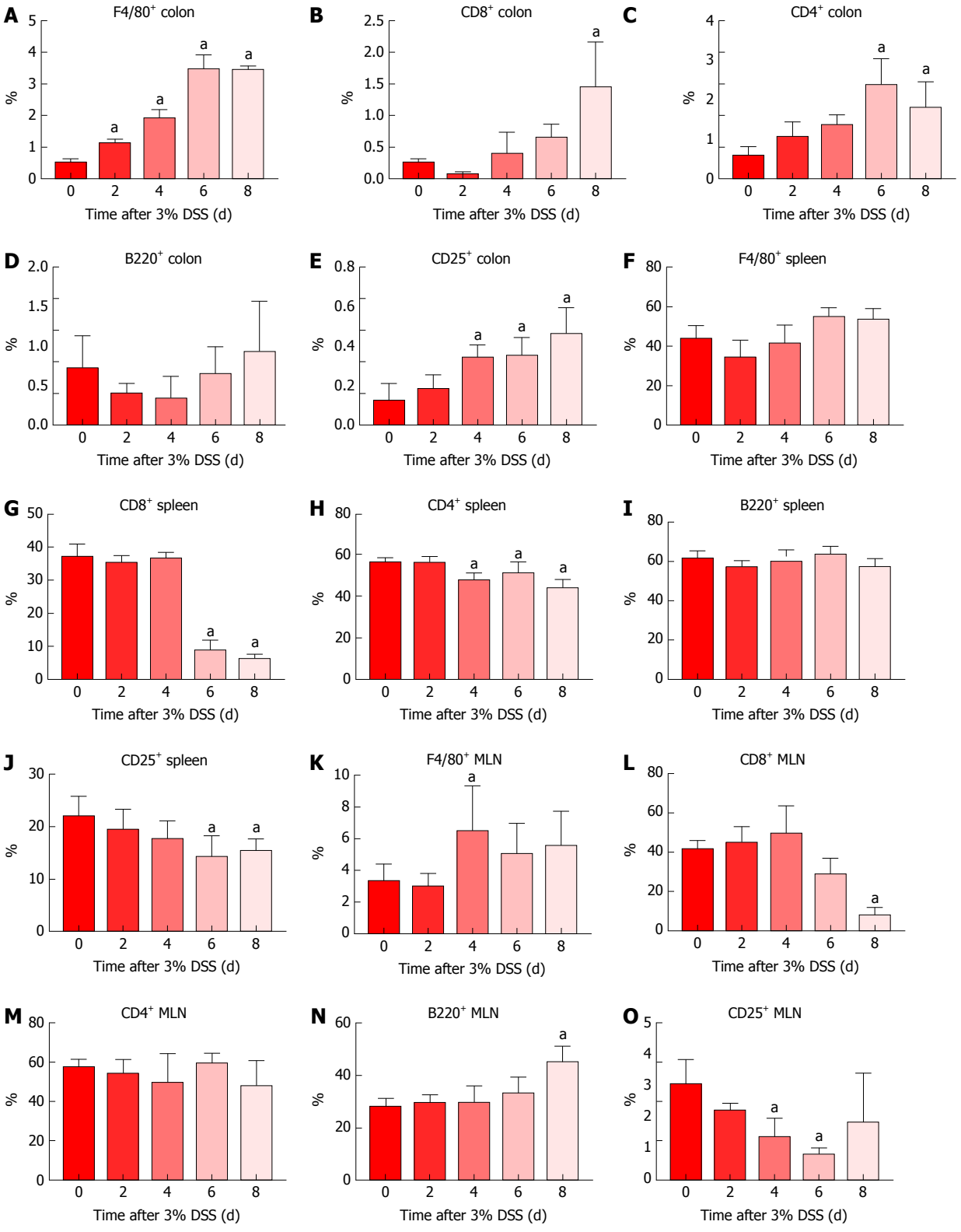


Figure 5 Immune cell profile in the colon, spleen and mesenteric lymph nodes of 3% dextran sulfate sodium animals. Tissue samples were collected at different time points and IHC or flow cytometry were performed for temporal analysis of immune cells' profile. Results showed progressive increased presence of colonic (A) F4/80$^+$ macrophages, (B) CD8$^+$ Tcyt cells, (C) CD4$^+$ Th cells and (E) CD25$^+$ Treg cells and no change in (D) B220$^+$ B cells. There was no difference in splenic (F) F4/80$^+$ macrophages and (I) B220$^+$ B cells, however, there was a decrease in (G) CD8$^+$ Tcyt cells, (H) CD4$^+$ Th cells and (J) CD25$^+$ Treg cells starting at day 4. MLN analysis resulted in decreased levels of (L) CD8$^+$ Tcyt cells and (O) CD25$^+$ Treg cells, along with a slight increase of (K) F4/80$^+$ macrophages at day 4, (N) B220$^+$ B cells at day 8 and no changes in (M) CD4$^+$ Th cells. One-way ANOVA followed by Dunnett post-hoc test. $^a P < 0.05$ compared to control (day 0). n = 4/each time point analyzed through IHC (All colon samples and MLN F4/80$^+$ and CD25$^+$). n = 6/each time point analyzed through flow cytometry (All spleen samples and MLN CD8$^+$, CD4$^+$ and B220$^+$). MLN: Mesenteric lymph nodes; DSS: Dextran sulfate sodium.

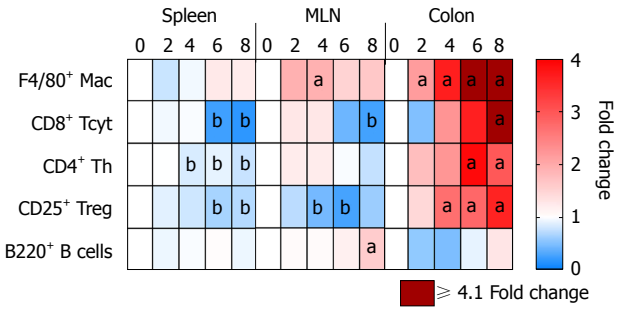


Figure 6 Immune cell population in the spleen, mesenteric lymph nodes and colon of 3% dextran sulfate sodium animals. Tissue samples were collected from 3% DSS animals at different time points and analyzed through either immunohistochemistry or flow cytometry. The heatmap shows a possible movement of different immune cell types from the spleen and MLN into the gut progressively during the 8 d of disease. [a]$P < 0.05$ increased fold changes compared to control (day 0). [b]$P < 0.05$ decreased fold changes compared to control (day 0). MLN: Mesenteric lymph nodes; DSS: Dextran sulfate sodium.

1α, IL1β, IL-5, IL-17, IFN-γ, LIX, MIG, MIP-1a MIP-1b, and RANTES in the colon on Day 8. It is unclear how Pattern C relates to the removal of the DSS and the apparent rebound of primarily pro-inflammatory CCTF in the microenvironment. IL-1α and IL-1β can be potent chemoattractants for neutrophils and macrophages into the microenvironment by the induction and propagation of the inflammatory response[68]. IL-1α and IL-1β can induce increases in pro-inflammatory chemokines as well as cell adhesion molecules, activate macrophages, dendritic cells and neutrophils, and support Th17 cells' differentiation[69,70]. IL-5 is characteristic of a Th2 immune response in UC that stimulates eosinophil growth, development, survival and activation, mobilizing them from the bone marrow to the peripheral blood. IL-5 along with Eotaxin serve as chemoattractants of E to the gut[71-75]. IL17 may also play a dual role in DSS colitis models as it has been reported to stimulate the production of matrix metalloproteinase, increase the expression of other pro-inflammatory factors (*e.g.*, IL-6, IL-1β, TNFα, KC, MCP-1, MIP-2, GM-CSF), and to be involved in the proliferation, maturation and chemotaxis of N[76-80]. In IL-17A deficient mice, the DSS colitis model is associated with improved survival and histological scores with less epithelial damage and immune cell infiltration in the intestine, when compared to WT mice[13]. However, studies have shown IL-17 to stabilize the epithelial barrier and to aggravate colitis when absent in the animal[76,81,82]. In the current study, it is unclear if IL-17 contributes to the inflammatory responses in the colon or improvement in the clinical score at the end of 8 d.

IFN-γ expression was significantly increased at day 8 and is highly expressed by $CD8^+$ T cells from IBD patients, when in contact with colonic epithelial cells[83]. IFN-γ stimulates the disruption of the intestinal epithelial barrier and supports the exacerbated immune response in IBD[44,84]. It is also essential for DSS colitis model, since IFN-$\gamma^{-/-}$ knockout mice do not develop colitis when challenged with DSS[84]. In this study, high levels of Tcyt cells and F4/80 macrophages were found in the colon that may be responsible for the increased levels of IFN-γ and the observed inflammatory response. LIX was found to be elevated in UC patients and contributes to the inflammatory response in DSS colitis. Of note, pre-treatment of mice with antisense oligonucleotides to LIX in the DSS colitis model reduces neutrophils' infiltration and the severity of the disease[85]. MIG can act as a chemoattractant for activated Tcyt cells, E and natural killer (NK) cells, along with having an angiostatic effect on endothelial cells by inhibiting cell division in colitis models[86,87]. MIP1-α and MIP1-β are chemoattractants for T cells into the lamina propria that can lead to mucosal damage and worsening of colitis[88,89]. Finally, RANTES has been shown to be elevated in chronic experimental colitis[90] and in the colonic mucosa of IBD patients, supporting both innate and adaptive immune responses[61,90-93]. Taken together, Pattern C appears to be associated with a rebound increased expression of inflammatory CCTF that contributed to colon pathology and higher histological scores.

Pattern D includes IL-4, IL-7, IL-9, IL-12(p40), IL-12(p70), M-CSF and VEGF that do not significantly change or were elevated at a random single time point over 8 d. IL-9 was elevated at day 3 coinciding when colon shortening was first detected. T cells expressing IL-9 are found in the intestinal mucosa in experimental colitis and UC patients. IL-9 is responsible for disruption of the intestinal barrier and the impairment of mucosal tissue repair through suppression of epithelial cell proliferation[94-96], which relates to the mucosal injury during the disease course. M-CSF, which mainly induces M2 macrophage phenotype[97-99], that was significantly ($P < 0.05$) decreased at day 6, when the pro-inflammatory CCTFs in Pattern B were increasing, associated with a possible predominance of M1 macrophages. M-CSF has also been proposed as an alternative therapy in treating UC and DSS colitis[100,101]. VEGF was found downregulated at day 4 and is usually found elevated in DSS model, however, it is also associated with lymphangiogenesis, which would aid in the clearance of interstitial fluid and immune cells from the colon[102-104]. With low VEGF levels, there could be impaired drainage function and lymphatic obstruction[103,105], leading to the accumulation of immune cells in the gut and disease worsening. In summary, the CCTF profiles observed following DSS are consistent of a pro-inflammatory microenvironment. The changes in CCTF over time serve as chemoattractants for neutrophils, eosinophils and macrophages as well as disrupting the integrity of the epithelial barrier and production of mucin. The inflammatory response in the colon leads to a clinical course that includes a malabsorption like syndrome accompanied by weight loss, bloody stools and diarrhea.

Analysis of immune cell populations revealed a progressive accumulation of macrophages and T cells (Th, Tcyt and Treg cells) in the colon compared to controls. It has been previously shown[16] that colonic $CD3^+$ T cells and $F4/80^+$ macrophages were upregulated in later time

points when evaluating acute and chronic DSS colitis, as compared to the current study. In addition, splenic and MLN F4/80$^+$ population were highly elevated whereas we did observe little or no change compared to control mice. Another study has reported that intestinal inflammation in UC presents as the initial fast response with increased number of macrophages originating from tissue-resident or infiltrating systemic macrophages in the intestinal mucosa[106]. The early arrival of macrophages in the gut at day 2 contributes to the initiation of inflammation coinciding with increase in pro-inflammatory CCTF and translating into clinical symptoms.

In the current study, T-cell phenotypes in the colon begin to increase 4 d after initiation of 3% DSS, which contributes to the perpetuation of inflammation and the high levels of CCTF seen at day 8. Decreased levels of Th and Tcyt cells were observed in the spleen, and to lesser extent in the MLN, starting at day 6 when compared to controls. Moreover, there were no significant differences in percentage of B220$^+$ cells in the colon or lymphoid organs compared to control levels, other than an increase in the MLN at day 8, suggesting the transition to a chronic state. Although T and B cells are not required for the development of DSS colitis[6], in this study T cells appear to contribute to the activation of the inflammatory response in the colon. In addition, Treg cells seem to be leaving the secondary lymphoid organs migrating towards the colon in an attempt to contain the exacerbated immune response.

Conclusion

There have been few reports describing the temporal distribution of immune cells along with proteomic changes in the colon in DSS colitis model[16,17]. In this study we observed that severity of colitis is dependent on DSS concentration, while presenting discrepancies amongst clinical and pathological results. When mice were administered 3% DSS, we observed increased clinical and histological scores that were accompanied by changes in CCTF and immune cell infiltration in the colon, with important participation of the secondary lymphoid organs. One limitation of this study is that DSS colitis was induced in C57Bl/6 mice from a single vendor and it is unclear if the same mouse strain containing a different microbiota may have influenced the temporal clinical, proteomic and pathological changes we observed in the current study. Further investigations are needed to determine the role of the gut flora in the development of colitis and the response to novel therapeutic interventions that could translate to clinical trials. Furthermore, acknowledging the time frame where these factors play a role in developing novel therapies for treating ulcerative colitis.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is an inflammatory bowel disease that affects the colon and the rectum, being characterized by uncontrolled immune response and inflammation. There is no specific cause for this disease and no current treatment that provides sustained remission. The animal model of colitis induced by dextran sulfate sodium (DSS) is largely used as a tool to better investigate human UC. Although not completely understood, DSS induces an uncontrolled immune response through disruption of the epithelial layer, providing a higher access of antigens to the colonic mucosa, this way perpetuating inflammation and tissue destruction.

Research motivation

There is no current study providing a detailed integrative temporal analysis of DSS-induced acute colitis regarding clinical symptoms, proteomics, immune cell profile and histology. Understanding the interaction of these factors may contribute to the research of novel UC therapies.

Research objectives

The aim of this study was to compare different concentrations of DSS in the induction of acute colitis, followed by a temporal analysis of clinical symptoms, colon proteomics, immune cell profile and histology of the most characteristic presentation of colitis amongst the different DSS concentrations. The changes seen throughout the 8 d may provide a clearer understanding of the DSS model mechanisms.

Research methods

1%, 2% and 3% DSS in drinking water was used for the induction of acute colitis. Clinical symptoms were daily scored for weight loss, stool consistency and blood in the stool. After 8 d, colon, spleen and mesenteric lymph nodes (MLN) were collected. Histological scores were evaluated through HE staining and grading of colonic samples for inflammation, extent, regeneration, crypt damage and percent of involvement. Colon proteomics was analyzed through multiplex ELISA for 3% DSS at different time points, in addition to immune cell profiling of the colon, spleen and MLN through immunohistochemistry and flow cytometry for B220$^+$, CD4$^+$, CD8$^+$, CD25$^+$ and F4/80$^+$ cells.

Research results

Severity of colitis is related to the increase in DSS concentration. When analyzing 3% DSS-induced colitis, worsening of histological inflammation agrees with an increase of immune cells' influx to the colon and changes in the pro- and anti-inflammatory cytokines colonic profile. Macrophages are the first ones to respond to the damage caused by DSS, followed by changes in the colonic cytokine profile and influx of CD25$^+$ T cells. Next, there is an increase in colonic CD4$^+$ and CD8$^+$ T cells and the highest level of pro-inflammatory cytokines is seen at day 8. Levels of T cells are progressively decreased in the spleen and MLN, while worsening of clinical symptoms corresponds with the progressive increase in histological inflammation, with exception of day 8.

Research conclusions

Our study demonstrates the correlated temporal changes of clinical, proteomic, immunological and histological characteristics of DSS-induced acute colitis. There is an important initial response by the innate immune system, mainly coordinated by macrophages, followed by increasing inflammation, further tissue damage and influx of T cells. T cells may be leaving the secondary lymphoid organs progressively towards the gut, as a response to the changes in colonic cytokine levels. There is a mixed response of pro- and anti-inflammatory cytokines in the colon, with the highest increase occurring after DSS withdrawal. Interestingly, amelioration of clinical symptoms is seen on day 8, demonstrating a mismatch to the histological/immunological/proteomic worsening of the disease. Since histological inflammation is seen in UC patients with endoscopic and clinical remission, this model could be used as a tool for the development of novel therapies targeting complete remission and prevention of disease relapse.

Research perspectives

Our study demonstrates that no individual factor develops this disease model, but rather a coordination between anti- and pro-inflammatory cytokines. Therefore, researchers should seriously consider a temporal analysis before investigating new therapies. The disease course here described would be

highly recommended for the study of novel treatments aiming resolution of histological inflammation during disease remission. Further temporal analysis of DSS-induced chronic colitis would add to a better understanding of this animal model.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Programs of the Clinical Center, the National Institute of Biomedical Imaging and Bioengineering at the National Institutes of Health and CAPES (Coordination for the Training of Higher Education Personnel Ministry of Education) from Brazil.

REFERENCES

1 **Strober W**, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007; **117**: 514-521 [PMID: 17332878 DOI: 10.1172/JCI30587]
2 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434 [PMID: 17653185 DOI: 10.1038/nature06005]
3 **Low D**, Nguyen DD, Mizoguchi E. Animal models of ulcerative colitis and their application in drug research. *Drug Des Devel Ther* 2013; **7**: 1341-1357 [PMID: 24250223 DOI: 10.2147/DDDT.S40107]
4 **Mizoguchi A**. Animal models of inflammatory bowel disease. *Prog Mol Biol Transl Sci* 2012; **105**: 263-320 [PMID: 22137435 DOI: 10.1016/B978-0-12-394596-9.00009-3]
5 **Kiesler P**, Fuss IJ, Strober W. Experimental Models of Inflammatory Bowel Diseases. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 154-170 [PMID: 26000334 DOI: 10.1016/j.jcmgh.2015.01.006]
6 **Chassaing B**, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol* 2014; **104**: Unit 15.25 [PMID: 24510619 DOI: 10.1002/0471142735.im1525s104]
7 **Gonçalves FC**, Schneider N, Mello HF, Passos EP, Meurer L, Cirne-Lima E, Paz AH. Characterization of Acute Murine Dextran Sodium Sulfate (DSS) Colitis: Severity of InFammation is Dependent on the DSS Molecular Weight and Concentration. *Acta Scientiae Veterinariae* 2013; **41**: 1-9
8 **Padua D**, Vu JP, Germano PM, Pisegna JR. The Role of Neuropeptides in Mouse Models of Colitis. *J Mol Neurosci* 2016; **59**: 203-210 [PMID: 26646243 DOI: 10.1007/s12031-015-0688-1]
9 **Yan Y**, Kolachala V, Dalmasso G, Nguyen H, Laroui H, Sitaraman SV, Merlin D. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. *PLoS One* 2009; **4**: e6073 [PMID: 19562033 DOI: 10.1371/journal.pone.0006073]
10 **Okayasu I**, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990; **98**: 694-702 [PMID: 1688816 DOI: 10.1016/0016-5085(90)90290-H]
11 **Fuss IJ**, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 1996; **157**: 1261-1270 [PMID: 8757634]
12 **Sands BE**, Kaplan GG. The role of TNFalpha in ulcerative colitis. *J Clin Pharmacol* 2007; **47**: 930-941 [PMID: 17567930 DOI: 10.1177/0091270007301623]
13 **Ito R**, Kita M, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, Imanishi J, Iwakura Y, Okanoue T, Yoshikawa T, Kataoka K, Mazda O. Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. *Biochem Biophys Res Commun* 2008; **377**: 12-16 [PMID: 18796297 DOI: 10.1016/j.bbrc.2008.09.019]
14 **Sarra M**, Pallone F, Macdonald TT, Monteleone G. IL-23/IL-17 axis in IBD. *Inflamm Bowel Dis* 2010; **16**: 1808-1813 [PMID: 20222127 DOI: 10.1002/ibd.21248]
15 **Alex P**, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis* 2009; **15**: 341-352 [PMID: 18942757 DOI: 10.1002/ibd.20753]
16 **Hall LJ**, Faivre E, Quinlan A, Shanahan F, Nally K, Melgar S. Induction and activation of adaptive immune populations during acute and chronic phases of a murine model of experimental colitis. *Dig Dis Sci* 2011; **56**: 79-89 [PMID: 20467900 DOI: 10.1007/s10620-010-1240-3]
17 **Perše M**, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. *J Biomed Biotechnol* 2012; **2012**: 718617 [PMID: 22665990 DOI: 10.1155/2012/718617]
18 **Al-Haddad S**, Riddell RH. The role of eosinophils in inflammatory bowel disease. *Gut* 2005; **54**: 1674-1675 [PMID: 16284283 DOI: 10.1136/gut.2005.072595]
19 **Melgar S**, Karlsson A, Michaëlsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G1328-G1338 [PMID: 15637179 DOI: 10.1152/ajpgi.00467.2004]
20 **Fang K**, Grisham MB, Kevil CG. Application of Comparative Transcriptional Genomics to Identify Molecular Targets for Pediatric IBD. *Front Immunol* 2015; **6**: 165 [PMID: 26085826 DOI: 10.3389/fimmu.2015.00165]
21 **Fang K**, Bruce M, Pattillo CB, Zhang S, Stone R 2nd, Clifford J, Kevil CG. Temporal genomewide expression profiling of DSS colitis reveals novel inflammatory and angiogenesis genes similar to ulcerative colitis. *Physiol Genomics* 2011; **43**: 43-56 [PMID: 20923862 DOI: 10.1152/physiolgenomics.00138.2010]
22 **Gonçalves Fda C**, Schneider N, Pinto FO, Meyer FS, Visioli F, Pfaffenseller B, Lopez PL, Passos EP, Cirne-Lima EO, Meurer L, Paz AH. Intravenous vs intraperitoneal mesenchymal stem cells administration: what is the best route for treating experimental colitis? *World J Gastroenterol* 2014; **20**: 18228-18239 [PMID: 25561790 DOI: 10.3748/wjg.v20.i48.18228]
23 **Dieleman LA**, Palmen MJ, Akol H, Bloemena E, Peña AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998; **114**: 385-391 [PMID: 9844047 DOI: 10.1046/j.1365-2249.1998.00728.x]
24 **Kitajima S**, Takuma S, Morimoto M. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. *Exp Anim* 1999; **48**: 137-143 [PMID: 10480018 DOI: 10.1538/expanim.48.137]
25 **Kaplan GG**. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 720-727 [PMID: 26323879 DOI: 10.1038/nrgastro.2015.150]
26 **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]
27 **Roy U**, Gálvez EJC, Iljazovic A, Lesker TR, Błażejewski AJ, Pils MC, Heise U, Huber S, Flavell RA, Strowig T. Distinct Microbial Communities Trigger Colitis Development upon Intestinal Barrier Damage via Innate or Adaptive Immune Cells. *Cell Rep* 2017; **21**: 994-1008 [PMID: 29069606 DOI: 10.1016/j.celrep.2017.09.097]
28 **Park YH**, Kim N, Shim YK, Choi YJ, Nam RH, Choi YJ, Ham MH, Suh JH, Lee SM, Lee CM, Yoon H, Lee HS, Lee DH. Adequate Dextran Sodium Sulfate-induced Colitis Model in Mice and Effective Outcome Measurement Method. *J Cancer Prev* 2015; **20**: 260-267 [PMID: 26734588 DOI: 10.15430/JCP.2015.20.4.260]
29 **Lobatón T**, Bessissow T, Ruiz-Cerulla A, De Hertogh G, Bisschops R, Guardiola J, Van Assche G, Vermeire S, Ferrante M. Prognostic value of histological activity in patients with ulcerative colitis in deep remission: A prospective multicenter study. *United European Gastroenterol J* 2018; **6**: 765-772 [PMID: 30083339 DOI: 10.1177/2050640617752207]
30 **Narang V**, Kaur R, Garg B, Mahajan R, Midha V, Sood N, Sood A. Association of endoscopic and histological remission with clinical course in patients of ulcerative colitis. *Intest Res* 2018; **16**: 55-61

[PMID: 29422798 DOI: 10.5217/ir.2018.16.1.55]
31 **Azad S**, Sood N, Sood A. Biological and histological parameters as predictors of relapse in ulcerative colitis: a prospective study. *Saudi J Gastroenterol* 2011; **17**: 194-198 [PMID: 21546723 DOI: 10.4103/1319-3767.80383]
32 **Bryant RV**, Winer S, Travis SP, Riddell RH. Systematic review: histological remission in inflammatory bowel disease. Is 'complete' remission the new treatment paradigm? An IOIBD initiative. *J Crohns Colitis* 2014; **8**: 1582-1597 [PMID: 25267173 DOI: 10.1016/j.crohns.2014.08.011]
33 **Hong HS**, Hwang DY, Park JH, Kim S, Seo EJ, Son Y. Substance-P alleviates dextran sulfate sodium-induced intestinal damage by suppressing inflammation through enrichment of M2 macrophages and regulatory T cells. *Cytokine* 2017; **90**: 21-30 [PMID: 27750083 DOI: 10.1016/j.cyto.2016.10.002]
34 **Da Silva AP**, Pollett A, Rittling SR, Denhardt DT, Sodek J, Zohar R. Exacerbated tissue destruction in DSS-induced acute colitis of OPN-null mice is associated with downregulation of TNF-alpha expression and non-programmed cell death. *J Cell Physiol* 2006; **208**: 629-639 [PMID: 16741956 DOI: 10.1002/jcp.20701]
35 **Morteau O**, Morham SG, Sellon R, Dieleman LA, Langenbach R, Smithies O, Sartor RB. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. *J Clin Invest* 2000; **105**: 469-478 [PMID: 10683376 DOI: 10.1172/JCI6899]
36 **Axelsson LG**, Landström E, Bylund-Fellenius AC. Experimental colitis induced by dextran sulphate sodium in mice: beneficial effects of sulphasalazine and olsalazine. *Aliment Pharmacol Ther* 1998; **12**: 925-934 [PMID: 9768537 DOI: 10.1046/j.1365-2036.1998.00357.x]
37 **Fiocchi C**, Hilfiker ML, Youngman KR, Doerder NC, Finke JH. Interleukin 2 activity of human intestinal mucosa mononuclear cells. Decreased levels in inflammatory bowel disease. *Gastroenterology* 1984; **86**: 734-742 [PMID: 6607860]
38 **Van Damme N**, De Keyser F, Demetter P, Baeten D, Mielants H, Verbruggen G, Cuvelier C, Veys EM, De Vos M. The proportion of Th1 cells, which prevail in gut mucosa, is decreased in inflammatory bowel syndrome. *Clin Exp Immunol* 2001; **125**: 383-390 [PMID: 11531945 DOI: 10.1046/j.1365-2249.2001.01638.x]
39 **Van Parijs L**, Abbas AK. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 1998; **280**: 243-248 [PMID: 9535647 DOI: 10.1126/science.280.5361.243]
40 **Sadlack B**, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 1993; **75**: 253-261 [PMID: 8402910 DOI: 10.1016/0092-8674(93)80067-O]
41 **Li B**, Alli R, Vogel P, Geiger TL. IL-10 modulates DSS-induced colitis through a macrophage-ROS-NO axis. *Mucosal Immunol* 2014; **7**: 869-878 [PMID: 24301657 DOI: 10.1038/mi.2013.103]
42 **Yoshihara K**, Yajima T, Kubo C, Yoshikai Y. Role of interleukin 15 in colitis induced by dextran sulphate sodium in mice. *Gut* 2006; **55**: 334-341 [PMID: 16162679 DOI: 10.1136/gut.2005.076000]
43 **Tosiek MJ**, Fiette L, El Daker S, Eberl G, Freitas AA. IL-15-dependent balance between Foxp3 and RORγt expression impacts inflammatory bowel disease. *Nat Commun* 2016; **7**: 10888 [PMID: 26964669 DOI: 10.1038/ncomms10888]
44 **Martini E**, Krug SM, Siegmund B, Neurath MF, Becker C. Mend Your Fences: The Epithelial Barrier and its Relationship With Mucosal Immunity in Inflammatory Bowel Disease. *Cell Mol Gastroenterol Hepatol* 2017; **4**: 33-46 [PMID: 28560287 DOI: 10.1016/j.jcmgh.2017.03.007]
45 **Nava P**, Koch S, Laukoetter MG, Lee WY, Kolegraff K, Capaldo CT, Beeman N, Addis C, Gerner-Smidt K, Neumaier I, Skerra A, Li L, Parkos CA, Nusrat A. Interferon-gamma regulates intestinal epithelial homeostasis through converging beta-catenin signaling pathways. *Immunity* 2010; **32**: 392-402 [PMID: 20303298 DOI: 10.1016/j.immuni.2010.03.001]
46 **Allocca M**, Jovani M, Fiorino G, Schreiber S, Danese S. Anti-IL-6 treatment for inflammatory bowel diseases: next cytokine, next target. *Curr Drug Targets* 2013; **14**: 1508-1521 [PMID: 24102406 DOI: 10.2174/13894501131466602224]
47 **Katsanos KH**, Papadakis KA. Inflammatory Bowel Disease: Updates on Molecular Targets for Biologics. *Gut Liver* 2017; **11**: 455-463 [PMID: 28486793 DOI: 10.5009/gnl16308]
48 **Chen W**, Paulus B, Shu D, Wilson, Chadwick V. Increased serum levels of eotaxin in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2001; **36**: 515-520 [PMID: 11346206 DOI: 10.1080/003655201750153377]
49 **Ahrens R**, Waddell A, Seidu L, Blanchard C, Carey R, Forbes E, Lampinen M, Wilson T, Cohen E, Stringer K, Ballard E, Munitz A, Xu H, Lee N, Lee JJ, Rothenberg ME, Denson L, Hogan SP. Intestinal macrophage/epithelial cell-derived CCL11/eotaxin-1 mediates eosinophil recruitment and function in pediatric ulcerative colitis. *J Immunol* 2008; **181**: 7390-7399 [PMID: 18981162 DOI: 10.4049/jimmunol.181.10.7390]
50 **Waddell A**, Ahrens R, Steinbrecher K, Donovan B, Rothenberg ME, Munitz A, Hogan SP. Colonic eosinophilic inflammation in experimental colitis is mediated by Ly6C(high) CCR2(+) inflammatory monocyte/macrophage-derived CCL11. *J Immunol* 2011; **186**: 5993-6003 [PMID: 21498668 DOI: 10.4049/jimmunol.1003844]
51 **Ohtsuka Y**, Sanderson IR. Dextran sulfate sodium-induced inflammation is enhanced by intestinal epithelial cell chemokine expression in mice. *Pediatr Res* 2003; **53**: 143-147 [PMID: 12508094 DOI: 10.1203/00006450-200301000-00024]
52 **Martins A**, Han J, Kim SO. The multifaceted effects of granulocyte colony-stimulating factor in immunomodulation and potential roles in intestinal immune homeostasis. *IUBMB Life* 2010; **62**: 611-617 [PMID: 20681025 DOI: 10.1002/iub.361]
53 **Smith AM**, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, Palmer CD, Wilde J, Foxwell BM, Gloger IS, Sweeting T, Marsh M, Walker AP, Bloom SL, Segal AW. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* 2009; **206**: 1883-1897 [PMID: 19652016 DOI: 10.1084/jem.20091233]
54 **Kudo T**, Matsumoto T, Nakamichi I, Yada S, Esaki M, Jo Y, Ohji Y, Yao T, Iida M. Recombinant human granulocyte colony-stimulating factor reduces colonic epithelial cell apoptosis and ameliorates murine dextran sulfate sodium-induced colitis. *Scand J Gastroenterol* 2008; **43**: 689-697 [PMID: 18569986 DOI: 10.1080/00365520701864627]
55 **Shea-Donohue T**, Thomas K, Cody MJ, Aiping Zhao, Detolla LJ, Kopydlowski KM, Fukata M, Lira SA, Vogel SN. Mice deficient in the CXCR2 ligand, CXCL1 (KC/GRO-alpha), exhibit increased susceptibility to dextran sodium sulfate (DSS)-induced colitis. *Innate Immun* 2008; **14**: 117-124 [PMID: 18713728 DOI: 10.1177/1753425908088724]
56 **Andoh A**, Zhang Z, Inatomi O, Fujino S, Deguchi Y, Araki Y, Tsujikawa T, Kitoh K, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y. Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology* 2005; **129**: 969-984 [PMID: 16143135 DOI: 10.1053/j.gastro.2005.06.071]
57 **Guimbaud R**, Abitbol V, Bertrand V, Quartier G, Chauvelot-Moachon L, Giroud J, Couturier D, Chaussade DC. Leukemia inhibitory factor involvement in human ulcerative colitis and its potential role in malignant course. *Eur Cytokine Netw* 1998; **9**: 607-612 [PMID: 9889404]
58 **Nicola NA**, Babon JJ. Leukemia inhibitory factor (LIF). *Cytokine Growth Factor Rev* 2015; **26**: 533-544 [PMID: 26187859 DOI: 10.1016/j.cytogfr.2015.07.001]
59 **Metcalfe SM**. LIF in the regulation of T-cell fate and as a potential therapeutic. *Genes Immun* 2011; **12**: 157-168 [PMID: 21368774 DOI: 10.1038/gene.2011.9]
60 **Li YW**, Yang CQ, Xiao YL, Li J, Xie CX, Zhang SH, Yu Q, Wang HL, Lu WM, Chen MH. The -A2518G polymorphism in the MCP-1 gene and inflammatory bowel disease risk: A meta-analysis. *J Dig Dis* 2015; **16**: 177-185 [PMID: 25620350 DOI: 10.1111/1751-2980.12232]
61 **Günaltay S**, Kumawat AK, Nyhlin N, Bohr J, Tysk C, Hultgren O, Hultgren Hörnquist E. Enhanced levels of chemokines and their receptors in the colon of microscopic colitis patients indicate mixed immune cell recruitment. *Mediators Inflamm* 2015; **2015**: 132458

[PMID: 25948880 DOI: 10.1155/2015/132458]
62 **Grimm MC**, Elsbury SK, Pavli P, Doe WF. Enhanced expression and production of monocyte chemoattractant protein-1 in inflammatory bowel disease mucosa. *J Leukoc Biol* 1996; **59**: 804-812 [PMID: 8691064 DOI: 10.1002/jlb.59.6.804]
63 **Banks C**, Bateman A, Payne R, Johnson P, Sheron N. Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *J Pathol* 2003; **199**: 28-35 [PMID: 12474223 DOI: 10.1002/path.1245]
64 **Reinecker HC**, Loh EY, Ringler DJ, Mehta A, Rombeau JL, MacDermott RP. Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology* 1995; **108**: 40-50 [PMID: 7806062 DOI: 10.1016/0016-5085(95)90006-3]
65 **Mazzucchelli L**, Hauser C, Zgraggen K, Wagner HE, Hess MW, Laissue JA, Mueller C. Differential in situ expression of the genes encoding the chemokines MCP-1 and RANTES in human inflammatory bowel disease. *J Pathol* 1996; **178**: 201-206 [PMID: 8683390 DOI: 10.1002/(SICI)1096-9896(199602)178:2<201::AID-PATH440>3.0.CO;2-4]
66 **Uguccioni M**, Gionchetti P, Robbiani DF, Rizzello F, Peruzzo S, Campieri M, Baggiolini M. Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. *Am J Pathol* 1999; **155**: 331-336 [PMID: 10433925 DOI: 10.1016/S0002-9440(10)65128-0]
67 **Maharshak N**, Hart G, Ron E, Zelman E, Sagiv A, Arber N, Brazowski E, Margalit R, Elinav E, Shachar I. CCL2 (pM levels) as a therapeutic agent in Inflammatory Bowel Disease models in mice. *Inflamm Bowel Dis* 2010; **16**: 1496-1504 [PMID: 20222120 DOI: 10.1002/ibd.21254]
68 **Rider P**, Carmi Y, Guttman O, Braiman A, Cohen I, Voronov E, White MR, Dinarello CA, Apte RN. IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. *J Immunol* 2011; **187**: 4835-4843 [PMID: 21930960 DOI: 10.4049/jimmunol.1102048]
69 **Lei-Leston AC**, Murphy AG, Maloy KJ. Epithelial Cell Inflammasomes in Intestinal Immunity and Inflammation. *Front Immunol* 2017; **8**: 1168 [PMID: 28979266 DOI: 10.3389/fimmu.2017.01168]
70 **Aguilera M**, Darby T, Melgar S. The complex role of inflammasomes in the pathogenesis of Inflammatory Bowel Diseases - lessons learned from experimental models. *Cytokine Growth Factor Rev* 2014; **25**: 715-730 [PMID: 24803013 DOI: 10.1016/j.cytogfr.2014.04.003]
71 **Bamias G**, Cominelli F. Role of type 2 immunity in intestinal inflammation. *Curr Opin Gastroenterol* 2015; **31**: 471-476 [PMID: 26376478 DOI: 10.1097/MOG.0000000000000212]
72 **Strober W**, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1756-1767 [PMID: 21530742 DOI: 10.1053/j.gastro.2011.02.016]
73 **Fulkerson PC**, Rothenberg ME. Origin, regulation and physiological function of intestinal oeosinophils. *Best Pract Res Clin Gastroenterol* 2008; **22**: 411-423 [PMID: 18492563 DOI: 10.1016/j.bpg.2007.10.023]
74 **Mishra A**, Hogan SP, Brandt EB, Wagner N, Crossman MW, Foster PS, Rothenberg ME. Enterocyte expression of the eotaxin and interleukin-5 transgenes induces compartmentalized dysregulation of eosinophil trafficking. *J Biol Chem* 2002; **277**: 4406-4412 [PMID: 11733500 DOI: 10.1074/jbc.M110424200]
75 **Collins PD**, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 1995; **182**: 1169-1174 [PMID: 7561691 DOI: 10.1084/jem.182.4.1169]
76 **Owaga E**, Hsieh RH, Mugendi B, Masuku S, Shih CK, Chang JS. Th17 Cells as Potential Probiotic Therapeutic Targets in Inflammatory Bowel Diseases. *Int J Mol Sci* 2015; **16**: 20841-20858 [PMID: 26340622 DOI: 10.3390/ijms160920841]
77 **Xu XR**, Liu CQ, Feng BS, Liu ZJ. Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 3255-3264 [PMID: 24695798 DOI: 10.3748/wjg.v20.i12.3255]
78 **Chen L**, Zou Y, Peng J, Lu F, Yin Y, Li F, Yang J. Lactobacillus acidophilus suppresses colitis-associated activation of the IL-23/Th17 axis. *J Immunol Res* 2015; **2015**: 909514 [PMID: 25973440 DOI: 10.1155/2015/909514]
79 **Bettelli E**, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 2007; **8**: 345-350 [PMID: 17375096 DOI: 10.1038/ni0407-345]
80 **Jovanovic DV**, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 1998; **160**: 3513-3521 [PMID: 9531313]
81 **Kinugasa T**, Sakaguchi T, Gu X, Reinecker HC. Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology* 2000; **118**: 1001-1011 [PMID: 10833473 DOI: 10.1016/S0016-5085(00)70351-9]
82 **Ogawa A**, Andoh A, Araki Y, Bamba T, Fujiyama Y. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin Immunol* 2004; **110**: 55-62 [PMID: 14962796 DOI: 10.1016/j.clim.2003.09.013]
83 **Bisping G**, Lügering N, Lütke-Brintrup S, Pauels HG, Schürmann G, Domschke W, Kucharzik T. Patients with inflammatory bowel disease (IBD) reveal increased induction capacity of intracellular interferon-gamma (IFN-gamma) in peripheral CD8+ lymphocytes co-cultured with intestinal epithelial cells. *Clin Exp Immunol* 2001; **123**: 15-22 [PMID: 11167992 DOI: 10.1046/j.1365-2249.2001.01443.x]
84 **Ito R**, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, Imanishi J, Kita M, Ueda Y, Iwakura Y, Kataoka K, Okanoue T, Mazda O. Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol* 2006; **146**: 330-338 [PMID: 17034586 DOI: 10.1111/j.1365-2249.2006.03214.x]
85 **Kwon JH**, Keates AC, Anton PM, Botero M, Goldsmith JD, Kelly CP. Topical antisense oligonucleotide therapy against LIX, an enterocyte-expressed CXC chemokine, reduces murine colitis. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G1075-G1083 [PMID: 16099872 DOI: 10.1152/ajpgi.00073.2005]
86 **Egesten A**, Eliasson M, Olin AI, Erjefält JS, Bjartell A, Sangfelt P, Carlson M. The proinflammatory CXC-chemokines GRO-alpha/CXCL1 and MIG/CXCL9 are concomitantly expressed in ulcerative colitis and decrease during treatment with topical corticosteroids. *Int J Colorectal Dis* 2007; **22**: 1421-1427 [PMID: 17703315 DOI: 10.1007/s00384-007-0370-3]
87 **Romagnani P**, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, Uguccioni M, Galli G, Cosmi L, Maurenzig L, Baggiolini M, Maggi E, Romagnani S, Serio M. Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest* 2001; **107**: 53-63 [PMID: 11134180 DOI: 10.1172/JCI9775]
88 **Li K**, Wang B, Sui H, Liu S, Yao S, Guo L, Mao D. Polymorphisms of the macrophage inflammatory protein 1 alpha and ApoE genes are associated with ulcerative colitis. *Int J Colorectal Dis* 2009; **24**: 13-17 [PMID: 18762952 DOI: 10.1007/s00384-008-0575-0]
89 **Pender SL**, Chance V, Whiting CV, Buckley M, Edwards M, Pettipher R, MacDonald TT. Systemic administration of the chemokine macrophage inflammatory protein 1alpha exacerbates inflammatory bowel disease in a mouse model. *Gut* 2005; **54**: 1114-1120 [PMID: 16009684 DOI: 10.1136/gut.2004.052779]
90 **Ajuebor MN**, Hogaboam CM, Kunkel SL, Proudfoot AE, Wallace JL. The chemokine RANTES is a crucial mediator of the progression from acute to chronic colitis in the rat. *J Immunol* 2001; **166**: 552-558 [PMID: 11123336 DOI: 10.4049/jimmunol.166.1.552]
91 **Tahara T**, Shibata T, Okubo M, Ishizuka T, Kawamura T, Yamashita H, Nakamura M, Nakagawa Y, Nagasaka M, Arisawa T, Ohmiya N, Hirata I. Effect of *RANTES* gene promoter genotypes in patients with ulcerative colitis. *Biomed Rep* 2014; **2**: 602-606 [PMID: 24944817 DOI: 10.3892/br.2014.287]
92 **Ansari N**, Abdulla J, Zayyani N, Brahmi U, Taha S, Satir AA. Comparison of RANTES expression in Crohn's disease and ulcerative colitis: an aid in the differential diagnosis? *J Clin Pathol* 2006; **59**: 1066-1072 [PMID: 16565224 DOI: 10.1136/jcp.2005.034983]
93 **Schall TJ**, Bacon K, Toy KJ, Goeddel DV. Selective attraction of

monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 1990; **347**: 669-671 [PMID: 1699135 DOI: 10.1038/347669a0]

94 **Gerlach K**, Hwang Y, Nikolaev A, Atreya R, Dornhoff H, Steiner S, Lehr HA, Wirtz S, Vieth M, Waisman A, Rosenbauer F, McKenzie AN, Weigmann B, Neurath MF. TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat Immunol* 2014; **15**: 676-686 [PMID: 24908389 DOI: 10.1038/ni.2920]

95 **Hufford MM**, Kaplan MH. A gut reaction to IL-9. *Nat Immunol* 2014; **15**: 599-600 [PMID: 24940947 DOI: 10.1038/ni.2916]

96 **Leake I**. IBD. TH9 cells might have a role in the pathogenesis of ulcerative colitis. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 455 [PMID: 24957010 DOI: 10.1038/nrgastro.2014.108]

97 **Zwicker S**, Bureik D, Bosma M, Martinez GL, Almer S, Boström EA. Receptor-Type Protein-Tyrosine Phosphatase ζ and Colony Stimulating Factor-1 Receptor in the Intestine: Cellular Expression and Cytokine- and Chemokine Responses by Interleukin-34 and Colony Stimulating Factor-1. *PLoS One* 2016; **11**: e0167324 [PMID: 27898738 DOI: 10.1371/journal.pone.0167324]

98 **Klebl FH**, Olsen JE, Jain S, Doe WF. Expression of macrophage-colony stimulating factor in normal and inflammatory bowel disease intestine. *J Pathol* 2001; **195**: 609-615 [PMID: 11745698 DOI: 10.1002/path.991]

99 **Makiyama K**, Tomonaga M, Nakamuta K, Oda H, Itsuno M, Hara K. Serum concentration of macrophage colony stimulating factor (M-CSF) in patients with inflammatory bowel disease. *Gastroenterol Jpn* 1993; **28**: 740 [PMID: 8224629 DOI: 10.1007/BF02806359]

100 **Lacey DC**, Achuthan A, Fleetwood AJ, Dinh H, Roiniotis J, Scholz GM, Chang MW, Beckman SK, Cook AD, Hamilton JA. Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. *J Immunol* 2012; **188**: 5752-5765 [PMID: 22547697 DOI: 10.4049/jimmunol.1103426]

101 **Hamilton JA**. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol* 2008; **8**: 533-544 [PMID: 18551128 DOI: 10.1038/nri2356]

102 **Linares PM**, Gisbert JP. Role of growth factors in the development of lymphangiogenesis driven by inflammatory bowel disease: a review. *Inflamm Bowel Dis* 2011; **17**: 1814-1821 [PMID: 21744436 DOI: 10.1002/ibd.21554]

103 **Becker F**, Potepalov S, Shehzahdi R, Bernas M, Witte M, Abreo F, Traylor J, Orr WA, Tsunoda I, Alexander JS. Downregulation of FoxC2 Increased Susceptibility to Experimental Colitis: Influence of Lymphatic Drainage Function? *Inflamm Bowel Dis* 2015; **21**: 1282-1296 [PMID: 25822012 DOI: 10.1097/MIB.0000000000000371]

104 **Halin C**, Tobler NE, Vigl B, Brown LF, Detmar M. VEGF-A produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. *Blood* 2007; **110**: 3158-3167 [PMID: 17625067 DOI: 10.1182/blood-2007-01-066811]

105 **Becker F**, Yi P, Al-Kofahi M, Ganta VC, Morris J, Alexander JS. Lymphatic dysregulation in intestinal inflammation: new insights into inflammatory bowel disease pathomechanisms. *Lymphology* 2014; **47**: 3-27 [PMID: 25109166]

106 **Kühl AA**, Erben U, Kredel LI, Siegmund B. Diversity of Intestinal Macrophages in Inflammatory Bowel Diseases. *Front Immunol* 2015; **6**: 613 [PMID: 26697009 DOI: 10.3389/fimmu.2015.00613]

P- Reviewer: Keshteli AH, Suzuki H **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4356-4368

DOI: 10.3748/wjg.v24.i38.4356

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

ORIGINAL ARTICLE

Basic Study

Analysis of the nitric oxide-cyclic guanosine monophosphate pathway in experimental liver cirrhosis suggests phosphodiesterase-5 as potential target to treat portal hypertension

Denise Schaffner, Adhara Lazaro, Peter Deibert, Peter Hasselblatt, Patrick Stoll, Lisa Fauth, Manfred W Baumstark, Irmgard Merfort, Annette Schmitt-Graeff, Wolfgang Kreisel

Denise Schaffner, Adhara Lazaro, Peter Deibert, Manfred W Baumstark, Institute for Exercise-und Occupational Medicine, Faculty of Medicine, University of Freiburg, Freiburg 79106, Germany

Peter Hasselblatt, Wolfgang Kreisel, Department of Medicine II, Gastroenterology, Hepatology, Endocrinology, and Infectious Diseases, Faculty of Medicine, University of Freiburg, Freiburg 79106, Germany

Patrick Stoll, Anaesthesiological Practice, Freiburg 79104, Germany

Lisa Fauth, Annette Schmitt-Graeff, Institute of Clinical Pathology, Faculty of Medicine, University of Freiburg, Freiburg 79106, Germany

Denise Schaffner, Irmgard Merfort, Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Freiburg 79104, Germany

ORCID number: Denise Schaffner (0000-0001-6173-9288); Adhara Lazaro (0000-0001-9023-1387); Peter Deibert (0000-0002-3291-884X); Peter Hasselblatt (0000-0002-1107-6516); Patrick Stoll (0000-0001-8133-2071); Lisa Fauth (0000-0001-5183-7140); Manfred W Baumstark (0000-0002-5442-1586); Irmgard Merfort (0000-0003-4716-5016); Annette Schmitt-Graeff (0000-0001-5082-7979); Wolfgang Kreisel (0000-0001-6884-0135).

Author contributions: Kreisel W and Deibert P designed the concept of the study; Schaffner D performed the experiments and statistical calculations and provided additional ideas; Lazaro A assisted in the laboratory experiments and in writing the manuscript; Hasselblatt P supervised the PCR study and helped in the interpretation of results; Stoll P supervised Schaffner D and Kreisel W in the initial performance the operative procedure for hemodynamic measurements; Fauth L performed the histological assessment of the degree of liver damage; Baumstark MW supervised the statistical calculations; Merfort I supervised the advanced training of Schaffner D; Schmitt-Graeff A performed and interpreted the immunohistochemical data; all authors contributed in writing the manuscript.

Institutional animal care and use committee statement: The animal research protocol was approved by the local institutional animal care and use committee (Regierungspräsidium Freiburg, Germany, ref. No. G-13/89). Animal care was performed in accordance to the rules of the German animal protection law and the animal care guidelines of the European community (2010/63/EU).

Conflict-of-interest statement: There was no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Manuscript source: Invited manuscript

Correspondence to: Wolfgang Kreisel, MD, Emeritus Professor, Department of Medicine II, Gastroenterology, Hepatology, Endocrinology, and Infectious Diseases, Faculty of Medicine, University of Freiburg, Hugstetter Str. 55, Freiburg 79106, Germany. wolfgang.kreisel@uniklinik-freiburg.de
Telephone: +49-761-27034010
Fax: +49-761-27074880

Received: July 16, 2018
Peer-review started: July 16, 2018
First decision: August 1, 2018
Revised: August 3, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 14, 2018

Abstract

AIM

To investigate the potential effect of inhibitors of phosphodiesterase-5 (PDE-5) for therapy of portal hypertension in liver cirrhosis.

METHODS

In the rat model of thioacetamide-induced liver fibrosis/cirrhosis the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway was investigated. Expression and localization of PDE-5, the enzyme that converts vasodilating cGMP into inactive 5'-GMP, was in the focus of the study. Hepatic gene expression of key components of the NO-cGMP pathway was determined by qRT-PCR: Endothelial NO synthase (eNOS), inducible NO synthase (iNOS), soluble guanylate cyclase subunits α1 and β1 (sGCa1, sGCb1), and PDE-5. Hepatic PDE-5 protein expression and localization were detected by immunohistochemistry. Serum cGMP concentrations were measured using ELISA. Acute effects of the PDE-5 inhibitor Sildenafil (0.1 mg/kg or 1.0 mg/kg) on portal and systemic hemodynamics were investigated using pressure transducers.

RESULTS

Hepatic gene expression of eNOS (2.2-fold; P = 0.003), sGCa1 (1.7-fold; P = 0.003), sGCb1 (3.0-fold; P = 0.003), and PDE-5 (11-fold; P = 0.003) was increased in cirrhotic livers compared to healthy livers. Overexpression of PDE-5 (7.7-fold; P = 0.006) was less pronounced in fibrotic livers. iNOS expression was only detected in fibrotic and cirrhotic livers. In healthy liver, PDE-5 protein was localized primarily in zone 3 hepatocytes and to a lesser extent in perisinusoidal cells. This zonation was disturbed in cirrhosis: PDE-5 protein expression in perisinusoidal cells was induced approximately 8-fold. In addition, PDE-5-expressing cells were also found in fibrous septa. Serum cGMP concentrations were reduced in rats with cirrhotic livers by approximately 40%. Inhibition of PDE-5 by Sildenafil caused a significant increase in serum cGMP concentrations [+ 64% in healthy rats (P = 0.024), + 85% in cirrhotic rats (P = 0.018)]. Concomitantly, the portal venous pressure was reduced by 19% in rats with liver cirrhosis.

CONCLUSION

Overexpression and abrogated zonation of PDE-5 likely contribute to the pathogenesis of cirrhotic portal hypertension. PDE-5 inhibition may therefore be a reasonable therapeutic approach for portal hypertension.

Key words: Portal hypertension; Thioacetamide; Nitric oxide; Liver cirrhosis; Cyclic guanosine monophosphate; Phosphodiesterase-5; Sildenafil; Hepatic stellate cells; Metabolic zonation

Core tip: A constriction of sinusoids plays an important role in the pathogenesis of cirrhotic portal hypertension, wherein the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway plays a pivotal role. In a rat model of liver cirrhosis phosphodiesterase-5 (PDE-5) was markedly overexpressed both on the mRNA and the protein level. PDE-5 converts the vasodilating cGMP to inactive 5'-GMP. In healthy liver a zonation of PDE-5 was found which is abrogated in cirrhosis. Serum cGMP was reduced in cirrhosis. Inhibition of PDE-5 by Sildenafil normalized serum cGMP levels and lowered portal venous pressure. Hence, the inhibition of PDE-5 may be a promising adjunct in portal hypertension therapy.

Schaffner D, Lazaro A, Deibert P, Hasselblatt P, Stoll P, Fauth L, Baumstark MW, Merfort I, Schmitt-Graeff A, Kreisel W. Analysis of the nitric oxide-cyclic guanosine monophosphate pathway in experimental liver cirrhosis suggests phosphodiesterase-5 as potential target to treat portal hypertension. *World J Gastroenterol* 2018; 24(38): 4356-4368 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4356.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4356

INTRODUCTION

Portal hypertension is among the most important complications of liver cirrhosis[1]. Several factors contribute to its pathogenesis[2,3]. Disturbed liver architecture resulting from fibrosis, scarring and nodule formation, angiogenesis, and vascular occlusion increase intrahepatic flow resistance. Furthermore, morphological, molecular, and functional changes within the hepatic sinusoids also substantially contribute to the increased intrahepatic flow resistance and consequently to pathogenesis of portal hypertension[3,4]. Generally, intrahepatic flow resistance is controlled by sinusoidal tone with liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) being the two central cellular elements within the sinusoids which are involved[4]. During cirrhosis development fenestrations of LSECs are lost, which is associated with an abnormal deposition of a basement membrane matrix, the so-called capillarization. Moreover, quiescent HSCs are activated and transformed into myofibroblasts, which are characterized by contractile elements, production of extracellular matrix, and loss of stored vitamin A.

The sinusoidal tone is regulated by the nitric oxide-

cyclic guanosine monophosphate (NO-cGMP) system[5]. NO is synthesized in LSECs by endothelial NO synthase (eNOS), diffuses to HCSs where it activates soluble guanylate cyclase (sGC) which in turn catalyzes the formation of cGMP. The latter mediates a signal transduction cascade leading to relaxation of HSCs. The action of cGMP is terminated by phosphodiesterase-5 (PDE-5) which catalyzes its conversion to inactive 5'-GMP.

In cirrhotic liver, NO formation in liver sinusoids is reduced, which results in sinusoidal constriction and increased intrahepatic flow resistance[6]. Activated HSCs exhibit increased contractility and impaired responsiveness to the vasodilator NO, while responsiveness to the vasoconstrictor endothelin (ET-1) is increased, both resulting in increased intrahepatic flow resistance[7]. In contrast, NO formation in the splanchnic system is increased which increases blood flow towards the liver, a process which further aggravates the increase of intrahepatic flow resistance[6,8].

The current mainstay of medical therapy of portal hypertension is non-selective beta-blockers (NSBB)[9,10]. β1 receptor blockade reduces portal flow by decreasing cardiac output while β2 blockade allows unopposed α1-adrenergic activity resulting in splanchnic vasoconstriction and decreased portal inflow. However, their therapeutic efficacy is limited by frequent side effects such as circulatory dysregulation, which prevent sufficient dosing in many cases, particularly in decompensated cirrhosis[11-13]. Therapy of portal hypertension by transjugular portosystemic shunts is effective, but may cause exacerbations of hepatic encephalopathy, at least in patients with advanced cirrhosis[9]. Organic nitrates deliver NO and reduce portal venous pressure. However, their action is unspecific and leads to arterial hypotension. Therefore, there is a need of novel medical therapies to treat portal hypertension.

Many approaches have been proposed to specifically target the reduced NO availability in diseased liver. Endothelial NOS gene transfer[14,15], neuronal NOS gene transfer[16], Akt (a kinase involved in NOS activation) gene transfer[17] and administration of tetrahydrobiopterin (BH4, a cofactor of NOS)[18], have not gone beyond preclinical testing. Liver specific NO delivering drugs (*e.g.*, NCX-1000) yielded disappointing results[19,20]. Statins lead to enhanced activity of endothelial NO synthase, mediated by an effect on the Rho/Rho-kinase-/Akt protein phosphorylation pathway[21]. Although statins reduced portal hypertension in a clinical setting[22], the effects on clinical outcome were modest[23]. Preclinical and clinical studies on inhibitors of PDE-5, restricting the inactivation of cGMP, yielded promising but variable results[24-30].

The aim of the current study was to determine liver disease-induced alterations in hepatic gene expression of eNOS, iNOS, sGC, and PDE-5, in hepatic protein expression of PDE-5, as well as in serum cGMP concentrations. Moreover, acute effects of the PDE-5 inhibitor Sildenafil on portal and systemic hemodynamics were tested. The model of thioacetamide-induced liver fibrosis/cirrhosis in rats was used.

MATERIALS AND METHODS

Laboratory animals

The animal research protocol was approved by the local institutional animal care and use committee (Regierungspräsidium Freiburg, Germany, ref. No. G-13/89). Animal care was performed in accordance to the rules of the German animal protection law and the animal care guidelines of the European community (2010/63/EU). A total of 141 male rats (Charles River, Sulzfeld, Germany) were studied. All were clearly recognizable from their permanent and unique identifiers. Rats were housed in individually ventilated cages in a laboratory animal facility and received daily human care. All had free access to food and water and were exposed to a 12:12-h light-dark cycle at an ambient temperature of 22 ℃ to 25 ℃. Before starting any experiments, the rats were allowed to acclimatize to the ambient conditions for one week.

For the biochemical investigations Wistar rats were used only. Hemodynamic measurements in healthy livers were performed in Sprague Dawley rats. Corresponding experiments in rats with with fibrotic or cirrhotic livers were performed in Wistar rats since the presence of cholangiocellular carcinomas in association with thioacetamide in Wistar rats is much lower than in Sprague Dawley rats.

Induction of liver disease with TAA

Among the current models of induction of liver disease in laboratory animals[31] the model of thioacetamide (TAA)-induced liver fibrosis/cirrhosis was chosen. Therefore, the protocol described previously by Li *et al*[32] was used but TAA exposure time was prolonged to 16 wk.

Histological assessment of liver damage

The median lobe of each rat's liver was excised, fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin, sirius red, and periodic acid-schiff diastase, and stained for reticulin and iron. Fibrosis was evaluated semiquantitatively by a blinded pathologist according to the Desmet score[33].

Immunohistochemistry for PDE-5

In order to detect PDE-5 protein expression and localization immunohistochemical staining of liver sections was performed with a rabbit polyclonal anti-PDE-5A-antibody (ab64179, Abcam, Cambridge, United Kingdom) at a 1:500 dilution. Antibody binding was detected by Dako REAL EnVision Detection System Peroxidase/DAB+, Rabbit/Mouse (K5007).

Table 1 Nucleotide sequences of forward and reverse primers

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Product length (bp)
eNOS	5´-AAGTGGGCAGCATCACCTAC-3´	5´-GCCTGGGAACCACTCCTTTT-3´	211
iNOS	5´-CTCACTGGGACTGCACAGAA-3´	5´-TGTTGAAGGGTGTCGTGAAA-3´	128
PDE-5	5´-GCGGAGGAAGAAACAAGGGA-3´	5´-ATCGGCAAAGAACCTCGTGT-3´	196
sGCa1	5´-GCCCCACGACATACAGGTTA-3´	5´-GCGGCTCACTAATCTACCCC-3´	229
sGCb1	5´-AATTACGGTCCCGAGGTGTG-3´	5´-ACCAGCATTGAGGTTGAGGAC-3´	147
18sRNA (reference)	5´-GTAACCCGTTGAACCCCATT-3´	5´-CCATCCAATCGGTAGTAGCG-3´	151
srsf4 (reference)	5´-GGTTCTGGACGCAGTGGATA-3´	5´-CTCCTTCGTTTTTGCGTCCC-3´	193

Quantification of PDE-5 protein along the sinusoids was performed in sections of respectively 4 healthy and cirrhotic livers.

Using Zeiss Axioplan microscope the number of stained perisinusoidal cells was counted in 20 random high power-fields (HPF) (400 × magnification) for each sample. PDE-5 staining around the central vein in healthy livers and in fibrous septa in cirrhotic livers was not considered.

Quantitative real-time PCR

The left lateral lobe of each rat's liver was excised, cut into pieces, snap frozen in liquid nitrogen, and stored at -80 ℃ until used for qRT-PCR. Hepatic total mRNA was extracted using the RNeasy® Mini Kit mRNA extraction kit (Qiagen, Hilden, Germany). Complementary DNA synthesis was performed using the First Strand cDNA synthesis kit (Thermo Fisher Scientific, MA, United States). qRT-PCR was performed with SYBR Green (Invitrogen, Karlsruhe, Germany) and 10% dimethylsulfoxide (Sigma-Aldrich, Schnelldorf, Germany) on a thermocycler (LightCyler® 480, Roche, Basel, Switzerland; 40 cycles: 30 s 95 ℃; 30 s 60 ℃; 40 s 72 ℃). Specificity of the PCR products was assessed by melting curve analysis. All primers (Table 1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Microsynth, Balgach, Switzerland.

Measurement of cyclic guanosine monophosphate

At the end of the invasive hemodynamic measurements blood samples were taken *via* the left carotid artery and stored at -80 ℃ until used for the quantification of serum cGMP concentrations by ELISA (ab133052, Abcam, Cambridge, United Kingdom).

Monitoring of systemic and portal hemodynamics

Before the invasive hemodynamic measurements were started rats were fasted for 1.5 h to avoid prandial effects on portal flow parameters. Anesthesia was initiated in an animal induction chamber using a mixture of 3% isoflurane and 97% oxygen. It was maintained by an intraperitoneally injected bolus of 0.3-0.4 mL pentobarbital (125 mg/mL). Rats were fixed on a homeothermic controlled operating table which kept body temperature stable at 37 ℃ ± 0.5 ℃. Vital parameters were monitored. Tracheotomy was performed and a tracheal cannula was inserted. Rats were mechanically ventilated (50 breaths/min) and muscle relaxation was induced by intraperitoneal injection of 0.5 mL pancuronium (0.4 mg/mL).

To monitor the central venous pressure (CVP) the right external jugular vein was cannulated with PE-10, which was positioned near the right atrium. A second PE-10 tubing was inserted and used for continuous infusion of isotone electrolyte solution (1 mL/h). The electrolyte solution was enriched with pentobarbital (15 mg/mL) to ensure continuous anesthesia. To monitor mean arterial pressure (MAP) the left carotid artery was cannulated with PE-50 tubing.

Median laparotomy was performed and the portal vein was exposed. To monitor portal venous pressure (PVP) a peripheral venous catheter was inserted into the portal vein. After a stabilization period of 10-15 min, basal values of all parameters were obtained and the intervention was administered through the second CVP-tubing. Rats were randomly allocated in one of three intervention groups: NaCl (0.9%), Sildenafil (Revatio®, Pfizer, Berlin, Germany) 0.1 mg/kg (Sil 0.1 mg/kg), and Sildenafil 1.0 mg/kg (Sil 1.0 mg/kg). The intervention was applied in a standardized volume of 0.6 mL.

Statistical analysis

Results were expressed as median ± interquartile range (IQR). Only results of the qRT-PCR experiments were expressed as mean ± standard deviation (SD) to enable the quantification of gene expression with the comparative C_t method[34].

To evaluate the effect of Sildenafil on hemodynamic parameters, absolute values were normalized (PVP_{norm}, MAP_{norm}, HR_{norm}). Hereby time point "10 min" was taken as baseline value and set to 100% since the administration of 0.6 mL liquid volume into the right atrium caused parameter variations for the next few minutes before they reached a new steady state. For all 9 groups the relative median of differences (RMD) was calculated to determine the change in parameters at time point "60 min" to baseline ("10 min").

To determine differences among groups the non-parametric Kruskal-Wallis test was used. Post-hoc pairwise comparisons between groups[35] were corrected for multiple comparisons according to Bonferroni. For reasons of consistency the non-parametric Kruskal-Wallis test and post-hoc pairwise comparisons with

Table 2 Mean ± standard deviation of enzyme gene expression

	CON (n = 11) Mean ± SD	FIB (n = 6) Mean ± SD	CIR (n = 8) Mean ± SD
eNOS (fold exp.)	1.0 ± 0.4	1.5 ± 0.3	2.2 ± 1.0
iNOS (fold exp.)	n.d.[1]	4.6 ± 3.0	5.3 ± 2.3
PDE5 (fold exp.)	1.0 ± 1.0	7.7 ± 0.9	11.0 ± 3.1
sGCa1 (fold exp.)	1.0 ± 0.3	1.4 ± 0.3	1.7 ± 0.4
sGCb1 (fold exp.)	1.0 ± 0.5	2.1 ± 0.3	3.0 ± 1.3

Fold expression is referring to CON. qRT-PCRs were performed in duplicates, thus values are based on mean values of duplicates. [1]Since iNOS expression in CON was below detection limit, it was arbitrarily set to "1.0". iNOS: Inducible NO synthase.

Bonferroni correction were also used for the qRT-PCR experiments. A two-tailed P-value of < 0.05 was considered as statistically significant.

For statistical analyses SPSS® software 23.0 (IBM Corp., Armonk, NY, United States) was used.

RESULTS

Increased hepatic gene expression of key enzymes of the NO-cGMP pathway and decreased serum cGMP concentrations in liver fibrosis/cirrhosis. Inhibition of PDE-5 by Sildenafil leads to renormalization of cGMP concentrations

In order to determine alterations in the key parameters of the NO-cGMP pathway and serum cGMP concentrations induced by liver fibrosis/cirrhosis different biochemical analyses were conducted. Fifty-three rats were included in these studies. However, the data sets of the 6 rats having no fibrosis after 16 wk of TAA exposure were excluded. For the statistical analysis of hepatic gene expression of eNOS, iNOS, PDE5, sGCa1 and sGCb1, and serum cGMP concentrations, the remaining 47 rats were classified depending on their histologically assessed degree of liver fibrosis: CON (healthy control, n = 11), FIB (fibrosis, n = 6), and CIR (cirrhosis, n = 8).

Moreover, to evaluate the effect of Sildenafil on serum cGMP concentrations two additional groups were analyzed: rats with healthy livers (CON + Sil, n = 12) and cirrhotic livers (CIR + Sil, n = 10) which have undergone the hemodynamic measurement with Sildenafil (1.0 mg/kg) as intervention.

qRT-PCR results showed that gene expression became the higher the more the rats became diseased (CON < FIB < CIR) (Figure 1 and Table 2). iNOS expression was detected in diseased rats only. In fibrotic livers gene expression analysis revealed significantly increased expression of PDE-5 (7.7-fold; P = 0.006), and sGCb1 (2.1-fold; P = 0.018) compared with healthy livers, whereas eNOS expression was significantly increased in non-adjusted pairwise comparisons only (Table 3).

In contrast, in cirrhotic livers a significantly increased expression of eNOS (2.2-fold; P = 0.003), PDE-5 (11-fold; P = 0.003), sGCa1 (1.7-fold; P = 0.003) and sGCb1 (3-fold; P = 0.003) was measured when compared with healthy livers.

No significant differences between fibrotic and cirrhotic livers were detected ($P \geq 0.05$).

Serum cGMP concentrations showed a nonsignificant decrease of 34% (P = 0.453) in rats with fibrotic livers and of 40% (P = 0.054) in rats with cirrhotic livers (Figure 1 and Table 4). Again, no differences between fibrotic and cirrhotic livers were observed (P = 1.000). Administration of Sildenafil led to a significant increase in serum cGMP concentrations of 64% (P = 0.024) in rats with healthy livers and of 85% (P = 0.018) in rats with cirrhotic livers.

In addition, the effect of the hemodynamic measurements and particularly the associated operative procedure on hepatic gene expression and serum cGMP concentration was analyzed. Only for eNOS a significant decrease was determined (data not shown).

Increased hepatic PDE-5 protein expression and disturbed PDE-5 zonation in liver cirrhosis

Immunohistochemistry was performed to analyze hepatic protein expression of PDE-5 in healthy and cirrhotic livers (Figure 2). In healthy livers, PDE-5 protein was predominantly expressed by perivenular hepatocytes (zone 3) and to a lesser extent by perisinusodial cells. In contrast, in cirrhotic livers hepatic zoning of PDE-5 was abrogated and bands of fibrous septa were formed. PDE-5 protein expression by perisinusoidal cells was increased, but positive cells were also present in fibrous septa.

Subsequent quantitative analysis of positive perisinusoidal cells revealed 3 stained cells per HPF in healthy livers and 23 stained cells per HPF in cirrhotic livers, what corresponds to a 7.7-fold increase.

Inhibition of PDE-5 by Sildenafil lowers portal venous pressure

To evaluate the effect of Sildenafil on portal and systemic blood pressure, hemodynamic measurements were performed. One hundred and ten rats were included in this study and sorted by their histological degree of liver fibrosis. However, the data sets of the 2 rats having no fibrosis after 16 wk of TAA exposure were excluded. For the statistical analysis of the hemodynamic parameters, the remaining 108 rats were classified into three groups depending on their histologically assessed degree of liver

Table 3 Median ± interquartile range of serum cyclic guanosine monophosphate concentrations

	CON (n = 11) Median ± IQR	FIB (n = 6) Median ± IQR	CIR (n = 8) Median ± IQR	CON + Sil (n = 12) Median ± IQR	CIR + Sil (n = 10) Median ± IQR
cGMP (pmol/mL)	152 ± 86	100 ± 68	91 ± 22	249 ± 153	168 ± 52

ELISAs were performed in duplicates, thus values are based on mean values of duplicates. IQR: Interquartile range; cGMP: Cyclic guanosine monophosphate.

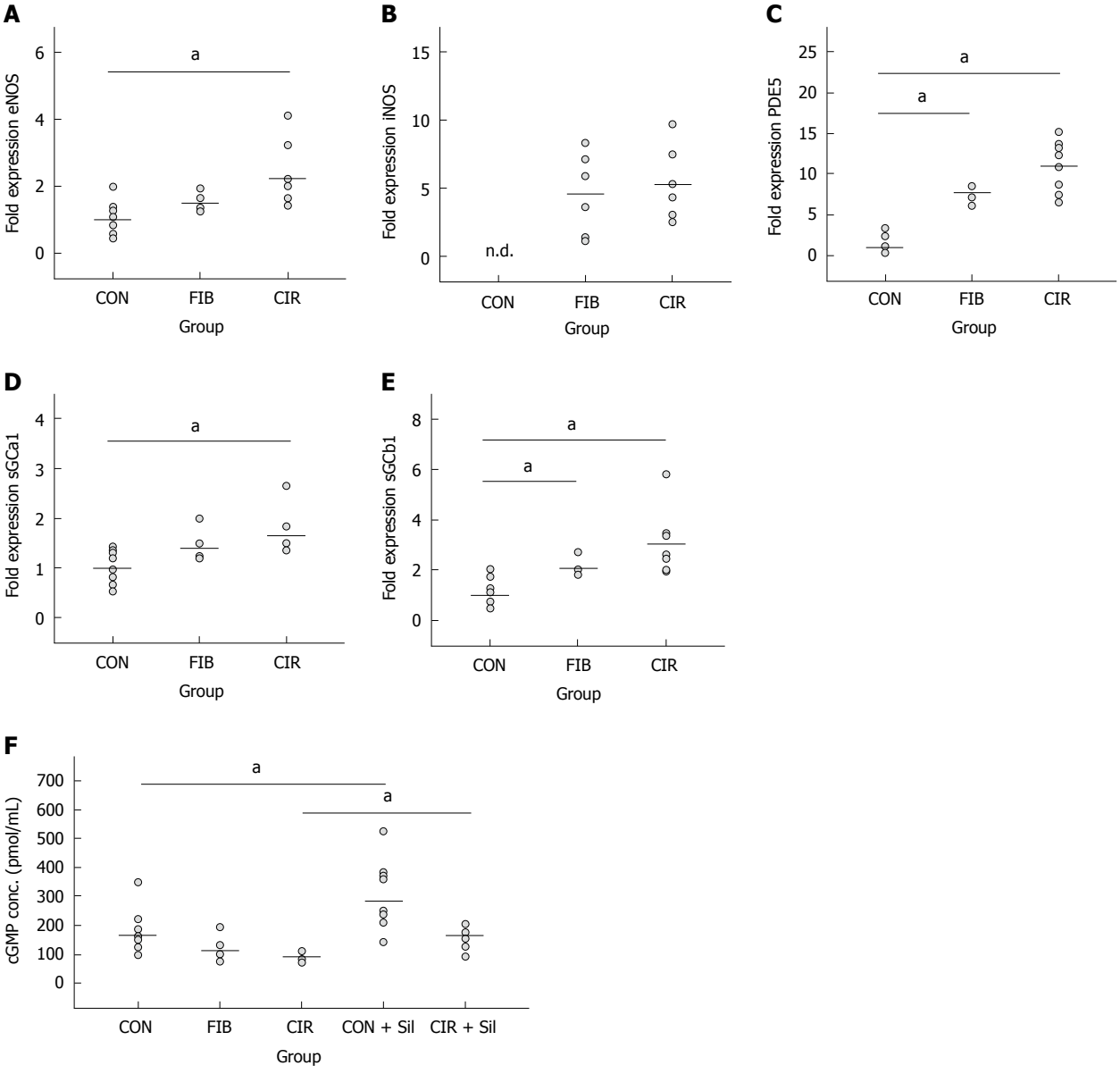


Figure 1 Dotplots showing hepatic gene expression of endothelial NO synthase (A), inducible NO synthase (B), phosphodiesterase-5 (C), soluble guanylate cyclase subunits α1 (D) and soluble guanylate cyclase subunits β1 (E), and serum cyclic guanosine monophosphate concentrations (pmol/mL) (F). Significant differences among groups are corrected for multiple comparisons and denoted by $^{a}P < 0.05$. Gene expression levels are given as fold expression compared to CON. Since iNOS expression in CON was below the detection limit, it was arbitrarily set to "1.0". iNOS: Inducible NO synthase.

fibrosis: CON (healthy control, n = 55), FIB (fibrosis, n = 29), and CIR (cirrhosis, n = 24). Each of those had 3 subgroups which are categorized based on intervention in form of NaCl, Sildenafil 0.1 mg/kg (Sil 0.1 mg/kg) or Sildenafil 1 mg/kg (Sil 1.0 mg/kg), which was applied in a standardized volume of 0.6 mL. Absolute values of the parameters are listed (Table 5).

All parameters of interest were normalized (PVP_{norm}, MAP_{norm}, and HR_{norm}) to compensate differences in absolute values between rats with healthy and diseased livers, the time point "10 min" being taken as baseline and set to 100% (see methods/statistical analysis).

Table 4 Pairwise comparisons between groups are determined according to Dunn[35]

		eNOS	iNOS	PDE5	sGCa1	sGCb1	cGMP
CON *vs* FIB	sig. (pairwise)	0.024[1]	-	0.002[1]	0.065	0.006[1]	0.151
	sig. (adjusted)	0.072	-	0.006[1]	0.195	0.018[1]	0.453
CON *vs* CIR	sig. (pairwise)	0.001[1]	-	0.001[1]	0.001[1]	0.001[1]	0.018[1]
	sig. (adjusted)	0.003[1]	-	0.003[1]	0.003[1]	0.003[1]	0.054
FIB *vs* CIR	sig. (pairwise)	0.280	0.732	0.201	0.194	0.267	0.495
	sig. (adjusted)	0.840	1.000	0.603	0.582	0.801	1.000
CON *vs* CON + Sil	sig. (pairwise)	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	0.012[1]
	sig. (adjusted)	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	0.024[1]
CIR *vs* CIR + Sil	sig. (pairwise)	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	0.009[1]
	sig. (adjusted)	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	n.m.[2]	0.018[1]

Adjusted comparisons are corrected according to Bonferroni. [1]Significant differences ($P < 0.05$) are marked by an. [2]Means: Not measured.

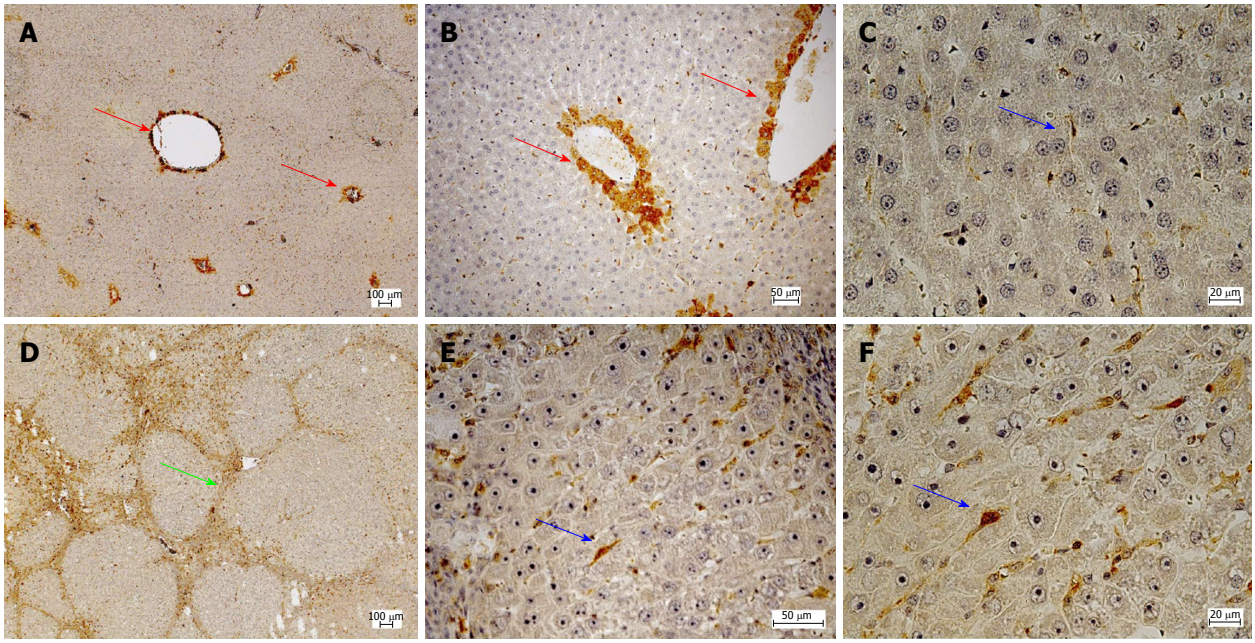


Figure 2 Immunohistochemical localization of phosphodiesterase-5. A-C: Healthy liver; D-F: Cirrhotic liver. Red arrows: Localization in zone 3 hepatocytes; Blue arrows: Localization in perisinusoidal cells; Green Arrow: Fibrous septa.

Regarding the course of hemodynamic parameters, a decrease in parameter values was observed for all groups regardless of intervention (Figure 3). In rats with diseased livers the decrease in PVP_{norm} (%) (blue curve) compared to the decrease in MAP_{norm} (%) (red curve) became more pronounced with Sildenafil and increasing dosage. CVP_{norm}, respiration $rate_{norm}$, oxygen $saturation_{norm}$ and body $temperature_{norm}$, remained unchanged in all groups (data not shown).

The effect of Sildenafil was evaluated by comparing the change in parameter values at time point "60 min" to baseline ("10 min") (Table 6). All parameters were normalized (PVPnorm, MAPnorm, HRnorm,) before differences were calculated for the interval 10 min to 60 min post-intervention with time point "10 min" being set to 100%. Significant differences ($P < 0.05$) between Sil 0.1 mg/kg and CON or Sil 1.0 mg/kg and CON are marked by an "[1]". No significant differences between Sil 0.1 mg/kg and Sil 1.0 mg/kg were observed.

In rats with healthy livers intragroup comparisons showed nonsignificant effects of Sildenafil on PVP_{norm} ($P = 0.399$), MAP_{norm} ($P = 0.867$), and HR_{norm} ($P = 0.664$). Also in rats with fibrotic livers intragroup comparisons revealed nonsignificant effects of Sildenafil on PVP_{norm} ($P = 0.320$), MAP_{norm} ($P = 0.272$), and HR_{norm} ($P = 0.311$). In contrast, the administration of Sildenafil caused a trend towards a lower PVP_{norm} ($P = 0.088$) in rats with cirrhotic livers. Moreover, Sildenafil showed nonsignificant effects on MAP_{norm} ($P = 0.915$) but for HR_{norm} a significant decrease of 8% (RMD) in Sil 1.0 mg/kg was found compared to NaCl ($P = 0.024$).

DISCUSSION

This study aimed to determine the alterations of key components in NO-cGMP pathway in the rat model of TAA-induced liver fibrosis/cirrhosis. Hemodynamic measurements were performed to further elucidate

Table 5 Median ± interquartile range of the absolute values of body weight, portal venous pressure, mean arterial pressure, and heart rate at different time points

	CON			FIB			CIR		
	NaCl (n = 19)	Sil 0.1 mg/kg (n = 18)	Sil 1.0 mg/kg (n = 18)	NaCl (n = 7)	Sil 0.1 mg/kg (n = 15)	Sil 1.0 mg/kg (n = 7)	NaCl (n = 7)	Sil 0.1 mg/kg (n = 7)	Sil 1.0 mg/kg (n = 10)
	Median ± IQR	Median ± IQR	Median ± IQR	Median ± IQR	Median ± IQR	Median ± IQR	Median ± IQR	Median ± IQR	Median ± IQR
Body weight (g)	375 ± 15	370 ± 28	380 ± 27	359 ± 35	363 ± 28	361 ± 24	336 ± 40	350 ± 46	337 ± 15
PVP_0 (mmHG)	6.4 ± 0.7	6.6 ± 0.4	6.3 ± 0.7	6.0 ± 0.9	6.2 ± 1.3	6.2 ± 0.9	6.8 ± 1.6	7.5 ± 1.7	7.7 ± 1.1
PVP_10 (mmHG)	6.0 ± 0.9	6.6 ± 0.6	6.6 ± 0.9	5.7 ± 1.2	5.5 ± 1.3	5.1 ± 1.4	6.8 ± 1.4	7.1 ± 1.6	7.1 ± 2.2
PVP_30 (mmHG)	5.9 ± 0.7	6.3 ± 0.7	6.4 ± 0.8	5.3 ± 1.4	5.4 ± 1.3	4.8 ± 1.4	6.5 ± 1.2	6.6 ± 1.0	6.3 ± 1.3
PVP_60 (mmHG)	5.9 ± 0.7	6.3 ± 0.8	6.4 ± 1.1	5.1 ± 1.2	5.2 ± 1.2	4.8 ± 1.5	6.6 ± 2.0	6.3 ± 1.0	5.8 ± 1.2
MAP_0 (mmHG)	89 ± 12	106 ± 14	101 ± 28	65 ± 30	59 ± 21	58 ± 28	49 ± 19	47 ± 15	60 ± 19
MAP_10 (mmHG)	83 ± 20	94 ± 14	85 ± 27	55 ± 25	45 ± 10	42 ± 8	42 ± 11	41 ± 5	38 ± 16
MAP_30 (mmHG)	79 ± 21	88 ± 17	77 ± 21	50 ± 14	43 ± 12	37 ± 6	41 ± 12	35 ± 8	36 ± 8
MAP_60 (mmHG)	76 ± 21	82 ± 23	74 ± 28	44 ± 11	40 ± 8	37 ± 7	38 ± 3	34 ± 5	34 ± 7
HR_0 (bpm)	373 ± 40	377 ± 47	368 ± 31	320 ± 30	335 ± 30	314 ± 22	307 ± 38	308 ± 76	365 ± 28
HR_10 (bpm)	362 ± 55	383 ± 44	380 ± 50	313 ± 23	339 ± 41	351 ± 29	286 ± 33	316 ± 71	374 ± 22
HR_30 (bpm)	356 ± 57	366 ± 52	373 ± 41	291 ± 20	310 ± 43	310 ± 21	268 ± 40	313 ± 57	350 ± 36
HR_60 (bpm)	347 ± 39	359 ± 43	362 ± 49	284 ± 26	304 ± 41	295 ± 33	254 ± 36	302 ± 47	324 ± 30

Table 6 Relative median of differences (%) ± interquartile range of the parameters portal venous pressure, mean arterial pressure, and heart rate

	CON			FIB			CIR		
	NaCl (n = 19)	Sil 0.1 mg/kg (n = 18)	Sil 1.0 mg/kg (n = 18)	NaCl (n = 7)	Sil 0.1 mg/kg (n = 15)	Sil 1.0 mg/kg (n = 7)	NaCl (n = 7)	Sil 0.1 mg/kg (n = 7)	Sil 1.0 mg/kg (n = 10)
	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR	RMD (%) ± IQR
PVP_{norm}	-3 ± 7	-6 ± 10	-3 ± 6	-9 ± 11	-8 ± 8	-7 ± 6	-3 ± 7	-13 ± 7	-19 ± 26
MAP_{norm}	-10 ± 17	-8 ± 16	-7 ± 19	-21 ± 24	-14 ± 21	-10 ± 10	-17 ± 16	-14 ± 11	-17 ± 23
HR_{norm}	-4 ± 6	-4 ± 5	-4 ± 5	-8 ± 5	-8 ± 14	-12 ± 13	-6 ± 6	-8 ± 11	-14 ± 10[1]

[1]Significant differences ($P < 0.05$) between Sil 0.1mg/kg and CON or Sil 1.0 mg/kg and CON are marked by an "[1]". RMD: Relative median of differences; IQR: Interquartile range; PVP: Portal venous pressure; MAP: Mean arterial pressure; HR: Heart rate.

the potential of PDE-5 inhibitors in portal hypertension therapy. An increased hepatic gene expression of key enzymes of the NO-cGMP pathway in diseased rats was demonstrated wherein a significant overexpression of PDE-5 was the most notable finding. Enhanced levels of PDE-5 protein expression were confirmed by immunohistochemical staining which revealed that PDE-5 zonation found in healthy livers was abrogated in diseased livers. With the increased PDE-5 expression, serum concentrations of cGMP, the most potent vasodilating compound in hepatic sinusoids, were reduced in rats with diseased livers. Administration of the PDE-5 inhibitor Sildenafil led to a significant increase of cGMP concentrations with accompanying reduction in portal venous pressure in rats with cirrhosis.

In healthy liver eNOS is constitutively expressed and uniformly distributed among the hepatic lobules (*i.e.*, LSECs, hepatocytes, endothelium of hepatic arteries, terminal venules, biliary epithelium). High concentrations of eNOS-derived NO attenuate HSC activity and have a protective role in liver function[36]. Activity of eNOS is reduced in cirrhosis contributing to decreased sinusoidal dilation[4,36]. The amount of eNOS is not lowered but the translational modification through Akt or interactions with caveolin prevent full functional capacity[37]. Present results revealed a 2.2-fold higher mRNA expression of eNOS in cirrhotic livers. However, this does not necessarily translate to a higher NO production.

In contrast, iNOS expression was observed only in fibrotic and cirrhotic livers. This is consistent with the hypothesis that multiple pathogenic factors like LPS, toxins,

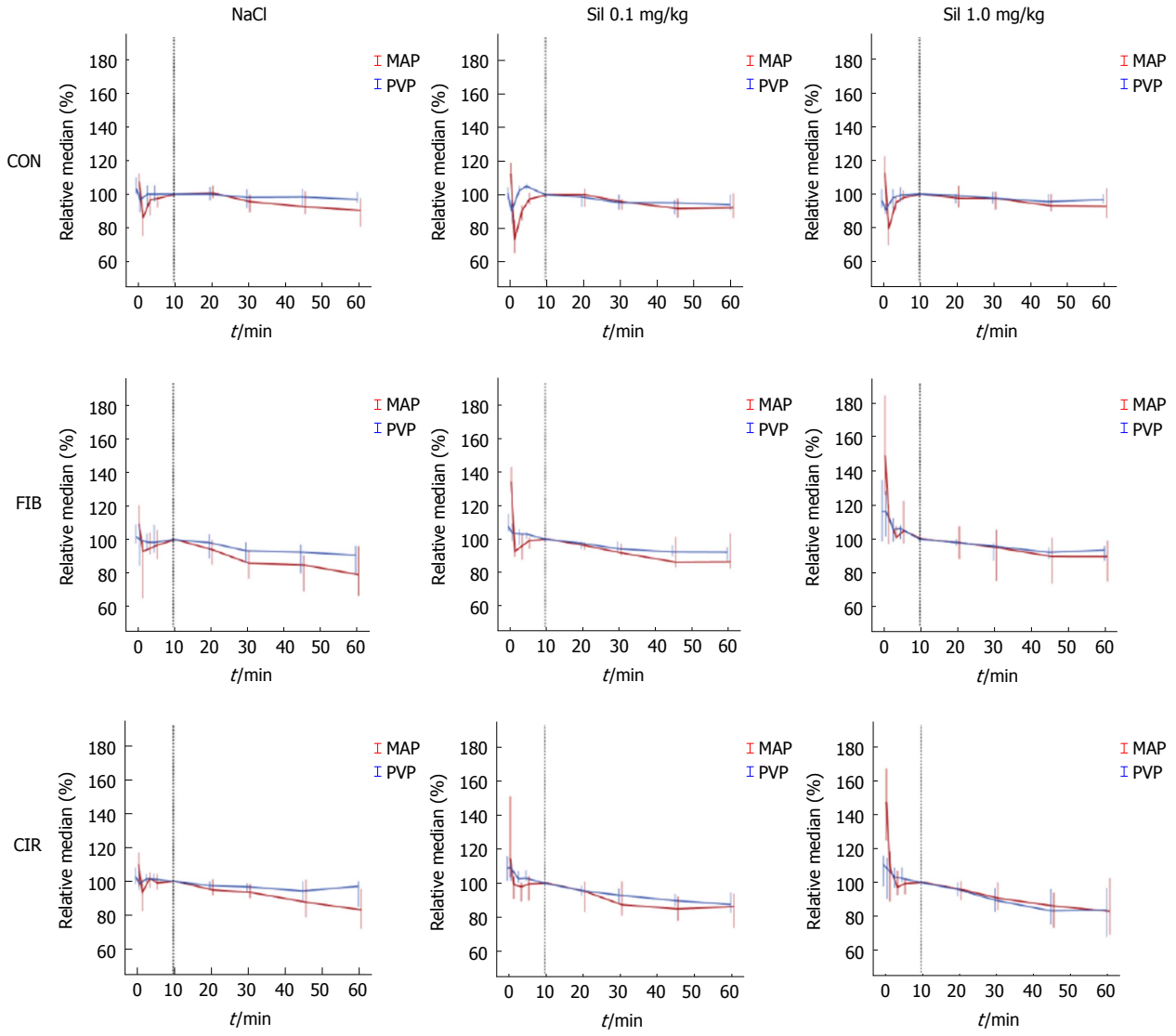


Figure 3 Relative median (%) of mean arterial pressure and portal venous pressure over 60 min with error bars representing 95% confidence intervals. The time point "10 min" was set to 100%.

or viruses are accompanied by overexpression of iNOS[38,39]. NO derived from iNOS activity may have pathogenic effect (probably mediated by peroxynitrite). It is assumed that iNOS competes with eNOS for the cofactor BH4, so that the effect of eNOS-derived NO, which maintains HSCs in the quiescent state, is abrogated[18,40]. Thus, the present results suggest that in the TAA model HSCs were activated and transformed into myofibroblasts.

A previous study from Theilig *et al*[41] showed that in healthy livers nearly all HSCs were immunostained for sGC in the periphery of the hepatic lobule, while the intensity decreased in the direction of the central vein. Around the central vein there was nearly no sGC detectable. A further preclinical study from Davies *et al*[42] in the model of bile duct ligation-induced cirrhosis demonstrated decreased hepatic sGC activity but the addition of NO donors increased its activity. These authors did not consider a zonation of sGC. Therefore, the lower sGC activity may indicate the loss of zonation in cirrhosis. cGMP concentrations were not measured in the study but the authors speculate that addition of substrates for eNOS and application of an phosphodiesterase inhibitor could be beneficial on vascular dysfunction in cirrhosis. Loureiro-Silva *et al*[43] used Western blotting and found 1.6-fold overexpression of the b1-subunit of sGC in the model of CCl4-induced cirrhosis. This corresponds to the present data of increased mRNA expression of both sGC subunits [a1 (1.7-fold) and b1 (3-fold)] in TAA-induced cirrhosis. A possible zonation of sGC was not investigated in the present study.

Likewise, Loureiro-Silva *et al*[43] demonstrated an overexpression of PDE-5. Using immunofluorescence a cellular mapping of the enzyme was not possible in this study. Moreover, Lee *et al*[25] found a markedly increased protein expression of PDE-5 and a slight overexpression of sGC. One week administration of Sildenafil induced a further overexpression of sGC and a reduction of PDE-5. This effect was accompanied by a reduction of portal venous pressure and portal perfusion pressure.

Finally, Angeli *et al*[44] found lowered cGMP content and increased PDE-5 activity in the cirrhotic liver but also in the kidney. The present study showed an 11-fold overexpression of PDE-5 on the mRNA level and an approximately 8-fold overexpression on the protein level in perisinusoidal cells (most probably HSCs) in cirrhosis. In healthy livers, a marked zonation was observed, low frequency of positive cells in perisinusoidal cells (most probably HCSs) and a marked expression of PDE-5 in perivenular hepatocytes (zone 3). In cirrhosis, the zonation was lost and a markedly increased staining was observed in perisinusoidal cells. The fibrous septa in cirrhosis also exhibited high staining, but a clear attribution to distinct cell types was not yet possible.

Regarding cGMP, Lee *et al*[26] found that in patients with liver cirrhosis after administration of Sildenafil cGMP in liver veins increases and intrahepatic flow resistance decreases. Levels of cGMP in humans with healthy liver were not determined. Other groups found elevated serum cGMP levels in animals and humans with advanced cirrhosis[45-47]. The increase of serum cGMP levels after administration of Sildenafil as demonstrated in the present study reflects the anticipated effect that Sildenafil reduces the PDE-5-induced inactivation of cGMP.

The opposing zonation of sGC and PDE-5 in the liver lobule of healthy rats suggests a physiological role in maintaining cGMP in the sinusoids at the appropriate level: Near the portal triad high sGC activity catalyzes the formation of high levels of cGMP which is not inactivated by the low activity of PDE-5. Near the terminal venule cGMP production is low and high PDE-5 activity inactivates cGMP before entering the systemic circulation. For many important metabolic processes in the liver lobule a zonation has been already found[48,49]. Generally, in cirrhosis the zonation of enzymes and metabolic functions is lost along with disturbed architecture of residual liver lobules. Using immunohistochemistry the present study showed that in cirrhosis the zonation of PDE-5 was lost and the number of PDE-5 positive perisinusoidal cells was largely increased. These data suggest that not only an altered expression but also a disturbed zonation of key components of the NO-cGMP system in cirrhosis may play an important role in the pathophysiology of portal hypertension.

To investigate whether pharmaceutical inhibition of PDE-5 might be a favorable measure in order to at least partly correct the dysregulation of the NO-cGMP pathway, hemodynamic measurements were performed in the next step to evaluate the acute effects of administration of either NaCl or Sildenafil (Sil 0.1 mg/kg or Sil 1.0 mg/kg). The parameters portal venous pressure (PVP), mean arterial pressure (MAP), and heart rate (HR) were monitored over 60 min in the same rat pool than before, *i.e.*, in rats with healthy, fibrotic and cirrhotic livers.

At the start of the measurements MAP was relatively low in diseased rats compared to healthy rats. However, it is known that advanced human liver cirrhosis is accompanied by a low MAP in the hyperdynamic circulatory state[50] and the patients are highly vulnerable to fluid loss and anesthesia, what might also explain the different baseline values in the present study. Consequently, PVP in diseased rats was relatively low in the present study. The most noticeable change was observed after high-dose Sildenafil (1.0 mg/kg) in rats with cirrhosis. This led to a trend towards decreased PVP by 19% and a nonsignificant lowering of MAP. Interestingly, Sildenafil showed no effect on PVP in rats with healthy livers and a physiological PDE-5 expression, supporting the hypothesis that the presence of high PDE-5 expression in a particular tissue reflects the effect of a PDE-5 inhibitor[51]. No statistical significance was observed in the present study, what might be attributed to the high variation of the decrease of PVP. Nevertheless, the present preclinical results support previous clinical findings[30] and provide additional arguments about the benefits of PDE-5 inhibitors in therapy of portal hypertension.

Limitations of the study

qRT-PCR data did not reflect the enzymatic activity. cGMP concentrations were measured from the carotid arterial instead of the portal venous serum.

The administration of 0.6 mL liquid into the right atrium caused parameter variations for some minutes due to volume overload. During the course of 60 min, a decrease in parameter values (*i.e.*, PVP, MAP, and HR) even in the NaCl groups could be observed, indicating that the anesthesia, as well as the operative conditions, could have added more variability and uncertainty to the measurements**.** The procedure itself - the operative procedure of the rats and the hemodynamic measurement - lasted about 2.5 to 3 h per rat. The use of different rat strains (Sprague Dawley for healthy liver, Wistar for diseased liver), as well as the fact that some of the diseased rats had CCCs could have influenced the results of the hemodynamic measurements.

In conclusion

This study provided further evidence that liver cirrhosis is associated with dysregulations in the NO - cGMP pathway. The most remarkable findings in the context of liver cirrhosis were hepatic overexpression and abrogated zonation of PDE-5 which might contribute to sinusoidal constriction and portal hypertension. Hence, the inhibition of PDE-5 may be a promising adjunct in portal hypertension therapy.

ARTICLE HIGHLIGHTS

Research background

Cirrhotic portal hypertension is partly caused by sinusoidal constriction, wherein the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway plays a pivotal role. Whereas in systemic circulation there is excess NO formation, NO availability is reduced inside the liver ("NO-paradox"). Currently non-selective beta-blockers (NSBB) are mainly used as medical

therapy for portal hypertension. β1 receptor blockade reduces portal flow by decreasing cardiac output while β2 blockade allows unopposed α1-adrenergic activity resulting in splanchnic vasoconstriction and decreased portal inflow. However, their therapeutic efficacy is limited by frequent side effects such as circulatory dysregulation, which often prevent sufficient dosing, particularly in decompensated cirrhosis.

Many approaches have been proposed to specifically target the reduced NO availability in diseased liver. Liver specific NO delivering drugs (*e.g.*, NCX-1000) yielded disappointing results. Statins lead to enhanced activity of endothelial NO synthase. Although statins reduced portal hypertension in a clinical setting, the effects on clinical outcome were modest. As vasodilating cGMP is converted to inactive 5'-GMP by phosphodiesterase-5 (PDE-5), PDE-5 inhibitors could exert a beneficial effect in portal hypertension therapy. However, preclinical and clinical studies on PDE-5 inhibition yielded promising but variable results.

Research motivation

Novel medical therapies of cirrhotic portal hypertension are clearly needed, because NSBB have only limited efficacy. Targeting the altered NO-cGMP pathway in liver cirrhosis may be an intriguing approach to solve this problem. The imbalance between vasodilating (cGMP) and vasoconstricting (*e.g.*, endothelins) compounds may be positively influenced by inhibiting the conversion of cGMP to inactive 5'-GMP by PDE-5 inhibitors. In this preclinical study, liver disease-induced alterations of biochemical components of the NO-cGMP pathway, in which cGMP represents the most potent vasodilating compound, were investigated. The current study aimed to demonstrate a potential rationale for therapy of cirrhotic portal hypertension using PDE-5 inhibitors.

Research objectives

The study investigated alterations of the NO-cGMP in an animal model of liver fibrosis/cirrhosis. The aim was to show whether or not the data yielded a rationale for therapy of cirrhotic portal hypertension using inhibitors of PDE-5.

Research methods

Gene expression of key components of the NO-cGMP pathway was investigated using PCR. Expression and localization of PDE-5 in healthy and cirrhotic livers was demonstrated by immunohistochemistry. The vasodilating compound cGMP was measured by ELISA. Systemic and portal hemodynamic parameters were monitored after application of NaCl, low-dose Sildenafil, and high-dose Sildenafil.

Research results

Key enzymes of the NO-cGMP system were overexpressed in cirrhosis: endothelial NO synthase (2.2-fold), soluble guanylate cyclase subunit α1 (1.7-fold) and soluble guanylate cyclase subunit β1 (3.0-fold), and PDE-5 (11-fold). Inducible NO synthase was expressed only in diseased livers. Immunohistochemistry showed a zonation of PDE-5 protein in healthy livers: Pronounced expression in zone 3 hepatocytes and slight expression in perisinusoidal cells (probably hepatic stellate cells). This zonation of PDE-5 was abrogated in cirrhotic livers. The enzyme was overexpressed in perisinusoidal cells. The vasodilating compound cGMP was reduced in liver cirrhosis. Inhibition of PDE-5 by Sildenafil could at least partly correct these disturbances: It led to a significant increase of cGMP and a reduction of portal venous pressure.

Research conclusions

The present study provided evidence that alterations of the NO-cGMP pathway may play an important role in the pathogenesis of cirrhotic portal hypertension. In healthy liver a zonation of PDE-5 was found, which may have a significant function in maintaining an appropriate level of the vasodilating compound cGMP in the sinusoids. This zonation was lost in cirrhosis and PDE-5 was overexpressed in perisinusoidal cells resulting in decreased cGMP serum levels. This disturbance was at least partly counteracted by inhibition of PDE-5. Sildenafil induced a normalization of cGMP levels and a reduction of portal venous pressure.

Research perspectives

The present study provides the rationale for the use of inhibitors of PDE-5 in the therapy of cirrhotic portal hypertension. Clinical studies are needed to test whether patients with portal hypertension in liver cirrhosis will benefit from application of PDE-5 inhibitors in terms of clinical endpoints such as prevention of (re)-bleeding from esophageal varices or prolonged survival.

REFERENCES

1 **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

2 **García-Pagán JC**, Gracia-Sancho J, Bosch J. Functional aspects on the pathophysiology of portal hypertension in cirrhosis. *J Hepatol* 2012; **57**: 458-461 [PMID: 22504334 DOI: 10.1016/j.jhep.2012.03.007]

3 **Bosch J**, Groszmann RJ, Shah VH. Evolution in the understanding of the pathophysiological basis of portal hypertension: How changes in paradigm are leading to successful new treatments. *J Hepatol* 2015; **62**: S121-S130 [PMID: 25920081 DOI: 10.1016/j.jhep.2015.01.003]

4 **Iwakiri Y**, Shah V, Rockey DC. Vascular pathobiology in chronic liver disease and cirrhosis - current status and future directions. *J Hepatol* 2014; **61**: 912-924 [PMID: 24911462 DOI: 10.1016/j.jhep.2014.05.047]

5 **Mónica FZ**, Bian K, Murad F. The Endothelium-Dependent Nitric Oxide-cGMP Pathway. *Adv Pharmacol* 2016; **77**: 1-27 [PMID: 27451093 DOI: 10.1016/bs.apha.2016.05.001]

6 **Langer DA**, Shah VH. Nitric oxide and portal hypertension: interface of vasoreactivity and angiogenesis. *J Hepatol* 2006; **44**: 209-216 [PMID: 16297493 DOI: 10.1016/j.jhep.2005.10.004]

7 **Rockey DC**. Hepatic blood flow regulation by stellate cells in normal and injured liver. *Semin Liver Dis* 2001; **21**: 337-349 [PMID: 11586464 DOI: 10.1055/s-2001-17551]

8 **Hennenberg M**, Trebicka J, Sauerbruch T, Heller J. Mechanisms of extrahepatic vasodilation in portal hypertension. *Gut* 2008; **57**: 1300-1314 [PMID: 18445644 DOI: 10.1136/gut.2007.144584]

9 **de Franchis R**; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]

10 **Garcia-Tsao G**, Bosch J. Management of varices and variceal hemorrhage in cirrhosis. *N Engl J Med* 2010; **362**: 823-832 [PMID: 20200386 DOI: 10.1056/NEJMra0901512]

11 **Ge PS**, Runyon BA. The changing role of beta-blocker therapy in patients with cirrhosis. *J Hepatol* 2014; **60**: 643-653 [PMID: 24076364 DOI: 10.1016/j.jhep.2013.09.016]

12 **Krag A**, Wiest R, Albillos A, Gluud LL. The window hypothesis: haemodynamic and non-haemodynamic effects of β-blockers improve survival of patients with cirrhosis during a window in the disease. *Gut* 2012; **61**: 967-969 [PMID: 22234982 DOI: 10.1136/gutjnl-2011-301348]

13 **Mandorfer M**, Bota S, Schwabl P, Bucsics T, Pfisterer N, Kruzik M, Hagmann M, Blacky A, Ferlitsch A, Sieghart W, Trauner M, Peck-Radosavljevic M, Reiberger T. Nonselective β blockers increase risk for hepatorenal syndrome and death in patients with cirrhosis and spontaneous bacterial peritonitis. *Gastroenterology* 2014; **146**: 1680-90.e1 [PMID: 24631577 DOI: 10.1053/j.gastro.2014.03.005]

14 **Van de Casteele M**, Omasta A, Janssens S, Roskams T, Desmet V, Nevens F, Fevery J. In vivo gene transfer of endothelial nitric oxide synthase decreases portal pressure in anaesthetised carbon tetrachloride cirrhotic rats. *Gut* 2002; **51**: 440-445 [PMID: 12171971]

15 **Shah V**, Chen AF, Cao S, Hendrickson H, Weiler D, Smith L, Yao J, Katusic ZS. Gene transfer of recombinant endothelial nitric oxide synthase to liver in vivo and in vitro. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G1023-G1030 [PMID: 11053000 DOI: 10.1152/ajpgi.2000.279.5.G1023]

16 **Yu Q**, Shao R, Qian HS, George SE, Rockey DC. Gene transfer of the neuronal NO synthase isoform to cirrhotic rat liver ameliorates portal hypertension. *J Clin Invest* 2000; **105**: 741-748 [PMID: 10727442 DOI: 10.1172/JCI7997]

17 **Morales-Ruiz M**, Cejudo-Martín P, Fernández-Varo G, Tugues S, Ros J, Angeli P, Rivera F, Arroyo V, Rodés J, Sessa WC, Jiménez W. Transduction of the liver with activated Akt normalizes portal pressure in cirrhotic rats. *Gastroenterology* 2003; **125**: 522-531 [PMID: 12891555]

18 **Matei V**, Rodríguez-Vilarrupla A, Deulofeu R, Colomer D, Fernández M, Bosch J, Garcia-Pagán JC. The eNOS cofactor tetrahydrobiopterin improves endothelial dysfunction in livers of rats with CCl4 cirrhosis. *Hepatology* 2006; **44**: 44-52 [PMID: 16799985 DOI: 10.1002/hep.21228]

19 **Berzigotti A**, Bellot P, De Gottardi A, Garcia-Pagan JC, Gagnon C, Spénard J, Bosch J. NCX-1000, a nitric oxide-releasing derivative of UDCA, does not decrease portal pressure in patients with cirrhosis: results of a randomized, double-blind, dose-escalating study. *Am J Gastroenterol* 2010; **105**: 1094-1101 [PMID: 19920806 DOI: 10.1038/ajg.2009.661]

20 **Fiorucci S**, Antonelli E, Tocchetti P, Morelli A. Treatment of portal hypertension with NCX-1000, a liver-specific NO donor. A review of its current status. *Cardiovasc Drug Rev* 2004; **22**: 135-146 [PMID: 15179450]

21 **Trebicka J**, Hennenberg M, Laleman W, Shelest N, Biecker E, Schepke M, Nevens F, Sauerbruch T, Heller J. Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. *Hepatology* 2007; **46**: 242-253 [PMID: 17596891 DOI: 10.1002/hep.21673]

22 **Pollo-Flores P**, Soldan M, Santos UC, Kunz DG, Mattos DE, da Silva AC, Marchiori RC, Rezende GF. Three months of simvastatin therapy vs. placebo for severe portal hypertension in cirrhosis: A randomized controlled trial. *Dig Liver Dis* 2015; **47**: 957-963 [PMID: 26321186 DOI: 10.1016/j.dld.2015.07.156]

23 **Abraldes JG**, Villanueva C, Aracil C, Turnes J, Hernandez-Guerra M, Genesca J, Rodriguez M, Castellote J, García-Pagán JC, Torres F, Calleja JL, Albillos A, Bosch J; BLEPS Study Group. Addition of Simvastatin to Standard Therapy for the Prevention of Variceal Rebleeding Does Not Reduce Rebleeding but Increases Survival in Patients With Cirrhosis. *Gastroenterology* 2016; **150**: 1160-1170.e3 [PMID: 26774179 DOI: 10.1053/j.gastro.2016.01.004]

24 **Clemmesen JO**, Giraldi A, Ott P, Dalhoff K, Hansen BA, Larsen FS. Sildenafil does not influence hepatic venous pressure gradient in patients with cirrhosis. *World J Gastroenterol* 2008; **14**: 6208-6212 [PMID: 18985812]

25 **Lee KC**, Yang YY, Huang YT, Lee FY, Hou MC, Lin HC, Lee SD. Administration of a low dose of sildenafil for 1 week decreases intrahepatic resistance in rats with biliary cirrhosis: the role of NO bioavailability. *Clin Sci* (Lond) 2010; **119**: 45-55 [PMID: 20132096 DOI: 10.1042/CS20090601]

26 **Lee KC**, Yang YY, Wang YW, Hou MC, Lee FY, Lin HC, Lee SD. Acute administration of sildenafil enhances hepatic cyclic guanosine monophosphate production and reduces hepatic sinusoid resistance in cirrhotic patients. *Hepatol Res* 2008; **38**: 1186-1193 [PMID: 18631254 DOI: 10.1111/j.1872-034X.2008.00388.x]

27 **Deibert P**, Schumacher YO, Ruecker G, Opitz OG, Blum HE, Rössle M, Kreisel W. Effect of vardenafil, an inhibitor of phosphodiesterase-5, on portal haemodynamics in normal and cirrhotic liver -- results of a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 121-128 [PMID: 16393289 DOI: 10.1111/j.1365-2036.2006.02735.x]

28 **Halverscheid L**, Deibert P, Schmidt R, Blum HE, Dunkern T, Pannen BH, Kreisel W. Phosphodiesterase-5 inhibitors have distinct effects on the hemodynamics of the liver. *BMC Gastroenterol* 2009; **9**: 69 [PMID: 19765284 DOI: 10.1186/1471-230X-9-69]

29 **Tandon P**, Inayat I, Tal M, Spector M, Shea M, Groszmann RJ, Garcia-Tsao G. Sildenafil has no effect on portal pressure but lowers arterial pressure in patients with compensated cirrhosis. *Clin Gastroenterol Hepatol* 2010; **8**: 546-549 [PMID: 20144739 DOI: 10.1016/j.cgh.2010.01.017]

30 **Kreisel W**, Deibert P, Kupcinskas L, Sumskiene J, Appenrodt B, Roth S, Neagu M, Rössle M, Zipprich A, Caca K, Ferlitsch A, Dilger K, Mohrbacher R, Greinwald R, Sauerbruch T. The phosphodiesterase-5-inhibitor udenafil lowers portal pressure in compensated preascitic liver cirrhosis. A dose-finding phase-II-study. *Dig Liver Dis* 2015; **47**: 144-150 [PMID: 25483910 DOI: 10.1016/j.dld.2014.10.018]

31 **Wallace MC**, Hamesch K, Lunova M, Kim Y, Weiskirchen R, Strnad P, Friedman SL. Standard operating procedures in experimental liver research: thioacetamide model in mice and rats. *Lab Anim* 2015; **49**: 21-29 [PMID: 25835735 DOI: 10.1177/0023677215573040]

32 **Li X**, Benjamin IS, Alexander B. Reproducible production of thioacetamide-induced macronodular cirrhosis in the rat with no mortality. *J Hepatol* 2002; **36**: 488-493 [PMID: 11943419]

33 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520 [PMID: 8188183]

34 **Pfaffl MW**. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45 [PMID: 11328886]

35 **Dunn OJ**. Multiple Comparisons Using Rank Sums. *Technometrics* 1964; **6**: 241-252 [DOI: 10.1080/00401706.1964.10490181]

36 **Iwakiri Y**. Pathophysiology of portal hypertension. *Clin Liver Dis* 2014; **18**: 281-291 [PMID: 24679494 DOI: 10.1016/j.cld.2013.12.001]

37 **Trebicka J**, Hennenberg M, Odenthal M, Shir K, Klein S, Granzow M, Vogt A, Dienes HP, Lammert F, Reichen J, Heller J, Sauerbruch T. Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation via decreased turnover of hepatic stellate cells. *J Hepatol* 2010; **53**: 702-712 [PMID: 20633948 DOI: 10.1016/j.jhep.2010.04.025]

38 **Iwakiri Y**. Nitric oxide in liver fibrosis: The role of inducible nitric oxide synthase. *Clin Mol Hepatol* 2015; **21**: 319-325 [PMID: 26770919 DOI: 10.3350/cmh.2015.21.4.319]

39 **Hasselblatt P**, Rath M, Komnenovic V, Zatloukal K, Wagner EF. Hepatocyte survival in acute hepatitis is due to c-Jun/AP-1-dependent expression of inducible nitric oxide synthase. *Proc Natl Acad Sci USA* 2007; **104**: 17105-17110 [PMID: 17940019 DOI: 10.1073/pnas.0706272104]

40 **Dai Y**, Cui J, Cun Y, Shi A. Tetrahydrobiopterin ameliorates hepatic ischemia-reperfusion Injury by coupling with eNOS in mice. *J Surg Res* 2012; **176**: e65-e71 [PMID: 22475351 DOI: 10.1016/j.jss.2011.12.034]

41 **Theilig F**, Bostanjoglo M, Pavenstädt H, Grupp C, Holland G, Slosarek I, Gressner AM, Russwurm M, Koesling D, Bachmann S. Cellular distribution and function of soluble guanylyl cyclase in rat kidney and liver. *J Am Soc Nephrol* 2001; **12**: 2209-2220 [PMID: 11675397]

42 **Davies NA**, Hodges SJ, Pitsillides AA, Mookerjee RP, Jalan R, Mehdizadeh S. Hepatic guanylate cyclase activity is decreased in a model of cirrhosis: a quantitative cytochemistry study. *FEBS Lett* 2006; **580**: 2123-2128 [PMID: 16563392 DOI: 10.1016/j.febslet.2006.02.080]

43 **Loureiro-Silva MR**, Iwakiri Y, Abraldes JG, Haq O, Groszmann RJ. Increased phosphodiesterase-5 expression is involved in the decreased vasodilator response to nitric oxide in cirrhotic rat livers. *J Hepatol* 2006; **44**: 886-893 [PMID: 16545481 DOI: 10.1016/j.jhep.2006.01.032]

44 **Angeli P**, Jiménez W, Veggian R, Fasolato S, Volpin R, MacHenzie HS, Craighero R, Libera VD, Sticca A, Arroyo V, Gatta A. Increased activity of guanosine 3'-5'-cyclic monophosphate phosphodiesterase in the renal tissue of cirrhotic rats with ascites. *Hepatology* 2000; **31**: 304-310 [PMID: 10655250 DOI: 10.1002/hep.510310207]

45 **Montoliu C**, Kosenko E, Del Olmo JA, Serra MA, Rodrigo JM, Felipo V. Correlation of nitric oxide and atrial natriuretic peptide changes with altered cGMP homeostasis in liver cirrhosis. *Liver Int* 2005; **25**: 787-795 [PMID: 15998430 DOI: 10.1111/j.1478-3231.2005.01066.x]

46 **Bezinover D**, Kadry Z, Uemura T, Sharghi M, Mastro AM,

Sosnoski DM, Dalal P, Janicki PK. Association between plasma cyclic guanosine monophosphate levels and hemodynamic instability during liver transplantation. *Liver Transpl* 2013; **19**: 191-198 [PMID: 23161851 DOI: 10.1002/lt.23570]

47 **Kirstetter P**, Moreau R, Vachiery F, Gadano A, Soupison T, Pilette C, Pussard E, Cailmail S, Takahashi H, Lebrec D. Plasma concentrations of cyclic 3', 5'-guanosine monophosphate in patients with cirrhosis: relationship with atrial natriuretic peptide and haemodynamics. *J Gastroenterol Hepatol* 1997; **12**: 233-236 [PMID: 9142641 DOI: 10.1111/j.1440-1746.1997.tb00414.x]

48 **Soto-Gutierrez A**, Gough A, Vernetti LA, Taylor DL, Monga SP. Pre-clinical and clinical investigations of metabolic zonation in liver diseases: The potential of microphysiology systems. *Exp Biol Med* (Maywood) 2017; **242**: 1605-1616 [PMID: 28467181 DOI: 10.1177/1535370217707731]

49 **Jungermann K**. Metabolic zonation of liver parenchyma. *Semin Liver Dis* 1988; **8**: 329-341 [PMID: 3062788 DOI: 10.1055/s-2008-1040554]

50 **Bolognesi M**, Di Pascoli M, Verardo A, Gatta A. Splanchnic vasodilation and hyperdynamic circulatory syndrome in cirrhosis. *World J Gastroenterol* 2014; **20**: 2555-2563 [PMID: 24627591 DOI: 10.3748/wjg.v20.i10.2555]

51 **Corbin JD**, Beasley A, Blount MA, Francis SH. High lung PDE5: a strong basis for treating pulmonary hypertension with PDE5 inhibitors. *Biochem Biophys Res Commun* 2005; **334**: 930-938 [PMID: 16023993 DOI: 10.1016/j.bbrc.2005.06.183]

P- Reviewer: Garbuzenko DV **S- Editor**: Wang XJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4369-4383

DOI: 10.3748/wjg.v24.i38.4369

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

ORIGINAL ARTICLE

Basic Study

Sex-specific effects of *Eugenia punicifolia* extract on gastric ulcer healing in rats

Larissa Lucena Périco, Vinícius Peixoto Rodrigues, Rie Ohara, Gabriela Bueno, Vânia Vasti Alfieri Nunes, Raquel Cássia dos Santos, Ana Carolina Lima Camargo, Luis Antônio Justulin Júnior, Sérgio Faloni de Andrade, Viviane Miranda Bispo Steimbach, Luísa Mota da Silva, Lúcia Regina Machado da Rocha, Wagner Vilegas, Catarina dos Santos, Clélia Akiko Hiruma-Lima

Larissa Lucena Périco, Vinícius Peixoto Rodrigues, Rie Ohara, Gabriela Bueno, Vânia Vasti Alfieri Nunes, Lúcia Regina Machado da Rocha, Clélia Akiko Hiruma-Lima, Department of Physiology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu 18618-970, São Paulo, Brazil

Raquel de Cássia dos Santos, Laboratory of Bioactive Compounds, São Francisco University, Bragança Paulista 12916-900, São Paulo, Brazil

Ana Carolina Lima Camargo, Luis Antônio Justulin Júnior, Department of Morphology, Institute of Biosciences, São Paulo State University (UNESP), Botucatu 18618-970, São Paulo, Brazil

Sérgio Faloni de Andrade, Viviane Miranda Bispo Steimbach, Luísa Mota da Silva, Programa de Pós-graduação em Ciências Farmacêuticas, Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí - UNIVALI, Itajaí 88302-901, Santa Catarina, Brazil

Wagner Vilegas, Coastal Campus of São Vicente, São Paulo State University (UNESP), São Vicente 11330-900, São Paulo, Brazil

Catarina dos Santos, Department of Biological Science, Faculty of Sciences and Languages, São Paulo State University (UNESP), Assis 19806-900, São Paulo, Brazil

ORCID number: Larissa Lucena Périco (0000-0002-0888-9911); Vinícius Peixoto Rodrigues (0000-0002-9312-2431); Rie Ohara (0000-0003-4430-0016); Gabriela Bueno (0000-0003-0502-3415); Vânia Vasti Alfieri Nunes (0000-0001-7354-471X); Raquel de Cássia dos Santos (0000-0002-3195-8188); Ana Carolina Lima Camargo (0000-0003-4993-2573); Luis Antônio Justulin Júnior (0000-0001-6142-3515); Sérgio Faloni de Andrade (0000-0001-9294-7290); Viviane Miranda Bispo Steimbach (0000-0002-2248-2464); Luísa Mota da Silva (0000-0001-5329-7301); Lúcia Regina Machado da Rocha (0000-0002-7911-3862); Wagner Vilegas (0000-0003-3032-2556); Catarina dos Santos (0000-0002-9881-527X); Clélia Akiko Hiruma-Lima (0000-0002- 8645-3777).

Author contributions: Périco LL, Rodrigues VP, Ohara R, Bueno G, Santos RC, Camargo AC, Justulin LA, de Andrade SF, Steimbach VM, da Silva LM performed most of the experiments; Nunes VV and dos Santos C prepared the extract of *Eugenia punicifolia*; Périco LL, dos Santos RC, da Rocha LR, Vilegas W and Hiruma-Lima CA, contributed to the conception, design and coordinated the study; Périco LL, Rodrigues VP, Ohara R, da Rocha LR and Hiruma-Lima CA participated in writing the manuscript; all authors approved the final version of the article to be published.

Supported by the São Paulo Research Foundation (FAPESP), No. 2015/14797-3 to Périco LL and No. 2009/52237-9 to Laboratory of Biological Assays with Natural Products.

Institutional animal care and use committee statement: All the experimental practices related to animal care and use agreed with the protocols approved by the Ethics Committee on the Use of Animals of the Institute of Biosciences, UNESP, Botucatu, São Paulo, Brazil (No. 675-CEUA).

Conflict-of-interest statement: The authors declare that they have no commercial, personal, political, intellectual, or religious interests related to the work presented herein.

Data sharing statement: All data are available from the corresponding author upon reasonable request.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Manuscript source: Unsolicited manuscript

Correspondence to: Clélia Akiko Hiruma-Lima, PhD, Adjunct Professor, Department of Physiology, Institute of Biosciences, Botucatu 18618-970, São Paulo, Brazil. clelia.hiruma@unesp.br
Telephone: +55-14-38800312
Fax: +55-14-3815-3744

Received: June 13, 2018
Peer-review started: June 13, 2018
First decision: July 4, 2018
Revised: July 11, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: October 14, 2018

Abstract

AIM

To evaluate the sex-specific effects of a hydroalcoholic extract from *Eugenia punicifolia* (HEEP) leaves on gastric ulcer healing.

METHODS

In this rat study involving males, intact (cycling) females, and ovariectomized females, gastric ulcers were induced using acetic acid. A vehicle, lansoprazole, or HEEP was administered for 14 d after ulcer induction. Body weight was monitored throughout the treatment period. At the end of treatment, the rats were euthanized and the following *in vivo* and *in vitro* investigations were performed: macroscopic examination of the lesion area and organ weights, biochemical analysis, zymography, and evaluation of protein expression levels. Additionally, the concentration-dependent effect of HEEP was evaluated in terms of subacute toxicity and cytotoxicity.

RESULTS

Compared to the vehicle, HEEP demonstrated a great healing capacity by substantially reducing the ulcerative lesion area in males (52.44%), intact females (85.22%), and ovariectomized females (65.47%), confirming that HEEP accelerates the healing of acetic acid-induced gastric lesions and suggesting that this effect is modulated by female sex hormones. The antiulcer effect of HEEP was mediated by prostaglandin E_2 only in male rats. Overall, the beneficial effect of HEEP was the highest in intact females. Notably, HEEP promoted the expression of vascular endothelial growth factor (intact *vs* ovariectomized females) and decreased the expression of Caspase-8 and Bcl-2 (intact female *vs* male or ovariectomized female). Additionally, HEEP enhanced fibroblast proliferation and migration into a wounded area *in vitro*, confirming its healing effect. Finally, no sign of subacute toxicity or cytotoxicity of HEEP was observed.

CONCLUSION

In gastric ulcers, HEEP-induced healing (modulated by female sex hormones; in males, mediated by prostaglandin) involves extracellular matrix remodeling, with gastric mucosa cell proliferation and migration.

Key words: *Eugenia punicifolia*; Gastric ulcer healing; Acetic acid; Sex difference; Myrtaceae; Toxicity

Core tip: Gastric ulcers, which occur due to an imbalance between protective and aggressive agents at the gastric mucosa surface, is a chronic disease that affects millions worldwide and has high rates of relapse. The conventional treatment for gastric ulcers is associated with several side effects and poor healing of the gastric mucosa. *Eugenia punicifolia* is a medicinal plant used to treat inflammation and wounds. The present study in rats with gastric ulcers confirms the healing effect of *Eugenia punicifolia* extract and clarifies its differential effect in males and females. These findings are useful for developing novel and safe therapies for gastric ulcers.

Périco LL, Rodrigues VP, Ohara R, Bueno G, Nunes VV, dos Santos RC, Camargo AC, Justulin LA, de Andrade SF, Steimbach VM, da Silva LM, da Rocha LR, Vilegas W, dos Santos RC, Hiruma-Lima CA. Sex-specific effects of *Eugenia punicifolia* extract on gastric ulcer healing in rats. *World J Gastroenterol* 2018; 24(38): 4369-4383 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4369.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4369

INTRODUCTION

Gastric ulcers, which result from an imbalance between the protective and aggressive agents at the surface of the gastric mucosa, is a chronic disease that affects millions around the world and has high relapse rates[1,2]. Alcohol consumption, prolonged treatment with non-steroidal anti-inflammatory drugs, stress, and *Helicobacter pylori* infection favor such an imbalance and represent key etiological factors of gastric ulcers[3]. Current treatment strategies for gastric ulcers involve the use of anti-secretory drugs, including antagonists of histamine receptor type 2 (*e.g.*, ranitidine) and proton pump inhibitors [*e.g.*, lansoprazole (LZ)], as well as antibiotics for the eradication of *Helicobacter pylori*. While such treatments are effective, prolonged use of anti-secretory drugs, especially ranitidine and proton pump inhibitors, is associated with several side effects[4] and poor healing of the gastric mucosa[5].

In 1991, Tarnawski *et al*[6] proposed the concept of quality of ulcer healing, which takes into consideration the fact that tissue regeneration within the ulcer scar is often incomplete. Within the quality of ulcer healing concept, the evaluation of gastric ulcer healing is focused on whether the structure and function of the mucosal and submucosal tissue have recovered completely, in addition to endoscopic examination and evaluation of ulcer size. It has been shown that ulcer recurrence is closely related to

the quality of ulcer healing[7].

Ulcers frequently recur following treatment with anti-secretory drugs[7,8]. Therefore, alternative therapies for gastric ulcers are desirable[9]. Herbal combination preparations are popular among traditional herbal medicine practitioners. The rationale behind such combinations is frequently questioned, and it remains challenging to assess the individual contribution of each component to the overall activity of the herbal combination preparation. This holds especially true when the preparation is used in the treatment of a chronic multifactorial disease such as gastric ulcers[10].

Eugenia punicifolia (Kunth) DC (Myrtaceae), popularly known as pedra-ume-caá, pedra-ume, murta, or muta, is a shrub found mainly in the Savanna biome and in the Amazon region. The leaves of *E. punicifolia* are popularly used as a natural remedy for inflammation[11], wounds, infections[12], diabetes[13], fever, and flu[14,15]. The gastroprotective activity of the hydroalcoholic extract of *E. punicifolia* (Kunth) DC leaves (HEEP) against ethanol- or non-steroidal anti-inflammatory drug-induced ulcers in rodents has been reported[16]. Nevertheless, it remains unclear whether HEEP has any beneficial effects in the healing of installed gastric ulcers, since the gastroprotective activities of an extract do not ensure their gastric healing effects in installed gastric ulcers[17]. Therefore, the present study aimed to evaluate the sex-specific effects of HEEP in the healing of gastric ulcers in a rat model. For this purpose, we employed a rat model of acetic acid-induced gastric ulcers and analyzed the curative action of HEEP in males, intact females, and ovariectomized females.

MATERIALS AND METHODS

Chemicals and reagents

The following chemicals and reagents were used: acetic acid, methanol (Dinamica Contemporary Chemicals™, Diadema, São Paulo, Brazil), LZ (Cruz Vermelha Pharmacy of Manipulation, Botucatu, São Paulo, Brazil), RIPA buffer, protease inhibitor cocktail, Ethylenediaminetetraacetic acid (EDTA), *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), ammonium persulfate (APS), sodium chloride, 400 g/L acrylamide/bis-acrylamide solution, Triton X-100, Tris-HCl, calcium chloride, zinc chloride, coomassie brilliant blue, tris base, glycine, sodium dodecyl sulfate (SDS), glycerol, Tween 20, β-mercaptoethanol, bromophenol blue, Ponceau (Sigma Chemical Co., St. Louis, MO, United States) gelatin (Merck KGaA, Darmstadt, Germany). Saline solution (9 g/L NaCl) was used to dilute the hydroalcoholic extract from the leaves of *E. punicifolia*. The same saline solution used as a vehicle served as a negative control.

Plant material

In December 2009, Dr. Catarina dos Santos collected the leaves of *E. punicifolia* from a site in Assis State Forest (latitude, 22°33′ to 22°37′ S; longitude, 50°21′ to 50°24′ W) located near one of the experimental stations of the Forestry Institute, Assis, state of São Paulo, Brazil. Dr. Antônio CG Melo identified the species and a voucher (No. 43322) was deposited in the Herbarium D Bento Pickel, available at the Forestry Institute in Assis, São Paulo, Brazil.

Preparation of the plant extract

The dried and crushed leaves (10 g of plant material) were dissolved in 100 mL of solvent consisting of a 70:30 mix of ethanol and water (v/v). Dynamic maceration of the solution was performed for 2 h at room temperature (25 ℃ ± 2 ℃). Thereafter, the solution was filtered, and the residue was extracted twice more. The solution was dried using a rotary evaporator (40 ℃). The extract yield was 45% (4.49 g) of the original plant material.

Animals

The animal protocol was designed to minimize pain or discomfort to the animals. The *in vivo* experiments used male (280 g) and female (220 g) Wistar albino rats obtained from the breeding facility of the State University of Campinas (Multidisciplinary Center for Biological Research). The HEEP dose (125 mg/kg) for the *in vivo* experiments was determined based on a dose-response curve previously obtained in a gastric injury induction test[16]. Male and female rats were kept in separate rooms, allocated into five animals per cage, fed with Presence® (Paulínia, SP, Brazil) rodents diet, and allowed free access to filtered water. The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy. The cages were kept under controlled conditions of illumination (12 h/12 h light/dark cycle) and temperature (22 ℃ ± 2 ℃).

The estrous cycle was verified through a vaginal smear performed daily starting on postnatal day 60. The material was observed under an optical microscope, and the estrous cycle phase was determined by cytology[18-20]. The duration of the estrous cycle was calculated as the number of days between one estrus phase and the next. Only female rats that showed two consecutive regular cycles of 4-5 d were included in the experiment. Additionally, ovariectomized (OVZ) females were included. We performed bilateral ovariectomies in ten-week-old female rats and we verified the absence of estrous cycle through a vaginal smear after 2 wk. The experiments were performed in 90 d old males as well as in intact and OVZ female rats.

The 84 animals included in the study (30 males, 30 intact females, 24 ovariectomized females) were divided into nine groups with 7-10 individuals each. Group allocation was performed according to sex (male *vs* female), hormonal status in females (intact *vs* OVZ), and treatment (negative control *vs* positive control *vs* HEEP). All subsequent analyses (except for the cell viability and migration assays) were performed in each of the nine groups. The nine groups were compared to assess the sex-specific effects of HEEP *vs* vehicle and *vs* LZ.

Establishing the rat model of acetic acid-induced gastric ulcers

After being fasted for 16 h, the animals were anesthetized with a solution of ketamine (80 mg/kg) and xylazine (8 mg/kg) injected intraperitoneally and received laparotomy through a midline epigastric incision. A plastic tube with an internal diameter of 4.2 mm was firmly applied to the serosal surface of the stomach wall, and 70 μL of concentrated acetic acid solution (80%) were delivered to the gastric mucosa through the tube. The acetic acid was left to act for 20 s and then completely removed. The stomach was bathed with saline to avoid adherence to the external surface of the ulcerated region, and the abdomen was closed. Subsequently, all animals were fed normally.

Treatment protocols

In order to determine the healing effect of HEEP, three 14-d treatment protocols were evaluated in this study. The three groups of negative controls (males, intact females, and OVZ females) received 0.9% NaCl solution dosed at 10 mL/kg. The three groups of positive controls (males, intact females, and OVZ females) received LZ dosed at 30 mg/kg. The remaining three groups (males, intact females, and OVZ females) received HEEP dosed at 125 mg/kg. All treatments were delivered orally once daily beginning one day after surgery and continuing for 14 d. One day after the last drug administration, the rats were euthanized through decapitation and the stomachs were removed to evaluate the lesions. The ulcer tissue was also removed and analyzed. The affected area, expressed in mm^2, was determined using the AvSoft Bioview™ Spectra 4.0 software, according to a previously established protocol[21,22]. The organs were removed and weighed. Additionally, analysis of blood collected upon euthanasia was performed. The animals were not anesthetized prior to decapitation, since anesthetics may interfere with the results of the biochemical parameters evaluated.

Cell viability assay (MTT assay)

L929 cells (1×10^5) were cultured in 96-well plates (in triplicate) in the presence of 10% DMSO (control), a vehicle (culture medium with 0.1% DMSO), or HEEP (0.3-30 μg/mL) at 37 ℃ for 24 h. MTT (0.5 mg/mL) was added to the cultures, the cells were incubated for 3 h at 37 ℃, and the absorbance at 570 nm was analyzed after solubilization of reduced formazan crystals with pure DMSO[23]. The percentage of viable cells was calculated as (Abs.sample - Abs.blank/ Abs.vehicle × 100), where "Abs." represents the absorbance.

Cell proliferation activity

The proliferation ability of fibroblasts exposed to HEEP was assessed using the scratch-wound assay, according to a protocol previously described by Balekar *et al*[24], with a few modifications. The scratch test measures the expansion of a cell population on surfaces. Monolayers of L929 cells were allowed to form in a 24-well plate containing enriched DMEM supplemented with 10% FBS. When nearly confluent, the plate was taken out and artificial wounds were created in the monolayers by making a linear scratch in the center of each well using the tip of a sterile 200 μL plastic pipette. Any cellular debris created while making the scratch was removed by gently washing the wells with PBS. The wells with scratch wounds were divided into three wells per group and treated with HEEP at low (3 μg/mL), intermediate (10 μg/mL), or high (30 μg/mL) concentration. DMEM supplemented with 5% FBS was used as a negative control. The plates were then incubated at 37 ℃ in a humidified incubator with 5% CO_2 atmosphere. The plates were evaluated after 24 h and 48 h of incubation to assess the closure of the scratch wounds. Micrographs were used to record the wound closure activity, which was captured using an inverted microscope (Olympus CK40; Olympus, Melville, NY, United States) with 100 × magnification. All experiments were performed in triplicate.

Determination of PGE_2 levels

Serum samples were obtained from all animals at the end of the 14-d treatment with either vehicle, LZ, or HEEP. The serum prostaglandin E_2 (PGE_2) levels were quantified using a suitable enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, United States).

Extraction of total protein from the ulcer tissue

Tissue containing the gastric ulcer was used to extract total protein. The extraction was carried out by crushing the tissue using a homogenizer (Polytron Benchtop Homogenizers, Daigger Scientific, Vernon Hills, IL, United States) in a RIPA buffer and protease inhibitor cocktail (1:5 ratio). The homogenate was centrifuged at 18620 g for 45 min at 4 ℃. The supernatant was collected and then centrifuged again at 13680 g for 15 min at 4 ℃. Finally, the protein content was quantified using the biuret method[25,26].

Zymography-based evaluation of matrix remodeling activity

The complete protocol for gelatin zymography was previously described by Justulin *et al*[27]. Briefly, samples containing extracted proteins (35 μg) obtained from the gastric ulcer tissue were subjected to non-reducing electrophoresis on 8% polyacrylamide gel copolymerized with 0.1% purified gelatin. After electrophoresis, the gels were subjected to two 15 min washes in a solution of 2.5% Triton X-100 to remove SDS, and to two 5 min washes in 50 mmol/L Tris-HCl buffer (pH 8.0). Subsequently, the gels were incubated for 20 h at 37 ℃ in 50 mmol/L Tris-HCl buffer (pH 8.0) containing 5 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$. Finally, the gels were stained with Coomassie Brilliant Blue R-250. The relative molecular weight of the bands was determined according to the molecular weight standard (Precision Plus Protein™; Bio-Rad Laboratories, Hercules, CA, United States) used in electrophoresis. The bands obtained

through zymography were scanned and analyzed by densitometry. The bands representative of the gelatinase activity of matrix metalloproteinase (MMP)-2 and MMP-9 were analyzed in terms of the integrated optical density (IOD) of the bands. Specifically, the band IODs were obtained using the Image J software (National Institutes of Health, Bethesda, MA, United States). The IOD values were then plotted in a histogram showing the IOD of the treated groups (LZ, HEEP) and control group (vehicle).

Evaluation of protein expression using western blots

For this test, the protein extracts were obtained as described above for zymography. After extraction, the samples were treated with Laemmili buffer (0.5 mol/L PB buffer; pH 6.8; glycerol, 10% SDS, 0.1% bromophenol, β-mercaptoethanol) in a ratio of 1:1. Equal amounts of protein (70 μg) were separated on 10% acrylamide gel using SDS-PAGE. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane or PVDF membrane (for epidermal growth factor (EGF)), blocked with 5% non-fat milk diluted in PBS, and incubated with the following primary antibodies: anti-cyclooxygenase (COX)-1 (1:10.000, ab133319); anti-COX-2 (1:1000, ab52237); anti-vascular endothelial growth factor (VEGF) (1:1.000, ab46154); anti-EGF (1:800, ab77851), anti-Bcl-2 (1:800, ab59348), anti-caspase-3 (1:10.000, ab32499), anti-caspase-8 (1:2.000, ab25901), and anti-caspase-9 (1:5.000, ab32539) (all from Abcam, Cambridge, MA, United States). The membranes were washed with PBS and incubated with a specific secondary antibody for 2 h (horseradish peroxidase, 1:10.000, ab 97051; Abcam). After washing, the reactions were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Westborough, MA, United States) and the signals were captured using a G:BOX system (Syngene, Cambridge, England). The IODs of the targeted protein bands were measured using Image J. The expression levels were normalized to that of actin (1:10000; ab179467; Abcam).

Toxicological evaluation

Body weight was recorded daily throughout the experimental period. Macroscopic analyses and weighing of the vital organs (liver, kidneys, heart, spleen, and lungs) were performed at the end of the treatment. Additionally, analysis of blood collected upon euthanasia was performed. The blood samples were centrifuged (at 3000 g for 15 min), and the serum obtained was frozen at -80 ℃ until biochemical analysis. Serum biochemical parameters including glucose, urea, creatinine, γ-glutamyl transpeptidase (γ-GT), aspartate aminotransferase (AST), and alanine amino transferase (ALT) were measured using an automated biochemical analyzer.

Statistical analysis

The statistical methods of this study were reviewed by Dr. Clélia Akiko Hiruma-Lima from the Institute of Biosciences, UNESP. The results were expressed as the mean ± standard error of the mean. Two-group comparisons were performed using Student's *t*-test, while comparisons of three or more groups were performed using one-way analysis of variance followed by Dunnett's or Tukey's test, or using two-way analysis of variance followed by Bonferroni's test. The minimal significance level considered was $P < 0.05$.

RESULTS

Healing effect of HEEP on acetic acid-induced gastric lesions

To evaluate the HEEP-mediated healing effect of gastric ulcers, we measured the gastric lesion area at the end of treatment. In male rats, 14-d treatment with HEEP was associated with significantly decreased lesion area (52.44%, $P < 0.01$ *vs* vehicle); the same effect, though less pronounced, was noted for LZ treatment (40.81%, $P < 0.05$ *vs* vehicle) (Figure 1). Among females, HEEP was associated with a very significant reduction in lesion area, both in cycling rats (85.22%, $P < 0.0001$ *vs* vehicle) and in OVZ rats (65.47%, $P < 0.001$ *vs* vehicle); the same effect was noted for LZ treatment (*P*-value *vs* vehicle: 84.21%, < 0.0001 and 49.40%, < 0.01, respectively) (Figure 1).

Compared to males, intact females showed higher benefit in terms of gastric lesion area reduction following treatment with HEEP or LZ ($P < 0.01$ for both comparisons), whereas no significant difference in lesion area reduction was noted between males and OVZ females treated with HEEP or LZ ($P > 0.05$). Compared to OVZ females, intact females had greater reduction in the lesion area ($P < 0.01$ for HEEP; $P < 0.001$ for LZ) (Figure 1).

Cell viability of L929 fibroblasts treated with HEEP

The cytotoxic potential of HEEP was evaluated in an MTT assay of L929 fibroblasts. No cytotoxicity signal was found after 24 h of incubation with HEEP, regardless of concentration (0.3-30 μg/mL) ($P > 0.05$ *vs* vehicle) (Figure 2).

Cell proliferation activity of L929 fibroblasts treated with HEEP

We performed an *in vitro* scratch-wound test to determine whether HEEP possesses cell proliferation activity. Indeed, HEEP promoted proliferation and migration of the cells to cover the scratch wounds made on the L929 cell monolayer (Figure 3A). The HEEP-induced enhancement of proliferation and coverage of the scratch wounds was found to decrease with increasing HEEP concentration (coverage achieved for HEEP concentration of 3 μg/mL, 10 μg/mL, and 30 μg/mL: 92.14%, 74.31%, and 75.25%, respectively; all significantly higher *vs* time-matched vehicle) (Figure 3B).

Involvement of PGE_2 in HEEP-mediated healing of gastric ulcers

To gauge whether PGE_2 plays a role in HEEP-mediated healing of gastric ulcers, we measured the levels of PGE_2 at the end of treatment. In males, 14-d treatment with

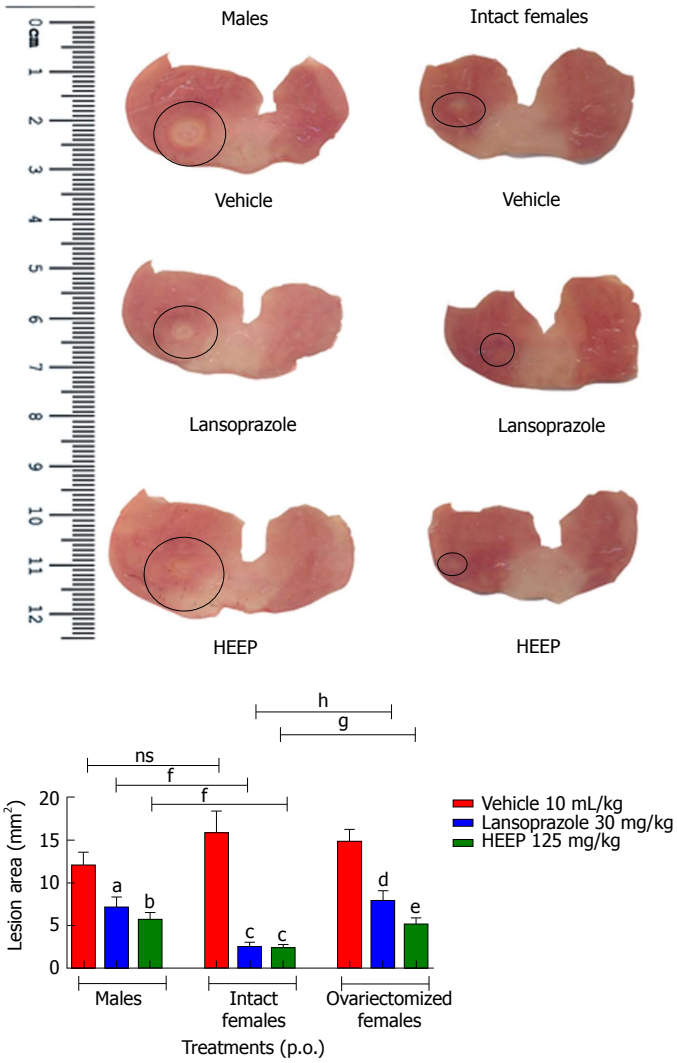


Figure 1 Effects of 14-d treatment with hydroalcoholic extract from the leaves of *Eugenia punicifolia* and lansoprazole on gastric ulcer healing in a rat model. Results were expressed as mean ± standard error of the mean in each group (n = 8-10). Values are given as a percentage indicating healing relative to the corresponding vehicle group. Statistical significance was determined using one-way ANOVA followed by Dunnett's test or Student's t-test. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* vehicle in males; $^{c}P < 0.0001$ *vs* vehicle in intact females (cycling); $^{d}P < 0.01$ and $^{e}P < 0.0001$ *vs* vehicle in ovariectomized females; $^{f}P < 0.01$ for HEEP and lansoprazole in intact females *vs* males; $^{g}P < 0.01$ and $^{h}P < 0.001$ for HEEP and lansoprazole in intact *vs* ovariectomized females; Ns: not significant; HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

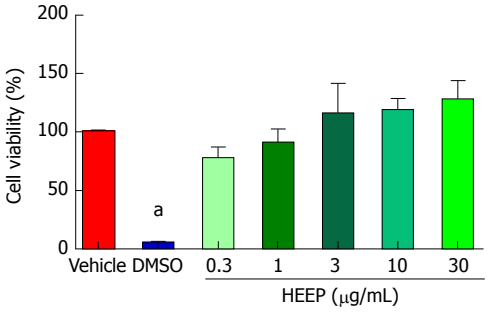


Figure 2 Effects of hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP) on the viability of L929 fibroblasts. L929 cells were treated with vehicle, DMSO, or HEEP of different concentrations and incubated for 24 h. Cell viability was measured using the MTT assay. The results represent mean ± standard error of the mean in triplicate experiments. Statistical significance was determined using one-way ANOVA followed by Dunnett's test. $^{a}P < 0.001$ *vs* vehicle.

HEEP or LZ was associated with elevation of serum PGE_2 levels (P-values *vs* vehicle: < 0.05 for HEEP, < 0.001 for LZ) (Figure 4). However, no increase in PGE_2 levels was noted in intact or OVZ females treated with HEEP or LZ ($P > 0.05$ *vs* vehicle).

Matrix remodeling in HEEP-mediated healing of gastric ulcers

To clarify the nature of the HEEP-mediated healing process of gastric ulcers, we performed zymography analysis of the gastric ulcer tissue and evaluated the activities of MMP-2 and MMP-9 on the gastric mucosa after treatment with vehicle, LZ, and HEEP. No band corresponding to MMP-9 was observed, indicating that MMP-9 activity is absent in all rats. In contrast, MMP-2 activity was present in all rats. Specifically, males (Figure 5), intact females (Figure 6), and OVZ females (Figure 7) exhibited pro-MMP-2, intermediate MMP-2, and active MMP-2 activities. There is no difference regarding MMP-2 activity between rats treated with vehicle and those treated with HEEP or LZ ($P > 0.05$ *vs* vehicle).

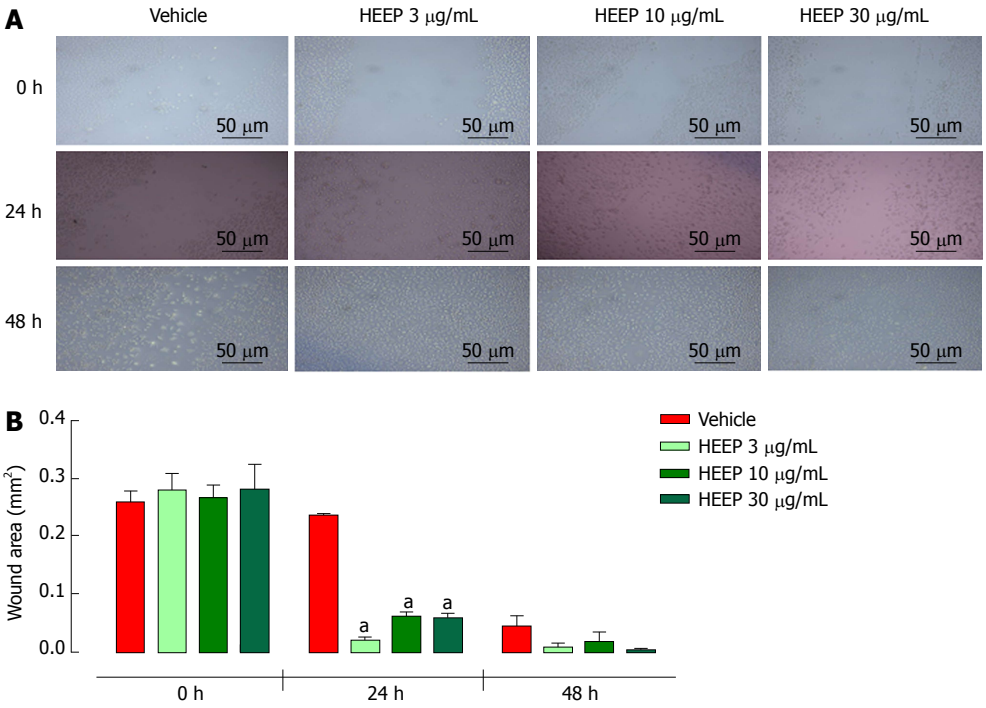


Figure 3 Effects of hydroalcoholic extract from the leaves of *Eugenia punicifolia* on cell proliferation and migration into the scratch-wound area. A monolayer of L929 fibroblasts was scratched and treated with vehicle or HEEP at different concentrations (3, 10, and 30 μg/mL). A: Images captured at 100 × magnification at 0, 24, and 48 h after incubation; B: Rate of migration, evaluated in terms of the total distance that the cells moved from the edge of the scratch toward the center. The results represent mean ± standard error of the mean in triplicate experiments. Statistical significance was established using analysis of variance (two-way ANOVA) followed by Bonferroni's test. $^{a}P < 0.001$ *vs* time-matched vehicle. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

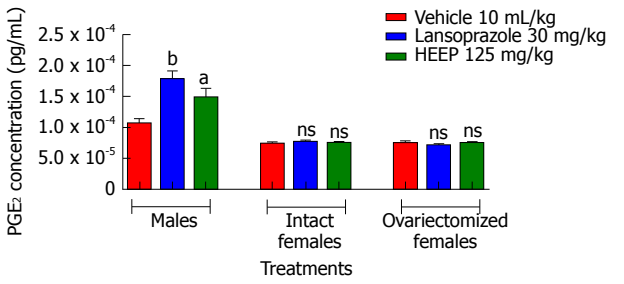


Figure 4 Prostaglandin E_2 levels in rats with acetic acid-induced gastric ulcers treated for 14 d with vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia*. The results are expressed as mean ± standard error of the mean in each group (n = 6-8). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. $^{a}P < 0.05$ and $^{b}P < 0.001$ *vs* vehicle in males; Ns: not significant; HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*; PGE_2: Prostaglandin E_2.

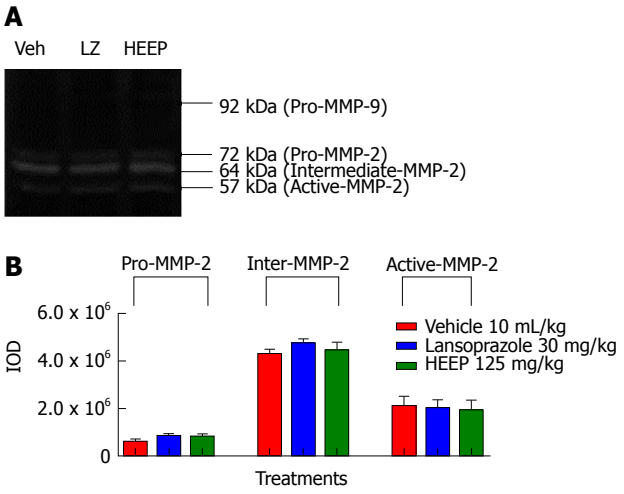


Figure 5 Representative zymography results of gastric ulcer tissue from male rats treated with vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. A: Zymography gel showing the typical clear bands of matrix metalloproteinase-2 (MMP-2) in its proenzyme (72 kDa), intermediate (64 kDa), and active form (57 kDa); B: Gelatinolytic activity of MMP-2 in the gastric mucosa after 14 d of treatment. The results represent mean ± standard error of the mean of the integrated optical density (IOD) for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*; MMP-2: Matrix metalloproteinase-2.

Protein expression levels in HEEP-mediated healing of gastric ulcers

To determine the protein expression profiles associated with HEEP-mediated healing of gastric ulcers, we examined the western blotting gels for several important contributors to cell growth and cell death. Western blotting revealed that oral treatment with HEEP or LZ for 14 d after gastric exposure to absolute acetic acid does not affect the expression of COX-1 (Figure 8) or COX-2 (Figure 9) in males, intact females, or OVZ females (*vs* corresponding vehicle). LZ treatment was associated with increased EGF expression in males and in OVZ females ($P < 0.01$ *vs* corresponding to the respective vehicle; $P < 0.05$ *vs* intact females) (Figure 10). No effect on EGF expression was noted for HEEP treatment.

Western blot analysis revealed that male rats and OVZ rats had low VEGF expression at 14 d after

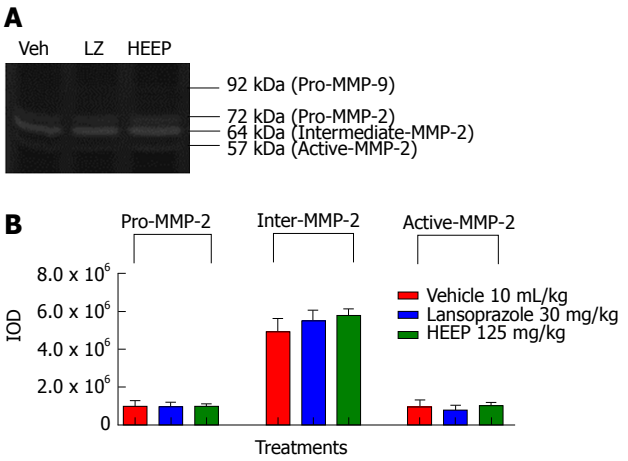


Figure 6 Representative zymography results of gastric ulcer tissue from intact female rats treated with vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. A: Zymography gel showing the typical clear bands of matrix metalloproteinase-2 (MMP-2) in its proenzyme (72 kDa), intermediate (64 kDa), and active form (57 kDa); B: Gelatinolytic activity of MMP-2 in the gastric mucosa after 14 d of treatment. The results represent mean ± standard error of the mean of the integrated optical density (IOD) for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*; MMP-2: Matrix metalloproteinase-2.

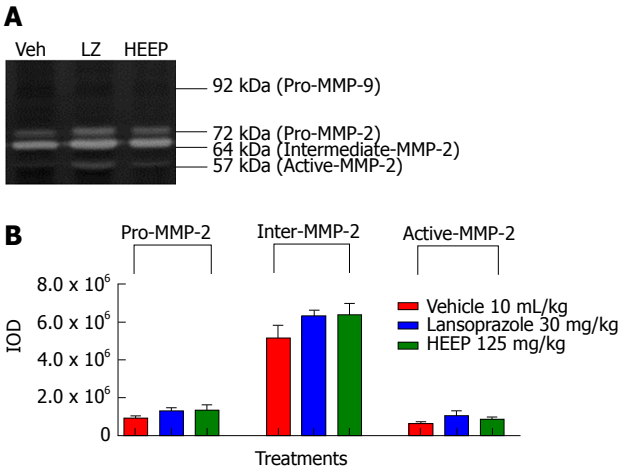


Figure 7 Representative zymography results of gastric ulcer tissue from ovariectomized rats treated with vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. A: Zymography gel showing the typical clear bands of matrix metalloproteinase-2 (MMP-2) in its proenzyme (72 kDa), intermediate (64 kDa), and active form (57 kDa); B: Gelatinolytic activity of MMP-2 in the gastric mucosa after 14 d of treatment. The results represent mean ± standard error of the mean of the integrated optical density (IOD) for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*; MMP-2: Matrix metalloproteinase-2.

induction of gastric ulcers, regardless of treatment (low-signal band; Figure 11). Neither HEEP nor LZ treatment induced significant changes in VEGF expression (P > 0.05 *vs* corresponding vehicle); however, higher VEGF expression was noted in intact females *vs* OVZ females treated with HEEP or LZ (P < 0.05).

In addition to growth factors, we evaluated apoptotic

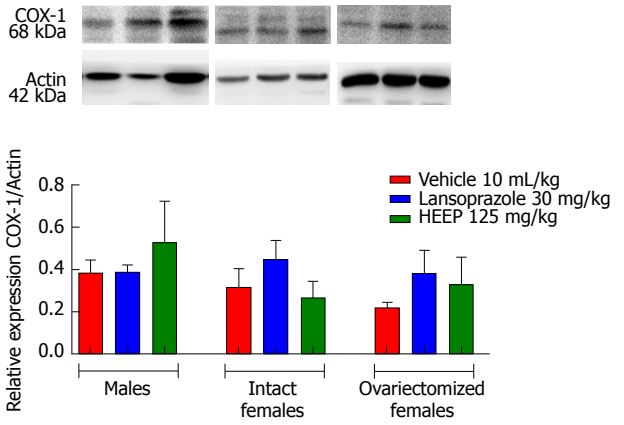


Figure 8 COX-1 expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP) for 14 d. The data represent relative expression of COX-1 normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No statistically significant differences were found. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

factors including caspase-8 (Figure 12), caspase-9 (Figure 13), and caspase-3 (Figure 14), as well as the anti-apoptotic factor Bcl-2 (Figure 15). Neither HEEP nor LZ treatment altered the expression of the apoptotic factors (P > 0.05 *vs* corresponding vehicle). However, expression of caspase-8 (Figure 12) was significantly lower in intact females *vs* males treated with HEEP or LZ. Furthermore, compared to intact females, OVZ females had higher expression of caspase-8 for all treatments (P < 0.01). Similarly, neither HEEP nor LZ treatment altered the expression of the non-apoptotic factor Bcl-2 (P > 0.05 *vs* corresponding vehicle). However, HEEP treatment was associated with higher expression of Bcl-2 in OVZ females *vs* intact females (Figure 15).

Toxicological potential of HEEP in rats with gastric ulcers

In male rats, oral administration of HEEP was not associated with any deaths or significant changes in body weight throughout the 14 d of treatment (Figure 16). Additionally, no significant changes in organ weight or biochemical parameters were noted for HEEP-treated males (P > 0.05 *vs* vehicle), whereas significantly higher glucose levels were noted in males treated with LZ (P < 0.05 *vs* vehicle) (Table 1).

Oral administration of HEEP (125 mg/kg) did not cause any sign of acute toxicity in intact females (Figure 16, Table 2) or OVZ females (Figure 16, Table 3). None of the female animals died during the treatment period, and no significant changes in body weight were noted. Finally, no differences in organ weights, hepatic enzyme levels (γ-GT, AST, and ALT), renal function (creatinine and urea levels), or glucose levels were observed in female rats at the end of the 14 d treatment period (P > 0.05 *vs* corresponding vehicle).

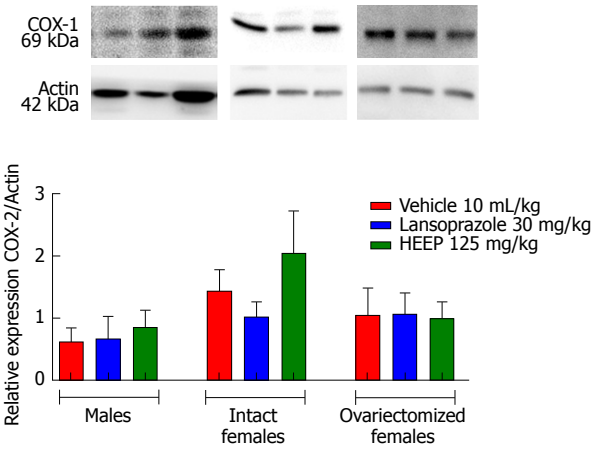


Figure 9 COX-2 expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. The data represent relative expression of COX-2 normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No statistically significant differences were found. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

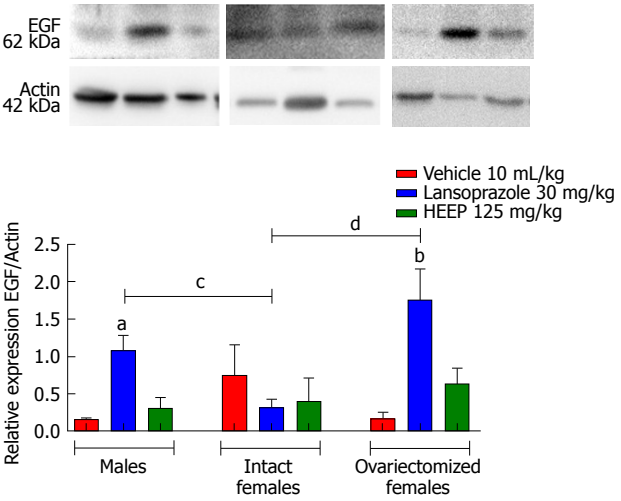


Figure 10 Epidermal growth factor expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. Data represent relative expression of EGF normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test or Student's t-test. $^{a}P < 0.01$ *vs* vehicle in males; $^{b}P < 0.01$ *vs* vehicle in ovariectomized females; $^{c}P < 0.05$ for lansoprazole in males *vs* intact females; $^{d}P < 0.05$ for lansoprazole in intact *vs* ovariectomized females. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

DISCUSSION

The acetic acid-induced gastrointestinal ulcer model is a classical model that has proven suitable for investigating the effect of treatment on the healing process of chronic gastrointestinal ulcers[28], provided that the wounds resemble human ulcers that do not heal naturally[29,30,31]. Such data can also help in the discovery of new anti-ulcer

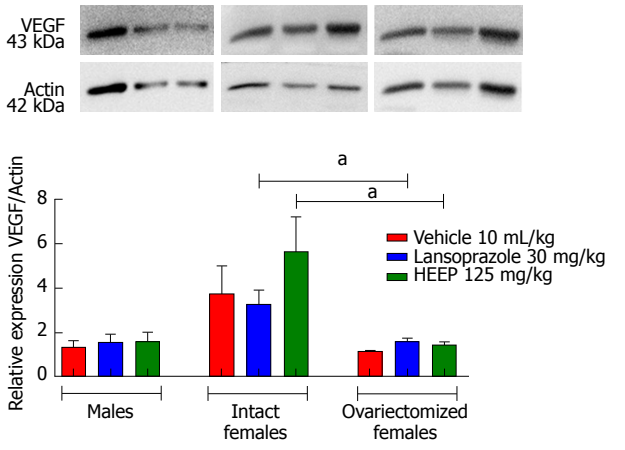


Figure 11 Vascular endothelial growth factor expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. The data represent relative expression of VEGF normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test or Student's t-test. $^{a}P < 0.05$ for lansoprazole and HEEP in intact *vs* ovariectomized females. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

drugs or treatment targets. This ulcer-induction model provides an easy and reliable technique for producing round, deep ulcers in the stomach of rats, and such ulcers do not heal naturally. Furthermore, ulcers obtained using this model resemble human ulcers, and this model was successfully used to assess agents with potential therapeutic effects in chronic gastric ulcers.

One of the least understood aspects of gastric ulcers is the chronicity of the disease, which is characterized by repeated episodes of healing and re-exacerbation, posing a challenge to physicians and a burden for patients[31]. The healing process of ulcers can be divided into three phases: ulcer development phase (0-3 d), involving tissue necrosis, ulcer implantation, inflammatory infiltration, and ulcer margin formation; rapid healing phase (3-10 d), involving migration of epithelial cells and contraction of the ulcer base; slow healing phase (10-20 d), involving angiogenesis, remodeling of granulation tissues, and complete re-epithelialization of the ulcer[32].

To the best of our knowledge, the present study is the first to show that 14 d treatment with HEEP achieves healing of established gastric ulcers in all groups of rats studied (males, females, and OVZ females) (Figure 1). Specifically, HEEP treatment achieved a 52.44% lesion area reduction in males, compared to 85.22% and 65.47% in intact and OVZ females, respectively (all $P < 0.05$ *vs* corresponding vehicle) (Figure 1). A significant decrease in lesion area was also noted for LZ treatment in females (84.21%), OVZ females (49.40%), and males (40.81%) (Figure 1). Intact females treated with HEEP or LZ showed significantly greater healing effects than that noted in males or OVZ females who received the same treatment. This beneficial effect of HEEP in intact females is confirmed by the increase

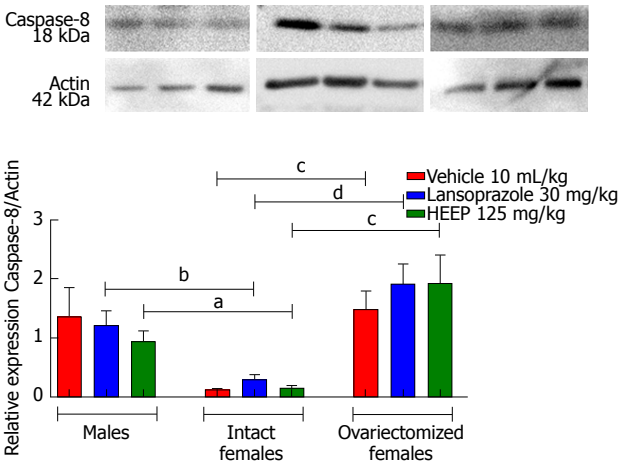


Figure 12 Caspase-8 expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. The data represent relative expression of caspase-8 normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test or Student's t-test. [a]P < 0.05 and [b]P < 0.01 for lansoprazole and HEEP in intact females *vs* males. [c]P < 0.01 and [d]P < 0.001 for vehicle, lansoprazole, and HEEP in intact *vs* ovariectomized females. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

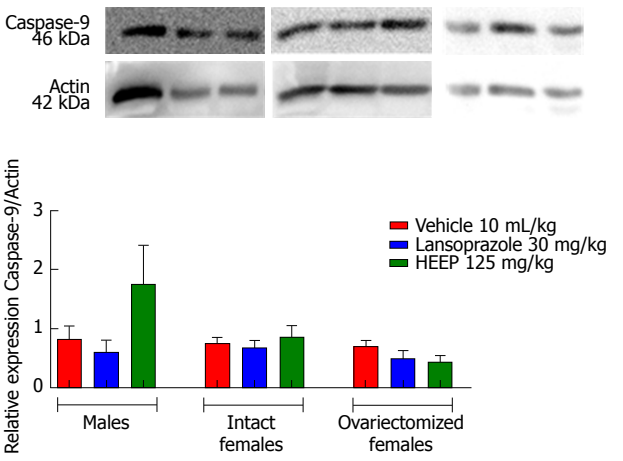


Figure 13 Caspase-9 expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. Data represent relative expression of caspase-9 normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No statistically significant differences were found. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

in VEGF expression associated with HEEP treatment in this group. Furthermore, there was no significant difference in lesion area between males and OVZ females who received the same treatment (Figure 1), suggesting that the absence of female sex hormones diminishes healing. Our results corroborate the findings of Günal *et al*[33], who demonstrated that the severity of gastric ulcers induced by the application of acetic acid

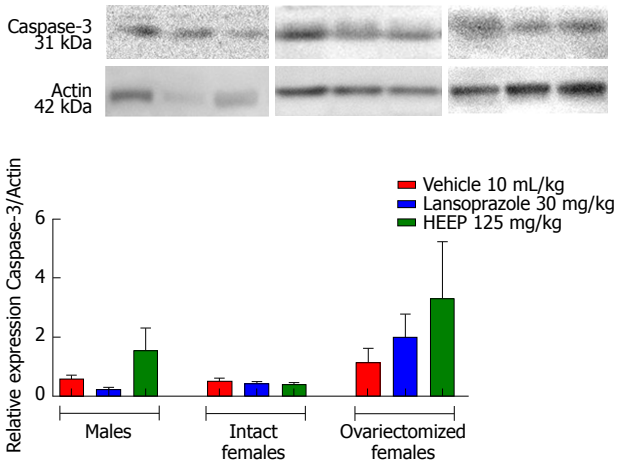


Figure 14 Caspase-3 expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* or 14 d. The data represent relative expression of caspase-3 normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No statistically significant differences were found. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

is attenuated by the effect of estrogen. The protective role of female sex hormones likely accounts for the higher incidence of gastric ulcers in men *vs* women[34]. The anti-ulcerogenic activity of estrogen was previously explained by its stimulating effect on parietal cell activity and maintenance of mucus integrity[34]. The protective effect of estrogens on endothelial function includes antioxidant properties, vasodilator action, prevention of the formation of platelet thrombi, and angiogenesis promotion[35]. Results from several studies support the idea that the estrogen-induced vasoprotective effect may be due to the release of nitric oxide from the vascular endothelium[36].

Gastric ulcer healing is a complex and genetically-programmed dynamic process[37]. It is well-established that myofibroblasts are an important component of wound healing[38], responsible for extracellular matrix production, morphogenesis, and the inflammatory process involved in tissue repair[38-40]. Previous studies confirmed that fibroblasts play an important role in gastric and esophageal ulcer healing in mice and rats[37,41-42]. Similar to da Silva *et al*[43] (2015), the murine fibroblast L929 (NCTC clone 929) cells were used as a complementary *in vitro* model to confirm the cell proliferative effect of HEEP. Fibroblast cell culture has been proposed as a useful method for testing wound healing activity *in vitro*[44]. We found that incubation with HEEP led to adequate coverage of the scratch-wound areas on the L929 cell monolayer after only 24 h (Figure 3), with no sign of cytotoxicity in the MTT assay (Figure 2). Thus, the HEEP effect of enhancing cell proliferation and migration to cover the scratch wounds reinforces the healing potential of HEEP.

Another step taking place in this process is the

Table 1 Organ weights following the 14-d treatment of male rats with acetic acid-induced gastric ulcers with lansoprazole and hydroalcoholic extract from the leaves of *Eugenia punicifolia*

	Treatment		
	Vehicle	Lansoprazole	HEEP
Organ weights, g			
Heart	3.15 ± 0.03	3.13 ± 0.03	3.14 ± 0.06
Kidneys	4.81 ± 0.05	4.79 ± 0.04	4.84 ± 0.05
Lung	3.62 ± 0.08	3.73 ± 0.10	3.59 ± 0.06
Liver	11.56 ± 0.18	11.55 ± 0.10	11.26 ± 0.13
Spleen	2.94 ± 0.02	2.98 ± 0.05	2.99 ± 0.04
Biochemical parameters			
Glucose, mg/dL	150.10 ± 6.04	170.90 ± 4.73[a]	154.70 ± 3.80
Creatinine, mg/dL	0.34 ± 0.02	0.34 ± 0.02	0.33 ± 0.01
Urea, mg/dL	54.75 ± 3.15	52.13 ± 1.71	54.13 ± 1.94
γ-GT, U/L	< 1.0	< 1.0	< 1.0
AST, U/L	137.80 ± 18.43	163.60 ± 22.33	144.50 ± 10.76
ALT, U/L	42.88 ± 3.55	48.88 ± 3.67	46.25 ± 3.09

Vehicle, lansoprazole, or HEEP was administered for 14 d after ulcer induction. Data were collected at the end of treatment. Results are expressed as the means ± standard error of the mean for each group (n = 7-8). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. [a]P < 0.05 *vs* vehicle; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

Table 2 Organ weights following the 14-d treatment of intact female rats with acetic acid-induced gastric ulcers with lansoprazole and hydroalcoholic extract from the leaves of *Eugenia punicifolia*

	Treatment		
	Vehicle	Lansoprazole	HEEP
Organ weights, g			
Heart	3.43 ± 0.05	3.50 ± 0.03	3.44 ± 0.06
Kidneys	4.74 ± 0.03	4.81 ± 0.04	4.69 ± 0.06
Lung	4.23 ± 0.07	4.53 ± 0.06	4.01 ± 0.30
Liver	10.67 ± 0.14	10.90 ± 0.10	10.61 ± 0.09
Spleen	2.56 ± 0.06	2.56 ± 0.07	2.48 ± 0.06
Biochemical parameters			
Glucose, mg/dL	119.10 ± 3.27	116.90 ± 3.21	122.30 ± 3.34
Creatinine, mg/dL	0.45 ± 0.02	0.41 ± 0.02	0.45 ± 0.01
Urea, mg/dL	49.50 ± 3.36	53.29 ± 1.11	53.70 ± 3.20
γ-GT, U/L	< 1.0	< 1.0	< 1.0
AST, U/L	202.70 ± 12.73	198.40 ± 18.84	162.10 ± 7.24
ALT, U/L	57.00 ± 3.40	60.57 ± 3.59	65.86 ± 2.79

Vehicle, lansoprazole, or HEEP was administered for 14 d after ulcer induction. Data were collected at the end of treatment. Results are expressed as the means ± standard error of the mean for each group (n = 7-8). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No significant differences *vs* vehicle were noted; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

mucosal reconstruction by formation of granulation tissue at the base of the ulcer, formation of new vessels, and restoration of the glandular architecture[45]. The integrity of the gastric mucosa is highly dependent on the continuous generation of prostaglandins by COX-1 and COX-2[46]. COX-1 is expressed constitutively in the gastric mucosa and mediates the synthesis of prostaglandins, which regulate blood flow in the mucosa and promote the secretion of mucus and bicarbonate. COX-2 also plays an important role in the healing of gastric ulcers by initiating cell proliferation, promoting angiogenesis, and restoring mucosal integrity[47,48]; COX-2 inhibition is associated with delayed healing. Upon evaluating PGE_2 levels in the present model of acetic acid-induced gastric ulcers, we found increased PGE_2 levels following HEEP or LZ treatment in male (P < 0.05 *vs* vehicle; Figure 4) but not female rats. This finding demonstrates the participation of PGE_2 in the healing process of gastric ulcers in male rats treated with HEEP for 14 consecutive days.

The injection of acetic acid into the gastric mucosa induces the development of deep gastric ulceration and gastric mucosal damage directly associated with extracellular matrix degradation, in which zinc-dependent MMPs play a crucial role. Several animal studies of gastric ulcers have focused on the role of MMPs, mainly MMP-2 and MMP-9[49]. MMPs are divided into several groups based on their substrate specificity and cellular localization; such groups include collagenases, gelatinases, stromelysins, membrane-type MMPs, and others[50]. MMP-2 (72 kDa) and MMP-9 (92 kDa) are gelatinases that function in wound formation and subsequent healing[51,52]. Wound formation and subsequent healing represent dynamic processes of extracellular matrix remodeling, mainly influenced by MMP-2 and MMP-9.

Li *et al*[49] reported enhanced expression of MMP-9 in the margin of the ulcer and suggested that this finding may be indicative of inflammation and poor wound healing. Our results suggest that treatment with HEEP or LZ may inhibit MMP-9 activity, as no band corresponding to MMP-9 was detected in males (Figure 5), intact females (Figure 6) and OVZ females (Figure 7). This finding may also be explained by the fact that, at 14 d after induction with acetic acid, the ulcers were in the slow-healing phase, whereas MMP-9 is important in the early phase of gastric ulcer formation[49]. Neither HEEP nor LZ treatment affected MMP-2 activity in this model of acetic acid-induced gastric ulcers in rats. These results corroborate the findings of Baragi *et al*[53], namely that the expression of MMP-2 remained constant throughout the ulcer healing phase. Fini and Girard[54] reported that the expression of MMP-2 was the same in controls and in animals injected with acetic acid, concluding that MMP-2 may not have a direct role in the formation or healing phases of the ulcer, but rather may help in maintaining the integrity of the matrix structure by aiding in the proper assembly of new collagen fibrils.

Ulcer healing requires filling of the defect with cells and connective tissue, which is accomplished by cell migration, proliferation (mediated by EGF), apoptosis (mediated by caspases and Bcl-2), angiogenesis (mediated by VEGF) and remodeling (mediated by MMPs), ultimately leading to scar formation. All of these processes are controlled by growth factors (prostaglandins

Table 3 Organ weights following the 14-d treatment of ovariectomized rats with acetic acid-induced gastric ulcers with lansoprazole and hydroalcoholic extract from the leaves of *Eugenia punicifolia*

	Treatment		
	Vehicle	Lansoprazole	HEEP
Organ weights, g			
Heart	3.35 ± 0.04	3.39 ± 0.05	3.49 ± 0.09
Kidneys	4.52 ± 0.07	4.56 ± 0.07	4.61 ± 0.05
Lung	4.28 ± 0.11	4.30 ± 0.10	4.47 ± 0.17
Liver	10.56 ± 0.16	10.61 ± 0.10	10.60 ± 0.11
Spleen	2.59 ± 0.06	2.68 ± 0.08	2.65 ± 0.05
Biochemical parameters			
Glucose, mg/dL	117.30 ± 4.36	126.70 ± 4.48	119.00 ± 5.46
Creatinine, mg/dL	0.42 ± 0.00	0.43 ± 0.01	0.43 ± 0.01
Urea, mg/dL	55.08 ± 2.24	48.97 ± 1.67	55.37 ± 2.92
γ-GT, U/L	< 1.0	< 1.0	< 1.0
AST, U/L	176.80 ± 15.37	192.70 ± 14.77	191.00 ± 21.04
ALT, U/L	62.17 ± 3.68	57.57 ± 2.36	56.43 ± 1.92

Vehicle, lansoprazole, or HEEP was administered for 14 d after ulcer induction. Data were collected at the end of treatment. Results are expressed as the means ± standard error of the mean for each group (n = 7-8). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No significant differences *vs* vehicle were noted; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

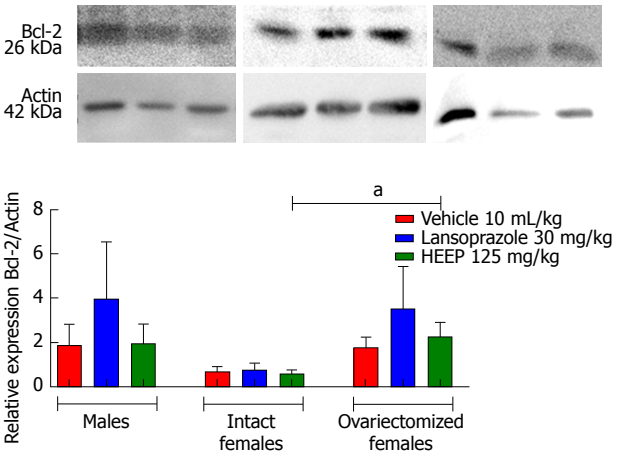


Figure 15 Bcl-2 expression in the gastric mucosa of rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. The data represent relative expression of Bcl-2 normalized to the expression of housekeeping genes (actin). The results represent mean ± standard error of the mean for each group (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett's test or Student's t-test. $^{a}P < 0.05$ for lansoprazole in intact *vs* ovariectomized females. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

produced by COX-1 and COX-2), which stimulate cell proliferation and division. At the ulcer margin, epithelial cells proliferate and migrate to the granulation tissue to cover (re-epithelialize) the ulcer and initiate reconstruction of the glands within the ulcer scar[55,56]. Hence, we performed western blotting to evaluate the expression levels of COX-1, COX-2, EGF, VEGF, caspase-8, caspase-9, caspase-3, and Bcl-2 after 14 d of treatment with HEEP or LZ in animals with gastric ulcers induced by acetic acid (Figures 8-15). While HEEP did not alter the levels of these proteins ($P > 0.05$ *vs* vehicle), VEGF expression was higher in intact females than in OVZ females treated with HEEP. On the other hand, LZ treatment was associated with increased expression of EGF in males and OVZ females ($P < 0.05$ *vs* corresponding vehicle) (Figure 10). Growth factors such as EGF activate epithelial cell migration and proliferation, accelerating wound and ulcer healing *in vivo* and *in vitro*[57,37]. The increased expression of EGF in rats treated with LZ may reflect the ongoing healing of gastric ulcers in these animals, with intact females having progressed further than males and OVZ females. Since no significant increase in EGF levels was noted in rats treated with HEEP, we may infer that healing had progressed further and was potentially stabilized in such rats; on the other hand, rats treated with LZ may have experienced re-exacerbation, which represents the main challenge in the treatment of lesions in poorly vascularized and epithelialized gastric tissue.

The results of western blotting analysis revealed that HEEP treatment was not associated with altered expression of cell growth and death factors at 14 d after ulcer induction, suggesting rather that the expression levels of such factors had already normalized. Furthermore, we found that female sex hormones interfered with the expression of EGF, VEGF, caspase-8, and Bcl-2 (Figures 10-12 and 15). Compared to males and OVZ females receiving the same treatment, intact females showed a greater healing effect, decreased expression of EGF, apoptotic factors such as caspase-8, and anti-apoptotic factors such as Bcl-2, as well as increased VEGF expression.

Little is known about gender differences in the gastrointestinal tract because the studies that correlate anti- and pro-apoptotic protein expression with female sex hormones in normal gastric mucosal tissue are limited. Qin *et al*[58] shows that estrogen can induce apoptosis in gastric cancer cells, and that Bcl-2 might be involved in this effect.

Despite the lack of studies that provide an explanation about the gastric healing action related to female sex hormones, Kumtepe *et al*[59] showed the beneficial effect of acute and chronic administration of progesterone, estrogen, FSH and LH in rat gastric tissue, indicating the interference of the hormonal factor in this process.

In this model of acetic acid-induced gastric ulcers, we also evaluated the safety of HEEP treatment. No deaths or changes in body mass were noted throughout the treatment (Figures 16). The weights of the organs were not detrimentally affected by HEEP administration (Tables 1-3). In addition, evaluation of biochemical parameters indicated no detrimental effect of HEEP administration on hepatic integrity (AST, ALT, and γ-GT), renal integrity (urea and creatinine), or glucose levels (Tables 1-3). We found that HEEP treatment (125 mg/kg daily) did not alter any of the biochemical parameters analyzed ($P > 0.05$ *vs* vehicle). In addition, LZ treatment (30

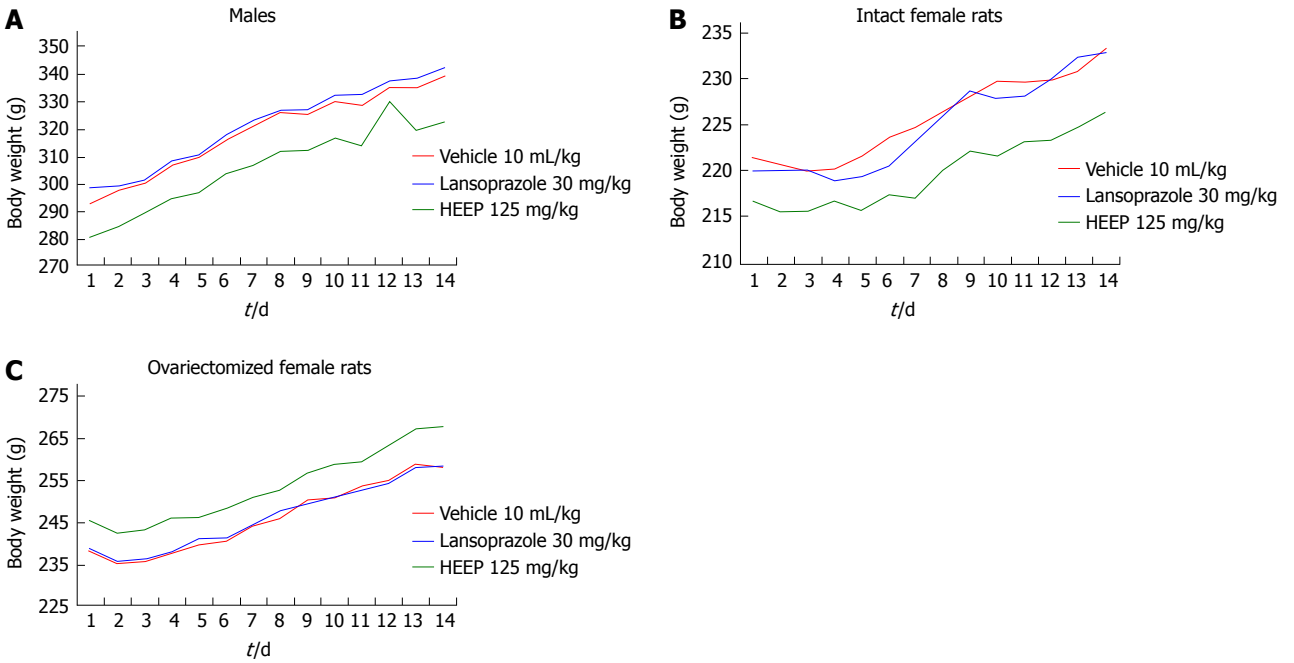


Figure 16 Body weight evolution in rats with acetic acid-induced gastric ulcers treated with oral administration of vehicle, lansoprazole, or hydroalcoholic extract from the leaves of *Eugenia punicifolia* for 14 d. A: Male rats; B: Intact female rats; C: Ovariectomized female rats. Results are expressed as the means of body weight (g) in each group (n = 8-10). Statistical significance was determined using one-way ANOVA followed by Dunnett's test. No statistically significant differences were found. HEEP: Hydroalcoholic extract from the leaves of *Eugenia punicifolia*.

mg/kg daily) was associated with significantly higher elevated serum glucose levels ($P < 0.05$ *vs* vehicle), but within the normal reference values[60]. According to the Clinical Laboratory Parameters for Crl:WI (Han) Rats, the reference values for glucose levels in male rats aged 8-16 wk range from 70-208 mg/dL. Thus, despite the variations between the groups (LZ *vs* vehicle), the values were within the normal range. Taken together, these findings indicate that 14 d oral treatment with HEEP (125 mg/kg daily) has no subacute toxicity in male or female (intact or OVZ) rats.

In conclusion, our findings suggest that HEEP treatment during 14 consecutive days can achieve the healing of gastric ulcer lesions in all groups defined in terms of sex (male *vs* female) and hormonal status (intact *vs* OVZ females). This healing effect is reinforced by the enhancement of cell proliferation and migration. The anti-ulcer effect of HEEP is mediated by PGE_2 only in males. Compared to OVZ females and males treated with HEEP, intact females treated with HEEP are expected to have improved healing (higher reduction in lesion area), higher VEGF expression, and decreased expression of caspase-8 and Bcl-2. Finally, HEEP treatment is safe and unlikely to exhibit subacute toxicity or cytotoxicity.

ARTICLE HIGHLIGHTS

Research background

Gastric ulcers refer to acid injury in the digestive tract, resulting in a mucosal break that reaches to the submucosal layer. Acid peptic disorders are very common in the United States, with four million individuals (new cases and recurrences) affected per year. This disease occurs more often in men than in women, but these sex differences are less pronounced after the age of 45 years, probably because there is an increased incidence of ulcers in menopausal women.

Research motivation

The conventional treatment of gastric ulcers is based on the inhibition of gastric acid secretion. However, this therapy is associated with several side effects and poor quality of ulcer healing. Therefore, alternative therapies are desirable. We investigated the effects of extracts from the leaves of *E. punicifolia* (HEEP), which is a medicinal plant popularly used to treat inflammation and wounds.

Research objectives

We evaluated the sex-specific effects of HEEP in the healing of gastric ulcers induced by acetic acid in rats.

Research methods

We used a rat model of acetic acid-induced gastric ulcers to evaluate the healing effect of HEEP. We measured prostaglandin levels, analyzed the extracellular matrix by zymography, evaluated the quality of ulcer healing by western blot, and assessed the healing activity by scratch assay. Subacute toxicity (*in vivo*) and cytotoxicity (*in vitro*) were also investigated.

Research results

HEEP demonstrated a high healing capacity, with substantial reduction of lesion area in all groups studied (males, intact females, ovariectomized females). HEEP accelerated the healing of gastric lesions, and this effect was modulated by female sex hormones. The curative role of HEEP is due to an increase in PGE_2 (only in males), as well as an inhibition (MMP-9) and maintenance (MMP-2) of the extracellular matrix in the gastric mucosa. The HEEP healing properties were also confirmed by the enhancement of proliferation and coverage of scratched wounds in a fibroblast monolayer (*in vitro*). No sign of toxicity was observed in this study.

Research conclusions

This was the first study to prove the healing activity of HEEP in acetic acid-induced gastric ulcers for both sexes (males *vs* females) and irrespective of hormonal status (cycling *vs* ovariectomized females). This effect is modulated by female sex hormones. The curative effect of HEEP is also mediated

by prostaglandin, the remodeling of the extracellular matrix, and both cell proliferation and migration in the gastric mucosa. Additionally, HEEP does not cause subacute toxicity or cytotoxicity.

Research perspectives

The present findings support the popular use of *E. punicifolia* in the treatment of gastrointestinal ulcers, and contribute to the search for new therapies for diseases of the gastrointestinal tract.

ACKNOWLEDGMENTS

We are grateful for the technical support kindly provided by the members of the Laboratory of Extracellular Matrix of the Department of Morphology, Institute of Biosciences, UNESP, Botucatu, São Paolo, Brazil.

REFERENCES

1 **Gurusamy KS**, Pallari E. Medical versus surgical treatment for refractory or recurrent peptic ulcer. *Cochrane Database Syst Rev* 2016; **3**: CD011523 [PMID: 27025289 DOI: 10.1002/14651858.CD011523.pub2]

2 **Venzon L**, Mariano LNB, Somensi LB, Boeing T, de Souza P, Wagner TM, Andrade SF, Nesello LAN, da Silva LM. Essential oil of Cymbopogon citratus (lemongrass) and geraniol, but not citral, promote gastric healing activity in mice. *Biomed Pharmacother* 2018; **98**: 118-124 [PMID: 29248831 DOI: 10.1016/j.biopha.2017.12.020]

3 **Monici LT**, Zeitune JMR, Lorena SLS, Mesquita MA, Magalhães AFN. de Úlcera péptica. *Rev Bras Med* 2003; 60: 25-32

4 **Yu LY**, Sun LN, Zhang XH, Li YQ, Yu L, Yuan ZQ, Meng L, Zhang HW, Wang YQ. A Review of the Novel Application and Potential Adverse Effects of Proton Pump Inhibitors. *Adv Ther* 2017; **34**: 1070-1086 [PMID: 28429247 DOI: 10.1007/s12325-017-0532-9]

5 **Young Oh T**, Ok Ahn B, Jung Jang E, Sang Park J, Jong Park S, Wook Baik H, Hahm KB. Accelerated Ulcer Healing and Resistance to Ulcer Recurrence with Gastroprotectants in Rat Model of Acetic Acid-induced Gastric Ulcer. *J Clin Biochem Nutr* 2008; **42**: 204-214 [PMID: 18545642 DOI: 10.3164/jcbn.2008030]

6 **Tarnawski A**, Stachura J, Krause WJ, Douglass TG, Gergely H. Quality of gastric ulcer healing: a new, emerging concept. *J Clin Gastroenterol* 1991; **13** Suppl 1: S42-S47 [PMID: 1719066 DOI: 10.1097/00004836-199112001-00007]

7 **Kangwan N**, Park JM, Kim EH, Hahm KB. Quality of healing of gastric ulcers: Natural products beyond acid suppression. *World J Gastrointest Pathophysiol* 2014; **5**: 40-47 [PMID: 24891974 DOI: 10.4291/wjgp.v5.i1.40]

8 **Fornai M**, Colucci R, Antonioli L, Awwad O, Ugolini C, Tuccori M, Fulceri F, Natale G, Basolo F, Blandizzi C. Effects of esomeprazole on healing of nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers in the presence of a continued NSAID treatment: Characterization of molecular mechanisms. *Pharmacol Res* 2011; **63**: 59-67 [PMID: 20969958 DOI: 10.1016/j.phrs.2010.10.013]

9 **Yang RQ**, Mao H, Huang LY, Su PZ, Lu M. Effects of hydrotalcite combined with esomeprazole on gastric ulcer healing quality: A clinical observation study. *World J Gastroenterol* 2017; **23**: 1268-1277 [PMID: 28275307 DOI: 10.3748/wjg.v23.i7.1268]

10 **Abdel-Aziz H**, Kelber O, Lorkowski G, Storr M. Evaluating the Multitarget Effects of Combinations through Multistep Clustering of Pharmacological Data: the Example of the Commercial Preparation Iberogast. *Planta Med* 2017; **83**: 1130-1140 [PMID: 28859216 DOI: 10.1055/s-0043-116852]

11 **Estrella E.** Plantas Medicinales Amazonicas: realidad y perspectivas. Lima, Peru. *Secretaria Pro Tempore* 1995; 1995: 302

12 **de Oliveira RN**, Dias IJM, Câmara CAG. Estudo comparativo do óleo essencial de Eugenia punicifolia (HBK) DC. *de diferentes localidades de Pernambuco* 2005; **15**: 39-43 [DOI: 10.1590/S0102-695X2005000100009]

13 **Maia JGS**, Zoghbi MGB, Andrade EHA. Plantas aromáticas na Amazônia e seus óleos essenciais. *Belém: Museu Paraense Emílio Goeldi* 2001; 1-173

14 **Chaves EMF**, Barros RFM. Diversidade e uso de recursos medicinais do carrasco na APA da Serra da Ibiapaba, Piauí, Nordeste do Brasil. *Rev Bras Plantas Med* 2012; **14**: 476-486 [DOI: 10.1590/S1516-05722012000300009]

15 **Silva S**. Plantas do cerrado utilizadas pelas comunidades da região do Grande Sertão Veredas. *Funatura* 1998; 1-109

16 **Basting RT**, Nishijima CM, Lopes JA, Santos RC, Lucena Périco L, Laufer S, Bauer S, Costa MF, Santos LC, Rocha LR, Vilegas W, Santos AR, Dos Santos C, Hiruma-Lima CA. Antinociceptive, anti-inflammatory and gastroprotective effects of a hydroalcoholic extract from the leaves of Eugenia punicifolia (Kunth) DC. in rodents. *J Ethnopharmacol* 2014; **157**: 257-267 [PMID: 25311275 DOI: 10.1016/j.jep.2014.09.041]

17 **Périco LL**, Heredia-Vieira SC, Beserra FP, de Cássia Dos Santos R, Weiss MB, Resende FA, Dos Santos Ramos MA, Bonifácio BV, Bauab TM, Varanda EA, de Gobbi JI, da Rocha LR, Vilegas W, Hiruma-Lima CA. Does the gastroprotective action of a medicinal plant ensure healing effects? An integrative study of the biological effects of Serjania marginata Casar. (Sapindaceae) in rats. *J Ethnopharmacol* 2015; **172**: 312-324 [PMID: 26099637 DOI: 10.1016/j.jep.2015.06.025]

18 **Long JA**, Evans HM. The oestrous cycle in the rat and its associated phenomena. Memories of University of California. *University of California Press* 1922; **6**: 1-148.

19 **Mandl AM**. The Phases of the Oestrous Cycle in the Adult White Rat. *J Exp Biol* 1951; **28**: 576-584

20 **Marcondes FK**, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol* 2002; **62**: 609-614 [PMID: 12659010 DOI: 10.1590/S1519-69842002000400008]

21 **Okabe S**, Roth JL, Pfeiffer CJ. A method for experimental, penetrating gastric and duodenal ulcers in rats. Observations on normal healing. *Am J Dig Dis* 1971; **16**: 277-284 [PMID: 5554507 DOI: 10.1007/BF02235252]

22 **Konturek SJ**, Dembinski A, Warzecha Z, Brzozowski T, Gregory H. Role of epidermal growth factor in healing of chronic gastroduodenal ulcers in rats. *Gastroenterology* 1988; **94**: 1300-1307 [PMID: 2896137 DOI: 10.1016/0016-5085(88)90667-1]

23 **Chen W**, Wang Z, Zhang Y. The effect of zinc on the apoptosis of cultured human retinal pigment epithelial cells. *J Huazhong Univ Sci Technolog Med Sci* 2003; **23**: 414-417 [PMID: 15015650 DOI: 10.1007/BF02829432]

24 **Balekar N**, Katkam NG, Nakpheng T, Jehtae K, Srichana T. Evaluation of the wound healing potential of Wedelia trilobata (L.) leaves. *J Ethnopharmacol* 2012; **141**: 817-824 [PMID: 22465731 DOI: 10.1016/j.jep.2012.03.019]

25 **Parvin R**, Pande SV, Venkitasubramanian TA. On the colorimetric biuret method of protein determination. *Anal Biochem* 1965; **12**: 219-229 [PMID: 14340012 DOI: 10.1016/0003-2697(65)90085-0]

26 **Brooks SP**, Lampi BJ, Sarwar G, Botting HG. A comparison of methods for determining total body protein. *Anal Biochem* 1995; **226**: 26-30 [PMID: 7785775 DOI: 10.1006/abio.1995.1186]

27 **Justulin LA Jr**, Della-Coleta HH, Taboga SR, Felisbino SL. Matrix metalloproteinase (MMP)-2 and MMP-9 activity and localization during ventral prostate atrophy and regrowth. *Int J Androl* 2010; **33**: 696-708 [PMID: 20059586 DOI: 10.1111/j.1365-2605.2009.01016.x]

28 **Takagi K**, Okabe S, Saziki R. A new method for the production of chronic gastric ulcer in rats and the effect of several drugs on its healing. *Jpn J Pharmacol* 1969; **19**: 418-426 [PMID: 5307474 DOI: 10.1254/jjp.19.418]

29 **Okabe S**, Amagase K. An overview of acetic acid ulcer models--the history and state of the art of peptic ulcer research. *Biol Pharm Bull* 2005; **28**: 1321-1341 [PMID: 16079471 DOI: 10.1248/bpb.28.1321]

30 **Okabe S**, Roth JL, Pfeiffer CJ. Differential healing periods of the acetic acid ulcer model in rats and cats. *Experientia* 1971; **27**: 146-148 [PMID: 5544720 DOI: 10.1007/BF02145860]

31 **Adinortey MB**, Ansah C, Galyuon I, Nyarko A. In Vivo Models

Used for Evaluation of Potential Antigastroduodenal Ulcer Agents. *Ulcers* 2013; **2013**: 1-12 [DOI: 10.1155/2013/796405]
32 **Schmassmann A**. Mechanisms of ulcer healing and effects of nonsteroidal anti-inflammatory drugs. *Am J Med* 1998; **104**: 43S-51S; discussion 79S-80S [PMID: 9572320 DOI: 10.1016/S0002-9343(97)00211-8]
33 **Günal O**, Oktar BK, Ozçinar E, Sungur M, Arbak S, Yeğen B. Estradiol treatment ameliorates acetic acid-induced gastric and colonic injuries in rats. *Inflammation* 2003; **27**: 351-359 [PMID: 14760943 DOI: 10.1023/B:IFLA.0000006703.53427.da]
34 **Aguwa CN**. Effects of exogenous administration of female sex hormones on gastric secretion and ulcer formation in the rat. *Eur J Pharmacol* 1984; **104**: 79-84 [PMID: 6542018 DOI: 10.1016/0014-2999(84)90371-6]
35 **Lindh A**, Carlström K, Eklund J, Wilking N. Serum steroids and prolactin during and after major surgical trauma. *Acta Anaesthesiol Scand* 1992; **36**: 119-124 [PMID: 1532279 DOI: 10.1111/j.1399-6576.1992.tb03436.x]
36 **Shan J**, Resnick LM, Liu QY, Wu XC, Barbagallo M, Pang PK. Vascular effects of 17 beta-estradiol in male Sprague-Dawley rats. *Am J Physiol* 1994; **266**: H967-H973 [PMID: 8160845 DOI: 10.1152/ajpheart.1994.266.3.H967]
37 **Tarnawski AS**. Cellular and molecular mechanisms of gastrointestinal ulcer healing. *Dig Dis Sci* 2005; **50** Suppl 1: S24-S33 [PMID: 16184417 DOI: 10.1007/s10620-005-2803-6]
38 **Chai J**, Norng M, Tarnawski AS, Chow J. A critical role of serum response factor in myofibroblast differentiation during experimental oesophageal ulcer healing in rats. *Gut* 2007; **56**: 621-630 [PMID: 17068115 DOI: 10.1136/gut.2006.106674]
39 **Hinz B**. The myofibroblast: paradigm for a mechanically active cell. *J Biomech* 2010; **43**: 146-155 [PMID: 19800625 DOI: 10.1016/j.jbiomech.2009.09.020]
40 **Mutoh H**, Sashikawa M, Hayakawa H, Sugano K. Monocyte chemoattractant protein-1 is generated via TGF-beta by myofibroblasts in gastric intestinal metaplasia and carcinoma without H. pylori infection. *Cancer Sci* 2010; **101**: 1783-1789 [PMID: 20557309 DOI: 10.1111/j.1349-7006.2010.01609.x]
41 **Konturek SJ**, Brzozowski T, Majka J, Szlachcic A, Bielanski W, Stachura J, Otto W. Fibroblast growth factor in gastroprotection and ulcer healing: interaction with sucralfate. *Gut* 1993; **34**: 881-887 [PMID: 8344573 DOI: 10.1136/gut.34.7.881]
42 **Nishida T**, Tsuji S, Kimura A, Tsujii M, Ishii S, Yoshio T, Shinzaki S, Egawa S, Irie T, Yasumaru M, Iijima H, Murata H, Kawano S, Hayashi N. Endothelin-1, an ulcer inducer, promotes gastric ulcer healing via mobilizing gastric myofibroblasts and stimulates production of stroma-derived factors. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1041-G1050 [PMID: 16384872 DOI: 10.1152/ajpgi.00462.2005]
43 **da Silva LM**, Boeing T, Somensi LB, Cury BJ, Steimbach VM, Silveria AC, Niero R, Cechinel Filho V, Santin JR, de Andrade SF. Evidence of gastric ulcer healing activity of Maytenus robusta Reissek: In vitro and in vivo studies. *J Ethnopharmacol* 2015; **175**: 75-85 [PMID: 26364940 DOI: 10.1016/j.jep.2015.09.006]
44 **Abe Y**, Inagaki K, Fujiwara A, Kuriyama K. Wound healing acceleration of a novel transforming growth factor-beta inducer, SEK-1005. *Eur J Pharmacol* 2000; **408**: 213-218 [PMID: 11080528 DOI: 10.1016/S0014-2999(00)00766-4]
45 **Perini RF**, Ma L, Wallace JL. Mucosal repair and COX-2 inhibition. *Curr Pharm Des* 2003; **9**: 2207-2211 [PMID: 14529401 DOI: 10.2174/1381612033454027]
46 **Laine L**, Takeuchi K, Tarnawski A. Gastric mucosal defense and cytoprotection: bench to bedside. *Gastroenterology* 2008; **135**: 41-60 [PMID: 18549814 DOI: 10.1053/j.gastro.2008.05.030]
47 **Brzozowski T**, Konturek PC, Konturek SJ, Sliwowski Z, Pajdo R, Drozdowicz D, Ptak A, Hahn EG. Classic NSAID and selective cyclooxygenase (COX)-1 and COX-2 inhibitors in healing of chronic gastric ulcers. *Microsc Res Tech* 2001; **53**: 343-353 [PMID: 11376495 DOI: 10.1002/jemt.1102]
48 **Tatsuguchi A**, Sakamoto C, Wada K, Akamatsu T, Tsukui T, Miyake K, Futagami S, Kishida T, Fukuda Y, Yamanaka N, Kobayashi M. Localisation of cyclooxygenase 1 and cyclooxygenase 2 in Helicobacter pylori related gastritis and gastric ulcer tissues in humans. *Gut* 2000; **46**: 782-789 [PMID: 10807888 DOI: 10.1136/GUT.46.6.782]
49 **Li SL**, Zhao JR, Ren XY, Xie JP, Ma QZ, Rong QH. Increased expression of matrix metalloproteinase-9 associated with gastric ulcer recurrence. *World J Gastroenterol* 2013; **19**: 4590-4595 [PMID: 23901237 DOI: 10.3748/wjg.v19.i28.4590]
50 **Parks WC**, Mecham RP. Matrix metalloproteinase. *Academic Press* 1998
51 **Lempinen M**, Inkinen K, Wolff H, Ahonen J. Matrix metalloproteinases 2 and 9 in indomethacin-induced rat gastric ulcer. *Eur Surg Res* 2000; **32**: 169-176 [PMID: 10878458 DOI: 10.1159/000008759]
52 **Gyenge M**, Amagase K, Kunimi S, Matsuoka R, Takeuchi K. Roles of pro-angiogenic and anti-angiogenic factors as well as matrix metalloproteinases in healing of NSAID-induced small intestinal ulcers in rats. *Life Sci* 2013; **93**: 441-447 [PMID: 23900029 DOI: 10.1016/j.lfs.2013.07.021]
53 **Baragi VM**, Qiu L, Gunja-Smith Z, Woessner JF Jr, Lesch CA, Guglietta A. Role of metalloproteinases in the development and healing of acetic acid-induced gastric ulcer in rats. *Scand J Gastroenterol* 1997; **32**: 419-426 [PMID: 9175201 DOI: 10.3109/00365529709025075]
54 **Fini ME**, Girard MT. The pattern of metalloproteinase expression by corneal fibroblasts is altered by passage in cell culture. *J Cell Sci* 1990; **97** (Pt 2): 373-383 [PMID: 2177480]
55 **Tarnawski A**, Tanoue K, Santos AM, Sarfeh IJ. Cellular and molecular mechanisms of gastric ulcer healing. Is the quality of mucosal scar affected by treatment? *Scand J Gastroenterol Suppl* 1995; **210**: 9-14 [PMID: 8578218 DOI: 10.3109/00365529509090261]
56 **Sánchez-Fidalgo S**, Martín-Lacave I, Illanes M, Motilva V. Angiogenesis, cell proliferation and apoptosis in gastric ulcer healing. Effect of a selective cox-2 inhibitor. *Eur J Pharmacol* 2004; **505**: 187-194 [PMID: 15556152 DOI: 10.1016/j.ejphar.2004.10.019]
57 **Tarnawski AS**, Jones MK. The role of epidermal growth factor (EGF) and its receptor in mucosal protection, adaptation to injury, and ulcer healing: involvement of EGF-R signal transduction pathways. *J Clin Gastroenterol* 1998; **27** Suppl 1: S12-S20 [PMID: 9872493 DOI: 10.1097/00004836-199800001-00004]
58 **Qin J**, Liu M, Ding Q, Ji X, Hao Y, Wu X, Xiong J. The direct effect of estrogen on cell viability and apoptosis in human gastric cancer cells. *Mol Cell Biochem* 2014; **395**: 99-107 [PMID: 24934239 DOI: 10.1007/s11010-014-2115-2]
59 **Kumtepe Y**, Borekci B, Karaca M, Salman S, Alp HH, Suleyman H. Effect of acute and chronic administration of progesterone, estrogen, FSH and LH on oxidant and antioxidant parameters in rat gastric tissue. *Chem Biol Interact* 2009; **182**: 1-6 [PMID: 19729004 DOI: 10.1016/j.cbi.2009.08.016]
60 **Giknis ML**, Clifford CB. Clinical laboratory parameters For Crl: WI (Han) rats. *Charles River Press* 2008; 1-17

P- Reviewer: Nakajima N, Soriano-Ursua MA **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4384-4392

DOI: 10.3748/wjg.v24.i38.4384

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

ORIGINAL ARTICLE

Retrospective Study

Practical fecal calprotectin cut-off value for Japanese patients with ulcerative colitis

Jun Urushikubo, Shunichi Yanai, Shotaro Nakamura, Keisuke Kawasaki, Risaburo Akasaka, Kunihiko Sato, Yosuke Toya, Kensuke Asakura, Takahiro Gonai, Tamotsu Sugai, Takayuki Matsumoto

Jun Urushikubo, Shunichi Yanai, Shotaro Nakamura, Keisuke Kawasaki, Risaburo Akasaka, Kunihiko Sato, Yosuke Toya, Kensuke Asakura, Takahiro Gonai, Takayuki Matsumoto, Division of Gastroenterology, Department of Internal Medicine, School of Medicine, Iwate Medical University, Morioka 020-8505, Japan

Tamotsu Sugai, Division of Molecular Diagnostic Pathology, Department of Pathology, School of Medicine, Iwate Medical University, Morioka 020-8505, Japan

ORCID number: Jun Urushikubo (0000-0002-4808-7614); Shunichi Yanai (0000-0003-1871-2412); Shotaro Nakamura (0000-0001-8166-2599); Keisuke Kawasaki (0000-0003-2645-4417); Risaburo Akasaka (0000-0001-7087-3016); Kunihiko Sato (0000-0002-0834-3353); Yosuke Toya (0000-0002-0990-9304); Kensuke Asakura (0000-0002-4653-3801); Takahiro Gonai (0000-0001-6759-2459); Tamotsu Sugai (0000-0002-4896-3557); Takayuki Matsumoto (0000-0001-9786-3854).

Author contributions: All authors helped to perform the research; Urushikubo J manuscript writing, performing procedures and data analysis; Yanai S manuscript writing, drafting conception and design, performing experiments, and data analysis; Nakamura S contribution to writing the manuscript, drafting conception and design; Kawasaki K, Akasaka R, Sato K, Toya Y, Asakura K and Gonai T contribution to writing the manuscript; Sugai T reviewed the histological specimens and was responsible for the pathological diagnosis; Matsumoto T critically reviewed and revised the manuscript; all authors have read and approved the final version of the manuscripts.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Iwate Medical University Hospital.

Informed consent statement: Patients were not required to give informed consent as this is a retrospective study.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Jun Urushikubo, MD, Doctor, Division of Gastroenterology, Department of Internal Medicine, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan. urujun50@gmail.com
Telephone: +81-19-6515111
Fax: +81-19-6526664

Received: July 10, 2018
Peer-review started: July 10, 2018
First decision: August 27, 2018
Revised: September 10, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 14, 2018

Abstract

AIM

To determine appropriate fecal calprotectin cut-off values for the prediction of endoscopic and histologic remission in Japanese patients with ulcerative colitis (UC).

METHODS

We performed a cross-sectional observational study of 131 Japanese patients with UC and measured fecal calprotectin levels by fluorescence enzyme immunoassay. The clinical activity of UC was assessed with the partial Mayo score (PMS). Relapse was defined as increase of

PMS by 2 points or more in stool frequency or rectal bleeding subscore. The endoscopic and histologic activities of UC were evaluated in 50 patients within a 2-mo period from fecal sampling. Endoscopic activity was determined by Mayo endoscopic subscore, Rachmilewitz endoscopic index, and ulcerative colitis endoscopic index of severity. The histologic grade of inflammation was evaluated with biopsy specimens obtained from the endoscopically most severely inflamed site, according to the scheme by Matts grade and Riley's score.

RESULTS

Fecal calprotectin levels varied from 1-20783 μg/g. There was a significant correlation between the partial Mayo score and fecal calprotectin levels (r = 0.548, P < 0.001). In 50 patients who underwent colonoscopy with biopsy, levels were significantly correlated with the Mayo endoscopic subscore (r = 0.574, P < 0.001), Rachmilewitz endoscopic index (r = 0.628, P < 0.001), ulcerative colitis endoscopic index of severity (r = 0.613, P < 0.001), Riley' s histologic score (r = 0.400, P = 0.006), and Matts grade (r = 0.586, P < 0.001). Receiver-operating characteristic analyses identified the best cut-off value for the prediction of endoscopic remission as 288 μg/g, with an area under the curve of 0.777 or 0.823, while that for histologic remission was 123 or 125 μg/g, with an AUC of 0.881 or 0918, respectively. Of the 131 study patients, 88 patients in clinical remission were followed up 6 mo. During the follow-up period, 19 patients relapsed. The best fecal calprotectin cut-off value for predicting relapse was 175 μg/g.

CONCLUSION

Fecal calprotectin is a predictive biomarker for endoscopic and histologic remission in Japanese patients with UC.

Key words: Ulcerative colitis; Remission; Mucosal healing; Colonoscopy; Histology; Biomarker; Feces; Calprotectin

Core tip: In recent years, fecal calprotectin (FC) has been reported as a reliable surrogate marker for clinical, endoscopic and histologic activity in ulcerative colitis (UC). The aim of the present study was to determine appropriate FC cut-off values measured by fluorescence enzyme immunoassay (FEI) for predicting endoscopic and histologic remission in Japanese patients with UC. The best FC cut-off values predictive of histologic remission were 125 μg/g for Riley histologic score and 123 μg/g for Matts histologic grade. FC measured by FEI is a useful biomarker for predicting histologic remission in UC.

Urushikubo J, Yanai S, Nakamura S, Kawasaki K, Akasaka R, Sato K, Toya Y, Asakura K, Gonai T, Sugai T, Matsumoto T. Practical fecal calprotectin cut-off value for Japanese patients with ulcerative colitis. *World J Gastroenterol* 2018; 24(38): 4384-4392 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4384.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4384

INTRODUCTION

Ulcerative colitis (UC) is an idiopathic chronic inflammatory disorder of the large intestine characterized by recurrent periods of clinical remission and disease relapse. In recent years, mucosal healing (MH) has been considered as an important therapeutic goal in inflammatory bowel diseases[1-7]. Achieving MH is associated with lower relapse rates, hospitalization rates, and surgery requirements. MH is often defined as a combination of clinical remission and endoscopic remission. However, histologic recovery is incomplete, even in patients with UC who achieve clinical and endoscopic remission[8-11]. Several reports have suggested that persistent active microscopic inflammation is associated with an increased risk of relapse[10-12]. In addition, the severity of such histologic inflammation is an important risk factor for colorectal neoplasia[4,13,14]. Hence, histologic remission should be an important goal in the management of UC.

Calprotectin is a calcium and zinc-binding protein produced mainly by neutrophils. It has been reported that fecal calprotectin (FC) levels reflect local inflammation of the gastrointestinal tract. The FC level has been reported as a reliable surrogate marker of endoscopic and histologic activity in UC[15-19]. However, appropriate FC cut-off values for the prediction of endoscopic and histologic remission remain to be established in Japanese patients with UC.

The aim of the present study was to determine appropriate FC cut-off values for predicting endoscopic and histologic remission in Japanese patients with UC.

MATERIALS AND METHODS

Patients

This was a cross-sectional observational study of 131 Japanese patients with UC for measurement of FC during the period from December 2015 to July 2017. All patients were recruited at the Division of Gastroenterology, Iwate Medical University Hospital, Morioka, Japan. The diagnosis of UC was based on established clinical, endoscopic, radiological, and histological criteria. Type of UC was classified into total colitis, left-sided colitis, proctitis and segmental colitis. Segmental colitis was regarded as a disease with typical mucosal lesion without rectal involvement[20]. Blood samples were collected for the measurement of white blood cell (WBC) counts, hemoglobin levels, platelet counts, erythrocyte sedimentation rate (ESR), serum albumin levels, and C-reactive protein (CRP) levels within a week from FC measurement.

The clinical activity of UC was assessed with the partial Mayo score (PMS)[21]; clinical remission was defined as a PMS of 0 without rectal bleeding and no requirement for steroid therapy in the previous 3 mo. Exclusion criteria were presence of infectious enterocolitis, colorectal cancer, Crohn's disease, or indeterminate colitis; inability to collect fecal samples; pregnancy, history of colorectal

resection, or regular intake of aspirin/non-steroidal anti-inflammatory drugs (NSAIDs), defined as ⩾ 2 tablets/week.

Definition of relapse

Of the 131 study patients with UC, 88 were in clinical remission (PMS = 0), and they were followed for a 6-mo period. Relapse was defined as increased stool frequency or rectal bleeding by a PMS increase of more than 2 points. Three patients who self-discontinued their medication were excluded.

FC assay

Stool samples were obtained on the morning of the scheduled day by patients themselves and stored at -20 ℃ until assay. FC was measured in a blind manner regarding the clinical and endoscopic profile, with a fluorescence enzyme immunoassay (FEI) (Phadia EliA™ Calprotectin 2).

Endoscopic and histological assessment

The endoscopic and histologic activity of UC were evaluated in 50 patients within a 2-mo period from fecal sampling. Because of the retrospective nature of the study, the indication for colonoscopy was heterogeneous among the study population. However, at least a single biopsy specimen was routinely obtained from the area of the most severe inflammation in subjects with active disease and from the rectum in subjects in remission.

Endoscopic activity was determined by Mayo endoscopic subscore (MES), Rachmilewitz endoscopic index (REI), and ulcerative colitis endoscopy index of severity (UCEIS)[21-23]. Endoscopic remission was defined as MES = 0, REI = 0, and UCEIS = 0. The histologic grade of inflammation was determined in biopsy specimens obtained from the endoscopically most severely inflamed site according to the scheme reported by Matts grade and Riley (Riley's score)[24,25]. For Riley's score, biopsy specimens were evaluated on a 5-point scale to measure the degree of chronic inflammatory cell infiltrate and tissue destruction[25]. The histologic grade was determined by a pathologist (TS), who was completely blinded to the endoscopic findings and FC levels. Histologic remission was defined as Matts grade = 1 and Riley's score = 0. Four cases in which histological evaluation was difficult or biopsy samples were taken from inappropriate sites were excluded.

Ethical considerations

The study protocol was approved by the Ethics Committee at Iwate Medical University Hospital (H29-172), and the study was conducted in accordance with the Helsinki Declaration (6th revision, 2008).

Statistical analysis

All of the statistical analyses were performed with the JMP® 13 (SAS Institute Inc., Cary, NC, United States) and SPSS version 22 software for MAC OS (SPSS Inc., Chicago, IL, United States). Numerical variables are presented as the median and interquartile range (IQR), while categorical variables are presented as frequencies. Associations between FC levels and blood tests, clinical disease activity, endoscopic activity or histologic activity were evaluated with the Spearman's rank sum test. A receiver-operating characteristic (ROC) curve was drawn to estimate the area under the curve (AUC) and the best cut-off levels for FC to predict relapse and clinical, endoscopic, and histological remission. According to the cut-off levels, test significance, including sensitivity, specificity, positive-predictive value, positive likelihood ratio, and accuracy were calculated. Associations between FC and other markers were examined by logistic regression analyses. Clinical characteristics were compared between relapsed patients and non-relapsed patients. Age and laboratory data were compared with the Wilcoxon test. Frequency by gender and medication were compared with the chi-square test. The types of disease extent were compared with the Kruskal-Wallis test. Relapse rate was compared between any two groups using Cox proportional hazard model. In each analysis, P values < 0.05 were considered statistically significant.

RESULTS

Baseline demographic characteristics of the study patients

The baseline demographic characteristics of the 131 patients included in the study are shown in Table 1. The median age was 41 (IQR 28-52) years, and 67 (51.1%) patients were male. The median duration of UC was 3.6 (IQR 2-10.1) years. Regarding disease extent, total colitis was seen in 76 (58.0%) patients, left-sided colitis in 24 (18.3%), proctitis in 29 (22.2%), and segmental colitis in 2 (1.5%). Among all 131 patients, FC levels varied from 1 to 20783 μg/g. The median FC level was 289 (IQR 78-785) μg/g. In most patients, medical treatment was administered; 112 patients were receiving oral mesalazine, 47 were receiving immunomodulators, 21 were receiving anti-tumor necrosis factor-α, and 17 were receiving a corticosteroid.

Association between fecal calprotectin levels and blood test results

The level of FC was correlated with the levels of CRP (Spearman's rank correlation coefficient r = 0.467, P < 0.001), ESR (r = 0.355, P = 0.0003), serum albumin (r = -0.447, P < 0.001), hemoglobin (r = -0.353, P = 0.0002), and platelets (r = 0.300, P = 0.0018), but not with WBC counts (r = 0.157, P = 0.104).

Association between fecal calprotectin levels and clinical, endoscopic, or histologic activity

When the clinical disease activity in all 131 patients was compared based on the PMS and FC level, a positive correlation was found between the FC level and the

Table 1 Baseline characteristics of the 131 study patients *n* (%)

Parameter	*n*
Age, yr, median (IQR)	41 (28-52)
Man/female	67/64
Disease duration, yr, median (IQR)	3.6 (2.0-10.1)
Actual disease extent	
Proctitis	29 (22.2)
Left-sided colitis	24 (18.3)
Total colitis	76 (58.0)
Segmental colitis	2 (1.5)
Partial Mayo score	
< 2	93 (71.0)
2-4	28 (21.4)
5-7	8 (6.1)
> 7	2 (1.5)
Laboratory data	
WBC, /μL, median (IQR)	6525 (4838-8140)
CRP, mg/dL, median (IQR)	0.1 (0.04-0.20)
ESR, mm/h, median (IQR)	7 (4-11)
Albumin, g/dL, median (IQR)	4.3 (4.0-4.6)
Hemoglobin, g/dL, median (IQR)	13.6 (12.1-14.3)
Platelets, × 1000/μL, median (IQR)	268 (190-320)
FC, μg/g, median (IQR)	289 (78-785)
Medication	
Mesalazine, oral	112 (85.5)
Mesalazine, topical	12 (9.2)
Immunomodulators	47 (35.9)
Anti-TNF	21 (16.0)
Corticosteroids	17 (13.0)

CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; FC: Fecal calprotectin; IQR: Interquartile range; TNF: Tumor necrosis factor; WBC: White blood cell.

grade of clinical activity (r = 0.548, P < 0.001). The best FC cut-off value was calculated to be 289 μg/g (AUC: 0.796; 95%CI: 0.714-0.878) for predicting clinical remission determined by PMS with a sensitivity of 72% and a specificity of 84%.

The correlation between endoscopic activity and FC could be examined in 50 subjects. The indication for colonoscopy was clinically active disease or exacerbation of clinical symptoms in 14 subjects, assessment of therapeutic efficacy in 12 subjects, and surveillance for colorectal cancer in 24 subjects. Among those subjects, FC levels increased along with increasing severity of endoscopic activity evaluated by MES (r = 0.574, P < 0.001), REI (r = 0.628, P < 0.001), and UCEIS (r = 0.613, P < 0.001). For predicting endoscopic remission, the best FC cut-off value was 490 μg/g for the MES (AUC: 0.823; 95%CI: 0.707-0.939) with a sensitivity of 100% and a specificity of 62% (Figure 1A), while it was 288 μg/g for both the REI (AUC: 0.780; 95%CI: 0.658-0.903) and the UCEIS (AUC: 0.777; 95%CI: 0.645-0.909) (Figure 1B and C). A logistic regression analysis including FC, CRP, WBC, ESR and platelet count as co-variables revealed that there was not a statistically significant correlation between FC and endoscopic remission defined as UCEIS = 0.

Biopsy specimens were obtained from 46 patients during colonoscopy. Among 46 subjects evaluated histologically, FC levels increased with increasing histologic inflammatory activity by both Matts grade (r = 0.586, P < 0.001), and Riley's score (r = 0.400, P = 0.006). For predicting histologic remission, the best FC cut-off values were 125 μg/g for the Riley score (AUC: 0.881; 95%CI: 0.780-0.981) and 123 μg/g for the Matts grade (AUC: 0.918; 95%CI: 0.831-1.000) (Figure 2A and B). A logistic regression analysis revealed that FC was a single and independent factor associated with histologic remission defined as Matts grade = 1 (P = 0.005).

Association between fecal calprotectin levels and relapse

Of the 131 study patients, 88 patients in clinical remission (PMS = 0) were followed up. During the 6-mo period after FC measurement, 19 (21.6%) of the 88 patients experienced a relapse of UC. Clinical characteristics of the relapsed and non-relapsed patients are compared in Table 2. Medication with oral mesalazine was more frequent in non-relapsed patients than in relapsed patients (P = 0.03). The ROC analysis estimated a FC cut-off value of 175 μg/g (AUC: 0.648; 95%CI: 0.517-0.778) for predicting relapse, with a sensitivity of 68% and a specificity of 61% (Figure 3). However, Cox proportional hazard model revealed that neither FC nor other co-variables was an independent predictive factor for clinical relapse within 6-mo period.

Table 3 summarizes the performance of FC levels using the appropriate cut-off values in our cohort of patients with UC.

DISCUSSION

In the present study, we investigated the association between FC levels and laboratory data and clinical, endoscopic, and histologic disease activity and risk of relapse. The results showed that FC was closely related to the laboratory data (CRP, ESR, serum albumin, hemoglobin, platelets) and to the clinical, endoscopic and histologic disease activity. While FC has been measured with enzyme-linked immunosorbent assays (ELISAs) in previous investigations[15,17-19], we measured FC levels with a FEI in the present study. Even though FC measured by FEI has been shown to be an appropriate marker for patients with UC[16,26,27], it has also been confirmed that measurement by FEI results in a wide range of the test value when compared to measurement by ELISA[27]. We thus presume that high cut-off values of FC as found in our study may be a consequence of FEI measurement.

Based on the interpretations of the ROCs, we obtained FC cut-off levels of 289 μg/g for predicting clinical remission, and 288 or 490 μg/g to predict endoscopic remission. The FC cut-off value for MES (490 μg/g) was higher than that for REI and UCEIS (288 μg/g). This is probably because the numbers of evaluated items are greater with the REI and UCEIS than with the MES. We obtained the FC cut-off levels of 123 μg/g and 125 μg/g

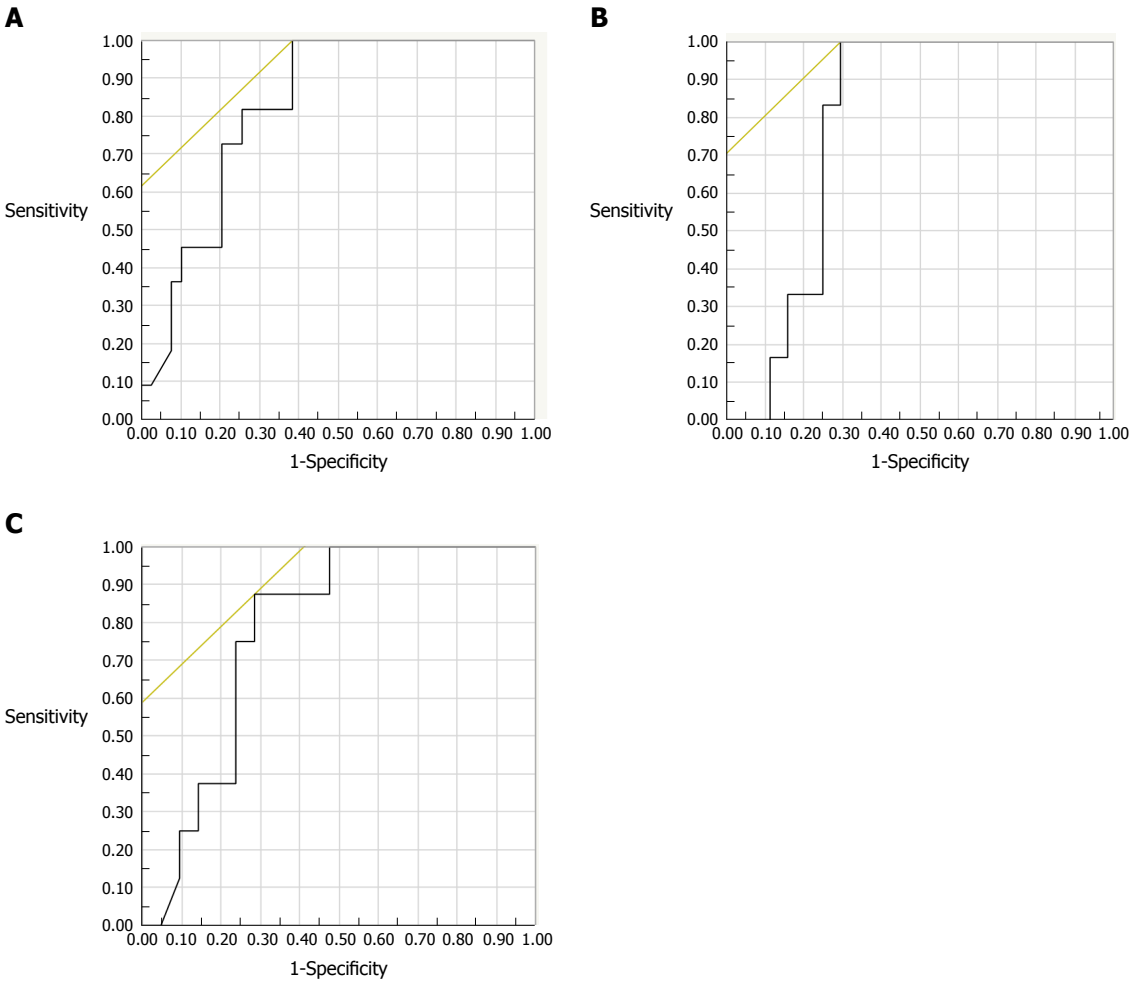


Figure 1 Receiver-operating characteristic curves for fecal calprotectin levels to predict endoscopic remission of ulcerative colitis (*n* = 50). A: ROC for FC levels in predicting remission by Mayo endoscopic subscore. The AUC was 0.823, 95%CI: 0.707-0.939. The best cut-off value of FC was 490 μg/g, with a sensitivity of 100% and a specificity of 62%; B: ROC for FC in predicting remission by the Rachmilewitz endoscopic index. The AUC was 0.780, 95%CI: 0.658-0.903. The best cut-off value of FC was 288 μg/g, with a sensitivity of 100% and a specificity of 70%; C: ROC for FC level in predicting remission by the ulcerative colitis endoscopy index of severity. The AUC was 0.777, 95%CI: 0.645-0.909. The best cut-off value of FC was 288 μg/g, with a sensitivity of 88% and a specificity of 71%. ROC: Receiver-operating characteristic; FC: Fecal calprotectin; AUC: Area under the curve.

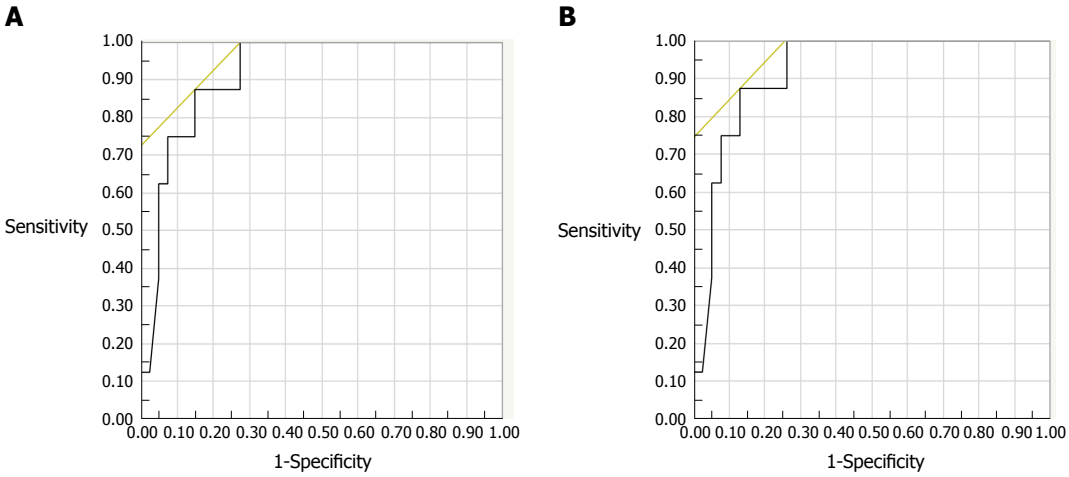


Figure 2 Receiver-operating characteristic curves for fecal calprotectin levels in predicting the histologic remission of ulcerative colitis (*n* = 46). A: ROC for FC in predicting remission by Riley's score. The AUC was 0.881, 95CI: 0.780-0.981. The best cut-off value of FC was 125 μg/g, with a sensitivity of 80% and a specificity of 86%; B: ROC for FC in predicting remission by Matts grade. The AUC was 0.918, 95%CI: 0.831-1.000. The best cut-off value of FC was 123 μg/g, with a sensitivity of 88% and a specificity of 87%. ROC: Receiver-operating characteristic; FC: Fecal calprotectin; AUC: Area under the curve.

Table 2 Clinical characteristics of relapsed and non-relapsed patients *n* (%)

Parameter	Relapsed patients (*n* = 19)	Non-relapsed patients (*n* = 69)	*P* value
Age, yr, median (IQR)	35 (23-53)	40 (29-52)	0.26
Male/female	6/13	37/32	0.08
Actual disease extent			
Proctitis	5 (26.3)	18 (26.1)	0.53
Left-sided colitis	1 (5.3)	12 (17.4)	
Total colitis	13 (68.4)	38 (55.1)	
Segmental colitis	0 (0)	1 (1.4)	
Laboratory data			
WBC, /μL, median (IQR)	6130 (5110-7745)	5585 (4640-7013)	0.48
CRP, mg/dL, median (IQR)	0.05 (0.01-0.10)	0.1 (0.04-0.14)	0.05
ESR, mm/h, median (IQR)	6 (4-8)	5 (3.0-10.0)	0.69
Albumin, g/dL, median (IQR)	4.4 (4.3-4.6)	4.4 (4.1-4.6)	0.75
Hemoglobin, g/dL, median (IQR)	13.3 (12.3-14.4)	13.9 (12.6-14.9)	0.25
Platelets, × 1000/μL, median (IQR)	238 (214-339)	256 (213-294)	0.92
FC, μg/g, median (IQR)	303 (94-846)	126 (55.5-485)	0.05
Medication			
Mesalazine, oral	14 (73.7)	64 (92.8)	0.03
Mesalazine, topical	1 (5.3)	3 (4.3)	0.87
Immunomodulators	6 (31.6)	18 (26.1)	0.98
Anti-TNF	4 (21.1)	12 (17.4)	0.71
Corticosteroids	3 (15.8)	5 (7.2)	0.28

CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; FC: Fecal calprotectin; IQR: Interquartile range; TNF: Tumor necrosis factor; WBC: White blood cell.

Table 3 Test characteristics of fecal calprotectin levels to predict relapse and remission

Variable	Cut-off value (μg/g)	AUC	95%CI	Sensitivity (%)	Specificity(%)	PPV (%)	PLR	Accuracy (%)
Relapse	175	0.648	0.517-0.778	68	61	33	1.75	63
PMS 0	289	0.796	0.714-0.878	72	84	88	4.48	76
MES 0	490	0.823	0.707-0.939	100	62	42	2.6	70
REI 0	288	0.78	0.658-0.903	100	70	32	3.38	74
UCEIS 0	288	0.777	0.645-0.909	88	71	37	3.06	74
Riley's score 0	125	0.881	0.780-0.981	80	86	62	5.76	85
Matts grade 1	123	0.918	0.831-1.000	88	87	58	6.65	87

AUC: Area under the curve; CI: Confidence interval; PPV: Positive-predictive value; PLR: Positive likelihood ratio; PMS: Partial Mayo score; MES: Mayo endoscopic subscore; REI: Rachmilewitz endoscopic index; UCEIS: Ulcerative colitis endoscopic index of severity.

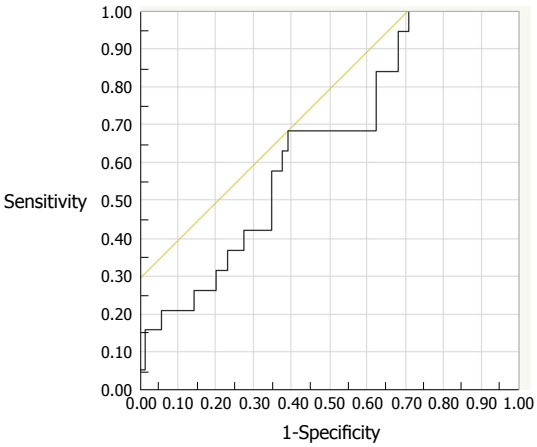


Figure 3 Receiver-operating characteristic curve for fecal calprotectin levels to predict relapse at the six-month follow-up assessment (AUC: 0.648; 95%CI: 0.517-0.778). The best cut-off value was 175 μg/g, with a sensitivity of 68% and a specificity of 61%. AUC: Area under the curve.

to predict histologic remission, as determined by the Matts grade and Riley score, respectively. Both of the determined FC values were close to each other, and the evaluation ability is considered nearly equivalent.

The therapeutic goal for patients with UC has recently shifted from symptom control to the combination of endoscopic and histologic remission with MH, because this has been found to be a better predictor of long-term remission and prevention of the need for hospitalizations and surgeries[1-7]. Previous studies showed that patients with a MES value of 1 are more likely to relapse and they are prone to colectomy than those with a MES of 0[28,29]. Further, achieving histological remission better predicts corticosteroid use and hospitalization[30]. Several previous studies reported that microscopic inflammation persists in some patients with endoscopic MH[31,32]. In the present study, we found that about 50% of patients had residual inflammation in the mucosa (Matts grade ⩾ 2) despite having been judged to be in endoscopic remission (UCEIS = 0). Accordingly, histological remission seems more important as a rational therapeutic target[33]. Previous studies reported the cut-off levels FC to range from 60-200 μg/g for histological remission[15,17,34-38]. We found that a lower FC cut-off value predicted histological

remission rather than clinical and endoscopic remission. Furthermore, logistic regression analyses revealed that FC was an independent factor associated with histological remission. While recent treat-to-target concept for UC itemized that either histology or FC is not a target for the treatment of UC, they are regarded as being adjunctive for the management of UC[39]. Our observation suggests that a simple measurement of FC is a substantial alternative for the evaluation of histologic severity of UC.

In our study, the FC cut-off value for prediction of relapse in UC was determined to be 175 μg/g. However, sensitivity and specificity for the prediction of relapse were less than 70%, and AUC for the prediction was less than 0.7. In addition, a multivariate analysis failed to identify FC as a predictive factor for clinical relapse. This observation does not conform to previous reports showing the accuracy of FC for the prediction of relapse in UC[19,34,35,40]. In our study population, however, the overall rate of relapse was high with a value of 14%. In addition, colonoscopy was not performed at the time when PMS was found to be zero. Therefore, it seems possible that we recruited subjects without mucosal healing, thus being prone to relapse, for the analysis.

Several limitations of this study should be acknowledged. First, our cross-sectional study design makes it possible to observe associations at a specific time point, but gives no information about longitudinal clinical end points, such as the prognostic value of FC. Second, we assessed endoscopic and histological activity by sigmoidoscopy or total colonoscopy. However, the original endoscopic items of the Mayo scoring system were evaluated by sigmoidoscopy[21]. In the case of sigmoidoscopy, the FC levels may have been affected by the degree of proximal colonic inflammation. Third, the clinical disease condition of the patients at the time of stool sampling varied. Finally, the study was performed at a single center and involved a limited number of patients.

In conclusion, our analysis indicates that FC measured by FEI is a useful biomarker in Japanese patients with UC. It is considered appropriate for the prediction of mucosal lesions and histologic activity rather than clinical activity of UC. However, the appropriateness of FC measured by FEI for the prediction of relapse of UC awaits further elucidation.

ARTICLE HIGHLIGHTS

Research background

Fecal calprotectin (FC) is a useful biomarker to assess disease activity in ulcerative colitis (UC). However, appropriate cut-off values of FC for the endoscopic and histologic remission has not yet been determined in Japanese patients with UC.

Research motivation

Calculating the FC cut-off value of the remissions will help to evaluate disease activity instead of invasive examination such as endoscopy.

Research objectives

To determine cut-off values of FC for endoscopic and histologic remission in Japanese patients with UC.

Research methods

We performed a retrospective study of Japanese patients with UC for measurement of FC that was measured by fluorescence enzyme immunoassay (FEI). We analyzed the relationship between FC and laboratory data, clinical activity, endoscopic score (Mayo endoscopic subscore: MES, Rachmilwitz endoscopic index: REI, ulcerative colitis endoscopic index of severity: UCEIS and histologic score (Matts grade, Riley's histologic score).

Research results

In 131 patients, there was a statistically significant correlation between PMS and FC ($P < 0.001$). FC levels were significantly correlated with the MES ($P < 0.001$), REI ($P < 0.001$), UCEIS ($P < 0.001$), Riley's histologic score ($P = 0.006$), and Matts grade ($P < 0.001$). Receiver-operating characteristic analyses identified the best cut-off value for the prediction of endoscopic remission as 288 μg/g, with an area under the curve (AUC) of 0.777 or 0.823, while that for histologic remission was 123 or 125 μg/g or 125 μg/g, with an AUC of 0.881 or 0.918.

Research conclusions

FC measured by FEI is considered a predictive biomarker for endoscopic and histologic remission in Japanese patients with UC.

Research perspectives

Our study showed that FC was useful biomarker for prediction of endoscopic and histologic activity. This research was a retrospective study, which is the maximum limitation. Further prospective studies are needed to confirm the reproducibility of the results of this research.

REFERENCES

1 **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 DOI: 10.1056/NEJMoa050516]

2 **Frøslie KF**, Jahnsen J, Moum BA, Vatn MH; IBSEN Group. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 2007; **133**: 412-422 [PMID: 17681162 DOI: 10.1053/j.gastro.2007.05.051]

3 **Ardizzone S**, Cassinotti A, Duca P, Mazzali C, Penati C, Manes G, Marmo R, Massari A, Molteni P, Maconi G, Porro GB. Mucosal healing predicts late outcomes after the first course of corticosteroids for newly diagnosed ulcerative colitis. *Clin Gastroenterol Hepatol* 2011; **9**: 483-489.e3 [PMID: 21195796 DOI: 10.1016/j.cgh.2010.12.028]

4 **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 DOI: 10.1053/j.gastro.2011.06.054]

5 **Meucci G**, Fasoli R, Saibeni S, Valpiani D, Gullotta R, Colombo E, D'Incà R, Terpin M, Lombardi G; IG-IBD. Prognostic significance of endoscopic remission in patients with active ulcerative colitis treated with oral and topical mesalazine: a prospective, multicenter study. *Inflamm Bowel Dis* 2012; **18**: 1006-1010 [PMID: 21830282 DOI: 10.1002/ibd.21838]

6 **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]

7 **Papi C**, Fascì-Spurio F, Rogai F, Settesoldi A, Margagnoni G, Annese V. Mucosal healing in inflammatory bowel disease: treatment efficacy and predictive factors. *Dig Liver Dis* 2013; **45**: 978-985 [PMID: 24018244 DOI: 10.1016/j.dld.2013.07.006]

8 **Baars JE**, Nuij VJ, Oldenburg B, Kuipers EJ, van der Woude CJ. Majority of patients with inflammatory bowel disease in clinical remission have mucosal inflammation. *Inflamm Bowel Dis* 2012; **18**:

1634-1640 [PMID: 22069022 DOI: 10.1002/ibd.21925]
9 **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 DOI: 10.1016/j.cgh.2013.02.030]
10 **Riley SA**, Mani V, Goodman MJ, Dutt S, Herd ME. Microscopic activity in ulcerative colitis: what does it mean? *Gut* 1991; **32**: 174-178 [PMID: 1864537 DOI: 10.1136/gut.32.2.174]
11 **Bitton A**, Peppercorn MA, Antonioli DA, Niles JL, Shah S, Bousvaros A, Ransil B, Wild G, Cohen A, Edwardes MD, Stevens AC. Clinical, biological, and histologic parameters as predictors of relapse in ulcerative colitis. *Gastroenterology* 2001; **120**: 13-20 [PMID: 11208709 DOI: 10.1053/gast.2001.20912]
12 **Bessissow T**, Lemmens B, Ferrante M, Bisschops R, Van Steen K, Geboes K, Van Assche G, Vermeire S, Rutgeerts P, De Hertogh G. Prognostic value of serologic and histologic markers on clinical relapse in ulcerative colitis patients with mucosal healing. *Am J Gastroenterol* 2012; **107**: 1684-1692 [PMID: 23147523 DOI: 10.1038/ajg.2012.301]
13 **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]
14 **Gupta RB**, Harpaz N, Itzkowitz S, Hossain S, Matula S, Kornbluth A, Bodian C, Ullman T. Histologic inflammation is a risk factor for progression to colorectal neoplasia in ulcerative colitis: a cohort study. *Gastroenterology* 2007; **133**: 1099-1105; quiz 1340-1341 [PMID: 17919486 DOI: 10.1053/j.gastro.2007.08.001]
15 **Guardiola J**, Lobatón T, Rodríguez-Alonso L, Ruiz-Cerulla A, Arajol C, Loayza C, Sanjuan X, Sánchez E, Rodríguez-Moranta F. Fecal level of calprotectin identifies histologic inflammation in patients with ulcerative colitis in clinical and endoscopic remission. *Clin Gastroenterol Hepatol* 2014; **12**: 1865-1870 [PMID: 24993368 DOI: 10.1016/j.cgh.2014.06.020]
16 **Yamaguchi S**, Takeuchi Y, Arai K, Fukuda K, Kuroki Y, Asonuma K, Takahashi H, Saruta M, Yoshida H. Fecal calprotectin is a clinically relevant biomarker of mucosal healing in patients with quiescent ulcerative colitis. *J Gastroenterol Hepatol* 2016; **31**: 93-98 [PMID: 26212346 DOI: 10.1111/jgh.13061]
17 **Theede K**, Holck S, Ibsen P, Ladelund S, Nordgaard-Lassen I, Nielsen AM. Level of Fecal Calprotectin Correlates With Endoscopic and Histologic Inflammation and Identifies Patients With Mucosal Healing in Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2015; **13**: 1929-1936. e1 [PMID: 26051392 DOI: 10.1016/j.cgh.2015.05.038]
18 **Taghvaei T**, Maleki I, Nagshvar F, Fakheri H, Hosseini V, Valizadeh SM, Neishaboori H. Fecal calprotectin and ulcerative colitis endoscopic activity index as indicators of mucosal healing in ulcerative colitis. *Intern Emerg Med* 2015; **10**: 321-328 [PMID: 25366383 DOI: 10.1007/s11739-014-1144-x]
19 **Theede K**, Holck S, Ibsen P, Kallemose T, Nordgaard-Lassen I, Nielsen AM. Fecal Calprotectin Predicts Relapse and Histological Mucosal Healing in Ulcerative Colitis. *Inflamm Bowel Dis* 2016; **22**: 1042-1048 [PMID: 26919460 DOI: 10.1097/MIB.0000000000000736]
20 **Park SH**, Yang SK, Park SK, Kim JW, Yang DH, Jung KW, Kim KJ, Ye BD, Byeon JS, Myung SJ, Kim JH. Atypical distribution of inflammation in newly diagnosed ulcerative colitis is not rare. *Can J Gastroenterol Hepatol* 2014; **28**: 125-130 [PMID: 24619632 DOI: 10.1155/2014/834512]
21 **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629 [PMID: 3317057 DOI: 10.1056/NEJM198712243172603]
22 **Rachmilewitz D**. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. *BMJ* 1989; **298**: 82-86 [PMID: 2563951 DOI: 10.1136/bmj.298.6666.82]
23 **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]
24 **MATTS SG**. The value of rectal biopsy in the diagnosis of ulcerative colitis. *Q J Med* 1961; **30**: 393-407 [PMID: 14471445]
25 **Riley SA**, Mani V, Goodman MJ, Herd ME, Dutt S, Turnberg LA. Comparison of delayed release 5 aminosalicylic acid (mesalazine) and sulphasalazine in the treatment of mild to moderate ulcerative colitis relapse. *Gut* 1988; **29**: 669-674 [PMID: 2899536 DOI: 10.1136/gut.29.5.669]
26 **Kittanakom S**, Shajib MS, Garvie K, Turner J, Brooks D, Odeh S, Issenman R, Chetty VT, Macri J, Khan WI. Comparison of Fecal Calprotectin Methods for Predicting Relapse of Pediatric Inflammatory Bowel Disease. *Can J Gastroenterol Hepatol* 2017; **2017**: 1450970 [PMID: 28491862 DOI: 10.1155/2017/1450970]
27 **Labaere D**, Smismans A, Van Olmen A, Christiaens P, D'Haens G, Moons V, Cuyle PJ, Frans J, Bossuyt P. Comparison of six different calprotectin assays for the assessment of inflammatory bowel disease. *United European Gastroenterol J* 2014; **2**: 30-37 [PMID: 24918006 DOI: 10.1177/2050640613518201]
28 **Manginot C**, Baumann C, Peyrin-Biroulet L. An endoscopic Mayo score of 0 is associated with a lower risk of colectomy than a score of 1 in ulcerative colitis. *Gut* 2015; **64**: 1181-1182 [PMID: 25550182 DOI: 10.1136/gutjnl-2014-308839]
29 **Barreiro-de Acosta M**, Vallejo N, de la Iglesia D, Uribarri L, Bastón I, Ferreiro-Iglesias R, Lorenzo A, Domínguez-Muñoz JE. Evaluation of the Risk of Relapse in Ulcerative Colitis According to the Degree of Mucosal Healing (Mayo 0 vs 1): A Longitudinal Cohort Study. *J Crohns Colitis* 2016; **10**: 13-19 [PMID: 26351390 DOI: 10.1093/ecco-jcc/jjv158]
30 **Bryant RV**, Burger DC, Delo J, Walsh AJ, Thomas S, von Herbay A, Buchel OC, White L, Brain O, Keshav S, Warren BF, Travis SP. Beyond endoscopic mucosal healing in UC: histological remission better predicts corticosteroid use and hospitalisation over 6 years of follow-up. *Gut* 2016; **65**: 408-414 [PMID: 25986946 DOI: 10.1136/gutjnl-2015-309598]
31 **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]
32 **Tontini GE**, Vecchi M, Neurath MF, Neumann H. Review article: newer optical and digital chromoendoscopy techniques vs. dye-based chromoendoscopy for diagnosis and surveillance in inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **38**: 1198-1208 [PMID: 24117471 DOI: 10.1111/apt.12508]
33 **Bryant RV**, Winer S, Travis SP, Riddell RH. Systematic review: histological remission in inflammatory bowel disease. Is 'complete' remission the new treatment paradigm? An IOIBD initiative. *J Crohns Colitis* 2014; **8**: 1582-1597 [PMID: 25267173 DOI: 10.1016/j.crohns.2014.08.011]
34 **García-Sánchez V**, Iglesias-Flores E, González R, Gisbert JP, Gallardo-Valverde JM, González-Galilea A, Naranjo-Rodríguez A, de Dios-Vega JF, Muntané J, Gómez-Camacho F. Does fecal calprotectin predict relapse in patients with Crohn's disease and ulcerative colitis? *J Crohns Colitis* 2010; **4**: 144-152 [PMID: 21122498 DOI: 10.1016/j.crohns.2009.09.008]
35 **Gisbert JP**, Bermejo F, Pérez-Calle JL, Taxonera C, Vera I, McNicholl AG, Algaba A, López P, López-Palacios N, Calvo M, González-Lama Y, Carneros JA, Velasco M, Maté J. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. *Inflamm Bowel Dis* 2009; **15**: 1190-1198 [PMID: 19291780 DOI: 10.1002/ibd.20933]
36 **Zittan E**, Kelly OB, Kirsch R, Milgrom R, Burns J, Nguyen GC, Croitoru K, Van Assche G, Silverberg MS, Steinhart AH. Low Fecal Calprotectin Correlates with Histological Remission and Mucosal Healing in Ulcerative Colitis and Colonic Crohn's Disease. *Inflamm*

Bowel Dis 2016; **22**: 623-630 [PMID: 26829408 DOI: 10.1097/MIB.0000000000000652]

37 **Patel A**, Panchal H, Dubinsky MC. Fecal Calprotectin Levels Predict Histological Healing in Ulcerative Colitis. *Inflamm Bowel Dis* 2017; **23**: 1600-1604 [PMID: 28590341 DOI: 10.1097/MIB.0000000000001157]

38 **Mak WY**, Buisson A, Andersen MJ Jr, Lei D, Pekow J, Cohen RD, Kahn SA, Pereira B, Rubin DT. Fecal Calprotectin in Assessing Endoscopic and Histological Remission in Patients with Ulcerative Colitis. *Dig Dis Sci* 2018; **63**: 1294-1301 [PMID: 29468374 DOI: 10.1007/s10620-018-4980-0]

39 **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV Jr, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]

40 **Jauregui-Amezaga A**, López-Cerón M, Aceituno M, Jimeno M, Rodríguez de Miguel C, Pinó-Donnay S, Zabalza M, Sans M, Ricart E, Ordás I, González-Suárez B, Cuatrecasas M, Llach J, Panés J, Pellise M. Accuracy of advanced endoscopy and fecal calprotectin for prediction of relapse in ulcerative colitis: a prospective study. *Inflamm Bowel Dis* 2014; **20**: 1187-1193 [PMID: 24874457 DOI: 10.1097/MIB.0000000000000069]

P- Reviewer: Eleftheriadis NP, Lankarani KB, Seicean A
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4393-4402

DOI: 10.3748/wjg.v24.i38.4393

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

ORIGINAL ARTICLE

Observational Study

Liver stiffness reversibly increases during pregnancy and independently predicts preeclampsia

Franziska J Ammon, Anna Kohlhaas, Omar Elshaarawy, Johannes Mueller, Thomas Bruckner, Christof Sohn, Gabriele Fluhr, Herbert Fluhr, Sebastian Mueller

Franziska J Ammon, Christof Sohn, Herbert Fluhr, Department of Obstetrics and Gynecology, University of Heidelberg, Heidelberg 69120, Germany

Anna Kohlhaas, Omar Elshaarawy, Johannes Mueller, Gabriele Fluhr, Sebastian Mueller, Department of Medicine and Center for Alcohol Research, Liver Disease and Nutrition, Salem Medical Center, University of Heidelberg, Heidelberg 69121, Germany

Thomas Bruckner, Institute of Medical Biometry und Informatics, University of Heidelberg, Heidelberg 69121, Germany

ORCID number: Franziska J Ammon (0000-0002-8877-9711); Anna Kohlhaas (0000-0001-7677-1619); Omar Elshaarawy (0000-0002-6945-6204); Johannes Mueller (0000-0002-5205-4129); Thomas Bruckner (0000-0001-9342-3456); Christof Sohn (0000-0001-9430-4037); Gabriele Fluhr (0000-0001-7415-2312); Herbert Fluhr (0000-0001-9300-4868); Sebastian Mueller (0000-0002-3577-8491).

Author contributions: Fluhr H and Mueller S designed research; Ammon FJ, Kohlhaas A and Fluhr G performed measurements; Ammon FJ, Mueller J and Mueller S analyzed the data and wrote the paper; Elshaarawy O revised the paper; Bruckner T reviewed statistics; Elshaarawy O, Sohn C and Fluhr H reviewed the paper.

Supported by the Dietmar Hopp Foundation (in part, DFG), No. MU 1373/9-1.

Institutional review board statement: The study was approved by the Ethical Committee of the University of Heidelberg.

Informed consent statement: All participants of the study, or their legal guardians, provided written informed consent prior to study enrollment.

Conflict-of-interest statement: The authors have declared no conflicts of interest.

Data sharing statement: Dataset and other information are available from the corresponding author at sebastian.mueller@urz.uni-heidelberg.de. Patients gave informed consent before inclusion in the study; the presented data are anonymized and the risk of identification is very low.

STROBE Statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Manuscript source: Invited manuscript

Correspondence to: Sebastian Mueller, MD, PhD, Professor, Department of Internal Medicine, Salem Medical Center and Center for Alcohol Research and Liver Disease, University of Heidelberg, Zeppelinstraße 11-33, Heidelberg 69121, Germany. sebastian.mueller@urz.uni-heidelberg.de
Telephone: +49-6221-483210
Fax: +49-6221-484494

Received: August 1, 2018
Peer-review started: August 1, 2018
First decision: August 24, 2018
Revised: September 20, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 14, 2018

Abstract

AIM

To study liver stiffness (LS) during pregnancy and its association with complications during pregnancy.

METHODS

In this observational, diagnostic study, 537 pregnant women were prospectively enrolled at the Department of Obstetrics and Gynecology, University hospital Heidelberg and Salem Medical Center. LS was measured using the Fibroscan device (Echosens, Paris) in all women and in 41 cases 24 h after delivery. Clinical and morphological data were recorded and abdominal ultrasound and standard laboratory tests were performed. No complications were observed in 475 women (controls) while preeclampsia and intrahepatic cholestasis of pregnancy (ICP) developed in 22 and 40 women, respectively.

RESULTS

In controls, LS increased significantly from initially 4.5 ± 1.2 kPa in the second trimester to 6.0 ± 2.3 kPa (P < 0.001) in the third trimester. In the third trimester, 41% of women had a LS higher than 6 kPa. Elevated LS in controls was significantly correlated with alkaline phosphatase, leukocytes, gestational age and an increase in body weight and body mass index (BMI). In women with pregnancy complications, LS was significantly higher as compared to controls (P < 0.0001). Moreover, in multivariate analysis, LS was an independent predictor for preeclampsia with an odds ratio of 2.05 (1.27-3.31) and a cut-off value of 7.6 kPa. In contrast, ICP could not be predicted by LS. Finally, LS rapidly decreased in all women within 24 h after delivery from 7.2 ± 3.3 kPa down to 4.9 ± 2.2 kPa (P < 0.001).

CONCLUSION

During pregnancy, LS significantly and reversibly increases in the final trimester of pregnant women without complications. In women with preeclampsia, LS is significantly elevated and an independent non-invasive predictor.

Key words: Pregnancy; Transient elastography; Liver stiffness; Pregnancy complications; Hemolysis, elevated liver enzymes and low platelets syndrome; Intrahepatic cholestasis of pregnancy; Preeclampsia

Core tip: Liver stiffness (LS) was measured by transient elastography during pregnancy in 537 healthy pregnant women without complications and in 62 women with pregnancy complications such as preeclampsia or intrahepatic cholestasis of pregnancy. Our results show that LS increases during pregnancy to levels above 6 kPa even in women without pregnancy complications and rapidly normalizes within 24 h after delivery. LS was significantly elevated in women with preeclampsia and, moreover, an independent predictor for preeclampsia in multivariate analysis. In conclusion, LS could be a novel non-invasive screening parameter to identify pregnant women at risk for complications of pregnancy.

Ammon FJ, Kohlhaas A, Elshaarawy O, Mueller J, Bruckner T, Sohn C, Fluhr G, Fluhr H, Mueller S. Liver stiffness reversibly increases during pregnancy and independently predicts preeclampsia. *World J Gastroenterol* 2018; 24(38): 4393-4402 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4393.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4393

INTRODUCTION

Over the last decades, maternal mortality has been drastically lowered in developed countries remaining now stable at a level of approximately 1 death per 4000 deliveries[1]. However, apart from postpartal bleeding and embolic complications, hypertension and preeclampsia remain the leading causes of maternal morbidity and mortality[2,3]. In total, up to 3 % of all pregnancies are complicated by liver disorders which can be related or unrelated to pregnancy and can have a high maternal and perinatal mortality[4-6]. Despite enormous research activities in the last decades, still little is known about the pathogenesis and efficient treatment options of pregnancy- related liver diseases[7]. Except hyperemesis gravidarum, most of the hepatic complications such as hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome and intrahepatic cholestasis of pregnancy (ICP) typically occur in the third trimester[8,9].

So far, rapid delivery is often the only choice to prevent life-threatening complications in mother and child[10]. At present, obstetricians routinely monitor laboratory markers such as aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyltransferase (GGT). Liver sonography is only performed if abnormalities are found. However, these routine tests provide limited information in predicting severe complications during pregnancy[11]. Moreover, no established screening tests exist for an early risk assessment of most of these pregnancy complications[11,12].

Over the last decade, liver stiffness (LS) as measured by transient elastography (TE) has become the preferred parameter to non-invasively assess liver fibrosis[13-15]. TE does not require dedicated ultrasound knowledge and has a fast learning curve[16]. In contrast to liver biopsy, TE is not only non-invasive but has a 10 times smaller sampling error (3% *vs* 30%) and a better inter-observer reliability rendering it ideal for follow-up studies[15,17]. While a physiological low LS below 6 kPa has an excellent predictive power to rule out fibrosis, several studies in adults have demonstrated an elevated LS in the absence of fibrosis[15,18-21] by conditions such as inflammation, cholestasis, congestion, elevated arterial pressure *e.g.* during exercise, rapid changes of the portal flow and food and alcohol intake[22-26]. Taken these observations together, LS seems to represent the sum of fibrosis and pressure-related factors being determined by the hepatic

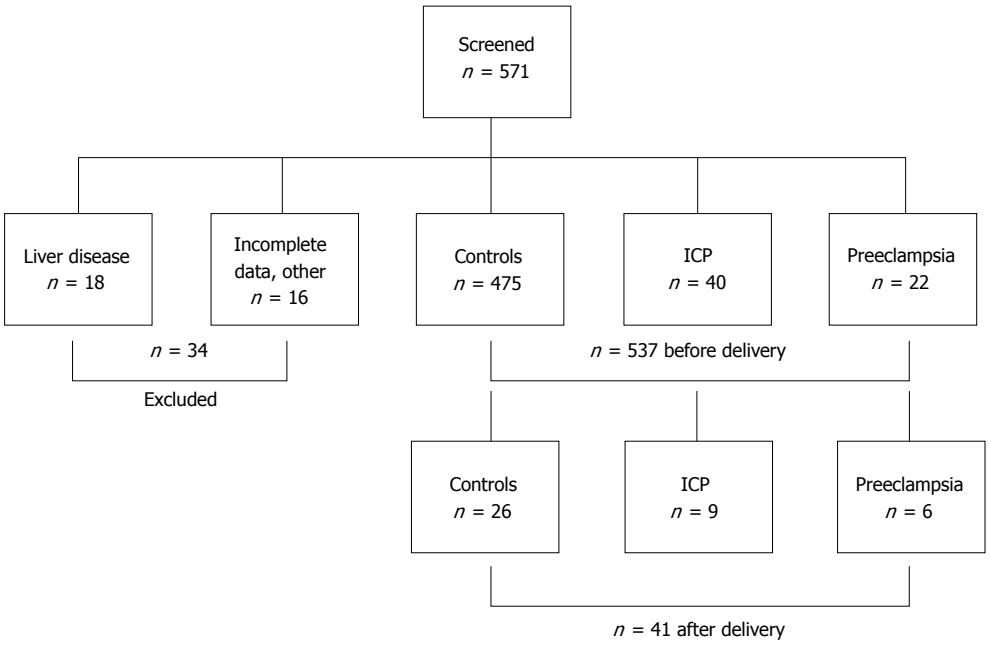


Figure 1 Study design. ICP: Intrahepatic cholestasis of pregnancy.

inflow and outflow balance[15,27]. In a preliminary study, we had recently demonstrated a significant elevation of LS during the third trimester and a rapid normalization in four cases after delivery[28].

We here present the final prospective data of LS in a large cohort of pregnant women using Fibroscan with the aim to investigate evolution of LS during pregnancy in healthy pregnant women and the association of LS to pregnancy complications. In addition, the performance of Fibroscan and its potential association with complications of pregnancy such as preeclampsia and ICP was studied. Our extended study confirms that LS is significantly elevated during the final trimester in women with uncomplicated pregnancy and rapidly normalizes after delivery. We further demonstrate, however, that LS is significantly elevated in patients with preeclampsia being an independent predictor of this complication.

MATERIALS AND METHODS

The study protocol (435/2006 and S201/2015) was reviewed and approved by the local Ethics Committee and all patients gave written informed consent prior to inclusion. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Prospective patient cohort

Figure 1 shows the study design. Three study aims were pursued: The primary aim was to measure LS in pregnant women with normal pregnancy and without preexisting liver disease. Here, we also studied the performance of both M and XL probes in order to obtain valid LS measurements and potential confounders that are correlated with an elevated LS. The second aim was to check whether LS is elevated in women with pregnancy-related complications such as preeclampsia or ICP and to analyze the associated conditions. Third, we studied the response of elevated LS directly 24 h after delivery.

Women were either recruited while undergoing prenatal ultrasound screening during gestational week 12 to 33 or presented to the prenatal outpatient's department with prenatal complications or abnormalities from gestational week 16 to 42.

Postpartum examination (n = 41) took place 24 h after delivery. For every participant of the study general data *i.e.* weight and age as well as obstetric and hepatic medical history were obtained in form of a personal interview. The 571 participants were recruited at the Department of Gynecology at the University of Heidelberg (n = 468) and at Salem Medical Center in Heidelberg (n = 103). Inclusion criteria were age ≥ 18 years, informed and written consent by the participant and an intact pregnancy at week 9 to 42 or status postpartum (see Figure 1). Exclusion criteria were presence of chronic liver disease either by the patient`s history and in addition by ruling out serum markers with evidence for viral hepatitis, autoimmune hepatitis, hemochromatosis or Wilson's disease. In addition, a normal routine laboratory tests before pregnancy and alcohol consumption less than 20 g/d were required. Fatty liver in the ultrasound was no exclusion criterium *per se*. Gynecological exclusion criteria included fatal abnormalities of the fetus or patients being in labor and maternal drug abuse. Patients were also excluded if data were not complete. Altogether, 34 women were excluded with evidence for preexisting liver disease in the majority of cases (see Figure 1).

Patients were considered as control group if no complications occurred and delivery was normal during follow-up. Preeclampsia (hypertonia during pregnancy and proteinuria > 300 mg per day) and ICP (elevated bile acids > 10 μmol/L and pruritus or bile acids > 40 μmol/L) according to standard guidelines[6] were considered as complications of pregnancy. Very rare complications such as Acute Fatty Liver of Pregnancy were not diagnosed during the inclusion period. Two cases of HELLP syndrome were excluded since LS could not be determined reliably due to intrahepatic hematoma.

Laboratory parameters

Blood tests on liver enzymes, bile acids, common electrolytes, coagulation and blood count were performed by the general laboratory of the University Hospital Heidelberg at the same day of TE measurement.

TE and transabdominal ultrasound

LS and controlled attenuation parameter (CAP) were measured by transient elastography (Fibroscan, Echosens, Paris, France) using both the M and XL probes according to the manufacturer's specifications in fasting conditions for no less than two hours. Briefly, TE was performed by physicians with at least 12 mo of experience in abdominal ultrasound and TE on the right lobe of the liver in intercostal position according to established protocols[26]. The LS values represent the median values of at least 10 consecutive measurements together with the corresponding IQR. LS data obtained by the XL probe was adjusted and standardized to M probe data according to previous findings[29]. In addition, liver size, spleen size, degree of hepatic steatosis (0-3), diameter of the vena cava inferior (VCI) and skin-to-liver capsule distance (SCD) were assessed by abdominal ultrasound.

Statistical analysis

The statistical analysis was performed using SPSS Statistics version 23.0 (IBM, United States) and Excel 2016 (Microsoft, United States). For group comparisons mean and standard deviations where calculated and to compare means, the *t*-test and non-parametric Mann-Whitney-*U*-test were used where appropriate. For conduction of a correlation analysis, the Spearman rank-order correlation coefficient was calculated. A nonlinear curve fit on data with a Boltzmann function was performed using OriginPro 8 (OriginLab Cooperation). Receiver operating curve (ROC) analysis was performed and to identify cutoff values the maximum of the Youden index was calculated. Multivariate binary logistic regression analysis was used to identify independent predictors for pregnancy complications. The statistical methods of this study were reviewed by Thomas Bruckner from Institute of Medical Biometry und Informatics, University of Heidelberg.

RESULTS

Patient characteristics

Out of 571 screened pregnant women, 34 were excluded either due to preexisting liver disease, insufficient information prior to pregnancy or other more rare exclusion criteria (Figure 1). Upon delivery and complete follow-up, the remaining 537 patients were stratified into a control group with uncomplicated pregnancies (*n* = 475, 88%), preeclampsia (*n* = 22, 4.5%) and ICP (*n* = 40, 7.4%). Table 1 shows the patient characteristics of the three different groups. Mean duration of pregnancy at date of LS measurement was 192 ± 67 d, 169 ± 59 d and 237 ± 18 d in controls, preeclampsia and ICP, respectively. Transaminase levels and LS values were significantly higher in both preeclampsia and ICP groups. Both groups also had a higher degree of steatosis as measured by ultrasound which could not be confirmed using the CAP parameter. Of note, women with preeclampsia had a significantly enlarged spleen and a higher mean arterial pressure (MAP) while body mass index (BMI) and levels of arterial pressure (AP) were higher in the ICP group. Taken together, important differences were observed with regard to LS between all three groups.

Performance of LS measurements

In all women, 10 successful measurements could be obtained. While 90.9% of women could be measured with the M-probe, all women were measurable with the XL-probe. Failure to measure LS with the M probe was related to a skin capsule difference > 2.4 cm or a BMI > 33.5 kg/m^2 (data not shown) while skin liver capsule distance (SCD) was more accurate for predicting the need for using the XL probe (Supplementary Table 1).

According to recently published criteria by Boursier *et al*[30], 16.7% of all measurements had an IQR to Median LS ratio (IQR/M) less or equal 10% (very reliable measurement), in 78.3%, IQR/M was between 10 and 30% (reliable measurement) and in 5.1% of cases, IQR/M was greater than 30% (poorly reliable). However and in line with previous findings[31], all data were used for further analysis. In summary, the Fibroscan device performs well in most pregnant women using the M probe and in all with the XL probe.

LS significantly increases in the third trimester of normal pregnancy

An elevated LS > 6 kPa was seen in 25% of healthy pregnant woman despite uncomplicated pregnancy. LS was higher than 8 and 12.5 kPa in 6% and 1.5%, respectively, the latter being typically regarded as cut-off value for cirrhosis[15,32]. LS increased almost exclusively in the third trimester (Figure 2). Here, 105 of 256 women (41%) showed an elevated LS > 6 kPa. Figure 2A shows LS averaged per week over the course of pregnancy and percentiles 5% and 95% calculated from the

Table 1 Patient characteristics

Parameter	Controls (n = 475)	Preeclampsia (n = 22)	[a]*P* value	ICP (n = 40)	[a]*P* value
Age (yr)	32.3 ± 5.0	33.4 ± 2.9	0.3069	27.8 ± 4.1	< 0.0001
Gestational age (d)	192 ± 67	169 ± 59	0.1330	237 ± 18	0.0007
Current BMI (kg/m²)	28.0 ± 6.1	29.0 ± 8.6	0.4601	32.0 ± 8.3	0.0001
BMI before pregnancy (kg/m²)	24.7 ± 5.8	25.9 ± 7.8	0.3569	28.4 ± 9.1	0.0002
MAP (kPa)	11.0 ± 1.6	12.1 ± 1.4	0.0279	10.4 ± 2.0	0.2747
Pruritus (present)	2%	0%	0.4948	53%	< 0.0001
Nulliparous	45%	50%	0.6345	37%	0.1883
AST (U/L)	24 ± 23	88 ± 122	< 0.0001	105 ± 122	< 0.0001
ALT (U/L)	15 ± 15	39 ± 35	< 0.0001	174 ± 174	< 0.0001
AP (U/L)	134 ± 126	115 ± 43	0.5955	179 ± 69	0.0382
GGT (U/L)	24 ± 48	35 ± 52	0.4439	32 ± 34	0.3863
Bilirubin total (mg/dL)	0.5 ± 0.3	0.5 ± 0.2	0.5374	0.5 ± 0.3	0.2561
Creatinine (mg/dL)	0.6 ± 0.1	0.6 ± 0.1	0.1308	0.8 ± 0.4	< 0.0001
Urea (mg/dL)	18 ± 5	23 ± 7	0.0006	22 ± 7	0.0001
Uric acid (mg/dL)	3.8 ± 1.1	5.0 ± 1.4	0.0019	5.8 ± 2.5	< 0.0001
Total protein (g/L)	66.1 ± 5.7	63.3 ± 4.3	0.1114	66.9 ± 5.2	0.5143
Albumin (g/L)	36.0 ± 2.2	25.5 ± 15.3	0.0046	36.4 ± 5.5	0.7729
Leukocytes (1/nL)	11.9 ± 4.2	9.6 ± 4.6	0.0599	10.1 ± 3.5	0.0158
Erythrocytes (1/pL)	3.9 ± 0.4	4.2 ± 0.4	0.0390	4.0 ± 0.5	0.4560
Hemoglobin (g/dL)	11.7 ± 1.3	12.4 ± 0.9	0.0632	11.2 ± 1.4	0.0313
Platelets (1/nL)	231 ± 63	173 ± 85	0.0022	231 ± 55	0.9790
INR	0.91 ± 0.03	1.01 ± 0.16	< 0.0001	0.91 ± 0.04	0.6616
Liver size (cm)	13.9 ± 1.8	13.2 ± 1.5	0.0786	14.4 ± 1.1	0.0812
Spleen size (cm)	10.9 ± 1.5	12.4 ± 2.8	< 0.0001	11.4 ± 1.8	0.0954
Diameter VCI (cm)	1.6 ± 0.3	1.8 ± 0.6	0.0075	1.5 ± 0.3	0.1713
Hepatic steatosis in sonography (0-3)	0.2 ± 0.4	0.2 ± 0.5	0.7334	0.4 ± 0.5	0.0174
Liver stiffness (kPa)	5.3 ± 2.0	17.9 ± 20.7	< 0.0001	6.9 ± 2.1	< 0.0001
> 6 kPa	120 (25%)	14 (63%)		23 (58%)	
> 8 kPa	30 (6%)	10 (45%)		8 (20%)	
> 12.5 kPa	7 (1.5%)	9 (40%)		1 (2.5%)	
CAP (dB/m)	226 ± 43	228 ± 47	0.7843	215 ± 40	0.1322

[a]*P*: *P*-value in comparison *vs* controls. ICP: Intrahepatic cholestasis of pregnancy; AST: Aspartate transaminase; ALT: Alanine transaminase; BMI: Body mass index; CAP: Controlled attenuation parameter; GGT: Gamma-glutamyltransferase; MAP: Mean arterial pressure; AP: Arterial pressure; INR: International normalized ratio; VCI: Vena cava inferior.

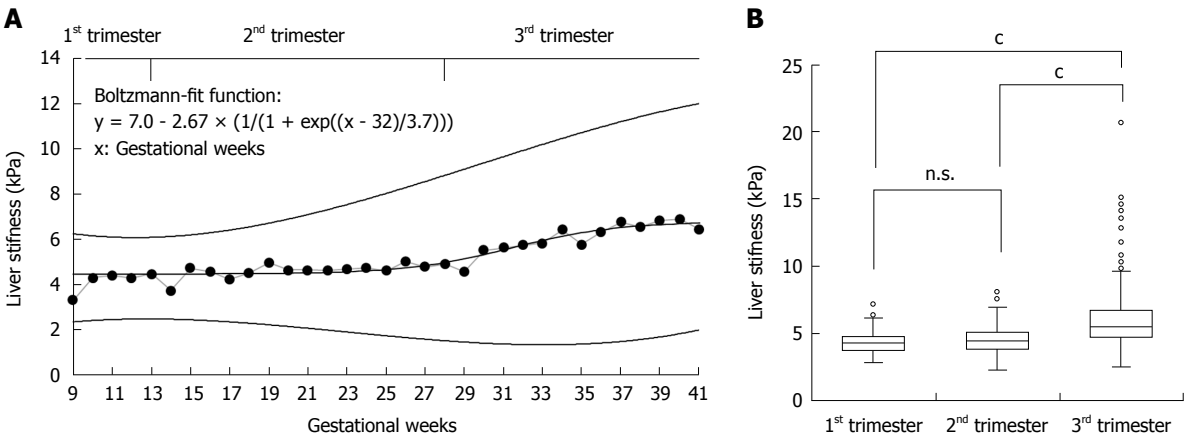


Figure 2 Liver stiffness increased almost exclusively in the third trimester of normal pregnancy. A: Mean LS as a function of gestational week (9 to 42) of healthy pregnant women without pregnancy complications. Displayed is the mean LS for every gestational week with 95% confidence bands from standard deviation and the nonlinear regression function from a Boltzmann fit with y = 7.0 - 2.67 × (1/(1 + exp((gest.week - 32)/3.7))). From gestational week 26 on LS starts to increase and settles at a plateau of 6.7 kPa at week 39. B: LS in healthy pregnant women without pregnancy complications such as preeclampsia or intrahepatic cholestasis dependent on the trimester of pregnancy where the measurement took place. LS is significantly increased in women in the third trimester of pregnancy compared to women in the first or second trimester. No significant difference between LS in the first and second trimester was observed. LS: Liver stiffness. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

standard deviation per week. LS stayed at mean values of 4.5 kPa until week 26 and increased thereafter. The values reached a plateau by gestational week 39 at a mean value of 6.7 kPa. To represent those changes, we calculated the mean values at each week using a non-linear regression fitting (modified Boltzmann fit). The Boltzmann fit showed a good R^2 of 0.86.

Figure 2B demonstrates the distribution of LS values

Table 2 Associations with liver stiffness in all women, controls, preeclampsia and cholestasis patients

Spearman correlations	All	Control	Preeclampsia	ICP
General				
Gestational age (d)	0.462[c]	0.499[c]	-0.372	0.017
Age (yr)	-0.071	-0.035	0.198	-0.249
Current body weight (kg)	0.296[c]	0.287[c]	0.195	0.193
Body weight before pregnancy (kg)	0.182[c]	0.161[c]	0.315	0.175
Increase in body weight (kg)	0.314[c]	0.325[c]	-0.184	0.233
Current BMI (kg/m²)	0.316[c]	0.313[c]	0.313	0.169
BMI before pregnancy (kg/m²)	0.188[c]	0.169[c]	0.686[c]	0.175
Increase in BMI (kg/m²)	0.322[c]	0.328[c]	-0.118	0.269
MAP (kPa)	0.154[c]	0.03	0.082	0.464
pruritus	0.194[c]	-0.034		0.471[b]
Nulliparous state	0.131[b]	0.129[b]	0.287	0.383[b]
Laboratory				
Creatinine (mg/dL)	0.138	0.045	-0.478	0.048
Urea (mg/dL)	0.064	-0.068	-0.459	0.063
AST (U/L)	0.267[c]	0.04	0.588[a]	0.325[a]
ALT (U/L)	0.286[c]	0.103	0.354	0.28
AP (U/L)	0.297[c]	0.313[c]	-0.091	0.128
GGT (U/L)	0.228[b]	0.164	0.717[b]	-0.038
Bilirubin total (mg/dL)	0.027	-0.128	0.754[b]	0.257
Uric acid (mg/dL)	0.283[c]	0.152	-0.157	0.059
Total protein (g/L)	-0.201[a]	-0.350[c]	-0.018	-0.018
Albumin (g/L)	-0.199	-0.195	-0.5	-0.21
Leukocytes (1/nL)	-0.346[c]	-0.205[a]	-0.834[c]	-0.595[c]
Erythrocytes (1/pL)	0.169[a]	0.133	-0.473	0.181
Hemoglobin (g/dL)	0.024	0.073	-0.767[b]	0.064
Platelets (1/nL)	-0.132	0.025	-0.790[b]	-0.480[b]
Quick-test (%)	-0.029	0.086	-0.814[b]	0.017
INR	-0.013	-0.132	0.802[b]	-0.038
Bile acid (μmol/L)	0.698[c]	0.393		0.698[c]
Sonography				
Liver size (cm)	0.031	0.027	0.357	-0.247
Spleen size (cm)	0.167[b]	0.112[a]	0.587[b]	-0.071
Diameter VCI (cm)	-0.009	-0.035	0.387	0.144
Level of hepatic steatosis (0-3)	0.211[c]	0.186[c]	0.343	0.309
CAP (dB/m)	0.075	0.081	0.775[c]	0.02
Cumulative estimated fetus weight (g)	0.244[a]	0.223		

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$. ICP: Intrahepatic cholestasis of pregnancy; AST: Aspartate transaminase; ALT: Alanine transaminase; BMI: Body mass index; CAP: Controlled attenuation parameter; GGT: Gamma-glutamyltransferase; MAP: Mean arterial pressure; AP: Arterial pressure; INR: International normalized ratio; VCI: Vena cava inferior.

for all three trimesters as box plot. LS was significantly elevated in the third trimester with a mean of 6.0 ± 2.3 kPa ($P < 0.0001$) in comparison to women in the first or second trimester with mean LS values of 4.3 ± 1.0 kPa and 4.5 ± 1.2 kPa, respectively. Taken together, our study demonstrates a significant increase of LS during uncomplicated pregnancy almost exclusively in the third trimester.

Parameters associated with LS elevation in healthy pregnant women without complications

Focusing on the control group, we analyzed morphological, laboratory and sonographic parameters to identify potential confounders of elevated LS. Correlation analysis (Table 2) showed that LS is not only positively correlated with gestational age (0.50, $P < 0.0001$), but also with related parameters such as increase in body weight (0.28, $P < 0.0001$), change of BMI (0.33, $P < 0.0001$) and current BMI (0.31, $P < 0.0001$). In addition, LS was also positively correlated with AP (0.31, $P < 0.001$) and negatively with total protein ($r = -0.35$, $P < 0.001$). A weak association was seen with spleen size and hepatic steatosis in ultrasound which could not be confirmed based on the non- invasive CAP parameter. In multivariate logistic regression analysis (Supplementary Table 2), using the binary state LS > 6 kPa as dependent variable, cumulative estimated fetus weight and gestational age were independent predictors for LS > 6 kPa [odds ratios exp(B) = 1.003, $P = 0.012$ and exp(B) = 1.016, $P = 0.027$, respectively], while nulliparous state, current body weight and increase in body weight were not significant.

LS is an independent predictor of preeclampsia

Women with preeclampsia and ICP had significantly higher LS as compared to controls (5.3 ± 1.9 kPa) with mean LS values of 17.9 ± 20.7 kPa ($P < 0.0001$) and 6.9 ± 2.1 kPa ($P < 0.0001$), respectively. This was also the case when only focusing on women in the third trimester (Figure 3). In multivariate logistic regression

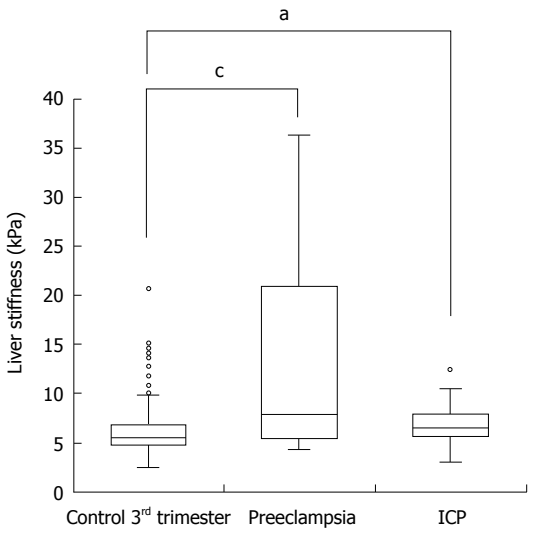


Figure 3 Liver stiffness in women without pregnancy complications and a measurement in the third trimester compared to liver stiffness in women with preeclampsia and intrahepatic cholestasis of pregnancy. LS is significantly higher in preeclampsia (17.9 ± 20.7 kPa, $P < 0.0001$) and cholestasis (6.9 ± 2.1 kPa, $P < 0.0001$) even when compared to controls in the third trimester (6.0 ± 2.3 kPa), where LS increases also in uncomplicated pregnancies. Two women with preeclampsia had LS measurements of 75 kPa, which are not shown for design reasons. LS: Liver stiffness; ICP: Intrahepatic cholestasis of pregnancy.

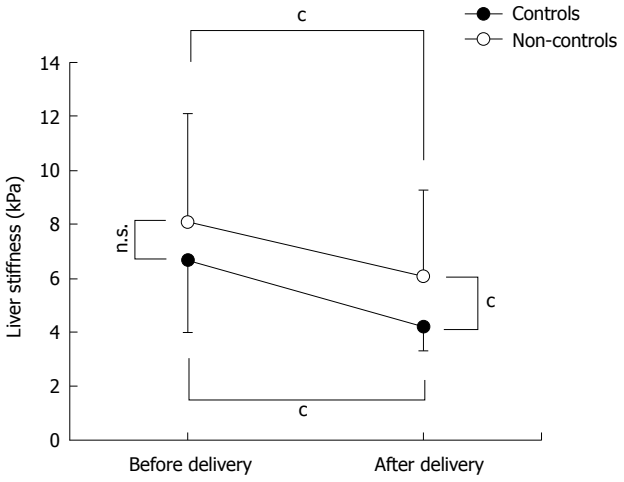


Figure 4 Mean liver stiffness and standard deviation in the third trimester of pregnancy. Twenty-four hours after delivery in healthy pregnant women without complications (controls, $n = 26$) and in non-control women with intrahepatic cholestasis of pregnancy ($n = 9$) and preeclampsia ($n = 6$). LS decreases significantly in both groups after delivery. All controls decreased after delivery to values < 6 kPa while two women with ICP and one woman with preeclampsia stayed above the 6 kPa level. $^{c}P < 0.001$ and $^{a}P < 0.05$. LS: Liver stiffness.

analysis, when comparing with important laboratory screening markers during pregnancy (Supplementary Figure 1A), LS and leukocyte count were independent predictors for preeclampsia with an odds ratio of 2.05 (1.27-3.31) and 1.39 (1.03-1.88), respectively. In contrast, the presence of pruritus and elevated ALT levels were highly predictive for ICP while no additional independent information could be obtained from LS. LS predicted preeclampsia with a fairly good area under the curve (AUROC) of 0.815 (0.722-0.907) and an optimal cut-off value of 7.6 kPa (Supplementary Figure 2). Using these values, preeclampsia was detected with a sensitivity of 0.55 (0.35-0.73) and specificity of 0.92 (0.89-0.94). In summary, an elevated LS seems to be an independent predictor of preeclampsia that is superior to established criteria such as arterial hypertension.

LS rapidly decreases after delivery

In a subgroup of 41 women, we were able to measure LS in the third trimester of pregnancy and 24 h after delivery (Figure 4). 26 women had uncomplicated pregnancy while ICP and preeclampsia were seen in 9 and 6 cases, respectively (Figure 1). Due to the small sample size, cases with preeclampsia and ICP are combined and depicted as non-controls in Figure 4. LS decreased significantly in all women after delivery from 7.2 ± 3.3 kPa to 4.9 ± 2.2 kPa ($P < 0.001$, Figure 3). Significant differences were observed between controls and non-controls after delivery but not before. Mean LS before delivery was 6.7 ± 2.7 kPa and 8.1 ± 4.0 kPa in controls and non-controls, respectively ($P = 0.18$). After delivery, mean LS in controls was significantly lower as compared to non-controls (4.2 ± 1.0 kPa *vs* 5.6 ± 1.3 kPa; $P < 0.001$). Of note, LS normalized in all controls to values below 6 kPa while LS remained > 6 kPa in three non-controls with 6.1, 8.1 and 16.8 kPa, respectively. In summary, LS rapidly decreases after delivery. It even normalized in all women without pregnancy complications.

DISCUSSION

We here show that LS can be easily measured in all women during pregnancy. We demonstrate, first, that LS significantly increases above 6 kPa in a large fraction of pregnant women without complications almost exclusively in the third trimester. Second, women with preeclampsia show even higher LS values and an elevated LS is an independent predictor of this complication. Finally, elevated pregnancy- associated LS rapidly decreased in all women after delivery.

Our data indicate that LS elevation during pregnancy should be interpreted with caution and not be mistaken for a manifest liver disease since it will most likely be reversible after delivery. Second, the significant elevation of a fundamental liver parameter such as LS in such a large fraction of pregnant women suggests that LS itself or its underlying causes could contribute to some of the rare but often fatal complications during late pregnancy[27]. Indeed, elevated LS was significantly higher in women with complications such as preeclampsia or ICP. Moreover, an elevated LS > 7.6 kPa was identified as independent predictor of preeclampsia with a fairly good AUROC of 0.815 being superior to other laboratory markers and arterial pressure. Thus, our data suggest that LS could be a valuable non-invasive tool to screen pregnant women for potential complications.

What are the mechanisms that underlie elevated LS during pregnancy? The rapid normalization of LS after delivery is suggestive of *e.g.* mechanic, pressure-related conditions of pregnancy in contrast to other *e.g.* inflammatory or apoptotic conditions. Thus, liver inflammation or apoptosis typically do not resolve within 24 h[33]. Indeed, in healthy women, we could not see any association of LS with laboratory parameters such as transaminases as observed in liver disease[31,33]. The rapid decrease of LS rather suggests potential mechanic factors such as an elevated intraabdominal pressure and its hemodynamic consequences that would rapidly normalize after deliver. Such an explanation would also be in line with the recently proposed inflow/outflow balance model of LS based on liver perfusion through the incoming portal vein and hepatic artery and the outflowing hepatic veins[21-24,27]. The significant association of LS with spleen size could be a further indirect link between portal hypertension and perfusion-associated LS elevation. Unfortunately, our study does not provide detailed insights into the hemodynamics since no information on blood flow and resistance was obtained.

Second, it is known for a long time that pregnancy causes water retention that can contribute to a large extent to weight gain in pregnant women. The underlying mechanisms involve the RAAS system and are comparable to heart failure and liver cirrhosis[34]. In addition, LS can be drastically elevated by water retention *e.g.* during heart failure[22,27]. Indirect evidence can be derived from the negative correlation between LS and serum protein levels. Unfortunately, however, we could not clearly discriminate between weight gain from water retention or obesity in our study.

Moreover, parameters of steatosis such as ultrasound (US) and CAP did not correlate significantly with LS. It also remains unclear while indirect markers of water retention such as an enlarged caval vein did not show a significant association with LS although it was related with complications. One explanation could be the drastically elevated intraabdominal pressure in pregnant women preventing an unfolding of the lower caval vein[35].

Third, an increased cardiac output of pregnant women may be also responsible for the elevated LS since exercise and arterial pressure have been recently identified as important determinants[23,35]. Notably, in our large study, no significant association could be observed between MAP and LS although a recent small study identified a relation between postpartum LS and arterial pressure in women with eclampsia[36]. Here, larger more detailed studies are required to shed light on the role of arterial pressure on LS during pregnancy.

Fourth, the strong association of elevated LS with parameters such as weight and BMI point towards an important role of elevated intraabdominal pressure. This is further underlined by the strong association with child-related parameters such as fetus weight. However, we have recently shown that an elevated intraabdominal pressure *per se* does not increase LS in the absence of liver congestion[37]. Thus, it is known that the uterus can compress *e.g.* the inferior caval vein during the continued growth of the fetus[38]. Again, our study was not able to provide further detailed insights regarding liver congestion or impaired hepatic vein outflow. Another fascinating argument for a mechanic explanation of LS elevation during pregnancy is the weak positive correlation of LS with the nulliparous state. It is well established in the literature that previous uncomplicated deliveries lower the risk for birth complications. The fact that pregnant women with their first pregnancy have a significantly higher LS could point towards a mechanic training or mechanic adaption that could be potentially obtained during the first pregnancies.

In conclusion, we here show that LS can be obtained easily in pregnant women. LS significantly increases in the third trimester despite normal pregnancy and rapidly normalizes after delivery. However, LS is significantly increased in women with preeclampsia and an independent predictor of this complication. Some findings and especially the rapid normalization of LS after delivery is highly suggestive for mechanic e.g. hemodynamic reasons as underlying mechanism while direct liver injury is less likely. More detailed prospective studies are needed to clarify LS elevation during pregnancy. Nevertheless, our data are highly encouraging to more frequently use non-invasive LS measurements in pregnant women with suspicion of pregnancy-related complications.

ARTICLE HIGHLIGHTS

Research background

Hypertension and preeclampsia remain the leading causes of maternal morbidity and mortality. Moreover, up to 3 % of all pregnancies are accompanied by liver complications which can have a high maternal and perinatal mortality. So far, no detailed studies existed on pregnancy and liver stiffness, a novel parameter and gold standard to early screen for liver fibrosis and other pathologies. We here present the first large cohort to study liver stiffness during pregnancy and its association with complications during pregnancy. Our data show that liver stiffness significantly and reversibly increases in the final trimester in pregnant women without complications. In women with preeclampsia, liver stiffness is significantly elevated and an independent non-invasive predictor. In conclusion, we think that liver stiffness could be an easy to use and fast non-invasive tool for the prediction and diagnosis of pregnancy complications.

Research motivation

Many liver-related complications during pregnancy are still poorly understood, life threatening and difficult to diagnose. The main motivation for this study, therefore, was to investigate the novel parameter liver stiffness for the first time in a large cohort of pregnant women and its relation to pregnancy complications. Based on first preliminary observations, it was our further goal to provide the normal range of liver stiffness values during pregnancy and its response to delivery.

Research objectives

The first aim was to test the performance and validity of liver stiffness measurements using FibroScan in pregnant women with and without pregnancy

complications. The second aim was to investigate liver stiffness in women with normal pregnancies at different gestational ages and its response to delivery. We eventually demonstrate that liver stiffness can be measured in pregnant women with sufficient accuracy using the XL probe. We further demonstrate an elevated liver stiffness in the final trimester of pregnant women without complications. Interestingly, liver stiffness can reach levels that could suggest the presence of advanced liver fibrosis. Finally, we show that liver stiffness is higher in patients with pregnancy complications such as preeclampsia or intrahepatic cholestasis of pregnancy and can even predict these complications. Our data pave the way for more detailed studies of liver stiffness in pregnant women in the future *e.g.* with the aim to investigate associations with more rare complications such as the hemolysis, elevated liver enzymes and low platelets (HELLP)-syndrome or acute fatty liver of pregnancy.

Research methods

We prospectively recruited 537 pregnant women including 22 with preeclampsia and 40 with intrahepatic cholestasis. Liver stiffness was measured by transient elastography (FibroScan, Paris) with the M and XL-probe. Additionally, transabdominal ultrasound and standard laboratory tests were performed. In some patients, liver stiffness was also measured 24 h after delivery. This study design allowed us to demonstrate elevated liver stiffness in the third trimester during uncomplicated pregnancy and its normalization after delivery. In addition, however, elevated liver stiffness was identified as sensitive and early prognostic parameter of pregnancy complications such as preeclampsia and intrahepatic cholestasis of pregnancy.

Research results

We first demonstrate that valid liver stiffness measurements can be obtained in most women (> 95%) using the XL-probe. Second, liver stiffness increases from ca. 4.5 kPa to 6 kPa ($P < 0.001$) in the third trimester of women with normal pregnancy. Importantly, 41% of women in the third trimester showed a liver stiffness above 6 kPa; in rare cases even higher than 20 kPa. Of note, elevated liver stiffness here was significantly correlated with alkaline phosphatase, leukocytes, gestational age and an increase in body weight and body mass index (BMI). Women with pregnancy complications such as preeclampsia or intrahepatic cholestasis had significantly higher liver stiffness measurements than women with uncomplicated pregnancy ($P < 0.0001$). Results even stayed significant when compared only to women in the third trimester ($P < 0.01$). Moreover, in multivariate analysis, liver stiffness could be identified as an independent predictor for preeclampsia with an odds ratio of 2.05 (1.27-3.31). Finally, liver stiffness rapidly decreased in all women within 24 h after delivery from 7.2 ± 3.3 kPa down to 4.9 ± 2.2 kPa ($P < 0.001$).

Research conclusions

For the first time, this study investigates liver stiffness in a large prospective cohort of pregnant women prior and after delivery. It shows that liver stiffness can be accurately measured in pregnant women. Surprisingly, liver stiffness significantly increased in the final trimester but normalized after delivery. Thus, an elevated liver stiffness during pregnancy should not be mistaken for a liver disease and *e.g.* prompt to further invasive diagnostic measures. Furthermore, liver stiffness is significantly higher in women with pregnancy complications and it can independently predict complications such as preeclampsia. Although an exact molecular cause of the liver stiffness elevation could not be identified, its rapid normalization after delivery in the absence of inflammation highly suggests mechanical factors *e.g.* hemodynamic changes as underlying cause. The findings are also in line with our recently introduced sinusoidal pressure hypothesis that highlights the role of pressure in modulating liver stiffness and eventually causing liver complications. Based on our findings we also suggest to routinely assess liver stiffness during pregnancy in order to early identify women at risk of preeclampsia.

Research perspectives

Transient elastography is a promising non-invasive, easy to use method to study liver stiffness during pregnancy. In future research, broader and more detailed studies are needed to investigate the cause for liver stiffness elevation in the third trimester and in complicated pregnancies and to investigate other aspects such as HELLP-syndrome or acute fatty liver of pregnancy.

REFERENCES

1 **Hill K**, AbouZhar C, Wardlaw T. Estimates of maternal mortality for 1995. *Bull World Health Organ* 2001; **79**: 182-193 [PMID: 11285661]
2 **Steegers EA**, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. *Lancet* 2010; **376**: 631-644 [PMID: 20598363 DOI: 10.1016/S0140-6736(10)60279-6]
3 **Clark SL**, Belfort MA, Dildy GA, Herbst MA, Meyers JA, Hankins GD. Maternal death in the 21st century: causes, prevention, and relationship to cesarean delivery. *Am J Obstet Gynecol* 2008; **199**: 36.e1-5; discussion 91-92.e7-11 [PMID: 18455140 DOI: 10.1016/j.ajog.2008.03.007]
4 **Hay JE**. Liver disease in pregnancy. *Hepatology* 2008; **47**: 1067-1076 [PMID: 18265410 DOI: 10.1002/hep.22130]
5 **Westbrook RH**, Dusheiko G, Williamson C. Pregnancy and liver disease. *J Hepatol* 2016; **64**: 933-945 [PMID: 26658682 DOI: 10.1016/j.jhep.2015.11.030]
6 **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]
7 **Mihu D**, Costin N, Mihu CM, Seicean A, Ciortea R. HELLP syndrome - a multisystemic disorder. *J Gastrointestin Liver Dis* 2007; **16**: 419-424 [PMID: 18193124]
8 **Kelly C**, Pericleous M. Pregnancy-associated liver disease: a curriculum-based review. *Frontline Gastroenterol* 2018; **9**: 170-174 [PMID: 30046419 DOI: 10.1136/flgastro-2017-100924]
9 **Kamimura K,** Abe H, Kawai H, Kamimura H, Kobayashi Y, Nomoto M, Aoyagi Y, Terai S. Advances in understanding and treating liver diseases during pregnancy: A review. *World J Gastroenterol* 2015; **21**: 5183-5190 [PMID: 25954092 DOI: 10.3748/wjg.v21.i17.5183]
10 **Kondrackiene J**, Kupcinskas L. Liver diseases unique to pregnancy. *Medicina* (Kaunas) 2008; **44**: 337-345 [PMID: 18541949]
11 **Suresh I**, Tr V, Hp N. Predictors of Fetal and Maternal Outcome in the Crucible of Hepatic Dysfunction During Pregnancy. *Gastroenterology Res* 2017; **10**: 21-27 [PMID: 28270873 DOI: 10.14740/gr787w]
12 **Poon LC**, Kametas NA, Chelemen T, Leal A, Nicolaides KH. Maternal risk factors for hypertensive disorders in pregnancy: a multivariate approach. *J Hum Hypertens* 2010; **24**: 104-110 [PMID: 19516271 DOI: 10.1038/jhh.2009.45]
13 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713 [PMID: 14698338]
14 **Foucher J**, Chanteloup E, Vergniol J, Castéra L, Le Bail B, Adhoute X, Bertet J, Couzigou P, de Lédinghen V. Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403-408 [PMID: 16020491 DOI: 10.1136/gut.2005.069153]
15 **Mueller S,** Sandrin L. Liver stiffness: a novel parameter for the diagnosis of liver disease *Hepat Med* 2010; **2**: 49-67 [PMID: 24367208]
16 **Nahon P**, Thabut G, Ziol M, Htar MT, Cesaro F, Barget N, Grando-Lemaire V, Ganne-Carrie N, Trinchet JC, Beaugrand M. Liver stiffness measurement versus clinicians' prediction or both for the assessment of liver fibrosis in patients with chronic hepatitis C. *Am J Gastroenterol* 2006; **101**: 2744-2751 [PMID: 17227522 DOI: 10.1111/j.1572-0241.2006.00816.x]
17 **Colletta C**, Smirne C, Fabris C, Toniutto P, Rapetti R, Minisini R, Pirisi M. Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferases. *Hepatology* 2005; **42**: 838-845 [PMID: 16121354]
18 **Friedrich-Rust M**, Ong MF, Martens S, Sarrazin C, Bojunga J, Zeuzem S, Herrmann E. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. *Gastroenterology* 2008;

134: 960-974 [PMID: 18395077 DOI: 10.1053/j.gastro.2008.01.034]
19 **Arena U**, Vizzutti F, Corti G, Ambu S, Stasi C, Bresci S, Moscarella S, Boddi V, Petrarca A, Laffi G, Marra F, Pinzani M. Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 2008; **47**: 380-384 [PMID: 18095306]
20 **Dechêne A**, Sowa JP, Gieseler RK, Jochum C, Bechmann LP, El Fouly A, Schlattjan M, Saner F, Baba HA, Paul A, Dries V, Odenthal M, Gerken G, Friedman SL, Canbay A. Acute liver failure is associated with elevated liver stiffness and hepatic stellate cell activation. *Hepatology* 2010; **52**: 1008-1016 [PMID: 20684020 DOI: 10.1002/hep.23754]
21 **Millonig G**, Reimann FM, Friedrich S, Fonouni H, Mehrabi A, Büchler MW, Seitz HK, Mueller S. Extrahepatic cholestasis increases liver stiffness (FibroScan) irrespective of fibrosis. *Hepatology* 2008; **48**: 1718-1723 [PMID: 18836992 DOI: 10.1002/hep.22577]
22 **Millonig G**, Friedrich S, Adolf S, Fonouni H, Golriz M, Mehrabi A, Stiefel P, Pöschl G, Büchler MW, Seitz HK, Mueller S. Liver stiffness is directly influenced by central venous pressure. *J Hepatol* 2010; **52**: 206-210 [PMID: 20022130 DOI: 10.1016/j.jhep.2009.11.018]
23 **Piecha F**, Peccerella T, Bruckner T, Seitz HK, Rausch V, Mueller S. Arterial pressure suffices to increase liver stiffness. *Am J Physiol Gastrointest Liver Physiol* 2016; **311**: G945-G953 [PMID: 27288426 DOI: 10.1152/ajpgi.00399.2015]
24 **Piecha F**, Paech D, Sollors J, Seitz HK, Rössle M, Rausch V, Mueller S. Rapid change of liver stiffness after variceal ligation and TIPS implantation. *Am J Physiol Gastrointest Liver Physiol* 2018; **314**: G179-G187 [PMID: 29051188 DOI: 10.1152/ajpgi.00239.2017]
25 **Mederacke I**, Wursthorn K, Kirschner J, Rifai K, Manns MP, Wedemeyer H, Bahr MJ. Food intake increases liver stiffness in patients with chronic or resolved hepatitis C virus infection. *Liver Int* 2009; **29**: 1500-1506 [PMID: 19732330 DOI: 10.1111/j.1478-3231.2009.02100.x]
26 **Mueller S**, Seitz HK, Rausch V. Non-invasive diagnosis of alcoholic liver disease. *World J Gastroenterol* 2014; **20**: 14626-14641 [PMID: 25356026 DOI: 10.3748/wjg.v20.i40.14626]
27 **Mueller S**. Does pressure cause liver cirrhosis? The sinusoidal pressure hypothesis. *World J Gastroenterol* 2016; **22**: 10482-10501 [PMID: 28082801 DOI: 10.3748/wjg.v22.i48.10482]
28 **Kohlhaas A,** Millonig G, Schütz F, Seitz HK, Mueller S. Liver stiffness during pregnancy. *J Hepatol* 2011; **54**: 134-135 [DOI: 10.1016/S0168-8278(11)60338-3]
29 **Durango E,** Dietrich C, Seitz HK, Kunz CU, Pomier-Layrargues GT, Duarte-Rojo A, Beaton M, Elkhashab M, Myers RP, Mueller S. Direct comparison of the FibroScan XL and M probes for assessment of liver fibrosis in obese and nonobese patients. *Hepat Med* 2013; **5**: 43-52 [PMID: 24696623 DOI: 10.2147/HMER.S45234]
30 **Jérôme B,** Jean-Pierre Z, Victor dL, Marie-Christine R, Nathalie S, Brigitte L, Isabelle F-H, Yves G, Frédéric O, Sandrine B, Paul C. Determination of reliability criteria for liver stiffness evaluation by transient elastography. *Hepatology* 2013; **57**: 1182-1191 [PMID: 22899556 DOI: 10.1002/hep.25993]
31 **Mueller S**, Englert S, Seitz HK, Badea RI, Erhardt A, Bozaari B, Beaugrand M, Lupsor-Platon M. Inflammation-adapted liver stiffness values for improved fibrosis staging in patients with hepatitis C virus and alcoholic liver disease. *Liver Int* 2015; **35**: 2514-2521 [PMID: 26121926 DOI: 10.1111/liv.12904]
32 **Castéra L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Lédinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350 [PMID: 15685546]
33 **Mueller S**, Millonig G, Sarovska L, Friedrich S, Reimann FM, Pritsch M, Eisele S, Stickel F, Longerich T, Schirmacher P, Seitz HK. Increased liver stiffness in alcoholic liver disease: differentiating fibrosis from steatohepatitis. *World J Gastroenterol* 2010; **16**: 966-972 [PMID: 20180235 DOI: 10.3748/wjg.v16.i8.966]
34 **Schrier RW**, Cadnapaphornchai MA, Ohara M. Water retention and aquaporins in heart failure, liver disease and pregnancy. *J R Soc Med* 2001; **94**: 265-269 [PMID: 11387413]
35 **Moll W**. [Physiological cardiovascular adaptation in pregnancy--its significance for cardiac diseases]. *Z Kardiol* 2001; **90** Suppl 4: 2-9 [PMID: 11373940]
36 **Frank Wolf M**, Peleg D, Kariv Silberstein N, Assy N, Djibre A, Ben-Shachar I. Correlation between changes in liver stiffness and preeclampsia as shown by transient elastography. *Hypertens Pregnancy* 2016; **35**: 536-541 [PMID: 27391875 DOI: 10.1080/10641955.2016.1197934]
37 **Kohlhaas A,** Durango E, Millonig G, Bastard C, Sandrin L, Golriz M, Mehrabi A, Büchler MW, Seitz HK, Mueller S. Transient elastography with the XL probe rapidly identifies patients with non-hepatic ascites. *Hepat Med* 2012; **4**: 11-18 [PMID: 24367229 DOI: 10.2147/HMER.S30256]
38 **Scott DB**. Inferior vena caval occlusion in late pregnancy and its importance in anaesthesia. *Br J Anaesth* 1968; **40**: 120-128 [PMID: 5645758]

P- Reviewer: Kamimura K **S- Editor**: Ma RY **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4403-4411

DOI: 10.3748/wjg.v24.i38.4403

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

ORIGINAL ARTICLE

Observational Study

Hepatitis C virus related cirrhosis decreased as indication to liver transplantation since the introduction of direct-acting antivirals: A single-center study

Alberto Ferrarese, Giacomo Germani, Martina Gambato, Francesco Paolo Russo, Marco Senzolo, Alberto Zanetto, Sarah Shalaby, Umberto Cillo, Giacomo Zanus, Paolo Angeli, Patrizia Burra

Alberto Ferrarese, Giacomo Germani, Martina Gambato, Francesco Paolo Russo, Marco Senzolo, Alberto Zanetto, Sarah Shalaby, Patrizia Burra, Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, via Giustiniani 2, Padua 35128, Italy

Umberto Cillo, Giacomo Zanus, Hepatobiliary Surgery and Liver Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, via Giustiniani 2, Padua 35128, Italy

Paolo Angeli, Internal Medicine, Department of Medicine, Padua University Hospital, via Giustiniani 2, Padua 35128, Italy

ORCID number: Alberto Ferrarese (0000-0002-3248-2038); Giacomo Germani (0000-0002-4332-2072); Martina Gambato (0000-0002-0101-1938); Francesco Paolo Russo (0000-0003-4127-8941); Marco Senzolo (0000-0002-7261-6520); Alberto Zanetto (0000-0002-6734-7178); Sarah Shalaby (0000-0002-8700-6282); Umberto Cillo (0000-0002-2310-0245); Giacomo Zanus (0000-0001-5489-3009); Paolo Angeli (0000-0002-3912-8242); Patrizia Burra (0000-0002-8791-191X).

Author contributions: Ferrarese A, Germani G and Burra P participated in research design, in the performance of the research and in data analysis. Russo FP, Gambato M, Zanetto A, Shalaby S, Senzolo M, Cillo U, Zanus G and Angeli P participated in research design. All Authors approved the final manuscript.

Institutional review board statement: The study protocol was approved by all members of the Local Ethical Committee "Comitato Etico per la Sperimentazione Clinica della Provincia di Padova" (protocol n. 4466/AO/18; code AOP1405).

Informed consent statement: Informed consent protocol was approved by the Local Ethical Committee. Informed consent was collected and signed by all patients included in the study.

Conflict-of-interest statement: All the Authors declare no conflict of interest regarding this paper.

Data sharing statement: Individual de-identified participant data have been anonymously collected.

STROBE statement: The manuscript has been prepared according to the STROBE statement.

Manuscript source: Unsolicited manuscript

Correspondence to: Patrizia Burra, MD, PhD, Professor, Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, via Giustiniani 2, Padova 35128, Italy. burra@unipd.it
Telephone: +39-49-8212892
Fax: +39-49-8218727

Received: June 7, 2018
Peer-review started: June 7, 2018
First decision: July 11, 2018
Revised: July 26, 2018
Accepted: August 1, 2018
Article in press: August 1, 2018
Published online: October 14, 2018

Abstract

AIM

To evaluate waiting list (WL) registration and liver transplantation (LT) rates in patients with hepatitis C

virus (HCV)-related cirrhosis since the introduction of direct-acting antivirals (DAAs).

METHODS

All adult patients with cirrhosis listed for LT at Padua University Hospital between 2006-2017 were retrospectively collected using a prospectively-updated database; patients with HCV-related cirrhosis were divided by indication for LT [dec-HCV *vs* HCV/hepatocellular carcinoma (HCC)] and into two interval times (2006-2013 and 2014-2017) according to the introduction of DAAs. For each patient, indications to LT, severity of liver dysfunction and the outcome in the WL were assessed and compared between the two different time periods. For patients receiving DAA-based regimens, the achievement of viral eradication and the outcome were also evaluated.

RESULTS

One thousand one hundred and ninty-four [male (M)/female (F): 925/269] patients were included. Considering the whole cohort, HCV-related cirrhosis was the main etiology at the time of WL registration (490/1194 patients, 41%). HCV-related cirrhosis significantly decreased as indication to WL registration after DAA introduction (from 43.3% in 2006-2013 to 37.2% in 2014-2017, $P = 0.05$), especially amongst dec-HCV (from 24.2% in 2006-2013 to 15.9% in 2014-2017, $P = 0.007$). Even HCV remained the most common indication to LT over time (289/666, 43.4%), there was a trend towards a decrease after DAAs introduction (from 46.3% in 2006-2013 to 39% in 2014-2017, $P = 0.06$). HCV patients (M/F: 43/11, mean age: 57.7 ± 8 years) who achieved viral eradication in the WL had better transplant-free survival (log-rank test $P = 0.02$) and delisting rate ($P = 0.002$) than untreated HCV patients.

CONCLUSION

Introduction of DAAs significantly reduced WL registrations for HCV related cirrhosis, especially in the setting of decompensated cirrhosis.

Key words: Liver transplantation; Hepatitis C; Cirrhosis; Sustained virological response

Core tip: All-oral direct-acting antivirals significantly modify the natural history of hepatitis C virus (HCV) infection. According to our study, liver transplantation for HCV decompensated cirrhosis will decrease in the next future.

Ferrarese A, Germani G, Gambato M, Russo FP, Senzolo M, Zanetto A, Shalaby S, Cillo U, Zanus G, Angeli P, Burra P. Hepatitis C virus related cirrhosis decreased as indication to liver transplantation since the introduction of direct-acting antivirals: A single-center study. *World J Gastroenterol* 2018; 24(38): 4403-4411 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4403.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4403

INTRODUCTION

Hepatitis C virus (HCV)-related liver disease has been the most common indication for liver transplantation (LT) in Western Europe for the last 20 years[1]. In Italy, due to the high prevalence of HCV in the general population[2,3], HCV-related decompensated cirrhosis and hepatocellular carcinoma (HCC) have been the main indications for LT to date[4]. The scenario of HCV treatment has evolved rapidly since 2011, due to the approval of oral direct-acting antiviral agents (DAAs). The first oral interferon-free drug available worldwide, sofosbuvir, was registered in Italy in 2014[5]. Since then, several highly-effective and well-tolerated DAA regimens have been approved, with real-life data confirming the high sustained virological response (SVR) rates seen in the registration trials, even in the setting of end-stage liver disease[6-8]. Improvement in liver function, as measured by Child-Turcotte-Pugh (CTP) and MELD scores, are reported in nearly two in three decompensated cirrhotic patients treated with DAAs[9,10]. These safe and effective new antiviral therapies will probably lead to a reduction of waiting list (WL) registrations due to HCV[11,12]. However, the role of DAAs in the Italian scenario has not been explored yet.

Therefore, the aims of our study were to evaluate the changing rates of waiting list (WL) registrations and LT for patients with HCV-related cirrhosis, with or without HCC, after introduction of DAAs.

MATERIALS AND METHODS

All patients registered on the WL for LT at Padua University Hospital between January 2006 and December 2017 were assessed. After WL registration, patients' data were collected prospectively in an electronic database.

Patients with acute liver failure, with indications for LT other than cirrhosis, re-LT, or patients younger than 18 years old were excluded from the study. Patients were divided into two main groups according to indication to WL registration: patients with HCC and compensated liver disease (*e.g.* MELD < 15; HCC-group), and patients with decompensated disease, independently from presence of HCC (*e.g.* MELD ⩾ 15 or complications of portal hypertension; dec-group). Patients with a combined diagnosis of HCV and other cause of liver disease (*e.g.*, alcohol, autoimmune liver disease) were classified as HCV. Alcohol-related or HBV-related (or HBV/HDV) liver disease were diagnosed according to current guidelines[13], and they were assessed in separate groups. Similarly, all patients with autoimmune, cholestatic liver diseases or less common indications to LT were grouped together. Patients listed for cryptogenic cirrhosis who had a body mass index (BMI) > 30 were

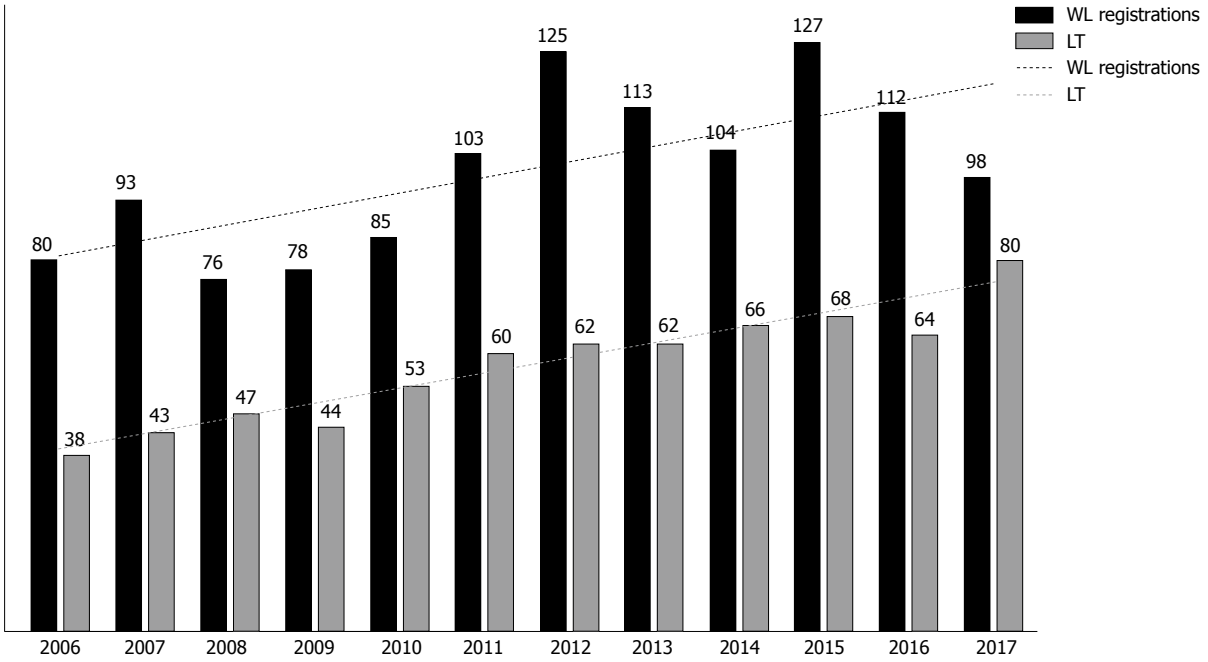


Figure 1 Overall trends in waiting list registrations and liver transplantations at Padua University Hospital in the study period. WL: Waiting list; LT: Liver transplantation.

classified as metabolic liver disease[14,15], and grouped together with patients having a known diagnosis of non-alcoholic steatohepatitis (NASH). Patients with diagnosis of HCC were received WL prioritization according to previously published policy[16], updated in 2016[16,17]. Patients with HCV-related liver disease were treated from 2014 with DAAs, according to the recommendations of the national regulatory agency[5]. Each patient was strictly followed-up during and after treatment with DAA; only patients with durable clinical improvements after SVR achievement were delisted. Patients were also divided into two different time intervals: pre-DAA period (2006-2013) and post-DAA period (2014-2017).

Outcome data were collected at the latest available follow-up for each patient. Informed consent was obtained, and the study was approved by the local Ethical Committee (Comitato Etico per la Sperimentazione Clinica della Provincia di Padova, n. AOP/1405).

Statistical analysis

Categorical and continuous variables were calculated as frequencies and means ± SD, respectively, and were compared using the Fisher's test or Student's *t*-test, as appropriate. *P* values < 0.05 were considered statistically significant. Survival analyses were performed using Kaplan-Meier curves (log-rank test). Analyses were performed using SPSS software version 18 (Chicago, IL, United States).

RESULTS

A total of 1469 patients were listed for LT at Padua University Hospital from January 2006 to December 2017. Two hundred seventy-five patients (18.7%) were excluded from the study for the following reasons: 100 (6.8%) patients were younger than 18 years old at WL registration, 87 (5.9%) patients had undergone previous LT, and 88 (6%) patients did not have cirrhosis as indication for LT, leaving 1194 (81.3%) patients for the current analysis.

Considering this cohort, overall WL registration rates increased from 94/year in 2006-2013 to 110/year in 2014-2017, with a concomitant rise in the number of LT performed overtime, from 51/year in 2006-2013 to 69.5/year in 2014-2017 (Figure 1).

Trajectories of WL registrations for HCV-related disease before and after DAA introduction

Overall, HCV related liver disease (490/1194, 41%), with or without HCC, was the main indication for WL registration over time, followed by alcohol and HBV (24% and 15.5%, respectively). At WL registration, there were no differences between HCV and non-HCV patients in terms of age, gender, BMI, however HCV patients had higher prevalence of HCC (61.8% *vs* 42%; *P* = 0.001), and lower MELD score (15 ± 6 *vs* 16.7 ± 6.8, *P* = 0.01) compared with non-HCV patients (Table 1).

When WL registration rates were compared in the whole cohort between pre-DAA and post-DAA periods, HCV significantly decreased as indication to LT (43.3% in 2006-2013 *vs* 37.2% in 2014-2017, *P* = 0.05). Notably, there was a significant drop in the WL registration rates for dec-HCV (from 24.2% in 2006-2013 to 15.9% in 2014-2017, *P* = 0.007), whereas HCV/HCC remained a stable indication (from 19% in 2006-2013 to 21% in 2014-2017, *P* = 0.4, Figure 2).

Dec-HCV patients had similar characteristics al WL registration between two different interval time

Table 1 Characteristics at waiting list registration between hepatitis C virus and non- hepatitis C virus patients

	HCV *n* = 490 (%)	Non-HCV *n* = 704 (%)	*P* value
Age (yr)	56 ± 7.8	55 ± 10	ns
Gender (male)	393 (80)	532 (75.5)	ns
BMI	25 ± 4	25 ± 4	ns
Blood group			ns
0	206 (42)	311 (44.2)	
A	199 (40.6)	282 (40)	
AB	19 (3.8)	30 (4.3)	
B	66 (13.4)	81 (11.5)	
HCC (yes)	303 (61.8)	295 (42)	0.001
Child-Pugh classes			0.04
A	137 (28)	163 (23)	
B	185 (38)	252 (36)	
C	168 (34)	289 (41)	
MELD at WL registration	15 ± 6	16.7 ± 6.8	0.01

HCV: Hepatitis C virus; BMI: Body mass index; HCC: Hepatocellular carcinoma; MELD: Model of End-Stage Liver Disease; WL: Waiting list.

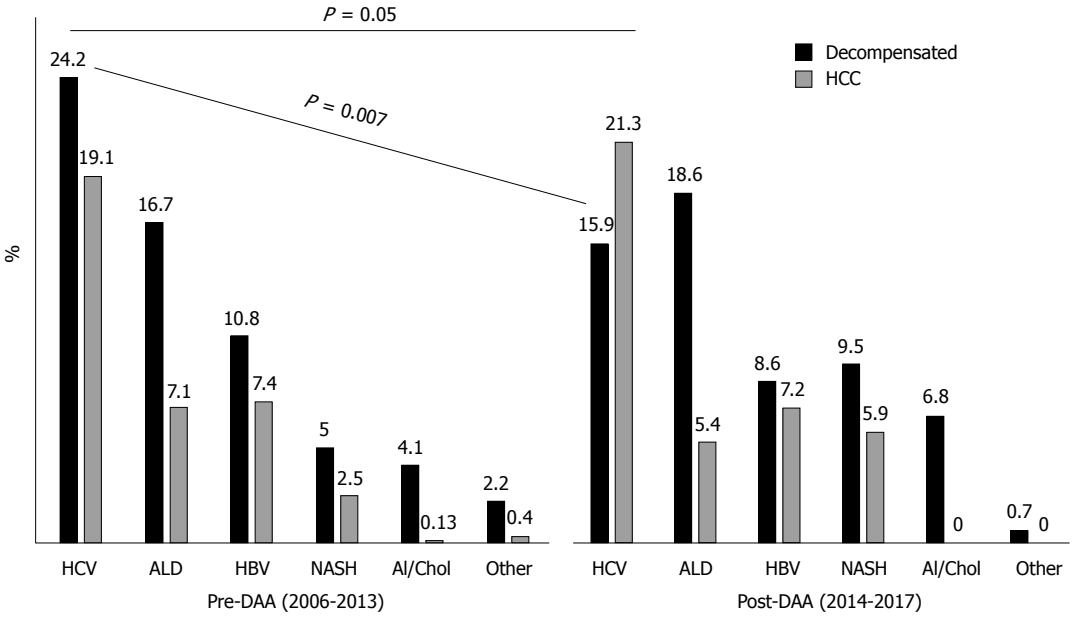


Figure 2 Trends in waiting list registration before and after direct-acting antiviral introduction. ALD: Alcoholic liver disease; DAA: Direct-acting antivirals; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis.

periods, whereas HCV/HCC patients in the last period were older (57 ± 7 years *vs* 59 ± 6 years, *P* = 0.03) and had lower CTP score (*P* = 0.01). The number of patients registered in the WL after achievement of SVR was higher in the post-DAA period, both amongst dec-HCV (7.3% in 2006-2013 *vs* 24.2% in 2014-2017, *P* = 0.004) and HCV/HCC (from 7% in 2006-2013 to 25.5% in 2014-2017, *P* = 0.001). Similarly, there was a significant increase in treatments while on the WL after DAA introduction, both amongst dec-HCV (from 4.9% in 2006-2013 to 20% in 2014-2017, *P* = 0.009) and HCV/HCC patients (from 4% in 2006-2013 to 26.5% in 2014-2017, *P* = 0.001) (Table 2).

Trajectories of LT for HCV-related disease before and after DAA introduction

Overall, HCV related disease remained the first indication to LT over time (43.4%), followed by alcohol and HBV (22.6% and 16%, respectively). At the time of LT, HCV patients were older than non-HCV patients (57 ± 7.5 *vs* 55 ± 10, *P* = 0.02), and had lower CTP score (*P* = 0.03) and MELD score (17 ± 8 *vs* 18 ± 8, *P* = 0.01) (Table 3).

Overall, there a was a trend towards a decrease in LTs for HCV related disease after DAA introduction (from 46.3% in 2006-2013 to 39% of overall LT in 2014-2017, *P* = 0.06). When HCV patients were stratified according to the main indication to transplantation, LT rates for decompensated disease (from 23.4% in 2006-2013 to 18.3% in 2014-2017, *P* = 0.1) and for HCC (from 22.9% in 2006-2013 to 20.8% in 2014-2017, *P* = 0.5) did not significantly change (Figure 3).

Characteristics of dec-HCV patients at time of LT did not differ across the two periods, except for a higher prevalence of HCC at the time of transplant in

Table 2 Characteristics of hepatitis C virus patients at waiting list registration before and after direct-acting antiviral introduction, according to indication to waiting list registration (decompensated liver disease or hepatocellular carcinoma)

	dec-HCV			HCV/HCC		
	Pre-DAA *n* = 181 (%)	Post-DAA *n* = 70 (%)	*P* value	Pre-DAA *n* = 144 (%)	Post-DAA *n* = 94 (%)	*P* value
Gender (male)	139 (77)	53 (75.7)	ns	118 (82)	82 (87)	ns
Age (yr)	55 ± 8	55 ± 9	ns	57 ± 7	59 ± 6	0.03
BMI	25 ± 3	25 ± 4	ns	24.7 ± 3	25 ± 3.6	ns
Refractory ascites (yes)	74 (40)	32 (45.7)	ns	-	-	
Blood group			ns			ns
0	86 (47.8)	32 (45.7)		62 (43)	35 (39)	
A	70 (38.4)	25 (35.7)		52 (36)	42 (44)	
AB	4 (2.2)	4 (5.7)		6 (4)	5 (5)	
B	21 (11.5)	9 (12.8)		24 (17)	12 (12)	
HCC (yes)	41 (22.5)	24 (34.2)	ns	-	-	
Comorbidities			ns			ns
None	134 (74.1)	44 (62.8)		104 (72.2)	76 (80)	
HBV	7 (3.8)	2 (2.8)		8 (5.5)	3 (3.2)	
Alcohol	34 (18.6)	19 (27.1)		26 (18)	10 (10.6)	
Metabolic	6 (3.2)	5 (7.3)		6 (4.3)	5 (5.3)	
Child-Pugh classes			ns			0.01
A	6 (3.3)	3 (4.2)		64 (44.5)	64 (68)	
B	67 (37)	25 (35.7)		67 (46.5)	26 (27)	
C	108 (59.6)	42 (60)		13 (9)	4 (4)	
MELD score	18.7 ± 6	19.3 ± 6	ns	11 ± 3.5	11 ± 3.4	ns
HCV Genotype[1]			ns			ns
1a	18 (15.1)	14 (23)		11 (10.2)	16 (18.8)	
1b	68 (57.1)	34 (55.7)		67 (62)	39 (45.8)	
2	12 (10)	3 (4.9)		10 (9.3)	4 (4.7)	
3	16 (13.4)	6 (9.8)		15 (13.9)	22 (25.8)	
4	5 (3.3)	4 (6.5)		5 (4.6)	4 (4.7)	
SVR achievement						
None	159 (87.8)	39 (55.7)		128 (89)	45 (47.8)	
Before WL registration	13 (7.1)	17 (24.2)	0.004	10 (7)	24 (25.5)	0.001
IFN based	13 (7.1)	2 (2.8)		10 (7)	6 (6.3)	
During WL registration	9 (4.9)	14 (20)	0.009	6 (4)	25 (26.5)	0.001

[1]HCV genotype was available in 373 patients at the time of WL registration. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; DAA: Direct-acting antivirals; BMI: Body mass index; HBV: Hepatitis B virus; MELD: Model of End-Stage Liver Disease; SVR: Sustained virological response; WL: Waiting list.

Table 3 Characteristics at the time of liver transplantation between hepatitis C virus and non-hepatitis C virus patients *n* (%)

	HCV *n* = 289	Non-HCV *n* = 377	*P* value
Age (yr)	57 ± 7.5	55 ± 10	0.02
Gender (male)	240 (83)	288 (76)	0.04
Blood group			ns
0	118 (40.8)	167 (44.2)	
A	124 (43)	147 (39)	
AB	12 (4.1)	19 (5)	
B	35 (12.1)	44 (11.8)	
Child-Pugh classes			
A	82 (25)	84 (22.2)	
B	99 (36.7)	116 (30.7)	0.03
C	108 (38.2)	177 (47)	
MELD score	17 ± 8	19 ± 8	0.01
Waiting time (mo)	9.6 ± 12	10 ± 14	ns

HCV: Hepatitis C virus; MELD: Model of End-Stage Liver Disease.

the post-DAA period (20.8% in 2006-2013 *vs* 37.3% in 2014-2017, *P* = 0.04). Similarly, no significant differences were found on characteristics of HCV/HCC patients at time of LT between the two periods. Prevalence of patients transplanted after achievement of SVR was significantly higher in the post-DAA period, both amongst dec-HCV (from 8.8% in 2006-2013 to 29.4% in 2014-2017, *P* = 0.01) and HCV/HCC (from 11% in 2006-2013 to 41% in 2014-2017, *P* = 0.0001) (Table 4).

Outcome of HCV patients treated with DAA in the setting of LT

Since 2014, 88 out of 164 (53.5%) patients with HCV

Table 4 Characteristics of hepatitis C virus patients at liver transplantation before and after direct-acting antiviral introduction, according to indication to waiting list registration (decompensated liver disease or hepatocellular carcinoma) *n* (%)

	dec-HCV			HCV/HCC		
	Pre-DAA *n* = 91	Post-DAA *n* = 51	*P* value	Pre-DAA *n* = 89	Post-DAA *n* = 58	*P* value
Gender (male)	74 (81)	40 (78.4)	ns	77 (86.5)	49 (84.5)	ns
Age (yr)	54.8 ± 8	56 ± 8	ns	57 ± 7	58 ± 7	ns
Refractory ascites (yes)	37 (40.6)	27 (53)	ns	-	-	
Blood group			ns			ns
0	41 (45)	21 (41.7)		37 (41.5)	19 (32.7)	
A	35 (38)	22 (43.3)		39 (43.8)	28 (54.9)	
AB	3 (3.3)	3 (5.8)		4 (4.5)	2 (3.4)	
B	12 (13)	5 (9.8)		9 (10.2)	9 (15.5)	
HCC (yes)	19 (20.8)	19 (37.3)	0.04	-	-	
Child-Pugh classes			ns			0.001
A	3 (3.2)	1 (1.9)		38 (42.6)	42 (72.4)	
B	27 (29.6)	17 (33.3)		42 (47.2)	12 (20.6)	
C	61 (67.1)	33 (64.7)		9 (10)	4 (6.8)	
MELD at LT	24 ± 6.5	23 ± 7.5	ns	11.7 ± 4	11.2 ± 3	ns
SVR at time of LT (yes)	8 (8.8)	15 (29.4)	0.002	10 (11)	24 (41)	0.0001
SVR after IFN-based regimens	7 (7.6)	2 (3.9)		9 (10.1)	1 (1.7)	
Waiting time (mo)	8.3 ± 12	10.6 ± 12	ns	9 ± 7.7	10 ± 11	ns

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; DAA: Direct-acting antivirals; BMI: Body mass index; MELD: Model of End-Stage Liver Disease; LT: Liver transplantation; SVR: Sustained virological response.

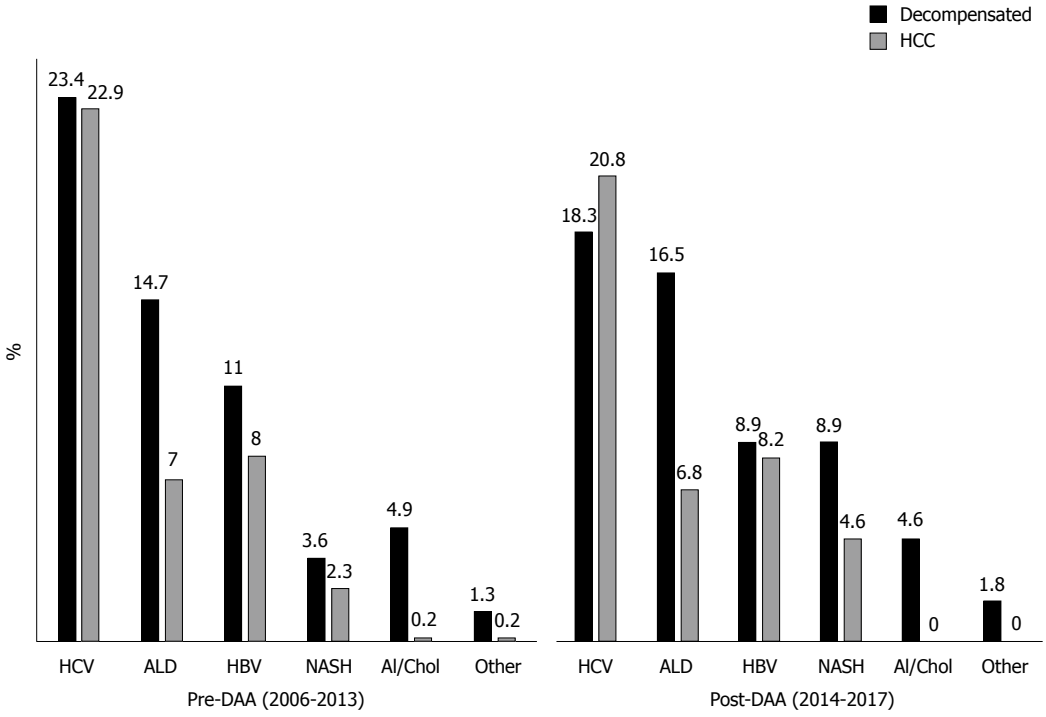


Figure 3 Trends in liver transplantation before and after direct-acting antiviral introduction. ALD: Alcoholic liver disease; DAA: Direct-acting antivirals; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis.

related cirrhosis were treated with DAA in the setting of LT (Table 5). Thirty-four (20%) were listed after achievement of SVR, whereas 54 (32.9%) were treated while on the WL. The SVR rate after first treatment with DAA was 88%. Ten patients relapsed after treatment with DAA and achieved SVR after a second treatment while they were on the WL. Sofosbuvir-based regimens were mostly used, whereas ribavirin was used in 43% of patients.

Considering only patients treated while in the WL, 28 (51%) patients underwent LT, whereas 10 (18%) were delisted due to improvement of liver disease, 8 (14.8%) died, and 2 (3.7%) dropped-out due to HCC progression. DAA-treated patients had a higher delisting rate due to improvement of liver disease than untreated HCV patients over time (18% *vs* 2.5%, $P = 0.002$), and

Table 5 Characteristics of hepatitis C virus patients who achieved sustained virological response after treatment with direct-acting antivirals while in the waiting list *n* (%)

	SVR after DAA before the WL *n* = 34	SVR after DAA in the WL *n* = 54
Age (yr)	58 ± 8	57.7 ± 8
Gender (male)	25 (73.5)	43 (79.6)
dec-HCV (yes)	15 (44)	44 (44.4)
MELD score before treament	-	13 ± 5
HCV genotype		
1a	6 (17.6)	6 (11)
1b	17 (50)	34 (63)
2	2 (5.8)	2 (3.7)
3	7 (20.5)	7 (13)
4	2 (5.8)	5 (9.3)
Treatment regimens		
Sofosbuvir	4 (11.7)	27 (50)
Sofosbuvir/Ledipasvir	23 (67.6)	18 (33.3)
Sofosbuvir/Daclatasvir	3 (8.8)	3 (5.5)
Sofosbuvir/Simeprevir	1 (2.9)	4 (7.4)
Other	5 (14.7)	2 (3.7)
Ribavirin use (yes)	1 (2.9)	37 (68.5)
MELD score after treament	12.3 ± 4	13 ± 6
Outcome		
Waiting for LT	16 (47)	6 (11)
Delisted	1 (2.9)	10 (18.5)
LT	10 (29.4)	28 (51.8)
Drop out	5 (14.7)	2 (3.7)
Dead	2 (5.8)	8 (14.8)

SVR: Sustained virological response; DAA: Direct-acting antivirals; HCV: Hepatitis C virus; MELD: Model of End-Stage Liver Disease; LT: Liver transplantation.

a better transplant free survival (log-rank $P = 0.02$).

DISCUSSION

In recent decades, HCV-related cirrhosis has been considered the most prevalent indication for LT in the Western Countries, for both decompensated liver disease and HCC. The introduction of DAAs has significantly modified the natural history of HCV infection, and viral eradication (especially in patients with compensated cirrhosis) has been associated with an improvement of liver function. We consequently explored dynamics of long-term changes in WL registrations and LTs in Our Centre.

Spanning more than a decade, we found that HCV remained the main indication for WL registration not only before, but also after DAA introduction. This might be because the first-generation protease inhibitors were approved in Italy since January 2013[18], but their use was initially limited by side effects and low rates of SVR achievement (less than 50% in cirrhotic patients)[19]. Extended approval of DAA therapies was granted in Italy in 2014, producing a significant drop in the WL registrations for HCV related cirrhosis, moving from 43.3% in the pre-DAA period to 37.2% in 2014-2017. These results are consistent with data recently published by another tertiary center in Italy[20], where the proportion of dec-HCV patients decreased from 49% to 36% in the last years. Two possible explanations may be hypothesized; first, a significant change in HCV epidemiology in Italy in recent years, as shown by several studies[3,21,22], with the highest prevalence of HCV in patients over 70 years old (who are not eligible for LT anymore); second, the achievement of SVR after DAA therapies[23-25], leading to improvement in liver function and reduction of portal-hypertensive complications[26]. Thus, previously decompensated patients, potentially suitable for LT, would therefore not be placed on the WL.

On the contrary, in the setting of HCV related cirrhosis, proportion of WL registration for HCC remained stable after DAA introduction (from 19% in 2006-2013 to 21% in 2014-2017; P = ns). Notably, in the post-DAA period, HCC became the first indication to LT amongst HCV patients. This trend was confirmed also in patients with decompensated disease (*e.g.* MELD $\geqslant$ 15), in whom the prevalence of HCC at the time of transplant rose from 20.8% in 2006-2013 to 37.3% in 2014-2017. Development of HCC in patients previously treated with DAA is debated. Several studies[6,27-29] recently showed that HCC may still occur or recur in patients with SVR achievement after DAA therapy (probably due to changes in the immunological microenvironment after viral clearance), and these patients would probably still need for LT.

In our experience, nearly 18% of HCV patients were delisted after achieving SVR, in a significantly higher proportion than in the pre-DAA period. This reinforces the concept that SVR achievement could significantly improve liver function and increase delisting rate in the next future, even if we'll expect that HCV patients will be cured before decompensation, without requiring WL registration. Furthermore, our data were consistent with those reported by Belli *et al*[30], who reported a delisting rate of 20% (though they only considered HCV-related decompensated cirrhosis patients without HCC).

Our study retrospectively collected all data regarding cirrhotic patients from WL registration to final outcome, using a prospectively updated electronic database. There were no significant changes in WL and LT policies during the study period, and the antiviral treatments were administered under the supervision of a tertiary center, in accordance with the changing criteria recommended by the Italian Regulatory Agency. This study has some limitations that need to be acknowledged, however. It was based on a single-center cohort and covered a lengthy period of time. These data, coming from a single-center cohort, might not be extended to the whole Italian scenario, due to its heterogenous epidemiology in terms of etiologies of liver disease. However, recently published data from Northern Italy seemed to be in accordance with our results[20]. Furthermore, criteria for NASH diagnosis significantly changed over, so we included patients with cryptogenic cirrhosis and a BMI > 30, as other Authors had done[15]. Lastly, we were assessing trends of the WL for LT, and some misdiagnosed comorbidities may have interfered

with the natural history of several patients.

In conclusion, our study showed the significant changes occurring in the LT scenario. Even if DAA therapies have significantly contributed to a decrease in the WL registration for dec-HCV, it will take more time for HCV to disappear as an indication for liver transplantation, especially in the setting of LT for HCC.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV)-related cirrhosis has been the first indication for liver transplantation in Western Countries in the last decades. Introduction of all-oral direct-acting antivirals (DAAs) significantly modified the natural history of HCV related liver disease.

Research motivation

Our study aimed at evaluating the change in waiting list registrations and in liver transplantation for HCV related cirrhosis after DAAs introduction.

Research objectives

To evaluate the outcome of patients with HCV related cirrhosis, listed for liver transplantation at Padua University Hospital between 2006 and 2017. Patients were further divided according to two different time periods (2006-2013 *vs* 2014-2017) and according to indication to liver transplantation (decompensated disease *vs* hepatocellular carcinoma).

Research methods

The outcome of patients listed for liver transplantation (LT) for HCV related cirrhosis was retrospectively analysed using a prospectively updated database.

Research results

After DAAs introduction, HCV-related cirrhosis significantly decreased as indication to waiting list registration, especially among patients with decompensated disease. Considering liver transplantation, even HCV remained the most common indication to LT over time (289/666, 43.4%), there was a trend towards a decrease in the last time period (2013-2017). Furthermore, HCV patients who achieved viral eradication had better transplant-free survival than untreated HCV patients.

Research conclusions

The study demonstrated that HCV related cirrhosis might be a decreasing indication to liver transplantation, especially for decompensated liver disease. Viral eradication achieved with DAA-based regimens should reduce decompensation rates and need to LT. This study confirmed what already known in literature about the beneficial role provided by DAAs in patients with HCV related cirrhosis. Viral eradication obtained after DAAs- based regimens should reduce decompensation rates amongst patients with HCV related cirrhosis. Future studies are needed to confirm the changing scenario regarding indications to LT in Western countries.

Research perspectives

Viral eradication obtained after DAA therapy should reduce decompensation rates amongst patients with HCV related cirrhosis. To further investigate trends in waiting list registrations and liver transplantations for HCV related cirrhosis, especially in the setting of hepatocellular carcinoma. Larger, multicentre, prospective studies are the best methods for the future research.

REFERENCES

1 **Adam R**, Karam V, Delvart V, O'Grady J, Mirza D, Klempnauer J, Castaing D, Neuhaus P, Jamieson N, Salizzoni M, Pollard S, Lerut J, Paul A, Garcia-Valdecasas JC, Rodríguez FS, Burroughs A; All contributing centers (www.eltr.org); European Liver and Intestine Transplant Association (ELITA). Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *J Hepatol* 2012; **57**: 675-688 [PMID: 22609307 DOI: 10.1016/j.jhep.2012.04.015]

2 **Scognamiglio P**, Piselli P, Fusco M, Pisanti FA, Serraino D, Ippolito G, Girardi E; Collaborating Study Group. Declining unawareness of HCV-infection parallel to declining prevalence in Southern Italy. *J Med Virol* 2017; **89**: 1691-1692 [PMID: 28464363 DOI: 10.1002/jmv.24840]

3 **Morisco F**, Loperto I, Stroffolini T, Lombardo FL, Cossiga V, Guarino M, De Feo A, Caporaso N. Prevalence and risk factors of HCV infection in a metropolitan area in southern Italy: Tail of a cohort infected in past decades. *J Med Virol* 2017; **89**: 291-297 [PMID: 27431017 DOI: 10.1002/jmv.24635]

4 **Angelico M**, Cillo U, Fagiuoli S, Gasbarrini A, Gavrila C, Marianelli T, Costa AN, Nardi A, Strazzabosco M, Burra P, Agnes S, Baccarani U, Calise F, Colledan M, Cuomo O, De Carlis L, Donataccio M, Ettorre GM, Gerunda GE, Gridelli B, Lupo L, Mazzaferro V, Pinna A, Risaliti A, Salizzoni M, Tisone G, Valente U, Rossi G, Rossi M, Zamboni F; Liver Match Investigators. Liver Match, a prospective observational cohort study on liver transplantation in Italy: study design and current practice of donor-recipient matching. *Dig Liver Dis*2011; **43**: 155-164 [PMID: 21185796 DOI: 10.1016/j.dld.2010.11.002]

5 Agenzia Italiana del Farmaco. Available from: URL: http://www.agenziafarmaco.gov.it

6 **Cheung MCM**, Walker AJ, Hudson BE, Verma S, McLauchlan J, Mutimer DJ, Brown A, Gelson WTH, MacDonald DC, Agarwal K, Foster GR, Irving WL; HCV Research UK. Outcomes after successful direct-acting antiviral therapy for patients with chronic hepatitis C and decompensated cirrhosis. *J Hepatol* 2016; **65**: 741-747 [PMID: 27388925 DOI: 10.1016/j.jhep.2016.06.019]

7 **Terrault NA**, McCaughan GW, Curry MP, Gane E, Fagiuoli S, Fung JYY, Agarwal K, Lilly L, Strasser SI, Brown KA, Gadano A, Kwo PY, Burra P, Samuel D, Charlton M, Pessoa MG, Berenguer M. International Liver Transplantation Society Consensus Statement on Hepatitis C Management in Liver Transplant Candidates. *Transplantation* 2017; **101**: 945-955 [PMID: 28437387 DOI: 10.1097/TP.0000000000001708]

8 **D'Ambrosio R**, Degasperi E, Colombo M, Aghemo A. Direct-acting antivirals: the endgame for hepatitis C? *Curr Opin Virol* 2017; **24**: 31-37 [PMID: 28419938 DOI: 10.1016/j.coviro.2017.03.017]

9 **Fernández Carrillo C**, Lens S, Llop E, Pascasio JM, Crespo J, Arenas J, Fernández I, Baliellas C, Carrión JA, de la Mata M, Buti M, Castells L, Albillos A, Romero M, Turnes J, Pons C, Moreno-Planas JM, Moreno-Palomares JJ, Fernández-Rodriguez C, García-Samaniego J, Prieto M, Fernández Bermejo M, Salmerón J, Badia E, Salcedo M, Herrero JI, Granados R, Blé M, Mariño Z, Calleja JL. Treatment of hepatitis C virus infection in patients with cirrhosis and predictive value of model for end-stage liver disease: Analysis of data from the Hepa-C registry. *Hepatology* 2017; **65**: 1810-1822 [PMID: 28170112 DOI: 10.1002/hep.29097]

10 **Chhatwal J**, Samur S, Bethea ED, Ayer T, Kanwal F, Hur C, Roberts MS, Terrault N, Chung RT. Transplanting hepatitis C virus-positive livers into hepatitis C virus-negative patients with preemptive antiviral treatment: A modeling study. *Hepatology* 2018; **67**: 2085-2095 [PMID: 29222916 DOI: 10.1002/hep.29723]

11 **Goldberg D**, Ditah IC, Saeian K, Lalehzari M, Aronsohn A, Gorospe EC, Charlton M. Changes in the Prevalence of Hepatitis C Virus Infection, Nonalcoholic Steatohepatitis, and Alcoholic Liver Disease Among Patients With Cirrhosis or Liver Failure on the Waitlist for Liver Transplantation. *Gastroenterology* 2017; **152**: 1090-1099.e1 [PMID: 28088461 DOI: 10.1053/j.gastro.2017.01.003]

12 **Flemming JA**, Kim WR, Brosgart CL, Terrault NA. Reduction in liver transplant wait-listing in the era of direct-acting antiviral therapy. *Hepatology* 2017; **65**: 804-812 [PMID: 28012259 DOI: 10.1002/hep.28923]

13 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Liver transplantation. *J Hepatol* 2016; **64**:

433-485 [PMID: 26597456 DOI: 10.1016/j.jhep.2015.10.006]
14 **Wong RJ**, Cheung R, Ahmed A. Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. *Hepatology* 2014; **59**: 2188-2195 [PMID: 24375711 DOI: 10.1002/hep.26986]
15 **Wong RJ**, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology* 2015; **148**: 547-555 [PMID: 25461851 DOI: 10.1053/j.gastro.2014.11.039]
16 **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]
17 **Cillo U**, Burra P, Mazzaferro V, Belli L, Pinna AD, Spada M, Nanni Costa A, Toniutto P; I-BELT (Italian Board of Experts in the Field of Liver Transplantation). A Multistep, Consensus-Based Approach to Organ Allocation in Liver Transplantation: Toward a "Blended Principle Model". *Am J Transplant* 2015; **15**: 2552-2561 [PMID: 26274338 DOI: 10.1111/ajt.13408]
18 **Ascione A**. Boceprevir in chronic hepatitis C infection: a perspective review. *Ther Adv Chronic Dis* 2012; **3**: 113-121 [PMID: 23251772 DOI: 10.1177/2040622312441496]
19 **Morgan TR**, Ghany MG, Kim HY, Snow KK, Shiffman ML, De Santo JL, Lee WM, Di Bisceglie AM, Bonkovsky HL, Dienstag JL, Morishima C, Lindsay KL, Lok AS; HALT-C Trial Group. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. *Hepatology* 2010; **52**: 833-844 [PMID: 20564351 DOI: 10.1002/hep.23744]
20 **Viganò R**, Mazzarelli C, Alberti AB, Perricone G. Change of liver transplantation list composition: Pre versus post direct-acting antivirals era. The Niguarda Hospital experience. *Dig Liver Dis* 2017; **49**: 317 [PMID: 28174002 DOI: 10.1016/j.dld.2017.01.145]
21 **Stroffolini T**, Sagnelli E, Gaeta GB, Sagnelli C, Andriulli A, Brancaccio G, Pirisi M, Colloredo G, Morisco F, Furlan C, Almasio PL; EPACRON study group. Characteristics of liver cirrhosis in Italy: Evidence for a decreasing role of HCV aetiology. *Eur J Intern Med*2017; **38**: 68-72 [PMID: 27836249 DOI: 10.1016/j.ejim.2016.10.012]
22 **Gardini I**, Bartoli M, Conforti M, Mennini FS, Marcellusi A, Lanati E. HCV - Estimation of the number of diagnosed patients eligible to the new anti-HCV therapies in Italy. *Eur Rev Med Pharmacol Sci* 2016; **20**: 7-10 [PMID: 28083865 DOI: 10.1016/j.jval.2016.09.948]
23 **Chhatwal J**, Samur S, Kues B, Ayer T, Roberts MS, Kanwal F, Hur C, Donnell DM, Chung RT. Optimal timing of hepatitis C treatment for patients on the liver transplant waiting list. *Hepatology* 2017; **65**: 777-788 [PMID: 27906468 DOI: 10.1002/hep.28926]
24 **Curry MP**, Charlton M. Sofosbuvir and Velpatasvir for Patients with HCV Infection. *N Engl J Med* 2016; **374**: 1688 [PMID: 27135094]
25 **Manns M**, Samuel D, Gane EJ, Mutimer D, McCaughan G, Buti M, Prieto M, Calleja JL, Peck-Radosavljevic M, Müllhaupt B, Agarwal K, Angus P, Yoshida EM, Colombo M, Rizzetto M, Dvory-Sobol H, Denning J, Arterburn S, Pang PS, Brainard D, McHutchison JG, Dufour JF, Van Vlierberghe H, van Hoek B, Forns X; SOLAR-2 investigators. Ledipasvir and sofosbuvir plus ribavirin in patients with genotype 1 or 4 hepatitis C virus infection and advanced liver disease: a multicentre, open-label, randomised, phase 2 trial. *Lancet Infect Dis* 2016; **16**: 685-697 [PMID: 26907736 DOI: 10.1016/S1473-3099(16)00052-9]
26 **Lens S**, Alvarado-Tapias E, Mariño Z, Londoño MC, LLop E, Martinez J, Fortea JI, Ibañez L, Ariza X, Baiges A, Gallego A, Bañares R, Puente A, Albillos A, Calleja JL, Torras X, Hernández-Gea V, Bosch J, Villanueva C, Forns X, García-Pagán JC. Effects of All-Oral Anti-Viral Therapy on HVPG and Systemic Hemodynamics in Patients With Hepatitis C Virus-Associated Cirrhosis. *Gastroenterology* 2017; **153**: 1273-1283.e1 [PMID: 28734831 DOI: 10.1053/j.gastro.2017.07.016]
27 **Conti F**, Buonfiglioli F, Scuteri A, Crespi C, Bolondi L, Caraceni P, Foschi FG, Lenzi M, Mazzella G, Verucchi G, Andreone P, Brillanti S. Early occurrence and recurrence of hepatocellular carcinoma in HCV-related cirrhosis treated with direct-acting antivirals. *J Hepatol* 2016; **65**: 727-733 [PMID: 27349488 DOI: 10.1016/j.jhep.2016.06.015]
28 **Foster GR**, Irving WL, Cheung MC, Walker AJ, Hudson BE, Verma S, McLauchlan J, Mutimer DJ, Brown A, Gelson WT, MacDonald DC, Agarwal K; HCV Research, UK. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. *J Hepatol* 2016; **64**: 1224-1231 [PMID: 26829205 DOI: 10.1016/j.jhep.2016.01.029]
29 **Reig M**, Boix L, Mariño Z, Torres F, Forns X, Bruix J. Liver Cancer Emergence Associated with Antiviral Treatment: An Immune Surveillance Failure? *Semin Liver Dis* 2017; **37**: 109-118 [PMID: 28388736 DOI: 10.1055/s-0037-1601349]
30 **Belli LS**, Berenguer M, Cortesi PA, Strazzabosco M, Rockenschaub SR, Martini S, Morelli C, Donato F, Volpes R, Pageaux GP, Coilly A, Fagiuoli S, Amaddeo G, Perricone G, Vinaixa C, Berlakovich G, Facchetti R, Polak W, Muiesan P, Duvoux C; European Liver and Intestine Association (ELITA). Delisting of liver transplant candidates with chronic hepatitis C after viral eradication: A European study. *J Hepatol* 2016; **65**: 524-531 [PMID: 27212241 DOI: 10.1016/j.jhep.2016.05.010]

P- Reviewer: Kamimura K **S- Editor**: Gong ZM **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 14; 24(38): 4412-4418

DOI: 10.3748/wjg.v24.i38.4412

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

CASE REPORT

FANCA D1359Y mutation in a patient with gastric polyposis and cancer susceptibility: A case report and review of literature

Jeffrey Peng Huang, Johnson Lin, Chi-Yuan Tzen, Wen-Yu Huang, Chia-Chi Tsai, Chih-Jen Chen, Yen-Jung Lu, Kuei-Fang Chou, Ying-Wen Su

Jeffrey Peng Huang, Johnson Lin, Kuei-Fang Chou, Ying-Wen Su, Division of Hematology and Medical Oncology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10491, Taiwan

Chi-Yuan Tzen, Department of Pathology, Mackay Memorial Hospital, Taipei 10491, Taiwan

Wen-Yu Huang, Laboratory of Good Clinical Research Center, Mackay Memorial Hospital, Tamsui Branch, New Taipei City 25160, Taiwan

Chia-Chi Tsai, Department of General Surgery, Mackay Memorial Hospital, Taipei 10491, Taiwan

Chih-Jen Chen, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10491, Taiwan

Yen-Jung Lu, ACT Genomics Co., Ltd., Taipei 11494, Taiwan

ORCID number: Jeffrey Peng Huang (0000-0001-9737-0785); Johnson Lin (0000-0002-5877-1508); Chi-Yuan Tzen (0000-0002-0568-0143); Wen-Yu Huang (0000-0001-7129-8420); Chia-Chi Tsai (0000-0002-6114-4230); Chih-Jen Chen (0000-0002-6702-5571); Yen-Jung Lu (0000-0003-3858-6767); Kuei-Fang Chou (0000-0003-0931-6055); Ying-Wen Su (0000-0003-3958-4202).

Author contributions: Su YW contributed to the conception and design of the work; Huang JP, Tzen CY, and Su YW collected the patient's clinical data and drafted the manuscript; Huang WY performed the research; Lu YJ and Su YW analyzed the data; Lin J, Tsai CC, Chen CJ and Chou KF helped to draft the manuscript; all authors read and approved the final manuscript.

Supported by the Mackay Memorial Hospital, No. MMH-106-62.

Informed consent statement: Informed consent was obtained from the patient.

Conflict-of-interest statement: All authors declare no conflict-of-interest related to this article.

Data sharing statement: No additional data are available.

CARE Checklist (2013) statement: The authors have read the CARE Checklist (2013), and the manuscript was prepared and revised according to the CARE Checklist (2013).

Manuscript source: Unsolicited manuscript

Correspondence to: Ying-Wen Su, MD, PhD, Doctor, Staff Physician, Division of Hematology and Medical Oncology, Department of Internal Medicine, Mackay Memorial Hospital, No. 92, Section 2, Zhongshan North Road, Taipei 10491, Taiwan. yingwensu.5896@mmh.org.tw
Telephone: +886-2-25433535
Fax: +886-2-28098746

Received: May 21, 2018
Peer-review started: May 21, 2018
First decision: June 21, 2018
Revised: August 2, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 14, 2018

Abstract

Gastric polyposis is a rare disease. Not all polyps progress to cancer. Monoallelic mutation in Fanconi anemia

(FA) genes, unlike biallelic gene mutations that causes typical FA phenotype, can increase risks of cancers in a sporadic manner. Aberrations in the FA pathway were reported in all molecular subtypes of gastric cancer. We studied a patient with synchronous gastric cancer from gastric polyposis by conducting a 13-year long-term follow up. *Via* pathway-driven massive parallel genomic sequencing, a germline mutation at *FANCA* D1359Y was identified. We identified several recurrent mutations in DNA methylation (*TET1*, V873I), the β-catenin pathway (*CTNNB1*, S45F) and RHO signaling pathway (*PLEKHG5*, R203C) by comparing the genetic events between benign and malignant gastric polyps. Furthermore, we revealed gastric polyposis susceptible genes and genetic events promoting malignant transformation using pathway-driven targeted gene sequencing.

Key words: Gastric polyposis; Gastric cancer; Adenocarcinoma; Fanconi's anemia; Malignant transformation

Core tip: The genetic events that predispose benign polyps to carcinoma are rarely explored in gastric cancer. We studied a rare case that progressed from benign gastric polyposis to gastric cancer and disclosed a monoallelic germline mutation (D1359Y) at Fanconi's anemia gene, which may contribute to the phenotype. Comparing the genetic events between benign and malignant gastric polyps, several recurrent mutations in DNA methylation (*TET1*, V873I), the β-catenin pathway (*CTNNB1*, S45F) and RHO signaling pathway (*PLEKHG5*, R203C) were identified.

Huang JP, Lin J, Tzen CY, Huang WY, Tsai CC, Chen CJ, Lu YJ, Chou KF, Su YW. *FANCA* D1359Y mutation in a patient with gastric polyposis and cancer susceptibility: A case report and review of literature. *World J Gastroenterol* 2018; 24(38): 4412-4418 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i38/4412.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i38.4412

INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. Most gastric cancers are associated with infectious agents such as *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV) and a minority (1%-3%) of gastric cancer cases are associated with germline mutations in E-cadherin (*CDH1*) or mismatch repair genes[1]. *H. pylori* infection and certain genetic conditions such as adenomatous polyposis coli (APC) gene mutation are also associated with benign gastric polyps either in sporadic or inherited form[2,3]. Although benign gastric polyps result from the accumulation of multiple genetic and epigenetic alterations, only a minority of cases progress to gastric cancer. Given that gastric polyposis is an exceedingly rare disease, the genetic cause of adenoma-adenocarcinoma consequence is unclear.

A recent large-scale investigation in gastric cancer genomics found that there are four major subtypes of gastric cancer[4]. Although the molecular processes driving tumorigenesis vary among patients, aberrations in the Fanconi anemia (FA) pathway were observed in all molecular subtypes of gastric cancer[5]. Germline mutations of genes encoding FA proteins are associated with FA, a rare hereditary autosomal recessive syndrome, characterized by progressive bone marrow failure, variable congenital malformations, and increased risk of cancers. Monoallelic mutation in FA genes, unlike biallelic or homozygous gene mutation that causes typical FA phenotype, can increase the susceptibility to a variety of solid tumors in a sporadic manner[6]. The major cellular function of the FA pathway is to maintain stability of genome during the process of DNA replication and damage repair.

In this report, we explore the genetic profiles in a patient with sporadic gastric polyposis and carcinoma. We hypothesize that multiple biopsies from recurrent gastric polyps will serve as an important model to study gastric homeostasis and gastric tumorigenesis. We have also sought to demonstrate the use of a pathway-driven massive parallel genetic sequencing approach for elucidating important tumoral characteristics.

CASE REPORT

Case description

A 65-year-old man, without significant medical history, presented with recurrent passage of black stool and iron-deficiency anemia (IDA) from the age of 48 (2002). Multiple polypoid tumors at the stomach with easy touch bleeding were noted by esophagogastroduodenoscopy (EGD) since 2002. Systemic survey showed that the polyposis was limited to the stomach and *H. pylori* infection was absent. There was only one sporadic polyp in the colon as shown by colonfibroscopy. The biopsy of the gastric polyps showed hyperplastic polyps, in which dilated, complex, tortuous, gastric, foveolar type glands were seen.

After medical treatment with iron supplement, he was essentially free of gastrointestinal symptoms and the levels of hemoglobin were kept in subnormal ranges (11.5-12 g/dL) until 2011 when recurrent passage of tarry stool with IDA developed. EGD was performed several times during 2011-2015 (between the ages of 57 and 61). The serial EGD showed the maximal size of gastric polypoid tumors gradually increased from 1.5 cm in 2011, to 2.5 cm in 2012, and to 5 cm in 2015. Microscopically, surface chronic ulceration and inflammation on the hyperplastic polyps were noted between 2011 and 2012. In later follow-ups in 2015, low-grade to high-grade intraepithelial neoplasia and intramucosal carcinoma in the background of tubular adenoma was found. CT of the abdomen in 2015 showed multiple intraluminal polypoid tumors and an exophytic tumor at the upper jejunum (Figure 1).

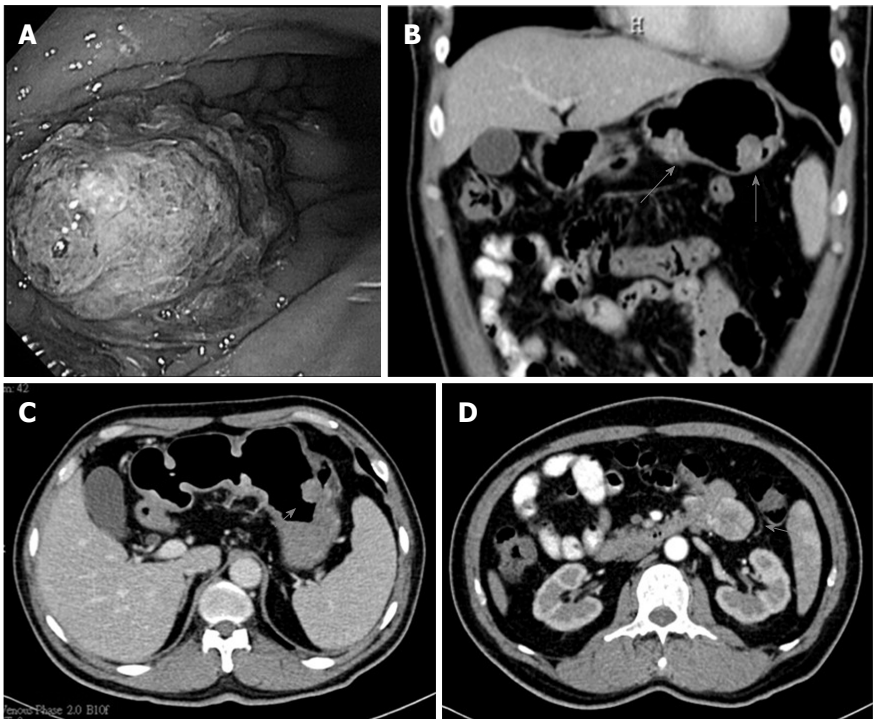


Figure 1 Endoscopic findings and computed tomography scans of the abdomen of the patient at the diagnosis of gastric cancer. A: Multiple polyps in the gastric body and duodenal bulb were demonstrated by EGD; B: The coronal and C: sagittal views from the CT scan; D: An exophytic mass measuring about 3.7 cm × 3.5 cm was incidentally found in the proximal jejunum by CT. CT: Computed tomography; EGD: Esophagogastroduodenoscopy.

Therefore, the patient underwent subtotal gastrectomy and resection of the jejunal tumor. In addition to benign gastric polyposis, adenocarcinoma at the stomach and gastrointestinal stromal tumor (GIST) at the jejunum were diagnosed after surgery. After the procedure, there was no further drop in the level of hemoglobin and no evidence of recurrence of cancer was noted in the subsequent follow-ups until the end of 2017.

The clinical course of gastric polyposis in this patient is summarized in Figure 2. To study the genetic association between benign gastric polyp, gastric adenocarcinoma, and jejunal GIST, we used a commercially available multiplexed gene sequencing panel (ACTOnco®, ACT Genomics Co., Ltd. Taipei, Taiwan) that detects mutations in 409 genes pertinent to cancer treatment, prognosis, and diagnosis.

Materials and methods

Ethic approval: The approval for conducting this study was granted by the Ethic Committee of Mackay Memorial Hospital, No. 15MMHIS190e. Informed consent was obtained from the patient.

Sample preparation: DNA from formalin-fixed paraffin-embedded tumor specimens (benign gastric polyp, gastric adenocarcinoma, and jejunal GIST tumor) were extracted using QIAamp DNA FFPE Tissue Kit (Qiagen Inc., Valencia, CA, United States) according to the manufacturer's protocol. DNA was quantified using Quant-iTTM dsDNA HS Assay (Invitrogen) and tested for the integrity of genomic DNA using Fragment Analyzer™ (Advanced Analytical Technologies, Inc.) according to the manufacturer's protocol.

ACTOnco comprehensive cancer panel sequencing: Eighty nanograms of genomic DNA from each sample were amplified using four pools of 15992 primer pairs (Ion AmpliSeq Comprehensive Cancer Panel, Thermo Fisher Scientific) to target all coding exons of 409 cancer-related genes (1688650 target bases). Amplicons were ligated with barcoded adaptors using the Ion Ampliseq Library Kit (Thermo Fisher Scientific). They were subsequently conjugated with sequencing beads by emulsion polymerase chain reaction and enriched using IonChef (Thermo Fisher Scientific) according to the manufacturer's protocol. Sequencing was performed on the Ion Proton Sequencer with the Ion PI chip (Thermo Fisher Scientific).

Raw data generated by the sequencer were mapped to the hg19 reference genome using Ion Torrent Suite (v. 4.4). Single nucleotide variants (SNVs) and short insertion/deletions (INDELs) were identified using the Torrent Variant Caller plug-in. Variant Effect Predictor (VEP) was used to annotate every variant. Genetic alterations of all coding exons and all exon-intron boundaries covered by the gene panel were analyzed. Identified variants were included both in protein-coding sequences and splice-site variants. For variants in protein-coding sequences, only non-synonymous mutations were analyzed. Any variant with less than 25 reads or lower than 5% variant frequency was filtered out. With the threshold for filtering, 99% of the captured region was covered in each case. Total numbers of mapped reads in the studied samples

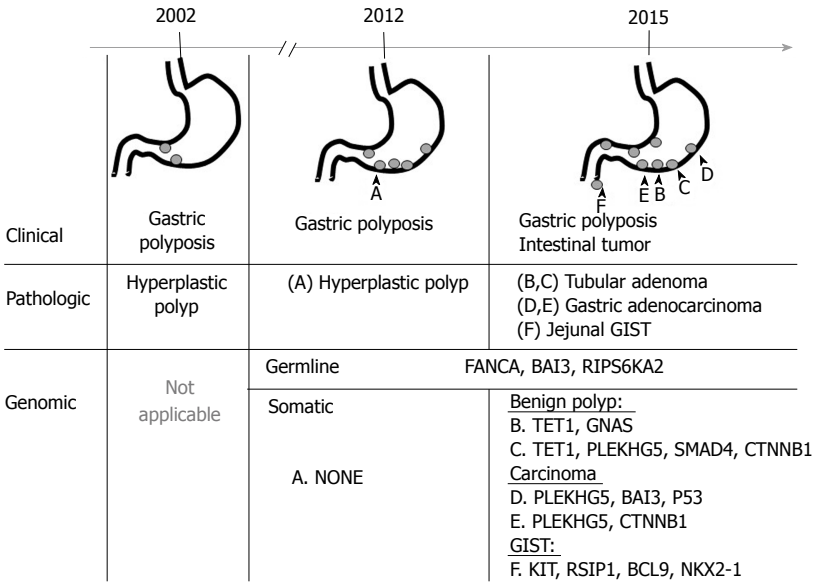


Figure 2 Chronological summary of the clinical, pathologic, and genomic findings of gastric polyps and tumor specimen labeling (A-F) for massive parallel sequencing. GIST: Gastro-intestinal stromal tumor.

were between 20907271 and 37358579. The mean base coverage depth ranged from 1355 to 2397. Variants that were included in 1000 Genomes project Phase 1 (Asian populations) with > 1% minor allele frequency (MAF) were considered as polymorphisms and excluded from further analysis.

Results

Mutational analysis of benign and malignant gastric polyps, and jejunal gastrointestinal stromal tumor: Massively parallel sequencing for a panel of 409 cancer-related genes in these tumors identified three germline mutations (*BAI3* p.S311W, *FANCA* pD1359Y, *RPS6KA2* p.T595I) and twelve somatic mutations in three benign and three malignant tumors (Table 1). Among the benign tumors, there was no identifiable somatic mutation in the hyperplastic polyp collected in 2011. However, several newly developed somatic mutations were found four years later: *TET1* p.V873I and *GNAS* p.R844C in one adenomatous polyp, and *TET1* p.V873I, *SMAD4* p.R361C, *CTNNB1* p.S45F, *and PLEKHG5* p.R203C in another adenomatous polyp with low-to-high-grade intraepithelial neoplasia. In the malignant gastric polyps, two new mutations (*BAI3* pL617P, and *P53* p.K164fs) were found, but no mutations in *TET1* or *SMAD4* were identified. The jejunal GIST tumor harbored unique somatic mutations in *KIT* p. S501_A502insAY, *RSIP1* p.S514N, *BCL9* p.A1191G, *BCL9* p.P1195A, and *NKX2-1* p.F209L, which were not observed in either benign or malignant gastric polyps.

DISCUSSION

We reported a patient with *H. pylori*-negative gastric polyposis presenting with bleeding and anemia from the age of 48. The polyp progressed from hyperplastic polyp to adenomatous polyp, then led to malignant transformation after 13 years of follow-up, and a jejunal GIST was identified incidentally. Because the polyps spread throughout the whole stomach and no other family members were affected by similar disease, the patient did not fulfill the diagnostic criteria of Gastric Adenocarcinoma and Proximal Polyposis of the Stomach (GAPPS), a recently described autosomal-dominant heritable syndrome characterized by isolated proximal gastric polyposis and risk of gastric cancer[7,8]. Therefore, we studied the genomic profiles from benign to malignant polyp by massive parallel sequencing and unexpectedly identified that the patient was a germline carrier of *FANCA*, *BAI3*, and *RPS6KA2* mutations. Comparing the genomic profiling from benign to malignant gastric polyp and jejunal GIST revealed clonal evolution between gastric polyposis and not jejunal GIST.

The adenoma-carcinoma sequence, first proposed in the 1990's, is now a widely accepted theory of step-wise carcinogenesis from normal epithelium to adenoma, carcinoma *in situ*, and invasive and metastatic tumor in colorectal cancer[9]. Genomic instability plays a critical role to initiate and facilitate the adenoma-carcinoma sequence[10]. In this case, the monoallelic germline mutation (D1359Y) in a DNA damage repair (DDR) gene, *FANCA*, might account for the predisposition to chromosomal instability and the development of tumors.

FA is a rare, genetically heterogeneous syndrome with an increased predisposition to cancers and bone marrow failure[11]. To date, mutations in 16 genes have been identified in FA, including *FANCA, B, C, D1/BRCA2, FANCD2, E, F, G, I, J, L, M, N/PALPB2, FANCO/RAD51C, FANCP/SLX4*, and *FANCQ*[12]. The above proteins work together in the FA pathway in response to DNA damage to coordinate DNA repair and maintain genomic stability. Therefore, mutation in any of the 16 FA genes may lead

Table 1 Single nucleotide variants and short insertion/deletions in benign and malignant gastric polyps, and jejunal gastrointestinal stromal tumor identified in a comprehensive cancer panel sequencing screen containing 409 cancer-related exons

Gene	DNA change	Amino acid change	A	B	C	D	E	F
			Benign polyp			Malignant polyp		
			Hyperplastic polyp	Tubular adenoma	Tubular adenoma with low-to-high-grade intraepithelial neoplasia	Tubular adenoma with signet ring cell gastric adenocarcinoma	Tubular adenoma with focal intramucosal carcinoma	Jejunal GIST
			1.5 cm	2.5 cm	N/A	2.5 cm	5 cm	N/A
[1]*FANCA*	c. 4075G>T	p. D1359Y	48.40%	54.00%	47.50%	52.00%	37.80%	45.70%
[1]*BAI3*	c. 932C>G	p. S311W	50.30%	46.10%	40.90%	50.40%	51.30%	49.30%
[1]*RPS6KA2*	c. 1784C>T	p. T595I	48.00%	49.40%	47.70%	45.70%	51.50%	48.40%
TET1	c. 2617G>A	p. V873I		10.10%	7.40%			
GNAS	c. 2530C>T	p. R844C		6.30%				
SMAD4	c. 1081C>T	p. R361C			6.40%			
CTNNB1	c. 134C>T	p. S45F			23.60%	4.20%		
PLEKHG5	c. 607C>T	p. R203C			40.70%	15.30%	35.40%	
BAI3	c. 1850T>C	p. L617P					37.70%	
P53	c. 489dupC	p. K164fs					49.90%	
KIT	C1502_1503insTGCCTA	p. S501_A502insAY						37.30%
PSIP1	c. 1541G>A	p. S514N						14.00%
BCL9	c. 3572C>G	p. A1191G						13.10%
BCL9	c. 3583C>G	p. P1195A						13.00%
NKX2-1	C627C>A	p. F209L						7.50%

[1]Somatic mutation. Percentage indicates variant frequency of the mutation. GIST: Gastro-intestinal stromal tumor.

to defects in DDR and chromosomal instability.

Germline *FANCA* mutation is the most frequent in patients with FA but less reported in other solid tumors[13]. In the TCGA database, somatic mutations in *FANC* genes were approximately 3% in stomach cancer[5,14]. Hierarchical clustering analysis showed that the DDR pathways, especially the FA pathway, were deranged across all subtypes of gastric cancer[5]. *FANCA* D1359Y, the amino acid substitution found in our case, results in a change from a charged to a hydrophobic amino acid[15]. It not only has a weaker interaction with *FANCC* and *FANCG* but also affects the ubiquitination of *FANCD2* and subsequent cellular response to DNA damage[16].

The association with clinical development of cancers is different in homozygous and heterozygous germline FA gene mutation carriers. In patients with inherited homozygous (biallelic) mutations of FA genes, the relative risk of nonhematologic malignancies is increased especially for squamous cell carcinomas (700-fold more affected than the normal population) and the median onset age of cancers is much earlier (16 years old in affected compared to 68 years old in non-FA population)[17,18]. In contrast, heterozygous (monoallelic) FA gene mutations somatically do not cause the FA phenotype but significantly increase cancer susceptibility in a sporadic manner, *e.g.* later onset without overt family history[6]. Some of the FA genes (*FANCD1, FANCD2, FANCJ, FANCN and FANCO*) have been described as breast and ovarian cancer susceptibility genes[19-22]. To our knowledge, our case is the first report of a *FANCA* mutation in gastric polyposis and cancer.

In addition to genetic susceptibility, environmental factors play critical roles in the development of gastric cancer, such as *H. pylori*, chronic use of a proton pump inhibitor, high salt diet, and smoking[23,24]. In response to tissue damage from the environmental stimuli, the stomach can initiate a regenerative program to accelerate stem cell division and lineage differentiation to replenish damaged cells[25]. In this study, two somatic mutations frequently found in gastric cancer were identified in malignant polyps: tumor suppressor genes *TP53* and oncogene *CTNNB1*. Interestingly, *PLEKHG5*, a less described mutation in gastric cancer, was found in three out of five examined samples, and *TET1* mutation was only identified in two benign polyps.

PLEKHG5, a member of pleckstrin homology and Rho guanine nucleotide exchange factor domain-containing G protein family, is currently considered to play a role in activating RhoA exchange factors and the nuclear factor kappa B ($NF_{\kappa}B$) signaling pathway[26]. The cellular function of *PLEKHG5* is to promote cell polarity, migration, adhesion, and degradation through the interaction with *RHO* downstream effectors[27,28]. In the molecular subtypes proposed by TCGA[5], *RHOA* mutation was found almost exclusively in genomically stable tumors. Although *PLEKHG5* mutation found in this study may theoretically activate the *RHOA*-driven signaling pathway and contribute to the invasive phenotype, its actual cellular function remains to be experimentally elucidated.

TET1, tet methylcytosine dioxygenase 1, belongs to the TET protein family and plays a role in DNA methylation and gene activation. One recent study reported that *TET1* can inhibit gastric cancer growth and AKT signaling through demethylation and re-expression of PTEN[29]. To our knowledge, only one breast cancer cell line, HCC1143, was reported to have this mutation (*TET1 V873I*) from dbEM (A database of epigenetic modifier,

www.crdd.osdd.net). In this study, *TET1* mutation was recurrently seen in benign polyps but not in malignant lesions. Whether this mutation can prevent malignant transformation requires more investigation.

To our knowledge, the association between an FA gene mutation and a gastric polyp or an adenocarcinoma has not been reported.

With the advent of new sequencing technologies, we were able to detect germline mutations/variations that may cause susceptibility to the development of gastric polyps and cancer, which might shed light in finding potential genetic events contributing to malignant transformation. Although more research is needed to elucidate the cellular phenotypes of the mutations observed in our study and their role in gastric carcinogenesis, the advent of a pathway-driven genomic profiling approach brings clinicians closer to the central cause of the disease and holds a great potential in providing better care for individuals.

ARTICLE HIGHLIGHTS

Case characteristics

A 65-year-old man had iron deficiency anemia from the age of 48 due to gastric polyposis with recurrent upper gastrointestinal bleeding.

Clinical diagnosis

Gastric polyposis

Differential diagnosis

Clinically, differential diagnoses for gastric polyposis include familial adenomatous polyposis syndrome, inherited autosomal-dominant syndrome "Gastric Adenocarcinoma and Proximal Polyposis of the Stomach" (GAPPS), or sporadic gastric polyposis. Histologically, gastric polyps should be differentiated between benign epithelial polyps (such as hyperplastic, fundic-gland, adenomatous, and inflammatory fibrinoid polyps), gastric neuroendocrine tumors (carcinoids), gastrointestinal stromal tumors (GIST), and malignant polyps (such as adenomatous carcinoma).

Laboratory diagnosis

Iron deficiency anemia

Imaging diagnosis

Polyposis at the stomach and upper jejunum by computed tomography scan

Pathological diagnosis

He was initially diagnosed with gastric hyperplastic polyp between the ages of 48 years to 58 years, and then gastric adenocarcinoma and jejunal GIST at the age of 61 years. Through comparing the genetic events between benign and malignant gastric polyps, a germline mutation at *FANCA* D1359Y and several recurrent mutations in DNA methylation (*TET1,* V873I), the β-catenin pathway (*CTNNB1,* S45F) and RHO signaling pathway (*PLEKHG5,* R203C) were identified in the polyps with malignant transformation.

Treatment

Endoscopic polypectomy for lesions larger than 0.5-1 cm, followed by annual upper endoscopic surveillance; subtotal gastrectomy for gastric adenocarcinoma and resection of the jejunal GIST.

Experiences and lessons

The adenoma-carcinoma sequence in gastric cancer is recapitulated in the case study. Germline mutation at *FANCA* D1359Y is identified as a potential gastric polyposis susceptible gene.

REFERENCES

1 **Boland CR**, Yurgelun MB. Historical Perspective on Familial Gastric Cancer. *Cell Mol Gastroenterol Hepatol* 2017; **3**: 192-200 [PMID: 28275686 DOI: 10.1016/j.jcmgh.2016.12.003]

2 **Li J**, Woods SL, Healey S, Beesley J, Chen X, Lee JS, Sivakumaran H, Wayte N, Nones K, Waterfall JJ, Pearson J, Patch AM, Senz J, Ferreira MA, Kaurah P, Mackenzie R, Heravi-Moussavi A, Hansford S, Lannagan TRM, Spurdle AB, Simpson PT, da Silva L, Lakhani SR, Clouston AD, Bettington M, Grimpen F, Busuttil RA, Di Costanzo N, Boussioutas A, Jeanjean M, Chong G, Fabre A, Olschwang S, Faulkner GJ, Bellos E, Coin L, Rioux K, Bathe OF, Wen X, Martin HC, Neklason DW, Davis SR, Walker RL, Calzone KA, Avital I, Heller T, Koh C, Pineda M, Rudloff U, Quezado M, Pichurin PN, Hulick PJ, Weissman SM, Newlin A, Rubinstein WS, Sampson JE, Hamman K, Goldgar D, Poplawski N, Phillips K, Schofield L, Armstrong J, Kiraly-Borri C, Suthers GK, Huntsman DG, Foulkes WD, Carneiro F, Lindor NM, Edwards SL, French JD, Waddell N, Meltzer PS, Worthley DL, Schrader KA, Chenevix-Trench G. Point Mutations in Exon 1B of APC Reveal Gastric Adenocarcinoma and Proximal Polyposis of the Stomach as a Familial Adenomatous Polyposis Variant. *Am J Hum Genet* 2016; **98**: 830-842 [PMID: 27087319 DOI: 10.1016/j.ajhg.2016.03.001]

3 **Elhanafi S**, Saadi M, Lou W, Mallawaarachchi I, Dwivedi A, Zuckerman M, Othman MO. Gastric polyps: Association with Helicobacter pylori status and the pathology of the surrounding mucosa, a cross sectional study. *World J Gastrointest Endosc* 2015; **7**: 995-1002 [PMID: 26265993 DOI: 10.4253/wjge.v7.i10.995]

4 **Tan P**, Yeoh KG. Genetics and Molecular Pathogenesis of Gastric Adenocarcinoma. *Gastroenterology* 2015; **149**: 1153-1162.e3 [PMID: 26073375 DOI: 10.1053/j.gastro.2015.05.059]

5 **Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; **513**: 202-209 [PMID: 25079317 DOI: 10.1038/nature13480]

6 **Chen H**, Zhang S, Wu Z. Fanconi anemia pathway defects in inherited and sporadic cancers. *Transl Pediatr* 2014; **3**: 300-304 [PMID: 26835350 DOI: 10.3978/j.issn.2224-4336.2014.07.05]

7 **Brosens LA**, Giardiello FM, Offerhaus GJ, Montgomery EA. Syndromic Gastric Polyps: At the Crossroads of Genetic and Environmental Cancer Predisposition. *Adv Exp Med Biol* 2016; **908**: 347-369 [PMID: 27573780 DOI: 10.1007/978-3-319-41388-4_17]

8 **Worthley DL**, Phillips KD, Wayte N, Schrader KA, Healey S, Kaurah P, Shulkes A, Grimpen F, Clouston A, Moore D, Cullen D, Ormonde D, Mounkley D, Wen X, Lindor N, Carneiro F, Huntsman DG, Chenevix-Trench G, Suthers GK. Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS): a new autosomal dominant syndrome. *Gut* 2012; **61**: 774-779 [PMID: 21813476 DOI: 10.1136/gutjnl-2011-300348]

9 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767 [PMID: 2188735 DOI: 10.1016/0092-8674(90)90186-I]

10 **Armaghany T**, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. *Gastrointest Cancer Res* 2012; **5**: 19-27 [PMID: 22574233]

11 **Brosh RM Jr**, Bellani M, Liu Y, Seidman MM. Fanconi Anemia: A DNA repair disorder characterized by accelerated decline of the hematopoietic stem cell compartment and other features of aging. *Ageing Res Rev* 2017; **33**: 67-75 [PMID: 27223997 DOI: 10.1016/j.arr.2016.05.005]

12 **Bogdanova N**, Helbig S, Dörk T. Hereditary breast cancer: ever more pieces to the polygenic puzzle. *Hered Cancer Clin Pract* 2013; **11**: 12 [PMID: 24025454 DOI: 10.1186/1897-4287-11-12]

13 **Wilkes DC**, Sailer V, Xue H, Cheng H, Collins CC, Gleave M, Wang Y, Demichelis F, Beltran H, Rubin MA, Rickman DS. A germline FANCA alteration that is associated with increased sensitivity to DNA damaging agents. *Cold Spring Harb Mol Case Stud* 2017; **3**: [PMID: 28864460 DOI: 10.1101/mcs.a001487]

14 **Kent WJ**, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler

AM, Haussler D. The human genome browser at UCSC. *Genome Res* 2002; **12**: 996-1006 [PMID: 12045153 DOI: 10.1101/gr.229102]
15 **Savino M**, Ianzano L, Strippoli P, Ramenghi U, Arslanian A, Bagnara GP, Joenje H, Zelante L, Savoia A. Mutations of the Fanconi anemia group A gene (FAA) in Italian patients. *Am J Hum Genet* 1997; **61**: 1246-1253 [PMID: 9399890 DOI: 10.1086/301632]
16 **Adachi D**, Oda T, Yagasaki H, Nakasato K, Taniguchi T, D'Andrea AD, Asano S, Yamashita T. Heterogeneous activation of the Fanconi anemia pathway by patient-derived FANCA mutants. *Hum Mol Genet* 2002; **11**: 3125-3134 [PMID: 12444097 DOI: 10.1093/hmg/11.25.3125]
17 **Alter BP**. Cancer in Fanconi anemia, 1927-2001. *Cancer* 2003; **97**: 425-440 [PMID: 12518367 DOI: 10.1002/cncr.11046]
18 **Kutler DI**, Singh B, Satagopan J, Batish SD, Berwick M, Giampietro PF, Hanenberg H, Auerbach AD. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). *Blood* 2003; **101**: 1249-1256 [PMID: 12393516 DOI: 10.1182/blood-2002-07-2170]
19 **Ghoussaini M**, Pharoah PDP, Easton DF. Inherited genetic susceptibility to breast cancer: the beginning of the end or the end of the beginning? *Am J Pathol* 2013; **183**: 1038-1051 [PMID: 23973388 DOI: 10.1016/j.ajpath.2013.07.003]
20 **Rosenberg PS**, Alter BP, Ebell W. Cancer risks in Fanconi anemia: findings from the German Fanconi Anemia Registry. *Haematologica* 2008; **93**: 511-517 [PMID: 18322251 DOI: 10.3324/haematol.12234]
21 **Levy-Lahad E**. Fanconi anemia and breast cancer susceptibility meet again. *Nat Genet* 2010; **42**: 368-369 [PMID: 20428093 DOI: 10.1038/ng0510-368]
22 **Meindl A**, Hellebrand H, Wiek C, Erven V, Wappenschmidt B, Niederacher D, Freund M, Lichtner P, Hartmann L, Schaal H, Ramser J, Honisch E, Kubisch C, Wichmann HE, Kast K, Deissler H, Engel C, Müller-Myhsok B, Neveling K, Kiechle M, Mathew CG, Schindler D, Schmutzler RK, Hanenberg H. Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nat Genet* 2010; **42**: 410-414 [PMID: 20400964 DOI: 10.1038/ng.569]
23 **Isomoto H**, Furusu H, Ohnita K, Takehara Y, Wen CY, Kohno S. Effect of Helicobacter pylori eradication on gastric hyperplastic polyposis in Cowden's disease. *World J Gastroenterol* 2005; **11**: 1567-1569 [PMID: 15770740 DOI: 10.3748/wjg.v11.i10.1567]
24 **Freeman HJ**. Proton pump inhibitors and an emerging epidemic of gastric fundic gland polyposis. *World J Gastroenterol* 2008; **14**: 1318-1320 [PMID: 18322941 DOI: 10.3748/wjg.14.1318]
25 **Kim TH**, Shivdasani RA. Notch signaling in stomach epithelial stem cell homeostasis. *J Exp Med* 2011; **208**: 677-688 [PMID: 21402740 DOI: 10.1084/jem.20101737]
26 **Kim HJ**, Hong YB, Park JM, Choi YR, Kim YJ, Yoon BR, Koo H, Yoo JH, Kim SB, Park M, Chung KW, Choi BO. Mutations in the PLEKHG5 gene is relevant with autosomal recessive intermediate Charcot-Marie-Tooth disease. *Orphanet J Rare Dis* 2013; **8**: 104 [PMID: 23844677 DOI: 10.1186/1750-1172-8-104]
27 **Iwatake M**, Nishishita K, Okamoto K, Tsukuba T. The Rho-specific guanine nucleotide exchange factor Plekhg5 modulates cell polarity, adhesion, migration, and podosome organization in macrophages and osteoclasts. *Exp Cell Res*2017; **359**: 415-430 [PMID: 28847484 DOI: 10.1016/j.yexcr.2017.08.025]
28 **Dachsel JC**, Ngok SP, Lewis-Tuffin LJ, Kourtidis A, Geyer R, Johnston L, Feathers R, Anastasiadis PZ. The Rho guanine nucleotide exchange factor Syx regulates the balance of dia and ROCK activities to promote polarized-cancer-cell migration. *Mol Cell Biol* 2013; **33**: 4909-4918 [PMID: 24126053 DOI: 10.1128/MCB.00565-13]
29 **Pei YF**, Tao R, Li JF, Su LP, Yu BQ, Wu XY, Yan M, Gu QL, Zhu ZG, Liu BY. TET1 inhibits gastric cancer growth and metastasis by PTEN demethylation and re-expression. *Oncotarget* 2016; **7**: 31322-31335 [PMID: 27121319 DOI: 10.18632/oncotarget.8900]

P- Reviewer: Ierardi E, Misra V, Pylkas K, Ranzani GN
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Bian YN

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 October 21; 24(39): 4419-4518

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 39 October 21, 2018

Contents

World Journal of Gastroenterology
Volume 24 Number 39 October 21, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Saadi Berkane, MD, PhD, Chief Doctor, Professor, Internal Medicine, Hepatology and Gastroenterology, Bologhine Hospital, Algiers 16000, Algeria

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
October 21, 2018

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4419-4427

DOI: 10.3748/wjg.v24.i39.4419

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

EDITORIAL

Portal vein thrombosis in cirrhotic patients - it is always the small pieces that make the big picture

Irina Gîrleanu, Anca Trifan, Carol Stanciu, Cătălin Sfarti

Irina Gîrleanu, Anca Trifan, Carol Stanciu, Cătălin Sfarti, Department of Gastroenterology, "Grigore T Popa" University of Medicine and Pharmacy, Iași 700115, Romania

Irina Gîrleanu, Anca Trifan, Carol Stanciu, Cătălin Sfarti, Institute of Gastroenterology and Hepatology, "St. Spiridon" University Hospital, Iași 700115, Romania

ORCID number: Irina Gîrleanu (0000-0001-5925-1232); Anca Trifan (0000-0001-9144-5520); Carol Stanciu (0000-0002-6427-4049); Cătălin Sfarti (0000-0001-7074-5938).

Author contributions: Gîrleanu I, Stanciu C, Sfarti C and Trifan A conceived and design the editorial; Gîrleanu I and Sfarti C wrote and revised the manuscript; Trifan A and Stanciu C critically revised the manuscript for important intellectual content and gave the final approval of the version to be published; all authors approved the final version of the manuscript.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Anca Trifan, MD, Doctor, Professor, Department of Gastroenterology, "Grigore T Popa" University of Medicine and Pharmacy, 16 Universitatii St, Iași 700115, Romania. ancatrifan@yahoo.com
Telephone: +40-72-6108428
Fax: +40-23-2211820

Received: July 27, 2018
Peer-review started: July 28, 2018
First decision: August 27, 2018
Revised: September 2, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

Portal vein thrombosis (PVT) is a frequent and serious complication in patients with liver cirrhosis (LC). Recently, a new classification of PVT was proposed, although the functional component was not completed included. The status of liver disease (compensated/decompensated) should be added to this classification. Reduced portal flow velocity and the acquired hypercoagulable status associated with LC are the main risk factors for PVT development, although endothelial dysfunction may play an important role that needs to be further evaluated. The European Association for the Study of the Liver and the American Association for the Study of Liver Disease recommend that the anticoagulant treatment should be consider in cirrhotic patients with PVT. Low molecular weight heparin and vitamin K antagonists proved their efficacy and relatively safety in PVT treatment, although in addition to recanalization rates, more complex end-points such as mortality and decompensation rate should be evaluated. The new oral anticoagulant therapies offers the advantage of oral administration in the absence of laboratory monitoring, however, there are a few reports regarding their use in cirrhotic patients, most of them referring to compensated isolated cases. Transjugular intrahepatic portosystemic shunt could be an alternative if thrombosis progresses despite anticoagulatant therapy and/or when PVT is associated with portal hypertension complications. The aim of this editorial is to discuss the different aspects of pathophysiology, clinical relevance, diagnosis and management of PVT in patients with LC.

Key words: Portal vein thrombosis; Liver cirrhosis; Classification; Risk factors; Anticoagulation

Core tip: Portal vein thrombosis is a frequent and serious complication in patients with liver cirrhosis. The new classification needs to be validated and should contain the pattern of thrombus evolution and the status of liver cirrhosis- compensated or decompensated. The two main risk factors - reduced portal flow velocity and the hypercoagulable state should be addressed more extensively in large studies, considering the stage of liver disease. The anticoagulant treatment could be considered in cirrhotics with portal vein thrombosis. The end-points of the anticoagulant treatment should consider the recanalization, decompensation, and the mortality rates.

Gîrleanu I, Trifan A, Stanciu C, Sfarti C. Portal vein thrombosis in cirrhotic patients - it is always the small pieces that make the big picture. *World J Gastroenterol* 2018; 24(39): 4419-4427 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4419.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4419

INTRODUCTION

Portal vein thrombosis (PVT) is a frequent and serious complication in patients with liver cirrhosis (LC).The prevalence of PVT in patients with LC ranges from 0.6% to 26%[1] compared to 0.7 -1/100000 in the general population[2]. PVT has been increasingly diagnosed in LC using noninvasive imaging techniques such as ultrasound (US), computed tomography (CT) or magnetic resonance imaging (MRI), with the highest prevalence at the time of liver transplantation[3]. PVT mechanisms in cirrhotic patients shifted from a hypocoagulable state, due to thrombocytopenia and decreased coagulation factors levels, towards an acquired hypercoagulable state characterized by decreased protein C, protein S and antithrombin III levels and increased factor VIII levels[4].

PVT clinical presentation in cirrhotic patients is heterogenous ranging from incidental diagnosis by US during routine follow-up screening for hepatocellular carcinoma (HCC) to life-threatening complications such esophageal/gastric variceal bleeding or intestinal infarction[5].

The diagnosis of PVT is based on imaging techniques: Doppler US the first choice method (widely available, cheap, without irradiation) or contrast enhanced US. Contrast enhanced CT and MRI are superior to Doppler US for the assessment of thrombotic extension to venous branches difficult to be assessed by US exam[6]. The optimal treatment of nonmalignant PVT in cirrhotic patients remains an unmet need. The European Association for the Study of the Liver (EASL)[7] guideline for vascular diseases of the liver, the American Association of the Study of the Liver Diseases (AASLD)[6] and BAVENO VI consensus[8] recommend that the anticoagulant treatment should be considered, without any strong clinical evidence The aim of this editorial is to discuss the different aspects of pathophysiology, clinical relevance, diagnosis and management of non-malignant PVT in patients with LC.

DEFINITIONS AND CLASSIFICATIONS: OLD AND NEW

PVT refers to partial or complete occlusion of the portal vein trunk which can includes its right and/or left intrahepatic branches, with the possibility to extend either to the superior mesenteric vein or to the splenic vein.

PVT includes two different entities: the first one is acute PVT defined by the sudden formation of a thrombus within portal vein and often involving mesenteric or splenic veins, and the second is chronic PVT (also known as cavernoma). The AASLD defined acute PVT as the sudden formation of a thrombus within the portal vein lumen, and chronic PVT as the replacement of the obstructed portion is replaced network of collaterals resulting in the cavernomatous transformation of the portal vein[6]. The EASL defined recent PVT as a recent formation of a thrombus within the portal vein and/or right or left branches, while chronic PVT is characterized by the absence of recanalization and development of porto-portal collaterals resulting in a cavernomatous transformation of the portal vein[7].

These definitions, otherwise simple and easily interpreted, are based only on anatomic findings, lacking any clinically significant consequences of thrombotic occlusion of the portal vein such as portal hypertension and ascites. Taking into account this aspect, Sarin *et al*[9] proposed that PVT should be defined as a clinical syndrome presenting itself either as an incidental finding or with variable signs and symptoms such as: abdominal pain, new onset of ascites, variceal bleeding or intestinal infarction.

During the past two decades at least eight classifications of PVT have been proposed; the first one by Stieber *et al*[10] in 1991, another by Yerdel *et al*[11] in 2000, and the last by de Franchis (Baveno VI classification) in 2015 (Table 1). All these classifications have several limitations: they are purely anatomic, with no functional relevance, or clear delineation between acute and chronic forms, no etiology assessment and, no clinical therapeutic decisiveness.

Sarin *et al*[9] have proposed a new classification of PVT in cirrhosis which is an anatomico-functional classification including the site of PVT, degree of portal venous system occlusion, extend of PVT, duration and presentation (recent, chronic asymptomatic, symptomatic), type and presence of underlying liver disease (cirrhotic, non-cirrhotic, post-transplant, HCC) (Table 2).

The prognostic value and the influence of this new

Table 1 Portal vein thrombosis - Baveno VI - classification[8]

Site of PVT	Type 1: Only trunk
	Type 2: Only branch: 2a - one branch, 2b - both branches
	Type 3: Trunk and branches
Presentation	R: Recent
	Ch: Chronic
Type of underlying liver disease	C: Cirrhotic
	N: Non-cirrhotic liver disease
	H: HCC and other local malignancies
	L: Post-liver transplant
	A: Absence of underlying liver disease
Degree of portal venous system occlusion	I: Incomplete: Flow visible in PV lumen through imaging
	T: Total: No flow visible in PV lumen on imaging
Extent of PV system occlusion	Splenic vein (S)
	Mesenteric vein (M)
	Both (SM)

PVT: Portal vein thrombosis; HCC: Hepatocellular carcinoma; PV: Portal vein.

Table 2 Anatomico-functional classification of portal vein thrombosis in cirrhosis[9]

Site of PVT	Type 1: Only trunk
	Type 2: Only branch: 2a - one branch, 2b - both branches
	Type 3: Trunk and branches
Duration and presentation	R: Recent (first time detected in previously patent PV)
	Asymptomatic: (As)
	Symptomatic: (S)- acute PVT features (with or without ABI)
	Ch: Chronic (previously diagnosed PVT on follow-up, portal cavernoma and clinical features of PHT)
	Asymptomatic
	Symptomatic: Features of portal hypertension
Type of underlying liver disease	Cirrhotic
	Non-cirrhotic liver disease
	HCC and other local malignancies
	Post-liver transplant
	Local malignancies
	Associated conditions
Degree of portal venous system occlusion	O: Occlusive: No flow visible in PV lumen on imaging/Doppler study
	NO: Nonocclusive: Flow visible in PV lumen through imaging/Doppler study
Extent of PV system occlusion	Splenic vein (S)
	Mesenteric vein (M)
	Both (SM)

PVT: Portal vein thrombosis; ABI: Acute bowel infarction; PHT: Portal hypertension; HCC: Hepatocellular carcinoma; PV: Portal vein.

classification in therapeutic decision remains to be confirmed by future prospective studies. In our opinion it is important to add the pattern of PVT evolution (spontaneous recanalization, extension or stable) with or without anticoagulant treatment, and the status of the liver cirrhosis (compensated or decompensated) in order to personalize the therapeutic approach.

PREVALENCE AND PREDICTORS FOR NON-MALIGNANT PVT: ETIOLOGY OF LIVER CIRRHOSIS

PVT prevalence is estimated 0.6%-26% in patients with liver cirrhosis, increasing proportionally with LC severity[1]. In patients with compensated cirrhosis reported prevalence ranges from 1% to 8%-26% in candidates for LT[3,4]. However, there are few studies that reported PVT incidence in LC. Thus, Nery *et al*[12] demonstrated a 5-year cumulative PVT incidence in LC of 10.7%, while Maruyama *et al*[13] performed a retrospective analysis on 150 patients with LC followed up for a median period of 66 mo and reported a 12.8% cumulative overall incidence of PVT at 1 year, 18.6% at 3 years, 20% at 5 years, and 38.7% at 8-10 years.

Multiple studies evaluated the predictive factors for PVT development in cirrhotic patients. Prior PVT[12], severe liver disease (Child-Pugh class A and B)[14], hypercoagulable status[15-17], recent surgical or invasive interventions of the abdomen[18], portal flow velocity < 15 cm/s[16], and HCC[12], were described as having predictive value for PVT development in cirrhotic patients. In a recent Italian national multicenter study, including 753 cirrhotic patients, Violi *et al*[19], demonstrated that previous portal vein thrombosis, Child-Pugh class B and C, HCC, prior upper gastrointestinal bleeding, and older age were independently associated with the presence of PVT.

The influence of cirrhosis ethiology on development

of PVT has not been yet clearly defined. Nonami *et al*[20] in a research on 885 candidates for LT with PVT demonstrated that alcoholic and hepatitis B virus related cirrhosis were found to be the most frequent etiologies of LC. The association between PVT and alcoholic etiology was recently confirmed by Scheiner *et al*[21]. Cruz *et al*[22] demonstrated that non-alcoholic steato-hepatitis was more frequently associated with PVT (40.48%), followed by hepatitis C virus (23.81%) and autoimmune hepatitis (19.05%), all of these etiologies being characterized by a significant pro-inflammatory status. By contrast, in another study with 199 candidates for LT, no relation was found between the etiology of liver disease and PVT prevalence[23].

MECHANISMS LEADING TO PVT IN LIVER CIRRHOSIS

The physiopathological mechanisms of PVT remain controversial, although many of them have been by now demonstrated. Even if, the interest in LC associated PVT have been increasing during these last years, considering the complex tests, such as the global test for coagulation assessment -thrombin generation assays or thrombelastometry used to characterize PVT[24-26] in cirrhotic patients, there are still a lot of missing pieces from the big picture of PVT.

PVT is a disease with multifactorial causes and, in some particular cases it is triggered by a genetic predisposition. The components of Virchow's triad (venous stasis, hypercoagulable state and endothelial dysfunction) are recognized as the main factors involved in PVT development in cirrhotic patients[4,13,15,16].

Reduced portal flow velocity was admitted to be as the most important risk factor for PVT development in LC, although this parameter varies significantly according to the degree of liver disease severity[16,19]. Zocco *et al*[16] demonstrated for the first time that portal vein velocity under 15 cm/s predisposes to PVT development and, recently, Stine *et al*[27] confirmed these results in a match case-control study. This theory started a long-debated argument that non-selective β-blockers (NSBBs) may induce PVT in liver cirrhosis. In a small study on 56 patients with liver cirrhosis, evaluated for PVT every 6 mo, Zampino *et al*[28] demonstrated that the use of NSBBs could be an independent predictor for PVT development, although no other study has yet confirmed this hypothesis.

Liver cirrhosis is associated with profound and complex coagulation defects, involving platelets number and function, pro- and anticoagulant factors, as well as fibrinolytic system[4].

It's a well known fact that patients diagnosed with LC present thrombocytopenia, mostly secondary to increased splenic destruction, but also due to thrombopoetin deficiency[29]. Although the platelet number is low, their function is not impaired, moreover platelet hyperreactivity is associated with increased levels of von Willebrand factor and factor VIII[30].

There is growing evidence that hypercoagulability is an important part of the hematological spectrum in cirrhosis. Tripodi *et al*[25] demonstrated, using global hemostasis assays, that in cirrhotic patients there is a normal or even increased thrombin generation. Liver cirrhosis is characterized by a decrease in procoagulant factors (fibrinogen, factor II, V, X, VII, IX, XI, XII) and anticoagulant factors (protein C, protein S and antithrombin III)[4,31,32]. In another study Tripodi *et al*[33] confirmed that protein C deficiency is the most important factor that contributes to the procoagulant status in LC. Rossetto *et al*[34] performed a more complex analysis of the coagulation cascade in cirrhotics with PVT and demonstrated that the complex factor VIIa-antithrombin was significantly higher in PVT patients compared to healthy volunteers. These data were not confirmed by two recent studies conducted by Chen *et al*[35] and Tang W *et al*[36], the results of their case-control analysis concluded that there was no difference regarding the pro- and anticoagulant factors between patients with and without PVT matched for age, sex and Child-Pugh score.

The fibrinolytic system also is involved in PVT development in cirrhotic patients. LC is characterized by increased tissue-type plasminogen activator and plasminogen activator inhibitor-1 levels, and decreased plasminogen, alpha 2-antiplasmin and thrombin-activable fibrinolysis inhibitor levels[37]. This abnormalities in the fibrinolytic cascade could explain the spontaneous recanalization of PVT as described in almost a quarter of the cirrhotic patients[38].

Inherited thrombophilic disorders are reported in up to 70% of patients with cirrhosis and PVT. The most important genetic abnormalities are factor II mutation (G20210A), factor V mutation and the homozygous polymorphisms of methylenetetrahydrofolate reductase (MTHFR) C677T gene mutation[39,40]. D'Amico *et al*[17] confirmed the influence of MTHFR gene mutation in PVT pathogenesis along with the plasminogen activator inhibitor-type 1 4G-4G mutation. Although, it should be mentioned that the prospective longitudinal found studies no clear relationship between inherited factors and PVT development.

Other inherited prothrombotic conditions as hyperhomocysteinemia[40] antiphospholipid syndrome[41] or myeloprolipherative disorders[42] were evaluated, but did not proved as major risk factors for PVT development in cirrhotic patients.

There is evidence that in cirrhotic patients, markers of endothelial dysfunction, including von Willebrand factor, P-selectin and isoprostanes, are up-regulated, suggesting that endothelial cells may favor the PVT development in cirrhosis[4]. Also, Carnevale *et al*[43] demonstrated that the lipopolysaccharide from *Escherichia coli* stimulates factor VIII production from the endothelial cells. Endotoxemia may play an important role in activating the clotting system in portal and systemic circulation and could represent an underlying mechanism for PVT.

The bacterial translocation determines inflammation which leads to hemodynamic alterations and ultimately to an increase in portal pressure[44]. There are studies that describe portal endotoxemia as a triggering factor of the coagulation cascade in cirrhotic patients, although a recent small study on 49 patients with cirrhosis found that endotoxemia and platelet activity were not associated to PVT[45]. Vascular endothelial dysfunction may play a role in the pathogenesis of PVT. All these risk factors could explain the favorable role of prophylactic administration of enoxaparin in delaying the hepatic decompensation and improving survival[46].

The two main risk factors for PVT in LC-reduced portal flow velocity and the procoagulant status should be addressed more extensively in large studies, considering two different scenarios: compensated and decompensated liver disease. This discrimination could influence not only the understanding of the physiopathological mechanism of PVT development, but also the indication for a certain anticoagulant therapy.

Obviously, data received while using the *in vitro* complex coagulation assessment should be considered carefully and not used for generalization[25]. Furthermore, data provided by the use of only one type of coagulation parameter should be partially considered, as there are several studies with different results in regard to pro- and anticoagulant factors levels in PVT[35,36]. As we advanced in understanding the underlying molecular mechanism of thrombosis, the coagulation investigation in cirrhotic patients becomes more complicated, time-consuming, and expensive, thus affordable only to large clinical laboratories. Unfortunately, this kind of comprehensive specific analysis of coagulation disorders in cirrhotic patients with PVT has not yet been conducted, while most of PVT and liver cirrhosis studies remain inconclusive, being based on a small sample size.

The screening for underlying thrombophilic conditions should be considered especially in patients with compensated liver disease in whom the vascular component of the Virchow's triad is not so important. A special category of cirrhotic patients with PVT is represented by those patients in whom PVT extends despite the administration of anticoagulant therapy or reappears after spontaneous recanalization. In such patients there are other risk factors which should be identified such as endothelial dysfunction, genetic thrombophilic disorders or undiagnosed neoplasia that could predispose to PVT.

WHEN AND HOW TO TREAT PVT IN LIVER CIRRHOSIS

The main goal of PVT treatment is to restore the portal blood flow and prevent the thrombus extension.

The Baveno VI Consensus[8], published in 2015, recommends the anticoagulant treatment in cirrhotic patients with PVT who are potential candidates for LT, while no recommendation is made for non-candidates, thus highlighting the need for individualized treatment and randomized trials on the benefit/risk ratio of anticoagulation in cirrhotic patients.

The EASL 2015[7] and 2018[47] guidelines for vascular diseases of the liver and for the management of patients with decompensated liver cirrhosis state that anticoagulant treatment must be considered in cirrhotic patients with PVT following the implementation of an adequate prophylaxis for gastrointestinal bleeding, while in 2009 the AASLD[6] recommended at least three months of anticoagulant use in the treatment of PVT, irrespective of the presence of cirrhosis.

Although the guidelines accepted the anticoagulant treatment or TIPS as therapeutic option for PVT in LC not all centers accepted the idea in the daily clinical practice, so that to treat or not to treat PVT in LC it still remains an open issue.

Low-molecular-weight heparin and vitamin K antagonists

The uncertainty regarding the real efficacy of an anticoagulant treatment derives from the data reporting the natural history of PVT in LC. Studies evaluating the anticoagulant treatment have reported that spontaneous recanalization of the portal vein in the absence of anticoagulant treatment is unusual[12,13]. In the study by Francoz *et al*[48] no patient achieved recanalization in the absence of anticoagulation, while 42% achieved recanalization while under anticoagulant therapy. Senzolo *et al*[49] reported thrombus progression in 75% of patients who did not receive anticoagulant treatment, compared to only 15% of treated patients. There are limited studies reporting on the use of anticoagulation for PVT in patients with cirrhosis. In all these studies, complete recanalization has been described in 33%-45% of cases, while partial portal vein recanalization was observed in 15%-35% of cases[48-53]. Small sample size is one of the major problems of nearly all such investigations. The most cited side effect was the bleeding from different sites: gastrointestinal (variceal bleeding, postligation ulcer, peptic ulcer), intracerebral hemorrhages, epistaxis and hematuria[48-53].

In order to overcome the small sample size bias and increase the efficacy and safeness of the anticoagulant treatment in patients with PVT and LC, two meta-analysis have recently been published[54,55]. Qi *et al*[54] concluded in 2015 that anticoagulation could achieve a relatively high rate of portal vein recanalization in cirrhotic patients with PVT, information confirmed by another meta-analysis published by Loffredo *et al*[55] in 2017.

The attendant optimism is, at least in part, based on the relatively safeness of the anticoagulant treatment, including the pleiomorphic effect of reducing fibrogenesis by thrombin antagonism[56]. For a better evaluation of the anticoagulant treatment in patients with LC other end-points should be established such as short-term and long-term mortality and decompensation or further

decompensation rate. Achieving PVT recanalization is only one of the goals of anticoagulant therapy in cirrhotics with PVT, and it is far more important to document the real impact of recanalization on LC evolution in order to confirm the benefits of this controversial treatment.

If the anticoagulant treatment is the first therapeutic option for cirrhotic patients with PVT, the ideal anticoagulanthas not been developed yet. Low-molecular-weight heparin (LMWH) and vitamin K antagonists (VKAs) are the anticoagulant drugs recommended for PVT treatment, but they have some disadvantages: efficacy of LMWH may be significantly decreased (up to 40%) due to lower levels of antithrombin III synthesis by the liver, and the coagulopathy secondary to liver disease frequently results in an elevated International Normalized Ratio (INR) and thus utilizing the INR to guide dosing of VKAs is particularly challenging[6-8].

Direct oral anticoagulants and PVT treatment

The direct oral anticoagulants (DOACs) - thrombin inhibitors (dabigatran) and activated factor X inhibitors (rivaroxaban, apixaban or edoxaban) overcame the numerous drawbacks of traditional anticoagulants and proved their efficacy and saffness in stroke prophylaxis in nonvalvular atrial fibrillation, venous thrombembolism prophylaxis in orthopaedic patients, and the treatment of acute pulmonary embolism and deep vein thrombosis[57]. DOACs offer the advantage of oral administration, the absence of laboratory monitoring, and an antithrombin III independent mechanism of action. Rivaroxaban and apixaban are 67% metabolized in the liver, with half-lives of 5-9 h and 12 h respectively[58], their concentration depending on the plasma bounding proteins. Edoxaban is 50% metabolized by the liver with a half-live of 10-15 h[58]. Dabigatran has limited hepatic metabolism, minimal binding to plasma proteins, and longer half-life (12-14 h)[57]. Another advantages of dabigatran is the development of an antidote - idarucizumab - monoclonal inhibitor antibody[59].

However, there are few reports regarding their use in cirrhotic patients, most of which in compensated isolated cases. Rivaroxaban is the most studied DOACs for the treatment of PVT[60]. There is little scientific evidence regarding the use of DOACs in cirrhosis with or without PVT, and even fewer well-designed prospective studies

In the VALDIG study, major bleeding requiring discontinuation of DOACs was seen in two of 258 (0.71%) patients without cirrhosis and in one of 36 (2.7%) patients with cirrhosis[61]. Intagliata *et al*[62] retrospectively evaluated class Child-Pugh A and B cirrhotic patients having received DOACs treatment for different conditions. Two thirds of the patients received DOACs for PVT treatment. There are no data reported on the recanalization rate. Major bleeding events occurred in 5% of the patients and a paradoxically PVT recurrence during the anticoagulant treatment was described. In a clinical trial assessing the efficacy of VKAs, Hafany *et al*[63] compared the DOAC rivaroxaban with warfarin in 80 patients with virus C compensated cirrhosis. They reported a 85% recanalization rate, in contrast with the 45% in patients treated with warfarin, higher short-term survival rate and fewer gastrointestinal bleeding events in patients treated with DOACs. Nagaoki *et al*[64] compared edoxaban and warfarin in cirrhotic patients with PVT and, concluded that edoxaban is an effective anticoagulant treatment, although most of the events involving the gastrointestinal bleeding were associated with the administration of edoxaban(15% *vs* 7%).

The recent literature does not establish with certainty the role of DOACs in treating PVT in cirrhotic patients, and further large clinical trials are needed confirm if the DOACs can be used effectively and safely in Child-Pugh A or B liver cirrhosis.

Transjugular intrahepatic portosystemic shunt

Classically considered contraindicated in PVT, TIPS could be an alternative particularly if thrombosis progresses despite satisfactory anticoagulation and/or when PVT is associated with severe portal hypertension complications[1,48]. However, in such cases, TIPS is expected to be technically challenging with a higher failure rate[48,65] and should be attempted only in experienced centres. TIPS may be a treatment option in patients with acute PVT. In chronic PVT or portal cavernoma TIPS is unsuccessful if the lumen of thrombosed portal vein is not catheterizable and cavernomatous vein is not amenable to dilatation.

FUTURES PERSPECTIVES: UNMET NEEDS

Although new data on the mechanisms of PVT development in cirrhotic patients was published and a new complex classification is proposed, there are still a lot of puzzle pieces missing in the big picture of PVT.

The prognostic value of the new PVT classification remains to be confirmed by future prospective studies, without omitting the pattern of PVT evolution and the status of the liver cirrhosis (compensated or decompensated).

Controversies persist regarding the mechanism leading to PVT in LC. The influence of each previously described risk factor, in the pathogenesis of PVT needs to be demonstrated. The role of microbiota and the influence of endotoxinemia in the development of PVT in compensated LC must also to be addressed. The natural history of PVT should be described in large multicenter studies in order to identify predictors for spontaneous recanalization and risk factors for rethrombosis. Updated complex and global dynamic coagulation tests should be developed and validated to assess the coagulation disorders in cirrhotic patients with PVT and even anticoagulant therapy monitoring.

The ideal anticoagulant treatment of PVT in cirrhotic patients is not yet described. DOACs are used off label for PVT treatment in LC despite the lack of randomized

control trials confirming the safety and efficacy. The end-points of these studies should also include short-term and long-term mortality rates together with decompensation outcomes.

No doubt, many advances have been made during the last decade regarding different aspects of PVT pathophysiology and treatment in cirrhotic patients, although this complication of liver cirrhosis still has more questions than answers.

REFERENCES

1 **Tsochatzis EA**, Senzolo M, Germani G, Gatt A, Burroughs AK. Systematic review: portal vein thrombosis in cirrhosis. *Aliment Pharmacol Ther* 2010; **31**: 366-374 [PMID: 19863496 DOI: 10.1111/j.1365-2036.2009.04182.x]

2 **Rajani R**, Björnsson E, Bergquist A, Danielsson A, Gustavsson A, Grip O, Melin T, Sangfelt P, Wallerstedt S, Almer S. The epidemiology and clinical features of portal vein thrombosis: a multicentre study. *Aliment Pharmacol Ther* 2010; **32**: 1154-1162 [PMID: 21039677 DOI: 10.1111/j.1365-2036.2010.04454.x]

3 **Francoz C**, Valla D, Durand F. Portal vein thrombosis, cirrhosis, and liver transplantation. *J Hepatol* 2012; **57**: 203-212 [PMID: 22446690 DOI: 10.1016/j.jhep.2011.12.034]

4 **Tripodi A**, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011; **365**: 147-156 [PMID: 21751907 DOI: 10.1056/NEJMra1011170]

5 **Cagin YF**, Atayan Y, Erdogan MA, Dagtekin F, Colak C. Incidence and clinical presentation of portal vein thrombosis in cirrhotic patients. *Hepatobiliary Pancreat Dis Int* 2016; **15**: 499-503 [PMID: 27733319]

6 **DeLeve LD**, Valla DC, Garcia-Tsao G; American Association for the Study Liver Diseases. Vascular disorders of the liver. *Hepatology* 2009; **49**: 1729-1764 [PMID: 19399912 DOI: 10.1002/hep.22772]

7 **European Association for the Study of the Liver.** EASL Clinical Practice Guidelines: Vascular diseases of the liver. *J Hepatol* 2016; **64**: 179-202 [PMID: 26516032 DOI: 10.1016/j.jhep.2015.07.040]

8 **de Franchis R**, Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]

9 **Sarin SK**, Philips CA, Kamath PS, Choudhury A, Maruyama H, Nery FG, Valla DC. Toward a Comprehensive New Classification of Portal Vein Thrombosis in Patients With Cirrhosis. *Gastroenterology* 2016; **151**: 574-577.e3 [PMID: 27575821 DOI: 10.1053/j.gastro.2016.08.033]

10 **Stieber AC**, Zetti G, Todo S, Tzakis AG, Fung JJ, Marino I, Casavilla A, Selby RR, Starzl TE. The spectrum of portal vein thrombosis in liver transplantation. *Ann Surg* 1991; **213**: 199-206 [PMID: 1998400]

11 **Yerdel MA**, Gunson B, Mirza D, Karayalçin K, Olliff S, Buckels J, Mayer D, McMaster P, Pirenne J. Portal vein thrombosis in adults undergoing liver transplantation: risk factors, screening, management, and outcome. *Transplantation* 2000; **69**: 1873-1881 [PMID: 10830225]

12 **Nery F**, Chevret S, Condat B, de Raucourt E, Boudaoud L, Rautou PE, Plessier A, Roulot D, Chaffaut C, Bourcier V, Trinchet JC, Valla DC; Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. Causes and consequences of portal vein thrombosis in 1,243 patients with cirrhosis: results of a longitudinal study. *Hepatology* 2015; **61**: 660-667 [PMID: 25284616 DOI: 10.1002/hep.27546]

13 **Maruyama H**, Okugawa H, Takahashi M, Yokosuka O. De novo portal vein thrombosis in virus-related cirrhosis: predictive factors and long-term outcomes. *Am J Gastroenterol* 2013; **108**: 568-574 [PMID: 23381015 DOI: 10.1038/ajg.2012.452]

14 **Chen H**, Trilok G, Wang F, Qi X, Xiao J, Yang C. A single hospital study on portal vein thrombosis in cirrhotic patients - clinical characteristics & risk factors. *Indian J Med Res* 2014; **139**: 260-266 [PMID: 24718401]

15 **Amitrano L**, Guardascione MA, Brancaccio V, Margaglione M, Manguso F, Iannaccone L, Grandone E, Balzano A. Risk factors and clinical presentation of portal vein thrombosis in patients with liver cirrhosis. *J Hepatol* 2004; **40**: 736-741 [PMID: 15094219 DOI: 10.1016/j.jhep.2004.01.001]

16 **Zocco MA**, Di Stasio E, De Cristofaro R, Novi M, Ainora ME, Ponziani F, Riccardi L, Lancellotti S, Santoliquido A, Flore R, Pompili M, Rapaccini GL, Tondi P, Gasbarrini GB, Landolfi R, Gasbarrini A. Thrombotic risk factors in patients with liver cirrhosis: correlation with MELD scoring system and portal vein thrombosis development. *J Hepatol* 2009; **51**: 682-689 [PMID: 19464747 DOI: 10.1016/j.jhep.2009.03.013]

17 **D'Amico M**, Pasta F, Pasta L. Thrombophilic genetic factors PAI-1 4G-4G and MTHFR 677TT as risk factors of alcohol, cryptogenic liver cirrhosis and portal vein thrombosis, in a Caucasian population. *Gene* 2015; **568**: 85-88 [PMID: 25987440 DOI: 10.1016/j.gene.2015.05.034]

18 **Li MX**, Zhang XF, Liu ZW, Lv Y. Risk factors and clinical characteristics of portal vein thrombosis after splenectomy in patients with liver cirrhosis. *Hepatobiliary Pancreat Dis Int* 2013; **12**: 512-519 [PMID: 24103282]

19 **Violi F**, Corazza GR, Caldwell SH, Perticone F, Gatta A, Angelico M, Farcomeni A, Masotti M, Napoleone L, Vestri A, Raparelli V, Basili S; PRO-LIVER Collaborators. Portal vein thrombosis relevance on liver cirrhosis: Italian Venous Thrombotic Events Registry. *Intern Emerg Med* 2016; **11**: 1059-1066 [PMID: 27026379 DOI: 10.1007/s11739-016-1416-8]

20 **Nonami T**, Yokoyama I, Iwatsuki S, Starzl TE. The incidence of portal vein thrombosis at liver transplantation. *Hepatology* 1992; **16**: 1195-1198 [PMID: 1427658]

21 **Scheiner B**, Stammet PR, Pokorny S, Bucsics T, Schwabl P, Brichta A, Thaler J, Lampichler K, Ba-Ssalamah A, Ay C, Ferlitsch A, Trauner M, Mandorfer M, Reiberger T. Anticoagulation in non-malignant portal vein thrombosis is safe and improves hepatic function. *Wien Klin Wochenschr* 2018; [PMID: 29916054 DOI: 10.1007/s00508-018-1351-y]

22 **Cruz-Ramón V**, Chinchilla-López P, Ramírez-Pérez O, Aguilar-Olivos NE, Alva-López LF, Fajardo-Ordoñez E, Ponciano-Rodríguez G, Northup PG, Intagliata N, Caldwell SH, Qi X, Méndez-Sánchez N. Thrombosis of the Portal Venous System in Cirrhotic vs. Non-Cirrhotic Patients. *Ann Hepatol* 2018; **17**: 476-481 [PMID: 29735798 DOI: 10.5604/01.3001.0011.7392]

23 **Hernández Conde M**, Llop Herrera E, de la Revilla Negro J, Pons Renedo F, Fernández Puga N, Martínez Porras JL, Trapero Marugan M, Cuervas-Mons V, Sánchez Turrión V, Calleja Panero JL. Prevalence and outcome of portal thrombosis in a cohort of cirrhotic patients undergoing liver transplantation. *Rev Esp Enferm Dig* 2016; **108**: 716-720 [PMID: 27756145 DOI: 10.17235/reed.2016.4211/2016]

24 **Chaireti R**, Rajani R, Bergquist A, Melin T, Friis-Liby IL, Kapraali M, Kechagias S, Lindahl TL, Almer S. Increased thrombin generation in splanchnic vein thrombosis is related to the presence of liver cirrhosis and not to the thrombotic event. *Thromb Res* 2014; **134**: 455-461 [PMID: 24913997 DOI: 10.1016/j.thromres.2014.05.012]

25 **Tripodi A**, Salerno F, Chantarangkul V, Clerici M, Cazzaniga M, Primignani M, Mannuccio Mannucci P. Evidence of normal thrombin generation in cirrhosis despite abnormal conventional coagulation tests. *Hepatology* 2005; **41**: 553-558 [PMID: 15726661 DOI: 10.1002/hep.20569]

26 **Rossetto V**, Spiezia L, Senzolo M, Rodriguez-Castro KI, Maggiolo S, Simioni P. Whole blood rotation thromboelastometry (ROTEM ®) profiles in subjects with non-neoplastic portal vein thrombosis. *Thromb Res* 2013; **132**: e131-e134 [PMID: 23810655 DOI: 10.1016/j.thromres.2013.06.009]

27 **Stine JG**, Wang J, Shah PM, Argo CK, Intagliata N, Uflacker A, Caldwell SH, Northup PG. Decreased portal vein velocity is predictive of the development of portal vein thrombosis: A matched case-control study. *Liver Int* 2018; **38**: 94-101 [PMID: 28632958 DOI: 10.1111/liv.13500]

28 **Zampino R**, Lebano R, Coppola N, Macera M, Grandone A, Rinaldi L, De Sio I, Tufano A, Stornaiuolo G, Adinolfi LE, Durante-Mangoni E, Battista GG, Niglio A. The use of nonselective beta blockers is a risk factor for portal vein thrombosis in cirrhotic patients. *Saudi J Gastroenterol* 2018; **24**: 25-29 [PMID: 29451181 DOI: 10.4103/sjg.SJG_100_17]

29 **Pradella P**, Bonetto S, Turchetto S, Uxa L, Comar C, Zorat F, De Angelis V, Pozzato G. Platelet production and destruction in liver cirrhosis. *J Hepatol* 2011; **54**: 894-900 [PMID: 21145808 DOI: 10.1016/j.jhep.2010.08.018]

30 **Lisman T**, Porte RJ. Platelet function in patients with cirrhosis. *J Hepatol* 2012; **56**: 993-994; author reply 994-995 [PMID: 22424439 DOI: 10.1016/j.jhep.2011.08.013]

31 **Caldwell SH**, Sanyal AJ. Coagulation disorders and bleeding in liver disease: future directions. *Clin Liver Dis* 2009; **13**: 155-157 [PMID: 19150319 DOI: 10.1016/j.cld.2008.09.011]

32 **Saner FH**, Gieseler RK, Akız H, Canbay A, Görlinger K. Delicate balance of bleeding and thrombosis in end-stage liver disease and liver transplantation. *Digestion* 2013; **88**: 135-144 [PMID: 24008288 DOI: 10.1159/000354400]

33 **Tripodi A**, Primignani M, Lemma L, Chantarangkul V, Mannucci PM. Evidence that low protein C contributes to the procoagulant imbalance in cirrhosis. *J Hepatol* 2013; **59**: 265-270 [PMID: 23583273 DOI: 10.1016/j.jhep.2013.03.036]

34 **Rossetto V**, Spiezia L, Senzolo M, Rodriguez K, Gavasso S, Woodhams B, Simioni P. Factor VIIa-antithrombin complexes in patients with non-neoplastic portal vein thrombosis with and without cirrhosis. *Int J Lab Hematol* 2013; **35**: 101-105 [PMID: 22958499 DOI: 10.1111/ijlh.12003]

35 **Chen H**, Qi X, He C, Yin Z, Fan D, Han G. Coagulation imbalance may not contribute to the development of portal vein thrombosis in patients with cirrhosis. *Thromb Res* 2013; **131**: 173-177 [PMID: 23157737 DOI: 10.1016/j.thromres.2012.11.003]

36 **Tang W**, Wang Y, Zhao X, Wang X, Zhang T, Ou X, Shou W, You H, Jia J. Procoagulant imbalance aggravated with falling liver function reserve, but not associated with the presence of portal vein thrombosis in cirrhosis. *Eur J Gastroenterol Hepatol* 2015; **27**: 672-678 [PMID: 25923942 DOI: 10.1097/MEG.0000000000000352]

37 **Violi F**, Ferro D. Clotting activation and hyperfibrinolysis in cirrhosis: implication for bleeding and thrombosis. *Semin Thromb Hemost* 2013; **39**: 426-433 [PMID: 23487343 DOI: 10.1055/s-0033-1334144]

38 **Girleanu I**, Stanciu C, Cojocariu C, Boiculese L, Singeap AM, Trifan A. Natural course of nonmalignant partial portal vein thrombosis in cirrhotic patients. *Saudi J Gastroenterol* 2014; **20**: 288-292 [PMID: 25253363 DOI: 10.4103/1319-3767.141687]

39 **Amitrano L**, Guardascione MA, Ames PR, Margaglione M, Iannaccone L, Brancaccio V, Balzano A. Increased plasma prothrombin concentration in cirrhotic patients with portal vein thrombosis and prothrombin G20210A mutation. *Thromb Haemost* 2006; **95**: 221-223 [PMID: 16493481 DOI: 10.1160/TH05-08-0555]

40 **Ventura P**, Venturelli G, Marcacci M, Fiorini M, Marchini S, Cuoghi C, Pietrangelo A. Hyperhomocysteinemia and MTHFR C677T polymorphism in patients with portal vein thrombosis complicating liver cirrhosis. *Thromb Res* 2016; **141**: 189-195 [PMID: 27065203 DOI: 10.1016/j.thromres.2016.03.024]

41 **Amitrano L**, Ames PR, Guardascione MA, Lopez LR, Menchise A, Brancaccio V, Iannaccone L, Balzano A. Antiphospholipid antibodies and antiphospholipid syndrome: role in portal vein thrombosis in patients with and without liver cirrhosis. *Clin Appl Thromb Hemost* 2011; **17**: 367-370 [PMID: 20530055 DOI: 10.1177/1076029610369797]

42 **Smalberg JH**, Arends LR, Valla DC, Kiladjian JJ, Janssen HL, Leebeek FW. Myeloproliferative neoplasms in Budd-Chiari syndrome and portal vein thrombosis: a meta-analysis. *Blood* 2012; **120**: 4921-4928 [PMID: 23043069 DOI: 10.1182/blood-2011-09-376517]

43 **Carnevale R**, Raparelli V, Nocella C, Bartimoccia S, Novo M, Severino A, De Falco E, Cammisotto V, Pasquale C, Crescioli C, Scavalli AS, Riggio O, Basili S, Violi F. Gut-derived endotoxin stimulates factor VIII secretion from endothelial cells. Implications for hypercoagulability in cirrhosis. *J Hepatol* 2017; **67**: 950-956 [PMID: 28716745 DOI: 10.1016/j.jhep.2017.07.002]

44 **Fukui H**. Gut-liver axis in liver cirrhosis: How to manage leaky gut and endotoxemia. *World J Hepatol* 2015; **7**: 425-442 [PMID: 25848468 DOI: 10.4254/wjh.v7.i3.425]

45 **Wosiewicz P**, Żorniak M, Hartleb M, Barański K, Hartleb M, Onyszczuk M, Pilch-Kowalczyk J, Kyrcz-Krzemień S. Portal vein thrombosis in cirrhosis is not associated with intestinal barrier disruption or increased platelet aggregability. *Clin Res Hepatol Gastroenterol* 2016; **40**: 722-729 [PMID: 27160816 DOI: 10.1016/j.clinre.2016.03.008]

46 **Villa E**, Cammà C, Marietta M, Luongo M, Critelli R, Colopi S, Tata C, Zecchini R, Gitto S, Petta S, Lei B, Bernabucci V, Vukotic R, De Maria N, Schepis F, Karampatou A, Caporali C, Simoni L, Del Buono M, Zambotto B, Turola E, Fornaciari G, Schianchi S, Ferrari A, Valla D. Enoxaparin prevents portal vein thrombosis and liver decompensation in patients with advanced cirrhosis. *Gastroenterology* 2012; **143**: 1253-1260.e4 [PMID: 22819864 DOI: 10.1053/j.gastro.2012.07.018]

47 **European Association for the Study of the Liver**. European Association for the Study of the Liver. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. *J Hepatol* 2018; **69**: 406-460 [PMID: 29653741 DOI: 10.1016/j.jhep.2018.03.024]

48 **Francoz C**, Belghiti J, Vilgrain V, Sommacale D, Paradis V, Condat B, Denninger MH, Sauvanet A, Valla D, Durand F. Splanchnic vein thrombosis in candidates for liver transplantation: usefulness of screening and anticoagulation. *Gut* 2005; **54**: 691-697 [PMID: 15831918 DOI: 10.1136/gut.2004.042796]

49 **Senzolo M**, M Sartori T, Rossetto V, Burra P, Cillo U, Boccagni P, Gasparini D, Miotto D, Simioni P, Tsochatzis E, A Burroughs K. Prospective evaluation of anticoagulation and transjugular intrahepatic portosystemic shunt for the management of portal vein thrombosis in cirrhosis. *Liver Int* 2012; **32**: 919-927 [PMID: 22435854 DOI: 10.1111/j.1478-3231.2012.02785.x]

50 **Werner KT**, Sando S, Carey EJ, Vargas HE, Byrne TJ, Douglas DD, Harrison ME, Rakela J, Aqel BA. Portal vein thrombosis in patients with end stage liver disease awaiting liver transplantation: outcome of anticoagulation. *Dig Dis Sci* 2013; **58**: 1776-1780 [PMID: 23314858 DOI: 10.1007/s10620-012-2548-y]

51 **Amitrano L**, Guardascione MA, Menchise A, Martino R, Scaglione M, Giovine S, Romano L, Balzano A. Safety and efficacy of anticoagulation therapy with low molecular weight heparin for portal vein thrombosis in patients with liver cirrhosis. *J Clin Gastroenterol* 2010; **44**: 448-451 [PMID: 19730112 DOI: 10.1097/MCG.0b013e3181b3ab44]

52 **Chen H**, Liu L, Qi X, He C, Wu F, Fan D, Han G. Efficacy and safety of anticoagulation in more advanced portal vein thrombosis in patients with liver cirrhosis. *Eur J Gastroenterol Hepatol* 2016; **28**: 82-89 [PMID: 26513611 DOI: 10.1097/MEG.0000000000000482]

53 **Dell'Era A**, Iannuzzi F, Fabris FM, Fontana P, Reati R, Grillo P, Aghemo A, de Franchis R, Primignani M. Impact of portal vein thrombosis on the efficacy of endoscopic variceal band ligation. *Dig Liver Dis* 2014; **46**: 152-156 [PMID: 24084343 DOI: 10.1016/j.dld.2013.08.138]

54 **Qi X**, De Stefano V, Li H, Dai J, Guo X, Fan D. Anticoagulation for the treatment of portal vein thrombosis in liver cirrhosis: a systematic review and meta-analysis of observational studies. *Eur J Intern Med* 2015; **26**: 23-29 [PMID: 25566699 DOI: 10.1016/j.ejim.2014.12.002]

55 **Loffredo L**, Pastori D, Farcomeni A, Violi F. Effects of Anticoagulants in Patients With Cirrhosis and Portal Vein

Thrombosis: A Systematic Review and Meta-analysis. *Gastroenterology* 2017; **153**: 480-487.e1 [PMID: 28479379 DOI: 10.1053/j.gastro.2017.04.042]

56 **Dhar A**, Sadiq F, Anstee QM, Levene AP, Goldin RD, Thursz MR. Thrombin and factor Xa link the coagulation system with liver fibrosis. *BMC Gastroenterol* 2018; **18**: 60 [PMID: 29739329 DOI: 10.1186/s12876-018-0789-8]

57 **Ageno W**, Gallus AS, Wittkowsky A, Crowther M, Hylek EM, Palareti G. Oral anticoagulant therapy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012; **141**: e44S-e88S [PMID: 22315269 DOI: 10.1378/chest.11-2292]

58 **DeWald TA**, Becker RC. The pharmacology of novel oral anticoagulants. *J Thromb Thrombolysis* 2014; **37**: 217-233 [PMID: 23860880 DOI: 10.1007/s11239-013-0967-z]

59 **Hu TY**, Vaidya VR, Asirvatham SJ. Reversing anticoagulant effects of novel oral anticoagulants: role of ciraparantag, andexanet alfa, and idarucizumab. *Vasc Health Risk Manag* 2016; **12**: 35-44 [PMID: 26937198 DOI: 10.2147/VHRM.S89130]

60 **Priyanka P**, Kupec JT, Krafft M, Shah NA, Reynolds GJ. Newer Oral Anticoagulants in the Treatment of Acute Portal Vein Thrombosis in Patients with and without Cirrhosis. *Int J Hepatol* 2018; **2018**: 8432781 [PMID: 29973997 DOI: 10.1155/2018/8432781]

61 **De Gottardi A**, Trebicka J, Klinger C, Plessier A, Seijo S, Terziroli B, Magenta L, Semela D, Buscarini E, Langlet P, Görtzen J, Puente A, Müllhaupt B, Navascuès C, Nery F, Deltenre P, Turon F, Engelmann C, Arya R, Caca K, Peck-Radosavljevic M, Leebeek FWG, Valla D, Garcia-Pagan JC; VALDIG Investigators. Antithrombotic treatment with direct-acting oral anticoagulants in patients with splanchnic vein thrombosis and cirrhosis. *Liver Int* 2017; **37**: 694-699 [PMID: 27778440 DOI: 10.1111/liv.13285]

62 **Intagliata NM**, Henry ZH, Maitland H, Shah NL, Argo CK, Northup PG, Caldwell SH. Direct Oral Anticoagulants in Cirrhosis Patients Pose Similar Risks of Bleeding When Compared to Traditional Anticoagulation. *Dig Dis Sci* 2016; **61**: 1721-1727 [PMID: 26725062 DOI: 10.1007/s10620-015-4012-2]

63 **Hanafy AS**, Abd-Elsalam S, Dawoud MM. Randomized controlled trial of rivaroxaban versus warfarin in the management of acute non-neoplastic portal vein thrombosis. *Vascul Pharmacol* 2018 [PMID: 29886103 DOI: 10.1016/j.vph.2018.05.002]

64 **Nagaoki Y**, Aikata H, Daijyo K, Teraoka Y, Shinohara F, Nakamura Y, Hatooka M, Morio K, Nakahara T, Kawaoka T, Tsuge M, Hiramatsu A, Imamura M, Kawakami Y, Ochi H, Chayama K. Efficacy and safety of edoxaban for treatment of portal vein thrombosis following danaparoid sodium in patients with liver cirrhosis. *Hepatol Res* 2018; **48**: 51-58 [PMID: 28342265 DOI: 10.1111/hepr.12895]

65 **Lv Y**, He C, Wang Z, Guo W, Wang J, Bai W, Zhang L, Wang Q, Liu H, Luo B, Niu J, Li K, Tie J, Yin Z, Fan D, Han G. Association of Nonmalignant Portal Vein Thrombosis and Outcomes after Transjugular Intrahepatic Portosystemic Shunt in Patients with Cirrhosis. *Radiology* 2017; **285**: 999-1010 [PMID: 28682164 DOI: 10.1148/radiol.2017162266]

P- Reviewer: Lutz P, Wu B **S- Editor**: Wang XJ **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4428-4435

DOI: 10.3748/wjg.v24.i39.4428

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

EDITORIAL

Novel targeting approaches and signaling pathways of colorectal cancer: An insight

Ankita Tiwari, Shivani Saraf, Amit Verma, Pritish Kumar Panda, Sanjay K Jain

Ankita Tiwari, Shivani Saraf, Amit Verma, Pritish Kumar Panda, Sanjay K Jain, Pharmaceutics Research Projects Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Central University, Sagar 470003, India

ORCID number: Ankita Tiwari (0000-0001-6433-7831); Shivani Saraf (0000-0001-7305-6054); Amit Verma (0000-0002-1376-0514); Pritish Kumar Panda (0000-0002-1422-5345); Sanjay K Jain (0000-0002-9241-3114).

Author contributions: Tiwari A drafted the manuscript after searching appropriate literature; Panda PK contributed in the writing work; Saraf S and Verma A contributed the related figures; Jain SK critically interpreted the findings of other scientists and revised the final version of the manuscript.

Conflict-of-interest statement: No conflict of interest exists.

Manuscript source: Invited manuscript

Correspondence to: Sanjay K Jain, PhD, Full Professor, Pharmaceutics Research Projects Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Central University, Sagar 470003, India. drskjainin@yahoo.com
Telephone: +91-7582-265457
Fax: +91-7582-264236

Received: July 10, 2018
Peer-review started: July 10, 2018
First decision: July 18, 2018
Revised: August 24, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018

Published online: October 21, 2018

Abstract

Colorectal cancer (CRC) is the third most common cancer of mortality in the world. Chemotherapy based treatment leads to innumerable side effects as it delivers the anticancer drug to both normal cells besides cancer cells. Sonic Hedgehog (SHH), Wnt wingless-type mouse mammary tumor virus/β-catenin, transforming growth factor-β/SMAD, epidermal growth factor receptor and Notch are the main signaling pathways involved in the progression of CRC. Targeted therapies necessitate information regarding the particular aberrant pathways. Advancements in gene therapies have resulted in the recognition of novel therapeutic targets related with these signal-transduction cascades. CRC is a step-wise process where mutations occur over the time and activation of oncogenes and deactivation of tissue suppressor genes takes place. Genetic changes which are responsible for the induction of carcinogenesis include loss of heterozygosity in tumor suppressor genes such as adenomatous polyposis coli, mutation or deletion of genes like p53 and K-ras. Therefore, many gene-therapy approaches like gene correction, virus-directed enzyme-prodrug therapy, immunogenetic manipulation and virotherapy are currently being explored. Development of novel strategies for the safe and effective delivery of drugs to the cancerous site is the need of the hour. This editorial accentuates different novel strategies with emphasis on gene therapy and immunotherapy for the management of CRC.

Key words: Colorectal cancer; Immunotherapy; Gene therapy; Signaling; Targeted therapy

Core tip: In spite of the advancements in the diagnosis and the treatment approaches for colorectal cancer (CRC), its survival rate is quite low. Therefore, there arises an urge to develop novel targeting strategies for its effective treatment. A meticulous apprehension of the signaling cascade is necessitated for better outcomes. In a nutshell, this editorial highlights various novel targeting approaches like gene therapy and immunotherapy which could usher better targeting of CRC.

Tiwari A, Saraf S, Verma A, Panda PK, Jain SK. Novel targeting approaches and signaling pathways of colorectal cancer: An insight. *World J Gastroenterol* 2018; 24(39): 4428-4435 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4428.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4428

INTRODUCTION

Colorectal cancer (CRC) is the third most predominant cancer amongst the world. In 2017, 97220 and 43030 new patients of colon and rectum cancers were reported in United States, respectively. CRC is manifested by the development of adenomatous polyps and malignant cells in the colon. These abnormal cells producing tumors are characterized by uncontrolled replication and the property of metastasis. The early detection, diagnosis, and the utilization of efficient and safe delivery systems would tremendously enhance the efficacy of therapy. The novel targeting approaches (Figure 1), of raising concern as manifested by cancer drugs in the past years, block transduction pathways leading to the cell death through apoptosis and triggering of the immune system, or deliver anticancer drugs to cancer cells, reducing the side effects. The major pathways which could be targeted for CRC therapy are, Sonic Hedgehog (SHH), Wnt/β-catenin, transforming growth factor-β (TGF-β)/SMAD, EGFR and Notch pathways[1,2] (Figure 2).

The Hh pathway is crucial in the normal development of various organs like gut epithelium. The Hh ligands bind to the Patched protein (Ptch) receptor, which subdues the activity of Smoothened (Smoh) receptor. Binding of the ligands to PTCH1 results in the Smoh-mediated activation of GLI transcription factors, which then modulates the expression of various Hh target genes. The expression of SHH, SMO, GLI1 mRNA in colon cancer tissues is remarkably enhanced as compared to the normal cells[3]. Vismodegib is an Hh inhibitor which acts by targeting Smoothened which is a modulator of the Hh pathway. In order to enquire novel Hh antagonists with apoptosis-triggering activity, a group of ~300 potential smoothened antagonists were screened. In colon cancer cells, Hh003 triggered caspase-dependent apoptosis whereas no apoptotic activity was depicted by vismodegib. In comparison to vismodegib, Hh003 displayed similar suppression on the Hh pathway. Hh003 depicted more suppression of the *in vitro* tumor forming colonies and colon cancer proliferation *in vivo*[4].

Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6) are the targets of the Wnt family of proteins. The primary element of the Wnt/β-catenin signaling pathway is the β-catenin destruction complex; which is comprised of a tumor suppressor protein encoded by the antigen-presenting cells (APC) gene, Axin, CKI, and GSK3. When the receptor binding does not occur, this complex undergoes binding with the β-catenin protein (encoded by *CTNNB1* gene), which then undergoes degradation through an ubiquitin-proteasome pathway. In contrary, binding of the receptor by Wnt ligands causes the deactivation of the β-catenin destruction complex and accumulation of β-catenin. It is then translocated to the nucleus for complex formation with T-cell factor/lymphoid enhancer factor, a transcription factor, causing the transcriptional actuation of the target genes. In majority of colon cancers (sporadic) mutation of both alleles of APC (a tumor suppressor gene) occurs which leads to stabilization of β-catenin and stimulation of WNT pathway genes, like TCF, which are needed for the maintenance of colon crypt. In few colon cancers identification of point mutation in β-catenin bearing wild-type alleles of APC has been done[5]. Aquaporin5 (AQP5), a water protein channel, has an oncogenic activity in many types of malignant cancers like CRC. The effect of AQP5 silencing on 5-fluorouracil (5-FU) sensitivity was inquired in cancer cells. It was observed that the Wnt/β-catenin pathway mediated the 5-FU chemosensitivity. AQP5 silencing suppressed the Wnt pathway. While, overexpression of the β-catenin (S33Y) mutant (which shows resistance to degradation) reversed the apoptosis process triggered by AQP5 silencing[6]. Berberine, which is an alkaloid derived from plants and its synthetic 13-arylalkyl derivatives have been accounted to possess antitumor potential; they were investigated for their involvement in Wnt/β-catenin signaling cascade. The cellular levels of active β-catenin were found to decrease accompanied by a rise in the expression of E-cadherin. The berberine derivatives depicted a 100-times reduced EC50 values in comparison to berberine for Wnt-repression[7]. Esculetin, (6, 7-dihydroxycoumarin) potentially inhibits the Wnt-β-catenin pathway. It interrupted the β-catenin-Tcf complex formation by binding with the Lys312, Gly307, Lys345, and Asn387 residues of β-catenin in tumor cells. Besides, esculetin efficaciously reduced the viability and suppressed the anchorage-independent proliferation of cancer cells[8]. Novel Wnt signaling inhibitors, isopropyl 9-ethyl-1- (naphthalen-1-yl)-9H-pyrido (3, 4-b) indole-3-carboxylate (Z86) have been recognized. Z86 suppressed the Wnt signaling functions and genes expression in mammalian cells. It suppressed the GSK3β (Ser9) phosphorylation, causing its overactivity and elevating the phosphorylation and β-catenin degradation[9].

TGF-β and BMP signaling pathways are often impaired in CRC. Ligand-induced oligomerization of the TGFBR1

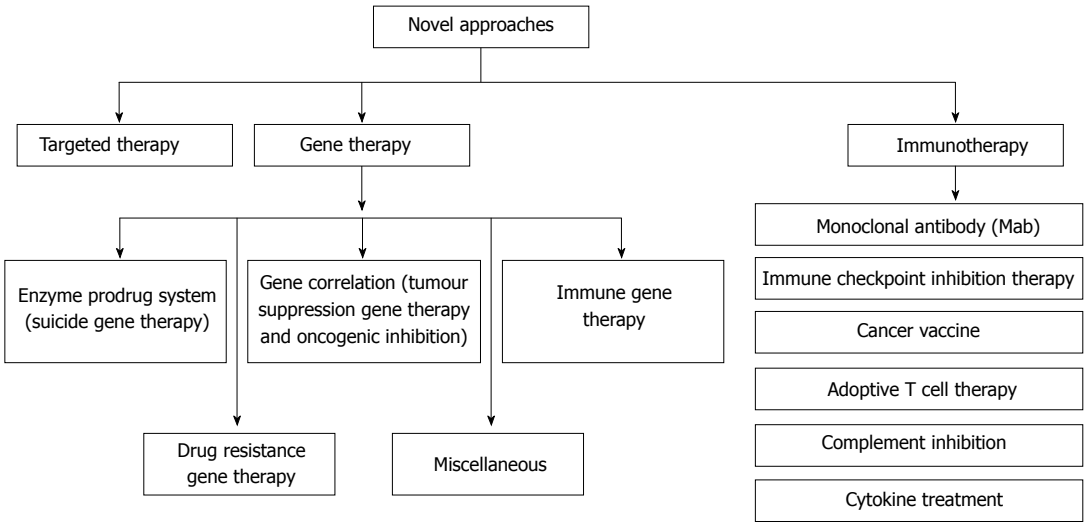


Figure 1 Various novel approaches for the treatment of colorectal cancer.

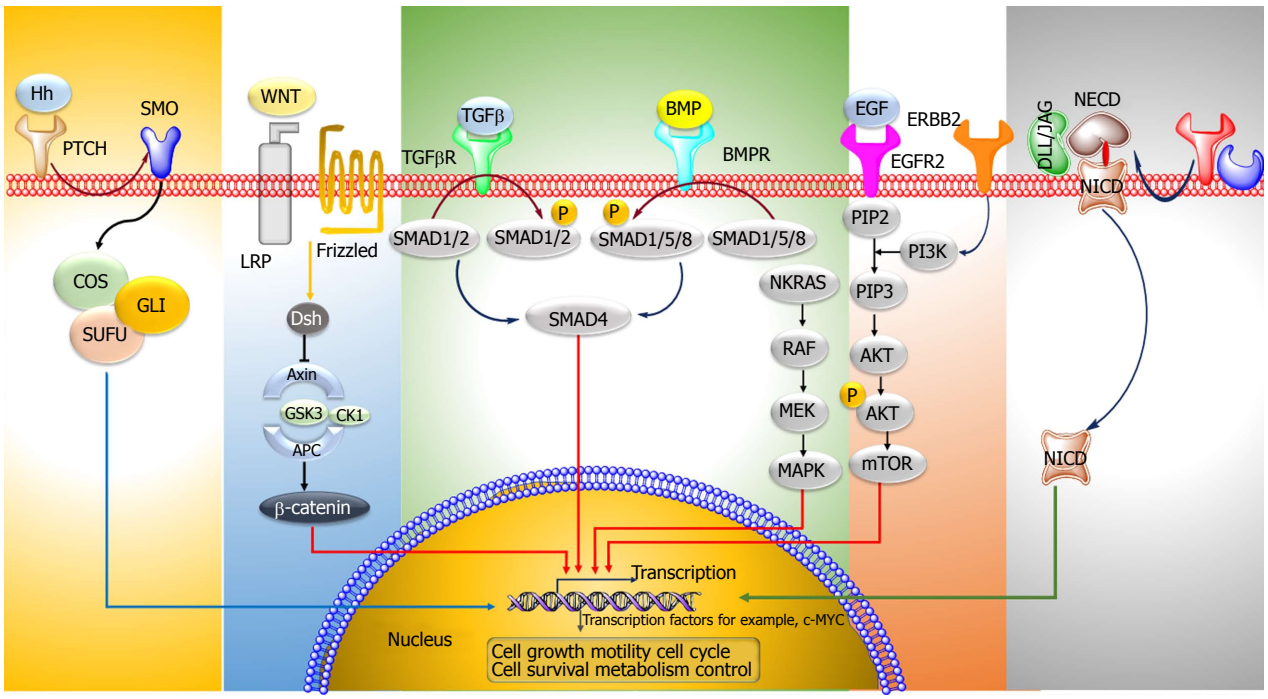


Figure 2 Signaling pathways involved in colorectal cancer. TGF-β: Transforming growth factor-β; LRP: Lipoprotein receptor-related protein; Dsh: Phosphoprotein Dishevelled; GSK3: Glycogen synthase kinase-3; CK1: Casein kinase 1; PI3K: Phosphoinositide 3-kinase; PIP2: Phosphatidylinositol biphosphate; PIP3: Phosphatidylinositol 3,4,5-triphosphate; EGF: Epidermal growth factor; EGFR2: Epidermal growth factor receptor 2; BMPR: Bone morphogenetic proteins receptor; BMP: Bone morphogenetic proteins; RAF: Rapidly Accelerated Fibrosarcoma; MEK: Mitogen-activated protein kinase; AKT: Protein kinase B; MAPK: Mitogen-activated protein kinases; SUFU: Suppressor of fused homolog.

serine/threonine receptor kinases leads to the initiation of the signal cascade succeeded by the phosphorylation of Smad1, Smad2 and Smad3 (signaling molecules). This leads to their association with Smad4 (signaling transducer) and translocation to the nucleus. Triggered Smads modulate various biological effects by binding to transcription factors and leading to the modulation of transcription. Juvenile polyposis is observed in colon cancer due to mutated Smad4 or BMPRI. In most of sporadic colon cancers, the phosphorylation of Smad1, Smad5 and Smad8 does not occur[10]. Genistein (obtained from soybean) is an isoflavone possessing an anticancer potential. A dose-dependent rise in TGF-beta1 mRNA expression was found in MC-26 cells in mouse. It stimulated the generation of Smad-DNA complexes and phosphorylated Smad2 and Smad3, depicting enhanced TGF-beta1 signaling[11].

The binding of epidermal growth factor and TGF to the EGFR, leads to the stimulation of homodimerization/heterodimerization of the receptor and phosphorylation of specific tyrosine residues (P). This in turn stimulates the downstream RAS/RAF/mitogen-activated protein

Table 1 Nanotechnology based drug delivery systems for colorectal cancer targeting

System	Chemotherapeutic agent	Significance	Ref.
Nanoparticles	Resveratrol (RSV)	Sustained release of RSV (over 72 h), and drug solubility enhancement	[17]
Micellar delivery system	Docetaxel	Enhanced the efficacy of hydrophobic chemotherapy and reduced systemic toxicity	[18]
Self-nanoemulsifying drug delivery systems (SNEDDS)	Sunitinib malate	Enhancement of *in vitro* dissolution rate and anticancer potential of drugs possessing low water solubility such as sunitinib malate	[19]
Small molecule-based theranostic system, Gal-Dox	Doxorubicin	Drug localization and site of action can be monitored	[20]
Polymeric micelles	Tanshinone IIA (TAN)	Improved efficacy of anticancer drugs and promoted the growth of beneficial commensal flora in the gut	[21]
Pressure-sensitive nanogels	5-Fluorouracil (5-FU)	Higher 5-FU intracellular accumulation and a significant cell death extension by apoptosis	[22]
Microspheres	Atorvastatin and celecoxib	Synergistic effect on colon cancer prevention and inhibition	[23]
Microbeads	Doxorubicin	Exhibited reduction-responsive character, release the DOX in reducing environments due to cleavage of the disulfide linkers	[24]
Carboxymethyl dextran (CMD) chitosan nanoparticles	Small interfering RNA	Significant changes of Epithelial mesenchymal transition genes and apoptosis	[25]
Liposomes	Apatinib	cRGD-modified liposomes displayed greater apoptosis	[26]

kinase (MAPK) and phosphoinositide 3'-kinase (PI3K) signaling pathways and expression of genes responsible for cell proliferation, angiogenesis and metastasis. KRAS2 and BRAF mutations have been seen in colon cancer. Mutations in PIK3CA which is the p110α catalytic subunit of PI3K have also been observed in few cases of colon cancers[12]. Everolimus (an inhibitor of mTOR) in combination with nilotinib (a platelet-derived growth factor receptors tyrosine kinase inhibitor) suppressed the growth and liver metastasis of colon cancer. The stromal reaction and cancer cell proliferation was reduced and apoptosis was stimulated in tumor cells[13].

The Notch signaling pathway is involved in the growth of intestinal epithelium. Notch ligands *i.e.*, Delta-like (DLL) bind to their transmembrane receptors (Notch 1-4) and induce the proteolytic breakdown of the receptors by the enzymes α-secretase and γ-secretase to release the intracellular domain of the Notch receptor. The cleaved Notch receptors (NICD) are then transferred into the nucleus which forms complexes with RBP-jk (CSL or CBF-1) and lead to the stimulation of Notch-target gene Transforming growth factor-β. An overexpression of ligands namely Jagged1, Jagged2, DLL1, DLL3, DLL4, Notch receptors 1-4 and genes like hairy-enhancer-of-split (Hes-1), Deltex and Notch intracellular domain (NICS) has been observed in colorectal cancer cells[14]. Withaferin-A is a natural compound (source Withania somnifera), which curbs Notch-1 signaling and downregulates various pathways like Akt/NF-kappa B/Bcl-2, in HCT-116, SW-480, and SW-620 cell lines. Besides, Withaferin-A downregulated the expression of mammalian target of rapamycin (mTOR) signaling components, pS6K and p4E-BP1, and stimulated c-Jun-NH (2)-kinase-mediated apoptosis in tumor cells[15].

TARGETED THERAPY

Nanotechnology is a rising arena in drug delivery which furnishes many advantages over the conventional system. Colon-specific novel delivery systems would allow for the local delivery of a high concentration of drugs in the colon to improve pharmacotherapy and reduce its potential systemic toxicity and side effects. Recently, theranostic nanocarriers are introduced to simultaneously monitor and treat the disease using a single delivery system[16]. Colon targeted nanocarriers have been described in brief in the Table 1[17-26].

GENE THERAPY

It involves introduction of genetic components for treating various diseases including cancer. The genetic component may be the nucleic acid *i.e.*, DNA or RNA which may help to replace or correct the malfunction due to defective genes. Gene therapy can also be utilized to actuate an immune response or itself used as a therapeutic agent.

Progression of colorectal cancer is mediated by mutation and aberration of genes. Modification and correction of these defective genes and prevention of those overexpressed genes can have the capability to prevent CRC. The alteration of multiple genes is involved in the development of colon carcinogenesis. Point mutation, formation of oncogenes, de-regulation or deletion of proto-oncogenes and lack of function of suppressor-oncogenes may lead to cancer.

Till November 2017, near about 2600 clinical trials had been conducted in 38 countries and more than 50% are in phase I clinical trial[27]. While 1309 gene therapy based trials which were performed across the

Table 2 Overview of clinical trials of colorectal cancer

Therapy	Agent	Clinical status	Ref.
Five peptides combination with oxaliplatin-based chemotherapy	Oxaliplatin	Phase II	[34]
Panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) *vs* FOLFOX4 alone	Fluorouracil, Oxaliplatin	Phase III trial	[35]
Checkpoint inhibitors	Nivolumab and pembrolizumab	Phase 2 study	[36]
Combination vaccine treatment of five therapeutic epitope-peptides	Fluorouracil, irinotecan or oxaliplatin	Phase I	[37]
Autologous dendritic cell based adoptive immunotherapy	-	Phase I-II	[38]
Autologous antigen-activated dendritic cells in the treatment of CRC	-	Phase I-II	[39]
Adjuvant chemotherapy (FOLFOX)	5-fluorouracil (FU)/leucovorin (LV)	Phase III	[40]

world, merely 45 reached the phase III. Eleven gene therapies for CRC are being subjected for trial in the United Kingdom[28]. There are about 50000 to 100000 genes which exist in the body and a few of them take part in the cell cycle. Defective genes could be most allied factors for CRC and it has been discovered that at least 30% of colon cancers are due to defective genes. Few of them are associated with familial colon cancers. The core benefit of gene therapy is the transfer of the specific genes to the specific tumors cells so that the abnormal function of mutated gene would be suppressed and tumor progression could be inhibited[29-32].

IMMUNOTHERAPY

Tumor immunotherapy has seized researchers in this scenario as it depicts remarkable clinical potential in CRC. Presently, there are various immunotherapies which are being subjected to clinical trials in human CRC. Various immunotherapy approaches employed in CRC are monoclonal antibody (mAb) therapy, immune checkpoint inhibitors therapy, cancer vaccines, adoptive cell therapy, complement inhibition and cytokine treatment. Majority of them are in phase I and II clinical trials and some of these trials showed promising results. So far, more than 24 immunotherapy-based clinical trials for human CRC have been completed and more than 40 clinical trials are recruiting or about to recruit patients[33]. Table 2[34-40] depicts various clinical studies of CRC.

Monoclonal antibody therapy

In this therapy, humanized antibodies like Cetuximab and Panitumumab which selectively recognize the epidermal growth factor receptor (EGFR) are employed for the treatment of metastatic CRC. There are some MAbs presently in various phases of clinical trials for CRC such as adecatumumab against EpCAM, labetuzumab against carcinoembryonic antigen (CEA), and pemtumomab against Mucins[41].

Immune checkpoint inhibitors therapy

T cell activation is down-regulated by CTLA-4 which is an immune checkpoint moiety by binding to CD80/CD86 entities on antigen-presenting cells (APC). T cell function is negatively regulated by programmed death receptor ligand 1/2 (PD-L1/L2) by binding to PD-1 receptor present on T cells usually stimulated by their various ligands which are expressed on either tumor cells (*e.g.*, PDL1/ L2→PD-1) or APCs (*e.g.*, CD80/86→CTLA-4; PD-L1/L2→PD-1), activated CTLA-4 and PD-1 immune checkpoint signaling pathways efficiently inhibit the tumor-reactive T cell activation and consequent tumor detection[42]. A phase II clinical trial of individual drug Nivolumab and also a combination of dual drugs like Nivolumab plus Ipilimumab is in undergoing process for CRC (ClinicalTrials.gov Identifier: NCT02060188).

Cancer vaccines

They have been designed to induce antigen specific T-cell or B-cell activity against cancer by rendering antigens to APC like dendritic cells (DCs). Besides, vaccines likewise include constituents proposed to activate DCs pulsated with antigens and aim them to move to a local lymph node.eg DC vaccine and OncoVAX.

DC vaccine: Because majority of CRCs express carcinoembryonic antigen (CEA) which is a tumor-associated antigen DCs, can be pulsed with CEA mRNA or CEA peptides. Most of the CRC patients who were administered with DC vaccine evoked CEA-specific T cell immune activities.

Oncovax: It has been developed to use patients' own cancer cells with an immune-stimulating adjuvant to evoke antitumor immune activities to evade the relapse of colon cancer after surgery. A combination of specific immunotherapy with surgery depicts a remarkable improvement in the survival of the patients[43].

Adoptive T cell therapy

This therapy possesses the potential to raise antitumor immunity and increase vaccine efficacy. Recent researches have riveted on endowing effector T cells with desired antigen receptors, like chimeric antigen receptor T cells. An *ex vivo* expanded human Vδ1 γδ T cells displayed a remarkable therapeutic activity in

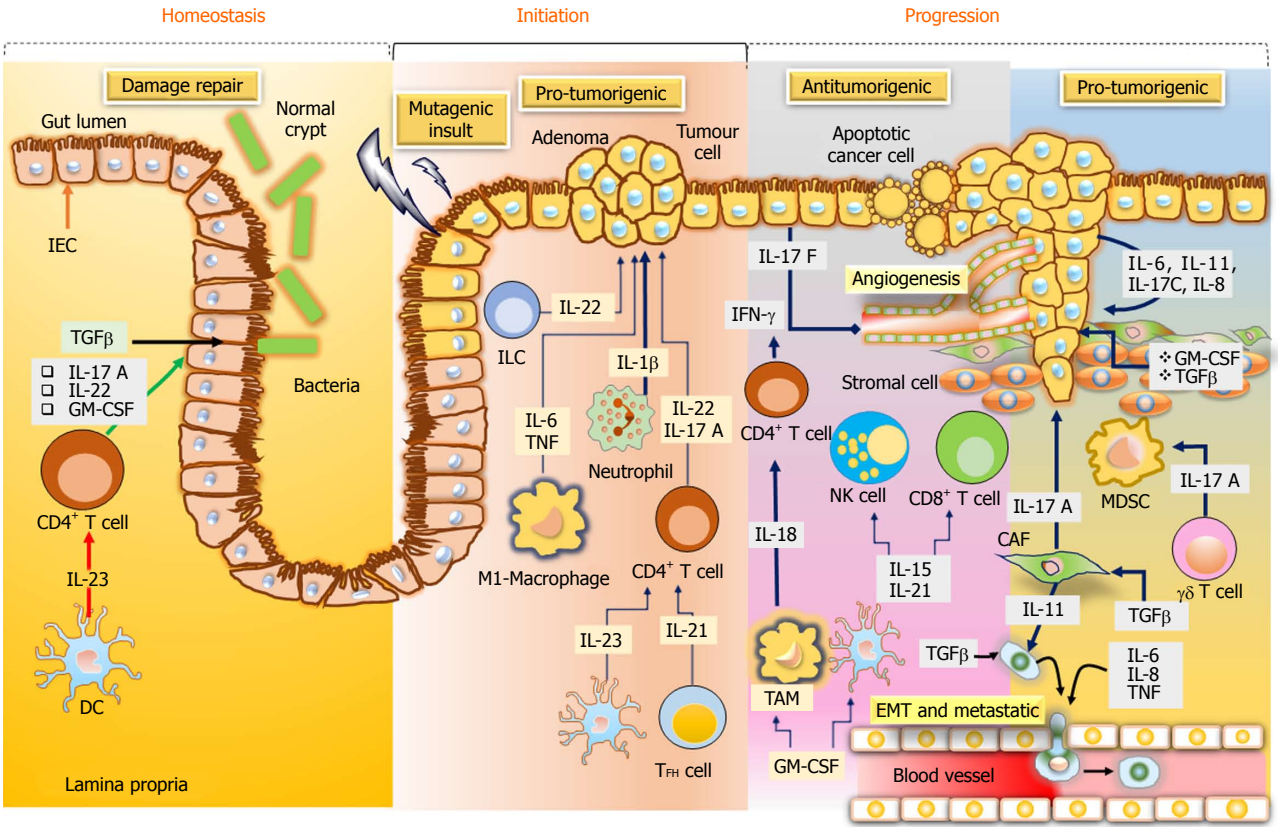


Figure 3 Cytokines involvement in the progression of colorectal cancer. IL: Interleukin; TNF: Tumour necrosis factor; TGF-β: Transforming growth factor-β; EMT: Epithelial to mesenchymal transition; TAM: Tumour-associated macrophage; ILC: Innate lymphoid cells; GM-CSF: Granulocyte–macrophage colony-stimulating factor; MDSCs: Myeloid-derived suppressor cells; CAF: Cancer-associated fibroblast; CIC: Cancer-initiating cell; IEC: Intestinal epithelial cell; DCs: Dendritic cells; TFH: T follicular helper cells; NK: Natural killer cells.

human colon cancer xenografted mouse mode[44].

Complement inhibition

Complement is a key part of immune system and its stimulation has been taken as an essential component of the immune surveillance response against CRC. Complement comprises of more than 30 proteins and fragments, is a part of the innate and adaptive immune system. Various protein inhibitors of complement such as cobra venom factor, humanized cobra venom factor, and recombinant *staphylococcus aureus* super antigen-like protein 7-have been assessed in murine colon cancer model. Complement depletion presents an efficient type of immunotherapy in CRC by its capability to vitiate tumor progression by raising the host's immune responses to cancer and reducing the immunosuppressive effect generated by the tumor microenvironment and finally could be employed as a constituent of combination immunotherapy[45].

Cytokine therapy

Cytokines are considered as essential aspects of tumour immunology, particularly for CRC, in which the tumor growth is determined by the inflammatory process and immunogenic responses. Cytokines like tumour necrosis factor and interleukin-6 are considered as important factors in CRC, triggering the stimulation of the central oncogenic factors nuclear factor-κB and inducer of transcription 3 (STAT3), respectively, in the intestinal cells to enhance the proliferation and the development of apoptosis resistance[46] (Figure 3).

CONCLUSION

Increasing evidences show that several signaling pathways play an essential role in the development and progression of CRC. Targeting these signaling cascades using nanocarriers might be advantageous for the treatment of CRC. The identification of various genes and other biomarkers improved the conventional therapy and target the specific tumor cells. The gene therapy and various immunotherapy including cytokine therapy, cancer vaccine, adoptive cell therapy, monoclonal antibody *etc*. have been recently introduced which may unravel new ways for the treatment of CRC and provide its efficient management in comparison to the conventional therapy.

REFERENCES

1 **Takebe N**, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol* 2015; **12**:

445-464 [PMID: 25850553 DOI: 10.1038/nrclinonc.2015.61]
2 **Gulbake A**, Jain A, Jain A, Jain A, Jain SK. Insight to drug delivery aspects for colorectal cancer. *World J Gastroenterol* 2016; **22**: 582-599 [PMID: 26811609 DOI: 10.3748/wjg.v22.i2.582]
3 **Rubin LL**, de Sauvage FJ. Targeting the Hedgehog pathway in cancer. *Nat Rev Drug Discov* 2006; **5**: 1026-1033 [PMID: 17139287 DOI: 10.1038/nrd2086]
4 **Chen Q**, Zhang H, Wu M, Wang Q, Luo L, Ma H, Zhang X, He S. Discovery of a potent hedgehog pathway inhibitor capable of activating caspase8-dependent apoptosis. *J Pharmacol Sci* 2018; **137**: 256-264 [PMID: 30064819 DOI: 10.1016/j.jphs.2018.07.001]
5 **Santoyo-Ramos P**, Likhatcheva M, García-Zepeda EA, Castañeda-Patlán MC, Robles-Flores M. Hypoxia-inducible factors modulate the stemness and malignancy of colon cancer cells by playing opposite roles in canonical Wnt signaling. *PLoS One* 2014; **9**: e112580 [PMID: 25396735 DOI: 10.1371/journal.pone.0112580]
6 **Li Q**, Yang T, Li D, Ding F, Bai G, Wang W, Sun H. Knockdown of aquaporin5 sensitizes colorectal cancer cells to 5-fluorouracil via inhibition of the Wnt/β-catenin signaling pathway. *Biochem Cell Biol* 2018 [PMID: 29390193 DOI: 10.1139/bcb-2017-0162]
7 **Albring KF**, Weidemüller J, Mittag S, Weiske J, Friedrich K, Geroni MC, Lombardi P, Huber O. Berberine acts as a natural inhibitor of Wnt/β-catenin signaling--identification of more active 13-arylalkyl derivatives. *Biofactors* 2013; **39**: 652-662 [PMID: 23982892 DOI: 10.1002/biof.1133]
8 **Lee SY**, Lim TG, Chen H, Jung SK, Lee HJ, Lee MH, Kim DJ, Shin A, Lee KW, Bode AM, Surh YJ, Dong Z. Esculetin suppresses proliferation of human colon cancer cells by directly targeting β-catenin. *Cancer Prev Res* (Phila) 2013; **6**: 1356-1364 [PMID: 24104353 DOI: 10.1158/1940-6207.CAPR-13-0241]
9 **Li X**, Bai B, Liu L, Ma P, Kong L, Yan J, Zhang J, Ye Z, Zhou H, Mao B, Zhu H, Li Y. Novel β-carbolines against colorectal cancer cell growth via inhibition of Wnt/β-catenin signaling. *Cell Death Discov* 2015; **1**: 15033 [PMID: 27551464 DOI: 10.1038/cddiscovery.2015.33]
10 **Biswas S**, Chytil A, Washington K, Romero-Gallo J, Gorska AE, Wirth PS, Gautam S, Moses HL, Grady WM. Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. *Cancer Res* 2004; **64**: 4687-4692 [PMID: 15256431 DOI: 10.1158/0008-5472.CAN-03-3255]
11 **Yu Z**, Tang Y, Hu D, Li J. Inhibitory effect of genistein on mouse colon cancer MC-26 cells involved TGF-beta1/Smad pathway. *Biochem Biophys Res Commun* 2005; **333**: 827-832 [PMID: 15963949 DOI: 10.1016/j.bbrc.2005.05.177]
12 **Vivanco I**, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002; **2**: 489-501 [PMID: 12094235 DOI: 10.1038/nrc839]
13 **Yuge R**, Kitadai Y, Shinagawa K, Onoyama M, Tanaka S, Yasui W, Chayama K. mTOR and PDGF pathway blockade inhibits liver metastasis of colorectal cancer by modulating the tumor microenvironment. *Am J Pathol* 2015; **185**: 399-408 [PMID: 25478811 DOI: 10.1016/j.ajpath.2014.10.014]
14 **Yin L**, Velazquez OC, Liu ZJ. Notch signaling: emerging molecular targets for cancer therapy. *Biochem Pharmacol* 2010; **80**: 690-701 [PMID: 20361945 DOI: 10.1016/j.bcp.2010.03.026]
15 **Koduru S**, Kumar R, Srinivasan S, Evers MB, Damodaran C. Notch-1 inhibition by Withaferin-A: a therapeutic target against colon carcinogenesis. *Mol Cancer Ther* 2010; **9**: 202-210 [PMID: 20053782 DOI: 10.1158/1535-7163.MCT-09-0771]
16 **Tiwari A**, Jain SK, Jain A, Verma A, Saraf S, Panda PK, Gour G. Application Potential of Polymeric Nanoconstructs for Colon-Specific Drug Delivery. *Contemporary Healthcare Applications* 2018 [DOI: 10.4018/978-1-5225-4781-5.ch002]
17 **Sukttham K**, Koobkokkruad T, Wutikhun T, Surassmo S. Efficiency of resveratrol-loaded sericin nanoparticles: Promising bionanocarriers for drug delivery. *Int J Pharm* 2018; **537**: 48-56 [PMID: 29229512 DOI: 10.1016/j.ijpharm.2017.12.015]
18 **Su CY**, Liu JJ, Ho YS, Huang YY, Chang VH, Liu DZ, Chen LC, Ho HO, Sheu MT. Development and characterization of docetaxel-loaded lecithin-stabilized micellar drug delivery system for improving the therapeutic efficacy and reducing systemic toxicity. *Eur J Pharm Biopharm* 2018; **123**: 9-19 [PMID: 29154834 DOI: 10.1016/j.ejpb.2017.11.006]
19 **Alshahrani SM**, Alshetaili AS, Alalaiwe A, Alsulays BB, Anwer MK, Al-Shdefat R, Imam F, Shakeel F. Anticancer Efficacy of Self-Nanoemulsifying Drug Delivery System of Sunitinib Malate. *AAPS PharmSciTech* 2018; **19**: 123-133 [PMID: 28620763 DOI: 10.1208/s12249-017-0826-x]
20 **Sharma A**, Kim EJ, Shi H, Lee JY, Chung BG, Kim JS. Development of a theranostic prodrug for colon cancer therapy by combining ligand-targeted delivery and enzyme-stimulated activation. *Biomaterials* 2018; **155**: 145-151 [PMID: 29175083 DOI: 10.1016/j.biomaterials.2017.11.019]
21 **Wang D**, Sun F, Lu C, Chen P, Wang Z, Qiu Y, Mu H, Miao Z, Duan J. Inulin based glutathione-responsive delivery system for colon cancer treatment. *Int J Biol Macromol* 2018; **111**: 1264-1272 [PMID: 29366899 DOI: 10.1016/j.ijbiomac.2018.01.071]
22 **Hosseinifar T**, Sheybani S, Abdouss M, Hassani Najafabadi SA, Shafiee Ardestani M. Pressure responsive nanogel base on Alginate-Cyclodextrin with enhanced apoptosis mechanism for colon cancer delivery. *J Biomed Mater Res A* 2018; **106**: 349-359 [PMID: 28940736 DOI: 10.1002/jbm.a.36242]
23 **Li W**, Liu D, Zhang H, Correia A, Mäkilä E, Salonen J, Hirvonen J, Santos HA. Microfluidic assembly of a nano-in-micro dual drug delivery platform composed of halloysite nanotubes and a pH-responsive polymer for colon cancer therapy. *Acta Biomater* 2017; **48**: 238-246 [PMID: 27815166 DOI: 10.1016/j.actbio.2016.10.042]
24 **Cheewatanakornkool K**, Niratisai S, Manchun S, Dass CR, Sriamornsak P. Characterization and in vitro release studies of oral microbeads containing thiolated pectin-doxorubicin conjugates for colorectal cancer treatment. *Asian J Pharm* 2017; **12**: 509-520 [DOI: 10.1016/j.ajps.2017.07.005]
25 **Song Z**, Lin Y, Zhang X, Feng C, Lu Y, Gao Y, Dong C. Cyclic RGD peptide-modified liposomal drug delivery system for targeted oral apatinib administration: enhanced cellular uptake and improved therapeutic effects. *Int J Nanomedicine* 2017; **12**: 1941-1958 [PMID: 28331317 DOI: 10.2147/IJN.S125573]
26 **Sadreddini S**, Safaralizadeh R, Baradaran B, Aghebati-Maleki L, Hosseinpour-Feizi MA, Shanehbandi D, Jadidi-Niaragh F, Sadreddini S, Kafil HS, Younesi V, Yousefi M. Chitosan nanoparticles as a dual drug/siRNA delivery system for treatment of colorectal cancer. *Immunol Lett* 2017; **181**: 79-86 [PMID: 27916629 DOI: 10.1016/j.imlet.2016.11.013]
27 **Ginn SL**, Amaya AK, Alexander IE, Edelstein M, Abedi MR. Gene therapy clinical trials worldwide to 2017: An update. *J Gene Med* 2018; **20**: e3015 [PMID: 29575374 DOI: 10.1002/jgm.3015]
28 **Durai R**, Yang SY, Seifalian AM, Winslet MC. Principles and applications of gene therapy in colon cancer. *J Gastrointestin Liver Dis* 2008; **17**: 59-67 [PMID: 18392246]
29 **Armaghany T**, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. *Gastrointest Cancer Res* 2012; **5**: 19-27 [PMID: 22574233]
30 **Cho KR**, Vogelstein B. Genetic alterations in the adenoma--carcinoma sequence. *Cancer* 1992; **70**: 1727-1731 [PMID: 1516027]
31 **Takami K**, Yana I, Kurahashi H, Nishisho I. Multistep carcinogenesis in colorectal cancers. *Southeast Asian J Trop Med Public Health* 1995; **26** Suppl 1: 190-196 [PMID: 8629105]
32 **Zhang J**, Kale V, Chen M. Gene-directed enzyme prodrug therapy. *AAPS J* 2015; **17**: 102-110 [PMID: 25338741 DOI: 10.1208/s12248-014-9675-7]
33 **Nappi A**, Berretta M, Romano C, Tafuto S, Cassata A, Casaretti R, Silvestro L, Divitiis C, Alessandrini L, Fiorica F, Ottaiano A, Nasti G. Metastatic Colorectal Cancer: Role of Target Therapies and Future Perspectives. *Curr Cancer Drug Targets* 2018; **18**: 421-429 [PMID: 28183254 DOI: 10.2174/1568009617666170209095143]
34 **Hazama S**, Nakamura Y, Tanaka H, Hirakawa K, Tahara K, Shimizu R, Ozasa H, Etoh R, Sugiura F, Okuno K, Furuya T, Nishimura T, Sakata K, Yoshimatsu K, Takenouchi H, Tsunedomi R, Inoue Y, Kanekiyo S, Shindo Y, Suzuki N, Yoshino S, Shinozaki

H, Kamiya A, Furukawa H, Yamanaka T, Fujita T, Kawakami Y, Oka M. A phase II study of five peptides combination with oxaliplatin-based chemotherapy as a first-line therapy for advanced colorectal cancer (FXV study). *J Transl Med* 2014; **12**: 108 [PMID: 24884643 DOI: 10.1186/1479-5876-12-108]

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

36 **Overman MJ**, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, Desai J, Hill A, Axelson M, Moss RA, Goldberg MV, Cao ZA, Ledeine JM, Maglinte GA, Kopetz S, André T. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol* 2017; **18**: 1182-1191 [PMID: 28734759 DOI: 10.1016/S1470-2045(17)30422-9]

37 **Hazama S**, Nakamura Y, Takenouchi H, Suzuki N, Tsunedomi R, Inoue Y, Tokuhisa Y, Iizuka N, Yoshino S, Takeda K, Shinozaki H, Kamiya A, Furukawa H, Oka M. A phase I study of combination vaccine treatment of five therapeutic epitope-peptides for metastatic colorectal cancer; safety, immunological response, and clinical outcome. *J Transl Med* 2014; **12**: 63 [PMID: 24612787 DOI: 10.1186/1479-5876-12-63]

38 **Hunyadi J**, András C, Szabó I, Szántó J, Szluha K, Sipka S, Kovács P, Kiss A, Szegedi G, Altorjay I, Sápy P, Antal-Szalmás P, Tóth L, Fazekas G, Rajnavölgyi É. Autologous dendritic cell based adoptive immunotherapy of patients with colorectal cancer-A phase I-II study. *Pathol Oncol Res* 2014; **20**: 357-365 [PMID: 24163303 DOI: 10.1007/s12253-013-9704-3]

39 **Riley JM**, Cross AW, Paulos CM, Rubinstein MP, Wrangle J, Camp ER. The clinical implications of immunogenomics in colorectal cancer: A path for precision medicine. *Cancer* 2018; **124**: 1650-1659 [PMID: 29315503 DOI: 10.1002/cncr.31214]

40 **Saleh K**, Khalife-Saleh N, Kourie HR, Chahine G. How and when adjuvant treatment should be intensified in stage III colorectal cancers? *Future Oncol* 2017 [PMID: 28829195 DOI: 10.2217/fon-2017-0197]

41 **Cunningham D**, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004; **351**: 337-345 [PMID: 15269313 DOI: 10.1056/NEJMoa033025]

42 **Sharma P**, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell* 2015; **161**: 205-214 [PMID: 25860605 DOI: 10.1016/j.cell.2015.03.030]

43 **Fong L**, Hou Y, Rivas A, Benike C, Yuen A, Fisher GA, Davis MM, Engleman EG. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc Natl Acad Sci USA* 2001; **98**: 8809-8814 [PMID: 11427731 DOI: 10.1073/pnas.141226398]

44 **Wu D**, Wu P, Wu X, Ye J, Wang Z, Zhao S, Ni C, Hu G, Xu J, Han Y, Zhang T, Qiu F, Yan J, Huang J. Ex vivo expanded human circulating Vδ1 γδT cells exhibit favorable therapeutic potential for colon cancer. *Oncoimmunology* 2015; **4**: e992749 [PMID: 25949914 DOI: 10.4161/2162402X.2014.992749]

45 **Downs-Canner S**, Magge D, Ravindranathan R, O'Malley ME, Francis L, Liu Z, Sheng Guo Z, Obermajer N, Bartlett DL. Complement Inhibition: A Novel Form of Immunotherapy for Colon Cancer. *Ann Surg Oncol* 2016; **23**: 655-662 [PMID: 26289805 DOI: 10.1245/s10434-015-4778-7]

46 **West NR**, McCuaig S, Franchini F, Powrie F. Emerging cytokine networks in colorectal cancer. *Nat Rev Immunol* 2015; **15**: 615-629 [PMID: 26358393 DOI: 10.1038/nri3896]

P- Reviewer: Bordonaro M, Caputo D **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4436-4447

DOI: 10.3748/wjg.v24.i39.4436

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

MINIREVIEWS

Carcinogenesis on the background of liver fibrosis: Implications for the management of hepatocellular cancer

Joanne Marie O'Rourke, Vandana Mridhu Sagar, Tahir Shah, Shishir Shetty

Joanne Marie O'Rourke, Vandana Mridhu Sagar, Shishir Shetty, Centre for Liver Research, Institute of Biomedical Research, Birmingham B15 2TT, United Kingdom

Joanne Marie O'Rourke, Vandana Mridhu Sagar, Tahir Shah, Shishir Shetty, NIHR Birmingham Biomedical Research Centre, University of Birmingham and University Hospitals Birmingham NHS Foundation Trust, United Kingdom

ORCID number: Joanne Marie O'Rourke (0000-0001-7851-0703); Vandana Mridhu Sagar (0000-0003-2662-755X); Tahir Shah (0000-0002-0420-0304); Shishir Shetty (0000-0002-4729-2173).

Author contributions: Shetty S contributed to review concept, literature review and writing the paper; O'Rourke JM contributed to the literature review and writing the paper; Sagar VM and Shah T provided critical intellectual revisions and editing of the manuscript.

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Shishir Shetty, MBChB, MRCP, PhD, Senior Research Fellow, Centre for Liver Research, Institute of Biomedical Research, University of Birmingham, Birmingham B15 2TT, United Kingdom. s.shetty@bham.ac.uk
Telephone: +44-121-3714852

Received: May 9, 2018
Peer-review started: May 9, 2018
First decision: June 15, 2018
Revised: September 3, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

Hepatocellular carcinoma (HCC) is now the second leading cause of cancer-related deaths globally and many patients have incurable disease. HCC predominantly occurs in the setting of liver cirrhosis and is a paradigm for inflammation-induced cancer. The causes of chronic liver disease promote the development of transformed or premalignant hepatocytes and predisposes to the development of HCC. For HCC to grow and progress it is now clear that it requires an immunosuppressive niche within the fibrogenic microenvironment of cirrhosis. The rationale for targeting this immunosuppression is supported by responses seen in recent trials with checkpoint inhibitors. With the impact of immunotherapy, HCC progression may be delayed and long term durable responses may be seen. This makes the management of the underlying liver cirrhosis in HCC even more crucial as studies demonstrate that measures of liver function are a major prognostic factor in HCC. In this review, we discuss the development of cancer in the setting of liver inflammation and fibrosis, reviewing the microenvironment that leads to this tumourigenic climate and the implications this has for patient management.

Key words: Hepatocellular cancer; Carcinogenesis; Inflammation; Fibrosis; Immunotherapy

Core tip: In this review, we discuss the development of hepatocellular carcinoma in the setting of liver inflammation and fibrosis, reviewing the microenviron-

ment that leads to this tumorigenic climate and the implications this has for patient management.

O'Rourke JM, Sagar VM, Shah T, Shetty S. Carcinogenesis on the background of liver fibrosis: Implications for the management of hepatocellular cancer. *World J Gastroenterol* 2018; 24(39): 4436-4447 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4436.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4436

INTRODUCTION

Hepatocellular carcinoma (HCC) is now the fifth most commonly diagnosed cancer in men worldwide, and in women it is ranked ninth. HCC is the second most common cause of cancer related deaths and is reported to have been responsible for nearly 745000 deaths in 2012[1]. Incidence rates are highest in Asia and Africa with Central Europe having intermediate rates[2]. Different risk factors predominate depending on the region of the world. In Africa and Asia infection with hepatitis B virus (HBV) and aflatoxin B1 exposure are the major risk factors. In developed countries the hepatitis C virus (HCV), alcohol and the metabolic syndrome have predominated[3].

Despite increasing knowledge on the aetiologies of cirrhosis and progress in diagnosing and managing risk factors, the incidence rates for HCC are increasing. In England, HCC incidence increased from 0.63 per 100000 in 1990 to 2.48 in 2009[4]. In the United States (US), HCC incidence increased by 4.5% (95%CI: 4.3-4.7) annually between 2000 and 2009 but only 0.7% annually (95%CI: 0.2-1.6) after that. The post 2009 slowing in overall rates, seen in the US, may represent a plateau created from increases in vaccination against HBV and improved chronic HBV antiviral treatment[5].

It is uncommon to see HCC in the absence of liver fibrosis but it does occur. Table 1 lists some of the aetiologies associated with non-cirrhotic HCC[6,7]. Chronic hepatitis B is a major risk factor for the development of HCC in the non-cirrhotic setting[8]. In Europe and the United States the 5-year cumulative incidence of developing HCC was found to be 1% in non-cirrhotic chronic HBV hepatitis. This incidence increased to 10% in HBV with cirrhosis[9]. Other causes of HCC in the non-cirrhotic setting include hereditary conditions for example porphyria and type 1 glycogen storage disease, metabolic syndromes and genotoxin exposure. Genotoxins are agents which damage the genetic information within a cell. For example, the aflatoxin B1, which is produced by Aspergillus flavus, is a pathogenic fungus and can lead to non-cirrhotic HCC induction[10]. The global epidemic of non-alcoholic fatty liver disease (NAFLD) which is characterised by macrovesicular steatosis can lead to cirrhosis. It is however observed that a significant proportion of patients with NAFLD develop HCC in the non-cirrhotic setting[11,12]. However worldwide at present the majority (70%-90%) of HCC cases occur on a background of cirrhosis[13].

Table 1 Conditions which have been associated with hepatocellular carcinoma development in the non-cirrhotic liver[6,7]

Viral	HBV
Metabolic	Porphyria
	Type 1 glycogen storage disease
	NAFLD
	A1 antitrypsin
	Haemochromatosis
	Type 1 hypercitrullinemia
Genotoxins	Aflatoxin B1
Congenital	Alagille syndrome
	Congenital hepatic fibrosis
Sex hormones	Anabolic steroids
	Hepatic adenoma transformation
Vascular	Hepatic vascular pathology, *e.g.*, Budd Chiari

HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease.

When data from the World Health Organization (WHO) mortality database was examined by Ascione *et al*[14], the age-standardised death rate for liver cirrhosis in European countries between 1970 and 2010 showed cause for concern for the United Kingdom (UK), Finland, Ireland and Denmark. Looking at percentage change in mortality, the UK in those four decades showed a high increase (+284.8%), Finland, Ireland and Denmark also saw increases. However these countries were the exceptions and in all other countries in Europe there was a reduction in mortality for liver cirrhosis. The same database provided comparable data, between 1980-2010 with a 85.4% increase in death from HCC over this period[14]. The overall decrease in liver cirrhosis related deaths in Europe and the increasing mortality for HCC is confounding and concerning.

Cirrhosis mortality in the UK has been the subject of extensive discussion and patterns of alcohol consumption may account for the discrepancies between the UK and other parts of Europe. The rise in HCC cases in Europe over the last 30 years seems confounding when it is reported that in many countries mortality from cirrhosis is reducing. However, our knowledge and the management of chronic liver disease has over this timeframe improved, and it is suggested that with increased survival we are seeing increased development of HCC[14]. This would be in keeping with our knowledge that cirrhosis creates a microenvironment for tumour development and is considered a precursor for HCC.

Over 3 decades ago the 5-year survival for HCC was 3%. Despite improvements in earlier detection 5-year survival is less than 20% for this cancer[15].

PATHOGENESIS

Setting of inflammation and fibrosis

Liver fibrosis is a risk factor for the development of HCC with up to 90% of cases occurring on the background

of a cirrhotic liver[16] and is a leading cause of death in this population. The major global causes of liver disease which are associated with HCC include viral hepatitis, alcoholism and non-alcoholic steatohepatitis (NASH). The effects of HBV infection have started to decline due to increased use of antivirals and immunisation programs. It is hoped that in the age of new direct acting antiviral agents with time we will see a reduction in HCV associated cirrhosis. The impact of alcohol and the development of NASH cirrhosis will prove to be more challenging to prevent and cases are predicted to continue to rise.

Fibrosis occurs when the liver is repeatedly and continuously injured. Liver volume is formed from 80% parenchymal and 20% non-parenchymal cells[17]. Hepatocytes are the parenchymal cells and they are the target for hepatotoxic agents. Damage to hepatocytes triggers the release of reactive oxygen species (ROS) and mediators of fibrosis inducing activation of hepatic stellate cells (HSCs). HSCs with phagocytic Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) are central players in fibrosis development[18]. The activation of HSCs, extracellular matrix (ECM) producing myofibroblasts, is said to be the key step in fibrosis development. Paracrine signals from injured hepatocytes and activated KCs play a prominent role in HSC activation. KCs also generate ROS in the liver and this enhances HSC activation and collagen synthesis leading to fibrosis[19,20].

In addition to the multitude of cells involved in the development of cirrhosis there are also several cytokines that have been identified to play significant roles. They include platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), interferon and interleukins (ILs). A variety of hepatotoxic agents can induce KC to synthesise PDGF[21] which binds to the HSC membrane and activates them. There are different isoforms of PDGF and two of these, PDGF B and D have been shown to have a role in activating HSCs leading to liver fibrosis[22]. TGF-β is the most potent stimulator of fibrogenesis and is produced by a variety of cells in the liver: HSCs, KCs, LSECs, and hepatocytes. The TGF-β family has multiple members and the one that has been implicated as a notable player in hepatic fibrosis is TGF-β1. It is reported to contribute not only to activation of HSCs but also the inhibition of ECM breakdown[23] and the induction of apoptosis of hepatocytes[24]. TNF-α has also been shown to activate HSCs to synthesise ECM[25], however, results from murine studies on TNF-α are complex and it appears to also have antifibrogenic effects in some reports[26]. ILs are expressed by many cells with the majority of ILs being produced by helper CD4 T lymphocytes. In the liver ILs have both pro-fibrogenic and antifibrogenic roles[18]. IL-1 can activate HSCs[27] and IL-17 has a role inducing fibrosis through the activation of HSCs and KCs[28]. ILs with antifibrogenic roles have been identified as IL-6, IL-10 and IL-22[29-31].

Setting for tumour development and progression in fibrosis

The inception and progression of HCC is described as being largely influenced by the microenvironment of the liver. This includes influences from chronic inflammation, liver remodelling, changes in genetics and cellular signalling. These pathways can be affected by chemical toxins, viruses, immune cells, hypoxia, ECM changes, microflora from the gastrointestinal tract and extra cellular microvesicles which carry altering signals, cytokines and oncogenic miRNAs.

Chronic inflammation and fibrosis are seen in the background of many HCCs and the most common aetiologies are viruses and ethanol. The immune mediated cell death seen in viral infections leads to increased production of ROS. This leads to increased hepatocellular oxidative stress which induces DNA mutations contributing to HCC development. Ethanol consumption is associated with increased ROS concentrations in hepatocytes resulting in hepatic DNA damage[32]. Chronic inflammation leads to increased proliferation of hepatocytes, shortening of telomeres and therefore chromosomal instability and a predisposition to malignant transformation[33]. Genomic alterations which have been identified in HCC and are considered to be drivers in progression include mutations affecting telomere maintenance, Wnt pathway activation, inactivation of p53, chromatin remodelling, Ras signalling, mechanistic target of rapamycin (mTOR) signalling and ROS pathway initiation[34].

Chronic inflammation can progress to fibrosis and cirrhosis and this in turn induces several further changes in the microenvironment. Firstly, it creates altered blood flow and hypoxic hepatocytes which produce reactive nitrogen species[35]. Areas of hypoxia in the liver parenchyma lead to changes in molecular signalling and we know that the response is to upregulate angiogenic factors including vascular endothelial growth factor (VEGF)[36]. In a tumour this facilitates angiogenesis and tumour growth. The hepatocytes may provide the genetic mutation but it is the unique surrounding microenvironment that enables the tumour to establish.

We have explained that chronic inflammation has effects on cytokine expression within the liver, ECM production by HSC, TNF-α receptors and also the mitogenic cytokine IL6 is significantly increased in advanced cirrhosis leading to a propensity towards cancer[37]. IL6 regulates immune cells and the growth of tumour cells[38] and this dual role therefore is an example of the association between the tumour and the microenvironment. The effects of IL6 are controlled by nuclear factor-κB signalling. Both pathways are altered in liver inflammation and hepatocarcinogenesis[39]. EGFR overexpression also promotes liver cancer progression when present in macrophages[40]. It has also been shown that hepatic stellate cells can promote the pro-

tumourigenic change in macrophages[41]. The expansion of liver progenitor cells to replace hepatocytes, in the presence of cytokines and increases in oxidative stress promotes the accruing of mutations[42].

Hepatocytes have great potential to regenerate but we know this predisposes cells to malignant transformation[43]. The cell underlying the inception of HCC is also critical to understand. The human liver is not just made up of hepatocytes but also adult stem cells and progenitor cells which maybe potential cells of origin for cancer[44].

The gut microbiota has in recent years received much attention in many disease processes including liver disease. It has been described that the microbes in our bodies encompass 100 trillion cells, with the majority residing in the gut[45]. It is increasingly recognised that the gastrointestinal tract plays a pivotal role in liver diseases, including HCC. As we have previously described HCC usually occurs in inflamed and fibrotic livers and intensive immune cell infiltration is seen. *Via* the portal vein the liver is exposed to gut-derived bacterial products and in advanced liver diseases there is increased intestinal permeability to gut-derived bacterial products including lipopolysaccharides (LPS)[46]. Accumulation of LPS is said to contribute to HCC development by generating inflammatory reactions in the hepatic environment[47], activating KCs and endothelial cells to release pro-inflammatory cytokines which contributes to liver injury. Levels of LPS are increased in animal models of hepatocarcinogenesis and in patients with HCC[47-49]. Dapito *et al*[50] found that toll like receptor 4 activation by LPS contributed to driving inflammation and tumour progression and that gut sterilisation supressed hepatocarcinogenesis. To date studies have been on preclinical animal models but there is potential that manipulating the microbiome may one day be an option in the prevention and perhaps treatment of HCC[51].

Tumour antigen tolerance promotes carcinogenesis

Dysregulation of the immune system has been implicated in the pathogenesis of HCC. Changes in the innate and adaptive immune system makes the immune system tolerant to cancer and facilitates tumour progression. Understanding these processes is therefore essential to tailor therapeutic approaches. Key cells implicated include T lymphocytes, myeloid-derived suppressor cells (MDSCs), dendritic cells and natural killer (NK) cells[52]. The innate immune system key players are dendritic cells, macrophages, MDSCs and NK cells. The adaptive immune system comprises the T lymphocyte subsets. Failure of HCC antigen presentation by dendritic cells is one defect in the immune system seen in HCC. Activated dendritic cells in HCC are not able to infiltrate cancer tissue effectively[53] and tumour associated macrophages express cytokines that favour tumour growth, invasion and suppress the anti-tumour immune response[54]. MDSCs possess strong immunosuppressive activities and expand in cancer and regulate T cell responses, increased quantities of these cells are seen in the tumour environment of a HCC[55]. NK cells are cytotoxic lymphocytes and they can modulate the activity of other immune cells, including dendritic cells and macrophages, *via* cytokine release. They are critical to the innate immune system and are capable of rapid responses and can destroy tumour cells without prior priming. In HCC a reduction in NK cell subsets has been reported with reduced cytotoxic ability[56].

The adaptive immune system has a significant role in thwarting the development and advancement of cancer. CD8+ cytotoxic T cells play a salient part in anti-tumour mechanisms and CD4+ helper cells have a role in generating CD8+memory T cells[57] which assist in the destruction of tumour cells. In the setting of cirrhosis there is a reduction in CD4+ cells[58]. Tregs expressing CD4+, CD25+ and forkhead box P3 (Foxp3) have an inhibitory role and they can suppress effective anti-tumour responses[59]. There are increased Tregs seen in patients with HCC and depletion can increase anti-tumour responses[60] and lead to a reduction in tumour growth[61]. In advanced HCC there are increased numbers of CD8+FoxP3+ regulatory T cells perhaps helping the tumour evade the immune system[62]. NK T cells accumulate in the tumour environment and they appear to be able to function either as anti-tumour cells or can promote tumour tolerance depending on the subset[63]. We can therefore conclude that a complex, partially understood, dysregulated immune environment has a key role in the development and evolution of liver tumours. An overview of the key responses to hepatic injury leading to fibrosis and HCC development together with therapeutic strategies are summarised in Figure 1.

THE SAME FIBROTIC ENVIRONMENT WHICH PROMOTES THE DEVELOPMENT OF HCC ALSO IMPACTS NEGATIVELY ON TREATMENT OPTIONS

Management of advanced HCC and the impact of cirrhosis

The fact that the majority of HCC arise in the cirrhotic liver, which affects liver function, can severely impact on therapeutics. A detailed review of the management of HCC has been covered elsewhere[64]. There are several algorithms for the management of HCC including TNM stage, the Japanese integrated system, Cancer of the Liver Italian group and the Hong Kong Liver Cancer staging system. The most well recognised being the Barcelona Liver Cancer (BCLC) criteria which is recommended by several international guidelines[65,66]. Considering the underlying liver disease is vital in HCC, the BCLC guidelines include both tumour stage and the severity of underlying liver cirrhosis (Child Pugh score) and helps guide treatment and to predict overall prognosis. The seminal study by Hoshida *et al*[67]

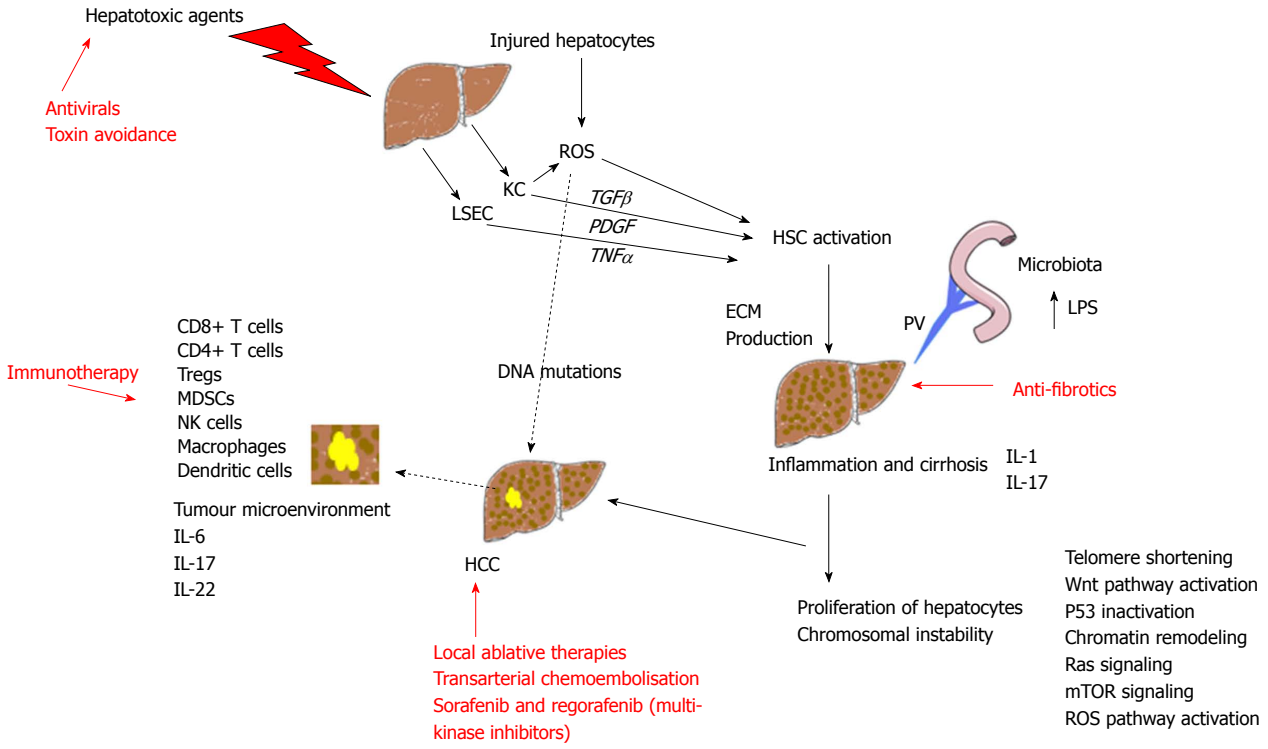


Figure 1 Overview of the key factors associated with fibrosis development and progression to hepatocellular carcinoma. Hepatotoxic agents damage key liver cells triggering reactive oxygen species and cytokine release culminating in hepatic stellate cell activation, the key step in fibrosis development. Chronic inflammation and fibrosis instigates several changes in the microenvironment predisposing to hepatocellular carcinoma (HCC) and creating distinct immune changes which promote HCC progression. Key therapeutic strategies are highlighted in red. LSEC: Liver sinusoidal endothelial cells; KC: Kupffer cells; ROS: Reactive oxygen species; HCC: Hepatocellular carcinoma; TGFβ: Transforming growth factor β; PDGF: Platelet-derived growth factor; HSC: Hepatic stellate cell; ECM: Extracellular matrix; PV: Portal vein; LPS: Lipopolysaccharides; IL: Interleukins; Treg: Regulatory T cell; MDSC: Myeloid-derived suppressor cells; NK: Natural killer cells.

highlighted the importance of underlying liver disease to overall prognosis in HCC. In this study the gene expression of the tumour was not associated with overall survival but the gene expression in adjacent non-tumour liver tissue correlated strongly with survival. More recently the severity of underlying liver disease as a prognostic marker has been highlighted with a study focusing on a scoring system based on bilirubin levels and albumin values. Johnson *et al*[68] developed a model incorporating just bilirubin and albumin levels called the ALBI score (Figure 2) which was an accurate discriminatory method for assessing liver function in HCC. Within the Child-Pugh class A patients, the ALBI score was able to differentiate patient groups with different prognoses. The model across a database of 3887 patients identified a median ten month difference in survival between ALBI grade 1 and ALBI grade 2 within the Child-Pugh class A group for European and US patients[64].

Patients who present with early HCC are amenable to curative treatments including surgery (resection or transplant) and local ablative therapies. Those with more intermediate stage HCC have non curative options such as transarterial chemoembolisaton which has been shown to improve survival in randomised control trials[69]. A significant proportion of patients present with advanced incurable disease; these patients have a poor prognosis and the only licensed medical treatment has been the multi-kinase inhibitor Sorafenib[70]. The SHARP trial demonstrated an improved survival in patients with advanced HCC who took Sorafenib but this was by only a median of three months. Several other agents have been studied in randomised trials but none have successfully demonstrated superiority to Sorafenib but most recently a phase 3 trial demonstrated Lenvatinib, an inhibitor of VEGF receptors 1-3, FGF receptors 1-4, PDGF receptor alpha, RET and KIT, had similar efficacy to Sorafenib[71]. Trials in which Sorafenib has been combined with other treatment such as TACE have not been successful in improving efficacy of these treatments[72]. Until recently no second line agents in randomised trials had demonstrated clinical benefit after Sorafenib therapy, but a recent trial with Regorafinib, another multikinase inhibitor, has finally demonstrated improved survival with a second line agent[73]. This has led to FDA approval for patients who have failed therapy with Sorafenib but overall survival for advanced HCC remains poor. These experiences have provided impetus to explore immunotherapy in HCC. We have already described that progressive HCC is associated with an immunosuppressive microenvironment. Recent early stage trials with checkpoint inhibitors which activate T cells have shown promising results. Checkpoint inhibitors currently include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockers and inhibitors of programmed cell death protein-1 (PD-1)/programmed cell death protein ligand-1 (PDL-1) interaction with

ALBI-score
[$\log_{10}$ bilirubin (μmol/L) × 0.66] + [albumin (g/L) × -0.085]
ALBI grade is defined by the resulting score:
Grade 1 ⩽ -2.60
Grade 2 > -2.60 to ⩽ -1.39
Grade 3 > -1.39

Figure 2 Formula to calculate the ALBI-score and translate the result to ALBI grade[64].

three studies being reported in the context of HCC. One study with 30 patients involved the administration of Tremelimumab, a CTLA-4 blocker, combined with ablative therapy, showed some patients did demonstrate immune responses and led to the accumulation of CD8+ T cells in tumours. A further study with the same drug was performed with 20 patients and demonstrated both anti-tumour and antiviral activity[74]. Recently, a large phase II trial (CheckMate-040 trial) with the agent Nivolumab (anti-PD-1 drug) has led to significant attention because of strong anti-tumour responses, leading to improved survival and led to accelerated approval by the FDA in 2017 for the treatment of patients after failure with Sorafenib[75].

Novel therapeutics which alter T-cell regulation are now being pursued for HCC. A study by Sia *et al*[76] looked at over 900 HCC samples and identified that around 25% exhibited high expression levels of programmed death ligand-1 (PD-L1) and programmed cell death protein 1 (PD-1) and a subgroup expressed many genes that are regulated by TGF β1, a cytokine which is linked to aggressive cancers and suppresses the immune response. TGF-β is involved in cell proliferation, angiogenesis, migration, immune infiltration, metastases dissemination, and drug resistance[77]. There are ongoing trials in HCC using the TGFβ1 inhibitor Galunisertib. The phase 2 trial using Galunisertib as monotherapy has shown promise with improved overall survival in AFP responders[78]. We are entering an era where we may be able to identify which tumours are most likely to respond to immunotherapy, tailoring treatment to the tumour biology.

With these advances in treatment of advanced HCC, clinicians will need to consider how best to manage the underlying chronic liver disease that is associated with HCC. Attempts to improve liver function will have the aim of (1) increasing the number of patients eligible for these novel therapies; (2) to minimise the potential liver related side effects of these novel agents and (3) to prolong the overall survival in patients whose tumours respond to these agents.

Management approaches of chronic liver disease for patients with HCC

Targeting the initiating factors of chronic liver disease can significantly improve liver function in patients even when they have established cirrhosis. Animal models have demonstrated that severe fibrosis can undergo resolution and healing through cell-mediated mechanisms including reducing the number of activated HSCs and the contribution of macrophages[79]. Patients with identifiable factors such as excess alcohol intake and metabolic syndrome benefit significantly from becoming abstinent and improving their metabolic risk factors respectively. Those with active chronic hepatitis B undergo significant improvement of liver function by suppressing viral replication. The treatment of HCV has seen dramatic improvements with the advent of direct acting antivirals[80]. Significant improvement of fibrosis has also been demonstrated in patients with HBV and HCV antiviral medication[81]. One assumption would be that the clearance of Hepatitis C would be beneficial in patients to improve liver function and reduction of future HCC recurrence. This is countered by recent reports suggesting that the viral clearance of HCV could alter immunological surveillance of tumour cells. Case series have described aggressive HCC in patients with cirrhosis after completing successful treatment of hepatitis C[82]. It is not possible to draw conclusions from these findings currently and further studies are required to clarify the situation.

In addition to treating the cause of chronic liver disease, there may also be benefit in preventing the complications of cirrhosis. Variceal bleeding is a complication of cirrhosis associated with a very high mortality rate (20%)[83]. It is well established that patients with HCC have a higher mortality rate compared to matched cirrhosis groups[84,85]. Ripoll *et al*[86] in their study confirmed this and furthermore demonstrated that less than half of HCC patients who were eligible for primary prophylaxis for bleeding were actually prescribed this medication. They also suggested that secondary prophylaxis improved survival in HCC patients.

Loss of muscle mass and function is provided the term sarcopenia and is frequently seen in advanced liver disease. The prevalence is estimated to be between 40%-70% in patients with cirrhosis alone[87]. The underlying mechanism is complex and not fully understood but includes inadequate intake, malabsorption and abnormal metabolism favouring proteolysis for gluconeogenesis. The addition of anti-cancer therapies can further compound the situation. A study looking at whether sarcopenia predicts the prognosis of patients treated with Sorafenib showed that skeletal muscle depletion is an independent prognostic factor[88]. The same has also been shown to apply to patients who have undergone transarterial chemoembolisation treatment for their HCC[89]. Although there is no evidence that sarcopenia is impacting on HCC progression, there is an impact on outcomes and therefore it should be recognised and addressed as part of the patient management pathway.

Carcinogenesis: Prevention is better than cure

With the aim to reduce the complications of cirrhosis

Table 2 Summary of some of the antifibrotic agents being actively pursued in clinical trials, outlining mechanism and key outcomes to date

Drug	Mechanism	Comment	Ref.
BMS-986263/ND-LO2-s0201	siRNA that inhibits HSP47, reducing type 1 collagen synthesis	A lipid nanoparticle containing siRNA that inhibits HSP47. Vitamin A conjugated to the nanoparticle surface target facilitating targeted delivery to HSC and preclinical studies suggest disruption of collagen synthesis which may reverse fibrosis. A phase 1 study has demonstrated tolerability	[94]
Simtuzumab	Inhibition of Lysyl oxidases (LOX) mediated collagen cross linking reduces the breakdown of collagen by proteases such as MMPs	Simtuzumab is a humanized monoclonal antibody. It binds to LOXL2 and acts as an immunomodulator. However in a large phase 2 clinical trial in patients with NASH fibrosis the results were disappointing and focus has been diverted to LOXL1 inhibition where expression appears constant in carbon tetrachloride induced fibrosis in mouse models	[95,96]
Selonsertib	Inhibits apoptosis signal-regulating kinase 1 which in the setting of oxidative stress activates pathways which lead to fibrosis	In patients with NASH has shown promise that it may lead to a reduction in fibrosis in a phase 2 trial where it was given with and without Simtuzumab and compared to Simtuzumab alone	[97,98]
Cenicriviroc	Dual antagonist of C-C motif chemokine receptor (CCR) types 2 and 5	Demonstrated anti-fibrotic activity in animal models of liver fibrosis. In a phase 2 study improvements were seen in noninvasive markers of hepatic fibrosis. Antagonism of CCR2 reduces pro-inflammatory monocytes and macrophages. CCR5 antagonism impairs the activation of HSCs	[99-101]
Emricasan	Inhibitor of apoptotic and inflammatory caspases	Emricasan in the murine NASH model attenuated HSC activation. In phase 2 clinical trials for the regression of hepatic fibrosis caused by HCV infection after liver transplantation, the study didn't reach its primary endpoint but the results from a phase 2 trial in NASH are awaited	[102]
GR-MD-02	Targets galectin-3	Phase 3 studies are already planned for GR-MD-02. Phase 1 and 2 have been completed in the NASH cohort. Preclinical data showed some reversal of fibrosis and a reduction in portal pressures in cirrhosis	[103]
Erlotinib	Epidermal growth factor (EGF) receptor inhibitor	In preclinical models the FDA approved inhibitor regressed fibrosis in some animals and blocked the development of HCC. A pilot phase1/2 trial is underway	[104]

including decompensation there has been progress in recent years to try to prevent or reverse liver fibrosis and some evidence that this reduces HCC risk[90]. The WHO recognise that the viral hepatitis pandemic contributes to an estimated 1.4 million deaths per year including HCC and cirrhosis[91]. The published strategy outlines aims of reducing transmission of HCV and creating global access to treatment by 2030.

Identification and addressing of specific aetiologies, such as viral hepatitis and addressing metabolic risk factors in those with NAFLD is clearly essential but there is also a drive to develop specific anti-fibrotic agents. Whether we can reduce carcinogenesis by interrupting or reversing fibrogenic process with specific anti-fibrotic agents remains to be seen but certainly improvement in liver function is desirable to facilitate management and improve outcomes when cancer occurs.

With a better understanding of the processes that lead to fibrogenesis and fibrolysis it is now possible to target the relevant cytokines and effector cells including HSCs, myofibroblasts, profibrogenic immune cells and other ECM targets including collagens and matrix metalloproteinases (MMP) inhibitors like tissue metallopeptidase inhibitor 1. We know that there are collagen types which are increased in liver fibrosis and are potentially targetable by small interfering RNA (siRNA) or antisense oligonucleotides. The therapeutic advantage of these nucleic acid based therapies is that they can now be delivered in vehicles which are preferentially taken up by the cell being targeted achieving knockdown effects only at the site of interest[92,93]

The identification of novel targets in the fibrotic environment has led to the development of therapeutic agents with efficacy in preclinical models and we have seen the first clinical trials take place. We summarise in Table 2[94-104] some of the therapeutic targets being actively pursued for their anti-fibrotic effects. Unfortunately trial design for direct anti-fibrotic agents are not at present designed to assess if they have an effect on HCC prevention.

Pre-clinical experiments have demonstrated inhibiting the pathways for pro-fibrogenic cytokines including

TGF-β and PDGF are also potential anti-fibrotic strategies to be explored further[105]. Integrins are transmembrane receptors that promote ECM adhesion. Some integrins can facilitate MMP activation and activate fibrogenic mediators such as TGF-β1. The identification of integrins which are important in fibrosis has led to mouse studies involving their direct and indirect inhibition[106]. Other preclinical studies are looking at targeting transmembrane collagen receptors, proteins expressed on myofibroblasts and manipulating the inflammatory environment to deactivate matrix producing myofibroblasts and hepatic stellate cells.

CONCLUSION

Hepatocellular cancer is a deadly complication of cirrhosis and remains difficult to diagnose at an early curative stage. The prognosis in advanced cases remains extremely poor despite significant changes in epidemiology and causes of chronic liver disease, with the rising epidemic of obesity. Nevertheless, new studies with novel agents are demonstrating increasing rates of tumour response and stability and there are exciting developments with immunotherapies. The challenge in patients who do respond to anti-cancer therapies is to maintain their underlying liver function in order that intolerable liver related side effects are minimised and patients have the best chance for a prolonged overall survival. Furthermore, standard of care for cirrhosis may not be met in patients with hepatocellular cancer because of assumptions of lack of benefit, but with new treatments leading to improved survival this will need to be reconsidered. There have been significant improvements in elucidating the underlying cellular mechanisms which drive fibrosis and cancer within the liver. Hopefully, new therapies will take advantage of these findings leading to personalised therapeutic combinations for these patients which have the dual effect of promoting fibrosis regression and anti-cancer effects.

ACKNOWLEDGMENTS

The NIHR Birmingham Biomedical Research Centre at the University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

REFERENCES

1 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

2 **Forman D**, Bray F, Brewster DH, Gombe Mbalawa C, Kohler B, Piñeros M, Steliarova-Foucher E, Swaminathan R, Ferlay J, editors. Cancer Incidence in Five Continents, Vol. X. IARC Scientific Publication 2014; No. 164. Lyon: International Agency for Research on Cancer

3 **McGlynn KA**, Petrick JL, London WT. Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. *Clin Liver Dis* 2015; **19**: 223-238 [PMID: 25921660 DOI: 10.1016/j.cld.2015.01.001]

4 **Konfortion J**, Coupland VH, Kocher HM, Allum W, Grocock MJ, Jack RH. Time and deprivation trends in incidence of primary liver cancer subtypes in England. *J Eval Clin Pract* 2014; **20**: 498-504 [PMID: 24902884 DOI: 10.1111/jep.12188]

5 **White DL**, Thrift AP, Kanwal F, Davila J, El-Serag HB. Incidence of Hepatocellular Carcinoma in All 50 United States, From 2000 Through 2012. *Gastroenterology* 2017; **152**: 812-820.e5 [PMID: 27889576 DOI: 10.1053/j.gastro.2016.11.020]

6 **Evert M**, Dombrowski F. [Hepatocellular carcinoma in the non-cirrhotic liver]. *Pathologe* 2008; **29**: 47-52 [PMID: 18057936 DOI: 10.1007/s00292-007-0953-3]

7 **Trevisani F**, Frigerio M, Santi V, Grignaschi A, Bernardi M. Hepatocellular carcinoma in non-cirrhotic liver: a reappraisal. *Dig Liver Dis* 2010; **42**: 341-347 [PMID: 19828388 DOI: 10.1016/j.dld.2009.09.002]

8 **Xiang X**, You XM, Zhong JH, Li LQ. Hepatocellular carcinoma in the absence of cirrhosis in patients with chronic hepatitis B virus infection. *J Hepatol* 2017; **67**: 885-886 [PMID: 28647566 DOI: 10.1016/j.jhep.2017.05.030]

9 **Fattovich G**, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol* 2008; **48**: 335-352 [PMID: 18096267 DOI: 10.1016/j.jhep.2007.11.011]

10 **Gaddikeri S**, McNeeley MF, Wang CL, Bhargava P, Dighe MK, Yeh MM, Dubinsky TJ, Kolokythas O, Lalwani N. Hepatocellular carcinoma in the noncirrhotic liver. *AJR Am J Roentgenol* 2014; **203**: W34-W47 [PMID: 24951228 DOI: 10.2214/AJR.13.11511]

11 **Mittal S**, Sada YH, El-Serag HB, Kanwal F, Duan Z, Temple S, May SB, Kramer JR, Richardson PA, Davila JA. Temporal trends of nonalcoholic fatty liver disease-related hepatocellular carcinoma in the veteran affairs population. *Clin Gastroenterol Hepatol* 2015; **13**: 594-601.e1 [PMID: 25148760 DOI: 10.1016/j.cgh.2014.08.013]

12 **Paradis V**, Zalinski S, Chelbi E, Guedj N, Degos F, Vilgrain V, Bedossa P, Belghiti J. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: a pathological analysis. *Hepatology* 2009; **49**: 851-859 [PMID: 19115377 DOI: 10.1002/hep.22734]

13 **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]

14 **Ascione A**, Fontanella L, Imparato M, Rinaldi L, De Luca M. Mortality from cirrhosis and hepatocellular carcinoma in Western Europe over the last 40 years. *Liver Int* 2017; **37**: 1193-1201 [PMID: 28111883 DOI: 10.1111/liv.13371]

15 **Altekruse SF**, McGlynn KA, Dickie LA, Kleiner DE. Hepatocellular carcinoma confirmation, treatment, and survival in surveillance, epidemiology, and end results registries, 1992-2008. *Hepatology* 2012; **55**: 476-482 [PMID: 21953588 DOI: 10.1002/hep.24710]

16 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50 [PMID: 15508101 DOI: 10.1053/j.gastro.2004.09.014]

17 **Werner M**, Driftmann S, Kleinehr K, Kaiser GM, Mathé Z, Treckmann JW, Paul A, Skibbe K, Timm J, Canbay A, Gerken G, Schlaak JF, Broering R. All-In-One: Advanced preparation of Human Parenchymal and Non-Parenchymal Liver Cells. *PLoS One* 2015; **10**: e0138655 [PMID: 26407160 DOI: 10.1371/journal.pone.0138655]

18 **Zhou WC**, Zhang QB, Qiao L. Pathogenesis of liver cirrhosis. *World J Gastroenterol* 2014; **20**: 7312-7324 [PMID: 24966602 DOI: 10.3748/wjg.v20.i23.7312]

19 **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]
20 **Casini A**, Ceni E, Salzano R, Biondi P, Parola M, Galli A, Foschi M, Caligiuri A, Pinzani M, Surrenti C. Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide. *Hepatology* 1997; **25**: 361-367 [PMID: 9021948 DOI: 10.1053/jhep.1997.v25.pm0009021948]
21 **Borkham-Kamphorst E**, Herrmann J, Stoll D, Treptau J, Gressner AM, Weiskirchen R. Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. *Lab Invest* 2004; **84**: 766-777 [PMID: 15077122 DOI: 10.1038/labinvest.3700094]
22 **Borkham-Kamphorst E**, van Roeyen CR, Ostendorf T, Floege J, Gressner AM, Weiskirchen R. Pro-fibrogenic potential of PDGF-D in liver fibrosis. *J Hepatol* 2007; **46**: 1064-1074 [PMID: 17397961 DOI: 10.1016/j.jhep.2007.01.029]
23 **Liu X**, Hu H, Yin JQ. Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. *Liver Int* 2006; **26**: 8-22 [PMID: 16420505 DOI: 10.1111/j.1478-3231.2005.01192.x]
24 **Kirmaz C**, Terzioglu E, Topalak O, Bayrak P, Yilmaz O, Ersoz G, Sebik F. Serum transforming growth factor-beta1(TGF-beta1) in patients with cirrhosis, chronic hepatitis B and chronic hepatitis C. *Eur Cytokine Netw* 2004; **15**: 112-116 [PMID: 15319169]
25 **Connolly MK**, Bedrosian AS, Mallen-St Clair J, Mitchell AP, Ibrahim J, Stroud A, Pachter HL, Bar-Sagi D, Frey AB, Miller G. In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha. *J Clin Invest* 2009; **119**: 3213-3225 [PMID: 19855130 DOI: 10.1172/JCI37581]
26 **Varela-Rey M**, Fontán-Gabás L, Blanco P, López-Zabalza MJ, Iraburu MJ. Glutathione depletion is involved in the inhibition of procollagen alpha1(I) mRNA levels caused by TNF-alpha on hepatic stellate cells. *Cytokine* 2007; **37**: 212-217 [PMID: 17485223 DOI: 10.1016/j.cyto.2007.03.013]
27 **Gieling RG**, Wallace K, Han YP. Interleukin-1 participates in the progression from liver injury to fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G1324-G1331 [PMID: 19342509 DOI: 10.1152/ajpgi.90564.2008]
28 **Hara M**, Kono H, Furuya S, Hirayama K, Tsuchiya M, Fujii H. Interleukin-17A plays a pivotal role in cholestatic liver fibrosis in mice. *J Surg Res* 2013; **183**: 574-582 [PMID: 23578751 DOI: 10.1016/j.jss.2013.03.025]
29 **Chou WY**, Lu CN, Lee TH, Wu CL, Hung KS, Concejero AM, Jawan B, Wang CH. Electroporative interleukin-10 gene transfer ameliorates carbon tetrachloride-induced murine liver fibrosis by MMP and TIMP modulation. *Acta Pharmacol Sin* 2006; **27**: 469-476 [PMID: 16539848 DOI: 10.1111/j.1745-7254.2006.00304.x]
30 **Kong X**, Feng D, Wang H, Hong F, Bertola A, Wang FS, Gao B. Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. *Hepatology* 2012; **56**: 1150-1159 [PMID: 22473749 DOI: 10.1002/hep.25744]
31 **Kishimoto T**. IL-6: from its discovery to clinical applications. *Int Immunol* 2010; **22**: 347-352 [PMID: 20410258 DOI: 10.1093/intimm/dxq030]
32 **Frank A**, Seitz HK, Bartsch H, Frank N, Nair J. Immunohistochemical detection of 1,N6-ethenodeoxyadenosine in nuclei of human liver affected by diseases predisposing to hepatocarcinogenesis. *Carcinogenesis* 2004; **25**: 1027-1031 [PMID: 14742317 DOI: 10.1093/carcin/bgh089]
33 **Plentz RR**, Caselitz M, Bleck JS, Gebel M, Flemming P, Kubicka S, Manns MP, Rudolph KL. Hepatocellular telomere shortening correlates with chromosomal instability and the development of human hepatoma. *Hepatology* 2004; **40**: 80-86 [PMID: 15239089 DOI: 10.1002/hep.20271]
34 **Llovet JM**, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016; **2**: 16018 [PMID: 27158749 DOI: 10.1038/nrdp.2016.18]
35 **Mohammed NA**, Abd El-Aleem S, Appleton I, Maklouf MM, Said M, McMahon RF. Expression of nitric oxide synthase isoforms in human liver cirrhosis. *J Pathol* 2003; **200**: 647-655 [PMID: 12898602 DOI: 10.1002/path.1377]
36 **Yu DC**, Chen J, Ding YT. Hypoxic and highly angiogenic non-tumor tissues surrounding hepatocellular carcinoma: the 'niche' of endothelial progenitor cells. *Int J Mol Sci* 2010; **11**: 2901-2909 [PMID: 21152281 DOI: 10.3390/ijms11082901]
37 **Girón-González JA**, Martínez-Sierra C, Rodriguez-Ramos C, Macías MA, Rendón P, Díaz F, Fernández-Gutiérrez C, Martín-Herrera L. Implication of inflammation-related cytokines in the natural history of liver cirrhosis. *Liver Int* 2004; **24**: 437-445 [PMID: 15482340 DOI: 10.1111/j.1478-3231.2004.0951.x]
38 **Park EJ**, Lee JH, Yu GY, He G, Ali SR, Holzer RG, Osterreicher CH, Takahashi H, Karin M. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* 2010; **140**: 197-208 [PMID: 20141834 DOI: 10.1016/j.cell.2009.12.052]
39 **Maeda S**, Hikiba Y, Sakamoto K, Nakagawa H, Hirata Y, Hayakawa Y, Yanai A, Ogura K, Karin M, Omata M. Ikappa B kinasebeta/nuclear factor-kappaB activation controls the development of liver metastasis by way of interleukin-6 expression. *Hepatology* 2009; **50**: 1851-1860 [PMID: 19821485 DOI: 10.1002/hep.23199]
40 **Lanaya H**, Natarajan A, Komposch K, Li L, Amberg N, Chen L, Wculek SK, Hammer M, Zenz R, Peck-Radosavljevic M, Sieghart W, Trauner M, Wang H, Sibilia M. EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation. *Nat Cell Biol* 2014; **16**: 972-977 [PMID: 25173978 DOI: 10.1038/ncb3031]
41 **Ji J**, Eggert T, Budhu A, Forgues M, Takai A, Dang H, Ye Q, Lee JS, Kim JH, Greten TF, Wang XW. Hepatic stellate cell and monocyte interaction contributes to poor prognosis in hepatocellular carcinoma. *Hepatology* 2015; **62**: 481-495 [PMID: 25833323 DOI: 10.1002/hep.27822]
42 **Tang Y**, Kitisin K, Jogunoori W, Li C, Deng CX, Mueller SC, Ressom HW, Rashid A, He AR, Mendelson JS, Jessup JM, Shetty K, Zasloff M, Mishra B, Reddy EP, Johnson L, Mishra L. Progenitor/stem cells give rise to liver cancer due to aberrant TGF-beta and IL-6 signaling. *Proc Natl Acad Sci USA* 2008; **105**: 2445-2450 [PMID: 18263735 DOI: 10.1073/pnas.0705395105]
43 **Marquardt JU**, Andersen JB, Thorgeirsson SS. Functional and genetic deconstruction of the cellular origin in liver cancer. *Nat Rev Cancer* 2015; **15**: 653-667 [PMID: 26493646 DOI: 10.1038/nrc4017]
44 **Tummala KS**, Brandt M, Teijeiro A, Graña O, Schwabe RF, Perna C, Djouder N. Hepatocellular Carcinomas Originate Predominantly from Hepatocytes and Benign Lesions from Hepatic Progenitor Cells. *Cell Rep* 2017; **19**: 584-600 [PMID: 28423321 DOI: 10.1016/j.celrep.2017.03.059]
45 **Ley RE**, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 2006; **124**: 837-848 [PMID: 16497592 DOI: 10.1016/j.cell.2006.02.017]
46 **Yu LX**, Yan HX, Liu Q, Yang W, Wu HP, Dong W, Tang L, Lin Y, He YQ, Zou SS, Wang C, Zhang HL, Cao GW, Wu MC, Wang HY. Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. *Hepatology* 2010; **52**: 1322-1333 [PMID: 20803560 DOI: 10.1002/hep.23845]
47 **Seki E**, Schnabl B. Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. *J Physiol* 2012; **590**: 447-458 [PMID: 22124143 DOI: 10.1113/jphysiol.2011.219691]
48 **Zeuzem S**. Gut-liver axis. *Int J Colorectal Dis* 2000; **15**: 59-82 [PMID: 10855547 DOI: 10.1007/s003840050236]
49 **Zhang HL**, Yu LX, Yang W, Tang L, Lin Y, Wu H, Zhai B, Tan YX, Shan L, Liu Q, Chen HY, Dai RY, Qiu BJ, He YQ, Wang C, Zheng LY, Li YQ, Wu FQ, Li Z, Yan HX, Wang HY. Profound impact of gut homeostasis on chemically-induced pro-tumorigenic inflammation and hepatocarcinogenesis in rats. *J Hepatol* 2012; **57**: 803-812 [PMID: 22727732 DOI: 10.1016/j.jhep.2012.06.011]
50 **Dapito DH**, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, Khiabanian H, Adeyemi A, Bataller

R, Lefkowitch JH, Bower M, Friedman R, Sartor RB, Rabadan R, Schwabe RF. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* 2012; **21**: 504-516 [PMID: 22516259 DOI: 10.1016/j.ccr.2012.02.007]

51 **Yu LX**, Schwabe RF. The gut microbiome and liver cancer: mechanisms and clinical translation. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 527-539 [PMID: 28676707 DOI: 10.1038/nrgastro.2017.72]

52 **Sachdeva M**, Chawla YK, Arora SK. Immunology of hepatocellular carcinoma. *World J Hepatol* 2015; **7**: 2080-2090 [PMID: 26301050 DOI: 10.4254/wjh.v7.i17.2080]

53 **Chen S**, Akbar SM, Tanimoto K, Ninomiya T, Iuchi H, Michitaka K, Horiike N, Onji M. Absence of CD83-positive mature and activated dendritic cells at cancer nodules from patients with hepatocellular carcinoma: relevance to hepatocarcinogenesis. *Cancer Lett* 2000; **148**: 49-57 [PMID: 10680592 DOI: 10.1016/S0304-3835(99)00312-2]

54 **Capece D**, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, Zazzeroni F, Alesse E. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *Biomed Res Int* 2013; **2013**: 187204 [PMID: 23533994 DOI: 10.1155/2013/187204]

55 **Hoechst B**, Ormandy LA, Ballmaier M, Lehner F, Krüger C, Manns MP, Greten TF, Korangy F. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. *Gastroenterology* 2008; **135**: 234-243 [PMID: 18485901 DOI: 10.1053/j.gastro.2008.03.020]

56 **Cai L**, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, Shi M, Zhang H, Yang Y, Wu H, Tien P, Wang FS. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. *Clin Immunol* 2008; **129**: 428-437 [PMID: 18824414 DOI: 10.1016/j.clim.2008.08.012]

57 **Wang JC**, Livingstone AM. Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo. *J Immunol* 2003; **171**: 6339-6343 [PMID: 14662830]

58 **McGovern BH**, Golan Y, Lopez M, Pratt D, Lawton A, Moore G, Epstein M, Knox TA. The impact of cirrhosis on CD4+ T cell counts in HIV-seronegative patients. *Clin Infect Dis* 2007; **44**: 431-437 [PMID: 17205454 DOI: 10.1086/509580]

59 **Ormandy LA**, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. *Cancer Res* 2005; **65**: 2457-2464 [PMID: 15781662 DOI: 10.1158/0008-5472.CAN-04-3232]

60 **Greten TF**, Ormandy LA, Fikuart A, Höchst B, Henschen S, Hörning M, Manns MP, Korangy F. Low-dose cyclophosphamide treatment impairs regulatory T cells and unmasks AFP-specific CD4+ T-cell responses in patients with advanced HCC. *J Immunother* 2010; **33**: 211-218 [PMID: 20139774 DOI: 10.1097/CJI.0b013e3181bb499f]

61 **Cany J**, Tran L, Gauttier V, Judor JP, Vassaux G, Ferry N, Conchon S. Immunotherapy of hepatocellular carcinoma: is there a place for regulatory T-lymphocyte depletion? *Immunotherapy* 2011; **3**: 32-34 [PMID: 21524167 DOI: 10.2217/imt.11.29]

62 **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]

63 **Bricard G**, Cesson V, Devevre E, Bouzourene H, Barbey C, Rufer N, Im JS, Alves PM, Martinet O, Halkic N, Cerottini JC, Romero P, Porcelli SA, Macdonald HR, Speiser DE. Enrichment of human CD4+ V(alpha)24/Vbeta11 invariant NKT cells in intrahepatic malignant tumors. *J Immunol* 2009; **182**: 5140-5151 [PMID: 19342695 DOI: 10.4049/jimmunol.0711086]

64 **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

65 **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 DOI: 10.1016/j.jhep.2011.12.001]

66 **Heimbach JK**. Overview of the Updated AASLD Guidelines for the Management of HCC. *Gastroenterol Hepatol* (NY) 2017; **13**: 751-753 [PMID: 29339953]

67 **Hoshida Y**, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, Reich M, Chan JA, Glickman JN, Ikeda K, Hashimoto M, Watanabe G, Daidone MG, Roayaie S, Schwartz M, Thung S, Salvesen HB, Gabriel S, Mazzaferro V, Bruix J, Friedman SL, Kumada H, Llovet JM, Golub TR. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 1995-2004 [PMID: 18923165 DOI: 10.1056/NEJMoa0804525]

68 **Johnson PJ**, Berhane S, Kagebayashi C, Satomura S, Teng M, Reeves HL, O'Beirne J, Fox R, Skowronska A, Palmer D, Yeo W, Mo F, Lai P, Iñarrairaegui M, Chan SL, Sangro B, Miksad R, Tada T, Kumada T, Toyoda H. Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach-the ALBI grade. *J Clin Oncol* 2015; **33**: 550-558 [PMID: 25512453 DOI: 10.1200/JCO.2014.57.9151]

69 **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]

70 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

71 **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

72 **Meyer T**, Fox R, Ma YT, Ross PJ, James MW, Sturgess R, Stubbs C, Stocken DD, Wall L, Watkinson A, Hacking N, Evans TRJ, Collins P, Hubner RA, Cunningham D, Primrose JN, Johnson PJ, Palmer DH. Sorafenib in combination with transarterial chemoembolisation in patients with unresectable hepatocellular carcinoma (TACE 2): a randomised placebo-controlled, double-blind, phase 3 trial. *Lancet Gastroenterol Hepatol* 2017; **2**: 565-575 [PMID: 28648803 DOI: 10.1016/S2468-1253(17)30156-5]

73 **Bruix J**, Qin S, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Breder V, Gerolami R, Masi G, Ross PJ, Song T, Bronowicki JP, Ollivier-Hourmand I, Kudo M, Cheng AL, Llovet JM, Finn RS, LeBerre MA, Baumhauer A, Meinhardt G, Han G; RESORCE Investigators. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **389**: 56-66 [PMID: 27932229 DOI: 10.1016/S0140-6736(16)32453-9]

74 **Sangro B**, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, Riezu-Boj JI, Larrea E, Alfaro C, Sarobe P, Lasarte JJ, Pérez-Gracia JL, Melero I, Prieto J. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; **59**: 81-88 [PMID: 23466307 DOI: 10.1016/j.jhep.2013.02.022]

75 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-

comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]
76 **Sia D**, Jiao Y, Martinez-Quetglas I, Kuchuk O, Villacorta-Martin C, Castro de Moura M, Putra J, Camprecios G, Bassaganyas L, Akers N, Losic B, Waxman S, Thung SN, Mazzaferro V, Esteller M, Friedman SL, Schwartz M, Villanueva A, Llovet JM. Identification of an Immune-specific Class of Hepatocellular Carcinoma, Based on Molecular Features. *Gastroenterology* 2017; **153**: 812-826 [PMID: 28624577 DOI: 10.1053/j.gastro.2017.06.007]
77 **Neuzillet C**, Tijeras-Raballand A, Cohen R, Cros J, Faivre S, Raymond E, de Gramont A. Targeting the TGFβ pathway for cancer therapy. *Pharmacol Ther* 2015; **147**: 22-31 [PMID: 25444759 DOI: 10.1016/j.pharmthera.2014.11.001]
78 **Faivre SJ**, Santoro A , Kelley RK, Merle P, Gane E, Douillard J, Waldschmidt D, Mulcahy MF, Costentin C, Minguez B, Papappicco P, Gueorgueva I, Cleverly A, Desaiah D, Lahn MM, Ameryckx S, Benhadji KA, Raymond E, Giannelli G. A phase 2 study of a novel transforming growth factor-beta (TGF-β1) receptor I kinase inhibitor, LY2157299 monohydrate (LY), in patients with advanced hepatocellular carcinoma (HCC). *Journal of Clinical Oncology* 2014; **32**: LBA173 [DOI: 10.1200/jco.2014.32.3_suppl.lba173]
79 **Campana L**, Iredale JP. Regression of Liver Fibrosis. *Semin Liver Dis* 2017; **37**: 1-10 [PMID: 28201843 DOI: 10.1055/s-0036-1597816]
80 **Chung RT**, Baumert TF. Curing chronic hepatitis C--the arc of a medical triumph. *N Engl J Med* 2014; **370**: 1576-1578 [PMID: 24720678 DOI: 10.1056/NEJMp1400986]
81 **Lee YA**, Wallace MC, Friedman SL. Pathobiology of liver fibrosis: a translational success story. *Gut* 2015; **64**: 830-841 [PMID: 25681399 DOI: 10.1136/gutjnl-2014-306842]
82 **Reig M**, Mariño Z, Perelló C, Iñarrairaegui M, Ribeiro A, Lens S, Díaz A, Vilana R, Darnell A, Varela M, Sangro B, Calleja JL, Forns X, Bruix J. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. *J Hepatol* 2016; **65**: 719-726 [PMID: 27084592 DOI: 10.1016/j.jhep.2016.04.008]
83 **El-Serag HB**, Everhart JE. Improved survival after variceal hemorrhage over an 11-year period in the Department of Veterans Affairs. *Am J Gastroenterol* 2000; **95**: 3566-3573 [PMID: 11151893 DOI: 10.1111/j.1572-0241.2000.03376.x]
84 **Lang BH**, Poon RT, Fan ST, Wong J. Outcomes of patients with hepatocellular carcinoma presenting with variceal bleeding. *Am J Gastroenterol* 2004; **99**: 2158-2165 [PMID: 15554997 DOI: 10.1111/j.1572-0241.2004.40336.x]
85 **Singal AK**, Jampana SC, Singal V, Kuo YF. Hepatocellular carcinoma predicts in-hospital mortality from acute variceal hemorrhage among patients with cirrhosis. *J Clin Gastroenterol* 2012; **46**: 613-619 [PMID: 22772741 DOI: 10.1097/MCG.0b013e318256b670]
86 **Ripoll C**, Genescà J, Araujo IK, Graupera I, Augustin S, Tejedor M, Cirera I, Aracil C, Sala M, Hernandez-Guerra M, Llop E, Escorsell A, Catalina MV, Cañete N, Albillos A, Villanueva C, Abraldes JG, Bañares R, Bosch J. Rebleeding prophylaxis improves outcomes in patients with hepatocellular carcinoma. A multicenter case-control study. *Hepatology* 2013; **58**: 2079-2088 [PMID: 23908019 DOI: 10.1002/hep.26629]
87 **Sinclair M**, Gow PJ, Grossmann M, Angus PW. Review article: sarcopenia in cirrhosis--aetiology, implications and potential therapeutic interventions. *Aliment Pharmacol Ther* 2016; **43**: 765-777 [PMID: 26847265 DOI: 10.1111/apt.13549]
88 **Imai K**, Takai K, Hanai T, Ideta T, Miyazaki T, Kochi T, Suetsugu A, Shiraki M, Shimizu M. Skeletal muscle depletion predicts the prognosis of patients with hepatocellular carcinoma treated with sorafenib. *Int J Mol Sci* 2015; **16**: 9612-9624 [PMID: 25927582 DOI: 10.3390/ijms16059612]
89 **Parikh ND**, Zhang P, Singal AG, Derstine BA, Krishnamurthy V, Barman P, Waljee AK, Su GL. Body Composition Predicts Survival in Patients with Hepatocellular Carcinoma Treated with Transarterial Chemoembolization. *Cancer Res Treat* 2018; **50**: 530-537 [PMID: 28602057 DOI: 10.4143/crt.2017.156]
90 **Li DK**, Chung RT. Impact of hepatitis C virus eradication on hepatocellular carcinogenesis. *Cancer* 2015; **121**: 2874-2882 [PMID: 26079399 DOI: 10.1002/cncr.29528]
91 **World Health Organisation**. Global health sector strategy on viral hepatitis 2016-2021. World Health Organisation. WHO reference No: WHO/HIV/2016.06. June 2016. Available from: URL: http://www.who.int/hepatitis/strategy2016-2021/ghss-hep/en/. Cited 28 August 2018
92 **Prakash TP**, Graham MJ, Yu J, Carty R, Low A, Chappell A, Schmidt K, Zhao C, Aghajan M, Murray HF, Riney S, Booten SL, Murray SF, Gaus H, Crosby J, Lima WF, Guo S, Monia BP, Swayze EE, Seth PP. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res* 2014; **42**: 8796-8807 [PMID: 24992960 DOI: 10.1093/nar/gku531]
93 **Zimmermann TS**, Karsten V, Chan A, Chiesa J, Boyce M, Bettencourt BR, Hutabarat R, Nochur S, Vaishnaw A, Gollob J. Clinical Proof of Concept for a Novel Hepatocyte-Targeting GalNAc-siRNA Conjugate. *Mol Ther* 2017; **25**: 71-78 [PMID: 28129130 DOI: 10.1016/j.ymthe.2016.10.019]
94 **Soule B**, Tirucherai G, Kavita U, Kundu S, Christian R. Safety, tolerability, and pharmacokinetics of BMS-986263/ND-L02-s0201, a novel targeted lipid nanoparticle delivering HSP47 siRNA, in healthy participants: A randomised, placebo-controlled, double-blind, phase 1 study. *J Hepatology* 2018; **68**: S112. [DOI: 10.1016/S0168-8278(18)30442-2]
95 **Harrison SA**, Abdelmalek MF, Caldwell S, Shiffman ML, Diehl AM, Ghalib R, Lawitz EJ, Rockey DC, Schall RA, Jia C, McColgan BJ, McHutchison JG, Subramanian GM, Myers RP, Younossi Z, Ratziu V, Muir AJ, Afdhal NH, Goodman Z, Bosch J, Sanyal AJ; GS-US-321-0105 and GS-US-321-0106 Investigators. Simtuzumab Is Ineffective for Patients With Bridging Fibrosis or Compensated Cirrhosis Caused by Nonalcoholic Steatohepatitis. *Gastroenterology* 2018; **155**: 1140-1153 [PMID: 29990488 DOI: 10.1053/j.gastro.2018.07.006]
96 **Perepelyuk M**, Terajima M, Wang AY, Georges PC, Janmey PA, Yamauchi M, Wells RG. Hepatic stellate cells and portal fibroblasts are the major cellular sources of collagens and lysyl oxidases in normal liver and early after injury. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G605-G614 [PMID: 23328207 DOI: 10.1152/ajpgi.00222.2012]
97 **Loomba R**, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, Diehl AM, Djedjos CS, Han L, Myers RP, Subramanian GM, McHutchison JG, Goodman ZD, Afdhal NH, Charlton MR; GS-US-384-1497 Investigators. The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: A randomized, phase 2 trial. *Hepatology* 2017 [PMID: 28892558 DOI: 10.1002/hep.29514.]
98 **Budas G**, Karnik S, Jonnson T, Shafizadeh T, Watkins S, Breckenridge D. Reduction of liver steatosis and fibrosis with an ASK1 inhibitor in a murine model of NASH is accomplished by improvements in cholesterol, bile acid and lipid metabolism. *J Hepatol* 2016; **64**: S170 [DOI: 10.1016/S0168-8278(16)01686-X]
99 **Friedman SL**, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, Francque S, Farrell G, Kowdley KV, Craxi A, Simon K, Fischer L, Melchor-Khan L, Vest J, Wiens BL, Vig P, Seyedkazemi S, Goodman Z, Wong VW, Loomba R, Tacke F, Sanyal A, Lefebvre E. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology* 2018; **67**: 1754-1767 [PMID: 28833331 DOI: 10.1002/hep.29477]
100 **Friedman S**, Sanyal A, Goodman Z, Lefebvre E, Gottwald M, Fischer L, Ratziu V. Efficacy and safety study of cenicriviroc for the treatment of non-alcoholic steatohepatitis in adult subjects with liver fibrosis: CENTAUR Phase 2b study design. *Contemp Clin Trials* 2016; **47**: 356-365 [PMID: 26944023 DOI: 10.1016/j.cct.2016.02.012]
101 **Marra F**, Tacke F. Roles for chemokines in liver disease. *Gastroenterology* 2014; **147**: 577-594.e1 [PMID: 25066692 DOI:

10.1053/j.gastro.2014.06.043]
102 **Barreyro FJ**, Holod S, Finocchietto PV, Camino AM, Aquino JB, Avagnina A, Carreras MC, Poderoso JJ, Gores GJ. The pan-caspase inhibitor Emricasan (IDN-6556) decreases liver injury and fibrosis in a murine model of non-alcoholic steatohepatitis. *Liver Int* 2015; **35**: 953-966 [PMID: 24750664 DOI: 10.1111/liv.12570]
103 **Harrison SA**, Marri SR, Chalasani N, Kohli R, Aronstein W, Thompson GA, Irish W, Miles MV, Xanthakos SA, Lawitz E, Noureddin M, Schiano TD, Siddiqui M, Sanyal A, Neuschwander-Tetri BA, Traber PG. Randomised clinical study: GR-MD-02, a galectin-3 inhibitor, vs. placebo in patients having non-alcoholic steatohepatitis with advanced fibrosis. *Aliment Pharmacol Ther* 2016; **44**: 1183-1198 [PMID: 27778367 DOI: 10.1111/apt.13816]
104 **Fuchs BC**, Hoshida Y, Fujii T, Wei L, Yamada S, Lauwers GY, McGinn CM, DePeralta DK, Chen X, Kuroda T, Lanuti M, Schmitt AD, Gupta S, Crenshaw A, Onofrio R, Taylor B, Winckler W, Bardeesy N, Caravan P, Golub TR, Tanabe KK. Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. *Hepatology* 2014; **59**: 1577-1590 [PMID: 24677197 DOI: 10.1002/hep.26898]
105 **Weiskirchen R**, Weiskirchen S, Tacke F. Recent advances in understanding liver fibrosis: bridging basic science and individualized treatment concepts. *F1000Res* 2018; **7** [PMID: 30002817 DOI: 10.12688/f1000research.14841.1]
106 **Schuppan D**, Ashfaq-Khan M, Yang AT, Kim YO. Liver fibrosis: Direct antifibrotic agents and targeted therapies. *Matrix Biol* 2018; **68-69**: 435-451 [PMID: 29656147 DOI: 10.1016/j.matbio.2018.04.006]

P- Reviewer: Boscá L, Chen YK, Corrales FJ, Guan YS, Reggiani GM
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4448-4461

DOI: 10.3748/wjg.v24.i39.4448

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

ORIGINAL ARTICLE

Basic Study

Sheng-jiang powder ameliorates obesity-induced pancreatic inflammatory injury *via* stimulating activation of the AMPK signalling pathway in rats

Yi-Fan Miao, Juan Li, Yu-Mei Zhang, Lv Zhu, Huan Chen, Ling Yuan, Jing Hu, Xiao-Lin Yi, Qiu-Ting Wu, Mei-Hua Wan, Wen-Fu Tang

Yi-Fan Miao, Juan Li, Yu-Mei Zhang, Lv Zhu, Huan Chen, Ling Yuan, Jing Hu, Xiao-Lin Yi, Qiu-Ting Wu, Mei-Hua Wan, Wen-Fu Tang, Department of Integrative Medicine, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

ORCID number: Yi-Fan Miao (0000-0002-3483-2345); Juan Li (0000-0002-5775-9355); Yu-Mei Zhang (0000-0001-9802-776X); Lv Zhu (0000-0002-4302-3339); Huan Chen (0000-0002-4763-6730); Ling Yuan (0000-0002-0921-713X); Jing Hu (0000-0002-8952-0357); Xiao-Lin Yi (0000-0003-0872-1155); Qiu-Ting Wu (0000-0002-6282-5791); Mei-Hua Wan (0000-0002-1237-9455); Wen-Fu Tang (0000-0001-9294-6634).

Author contributions: Tang WF designed the study; Miao YF, Li J, Zhang YM, Zhu L, Chen H, Yuan L, Hu J, Yi XL, Wu QT, and Wan MH were responsible for the acquisition of data; Miao YF, Li J, and Zhang YM were responsible for the analysis and interpretation of data; Miao YF and Li J were responsible for the drafting of the paper; Tang WF was responsible for critical revisions of the paper; Tang WF and Li J obtained funding and provided study supervision; all authors read and approved the final manuscript.

Supported by the National Natural Science Foundation of China, No. 81603519 and No. 81573857.

Institutional review board statement: This study was reviewed and approved by the Institutional Animal Care and Use Committee of West China Hospital of Sichuan University.

Institutional animal care and use committee statement: All rats were handled according to the University Guidelines and the Animal Care Committee Guidelines of West China Hospital (Chengdu, China) (protocol number, 2017052A).

Conflict-of-interest statement: None of the authors have any conflicts of interest to declare.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Wen-Fu Tang, PhD, Professor, Department of Integrative Medicine, West China Hospital, Sichuan University, No. 37, Guoxue Lane, Wuhou District, Chengdu 610041, Sichuan Province, China. tangwf@scu.edu.cn
Telephone: +86-28-85423546
Fax: +86-28-85423373

Received: July 9, 2018
Peer-review started: July 9, 2018
First decision: August 25, 2018
Revised: September 12, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

AIM

To investigate the mechanisms by which Sheng-jiang powder (SJP) ameliorates obesity-induced pancreatic inflammatory injury.

METHODS

Sprague-Dawley rats were randomized into three groups: normal group (NG), obese group (HLG), or SJP treatment group (HSG). Obesity was induced by feeding a high-fat diet in the HLG and HSG, while the NG received standard chow. Rats were euthanized after 12 wk, and blood and pancreatic tissues were collected for histopathological analyses. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transforming growth factor beta (TGF-β) expression, serum triglyceride and adiponectin levels, and apoptosis in pancreatic acinar cells were assessed. A high-fat AR42J acinar cell injury model was established using very low-density lipoprotein (VLDL). AR42J acinar cell culture supernatant, treated with different interventions, was applied to seven groups of pancreatic stellate cells (PSCs). The proliferation of PSCs and the expression of fibronectin and type I collagenase were assessed.

RESULTS

Compared with the NG, we found higher pathological scores for pancreatic tissues, lower serum adiponectin levels, higher expression levels of NF-κB in pancreatic tissues and TGF-β in pancreatic inflammatory cells, and increased apoptosis among pancreatic acinar cells for the HLG ($P < 0.05$). Compared with the HLG, we found reduced body weight, Lee's index scores, serum triglyceride levels, and pathological scores for pancreatic tissues; higher serum adiponectin levels; and lower expression levels of NF-κB, in pancreatic tissue and TGF-β in pancreatic inflammatory cells for the HSG ($P < 0.05$). The *in vitro* studies showed enhanced PSC activation and increased expression levels of fibronectin and type I collagenase after SJP treatment. An adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibitor inhibited PSC activation.

CONCLUSION

SJP may ameliorate obesity-induced pancreatic inflammatory injury in rats by regulating key molecules of the adiponectin-AMPK signalling pathway.

Key words: Obesity; Sheng-jiang powder; Adiponectin; Adenosine 5'-monophosphate-activated protein kinase; Pancreatic inflammatory injury

Core tip: Obesity is a risk factor for non-alcoholic fatty pancreas disease and induces pancreatic inflammatory injury. Sheng-jiang powder (SJP) can ameliorate obesity-induced pancreatic inflammatory injury; however, the specific mechanisms remain unclear. This study demonstrates that SJP may inhibit the inflammatory response, prevent pancreatic fibrosis, promote pancreatic acinar cell repair, and ultimately ameliorate obesity-induced pancreatic inflammatory injury in rats by regulating the key molecules of the AMPK signalling pathway.

Miao YF, Li J, Zhang YM, Zhu L, Chen H, Yuan L, Hu J, Yi XL, Wu QT, Wan MH, Tang WF. Sheng-jiang powder ameliorates obesity-induced pancreatic inflammatory injury *via* stimulating activation of the AMPK signalling pathway in rats. *World J Gastroenterol* 2018; 24(39): 4448-4461 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4448.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4448

INTRODUCTION

Obesity rates have increased sharply over the past 40 years, creating a global public health crisis[1]. According to the results of the Global Burden of Disease Study 2013, the number of overweight and obese individuals increased to 2.1 billion worldwide in 2013, which is 2.28 times more than that in 1980[2]. Obesity or excess weight can lead to high morbidity for many noncommunicable diseases, including 75% of hypertension, 44% of the diabetes burden, 23% of ischaemic heart disease, and 7%-41% of certain cancers[3]. Additionally, the prevalence of non-alcoholic fatty pancreas disease (NAFPD), which is characterized by pancreatic fat infiltration due to obesity, ranges from 16% to 35% in Asian populations[4,5]. In addition, NAFPD may play an important role in the development of type 2 diabetes (T2DM), acute pancreatitis, and even pancreatic cancer[5]. As a result, obesity- and excess weight-related complications have led to a considerable burden on patients and the society. Withrow and Alter (2011)[6] indicated that obesity accounted for between 0.7% and 2.8% of the total healthcare costs of a country. Therefore, due to the side effects of the current treatments for obesity and the lack of specific drugs, people have gradually begun to focus on interventions using traditional Chinese medicine (TCM), such as Sheng-jiang powder (SJP)[3,7].

The pathogenesis of obesity-induced tissue injury is complex and diverse. The most common pathogeneses are endoplasmic reticulum (ER) stress and the inflammatory response[5,8]. According to experimental reports, maternal obesity and postnatal obesogenic diets can result in NAFPD because of an ER imbalance and an alteration in circadian metabolic patterns[9]. Obesity-induced inflammation is a chronic and low-grade form of inflammation, which starts in adipose tissue, with abundant macrophage infiltration, followed by the increased secretion of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and C-reactive protein, while the production of anti-inflammatory cytokines, such as interleukin 10 (IL-10) and adiponectin, drastically decreases[10]. Gotoh *et al*[11] found that obesity reduced the production of spleen-derived IL-10, which can protect against the development of NAFPD. In addition, insulin resistance (IR) and β-cell dysfunction also play important roles[12]. IR decreases the inhibitory activity of insulin on peripheral

lipolysis, leading to an increase in circulating free fatty acids (FFAs). The chronic exposure of β-cells to elevated FFAs results in β-cell dysfunction and creates a vicious cycle resulting in the continuous deterioration of the glucometabolic state[13]. Although obesity-induced pancreatic injury is known to be related to the inflammatory response[7], the specific and detailed mechanisms involved remain unclear.

According to the TCM theory, obesity belongs to the category of "Turbidity", which is primarily caused by the "ascending and descending dysfunction" of the spleen[14]. As a classic representative formula for ascending lucidity and descending turbidity, SJP originates from a Nei-Fu-Xian-Fang decoction in *Wanbing Huichun*, which was compiled by Ting-Xian Gong during the Ming dynasty in China and is composed of Jiangchan (*Bombyx Batryticatus*), Chantui (*Periostracum cicada*), Jianghuang (*Curcuma longa* L.), and Dahuang (*Rheum palmatum* L.)[15]. Several clinical studies have confirmed that SJP is effective in regulating lipid metabolism and improving IR, and SJP is widely used to treat obesity-related diseases, such as hyperlipidaemia, fatty liver, and diabetes[16-18]. Our previous studies have demonstrated that SJP can ameliorate the inflammatory response and histopathological lesions in the pancreas of obese rats[7]. However, the specific mechanisms underlying the amelioration of obesity-induced pancreatic inflammatory injury by SJP are far from being sufficiently understood. Therefore, we designed this study to further investigate the specific mechanisms of SJP on obesity-induced pancreatic inflammatory injury.

MATERIALS AND METHODS

Preparation of SJP for oral administration to rats

The spray-dried drug particles of SJP ingredients, including Dahuang (batch No. 16110150), Jianghuang (batch No. 16080008), Jiangcan (batch No. 16100147), and Chantui (batch No. 16080020), were purchased from the Affiliated Hospital of Chengdu University of TCM (Chengdu, China) and authenticated by Professor Wang WM (Department of Herbal Pharmacy, West China Hospital, Sichuan University, China), according to the Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2010). Voucher specimens were deposited at our laboratory. The spray-dried drug particles were mixed in the proportions of 4:3:2:1, according to Ting-Xian Gong's *Wanbing Huichun*, a famous, classic TCM book from the Ming dynasty[15], and they were completely reconstituted with sterile double-distilled water (concentration: 1 g/mL). This SJP solution was stored at 4 ℃ until ready for use, and it was administered orally to the rats at a dose of 5 mL/kg of body weight (BW).

Preparation of SJP for cell treatment

Our previous study determined that the serum peak concentration of rhein in the plasma of rats that received orally administered SJP (Dahuang, Jianghuang, Jiangcan, and Chantui proportions: 12:9:6:3) was 4388 ± 957 μg/L; thus, for convenience, we used 5000 μg/L for calculations[19]. According to the above concentration and the content of rhein in the SJP compound formula (Dahuang, Jianghuang, Jiangcan, and Chantui proportions: 12:9:6:3; the ratio of rhein to the SJP compound formula was 0.5 mg/g)[20], we calculated the compound dosage for cell treatments as follows: 1 g of SJP compound formula was added to 100 mL of PBS (SH30256.01B, HyClone, Logan, UT, United States) to dilute to a 1 × working concentration. In this study, 1 mL of the above 1 g/mL SJP solution for rat oral administration was diluted to 100 ×, filtered, and sterilized to prepare the highest concentration of the compound for *in vitro* use.

Preparation of adenosine 5'-monophosphate-activated protein kinase inhibitor Compound C

One gram of Compound C (171260, Merck KGaA, Darmstadt, Hessen, Germany) was dissolved in 1000 mL of phosphate buffer solution (PBS), and the mixture was diluted to a 2.5 mmol/L stock solution (100 ×), sterilized by filtration, and stored at -20 ℃. Before use, the appropriate amount of the above stock solution was diluted 100 ×, for a final working concentration of 25 μmol/L.

Induction of obesity, animal treatments, and sample collection

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of West China Hospital of Sichuan University. Twenty-four male Sprague-Dawley rats, weighing 60-80 g, were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). The protocol was designed to minimize the pain and discomfort of the rats. All rats were acclimatized to laboratory conditions (22 ± 2 ℃, 65% ± 10% relative humidity, 12-h light/12-h dark cycle, *ad libitum* access to water and food) for one week prior to the special feeding. Special feeding meant that the rats had free access to a high-fat diet (HFD; 60% of calories derived from fat; TP23300; Trophic Animal Feed High-tech Co., Ltd., Nantong, China) to induce obesity, or to a control diet (16.7% of calories derived from fat; LAD3001G; Trophic Animal Feed High-tech Co., Ltd., Nantong, China).

All rats were randomly divided into a normal group (NG, control diet), an obese group (HLG, HFD), or an SJP treatment group (HSG, HFD plus SJP), with 8 rats in each group. The whole study lasted for 12 wk. Rats in the HSG were intragastrically administered with SJP (5 g/kg) once daily, beginning in the third week, while the rats in the other two groups were instead administered with equal volumes of normal saline. Food intake was monitored daily. After 12 wk of feeding, the rats were anesthetized (2% sodium pentobarbital, intraperitoneal injection, 40 mg/kg of BW), heart blood samples were taken to test the levels of triglyceride and adiponectin,

and the BW and naso-anal length were measured for Lee's index calculations, using the following formula[21]:

$$\text{Lee's index} = \sqrt[3]{body\ weight(g)} \times 10^3 / naso - anal\ length(cm)\ .$$

Pancreatic tissue samples were obtained for histopathological analyses, immunohistochemistry tests for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transforming growth factor-β (TGF-β), and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). Then, all rats were euthanized with a 2% sodium pentobarbital overdose (intraperitoneal injection, 200 mg/kg of BW).

Measurement of serum triglyceride and adiponectin levels

The blood samples were centrifuged at 1000 r/min for 5 min to collect supernatants for analysis. The levels of triglyceride were measured with a HITACHI automatic biochemical analyser (7170A, HITACHI, Tokyo, Japan), and the levels of adiponectin were measured with ELISA kits (EKT246253, eBio, Wuhan, China). According to the manufacturer's protocol, absorbance was measured at 450 nm with a High Throughput Universal Microplate Assay. The sample values were then read off the standard curve, and the relative concentrations were calculated.

Histopathological analysis of pancreatic tissues

Fresh pancreatic tissue samples were fixed with 40 g/L paraformaldehyde (AR1068, BOSTER, Wuhan, China), embedded in paraffin, sectioned into 5 μm sections, and stained with haematoxylin and eosin. All histopathological sections were observed and scored in a blinded manner by two independent pathologists using the scoring system described by Kusske *et al*[22] (0-4 points: oedema, inflammation, haemorrhage, and necrosis). The total histopathology score is the mean of the combined scores for each parameter from both investigators.

Immunohistochemistry

Paraffin-embedded pancreatic samples were deparaffinized and then rehydrated. Endogenous peroxidase was quenched for 10 min with 30 g/L H_2O_2 and washed three times with distilled water. Sections were immersed in 0.01 mol/L citric acid buffer (pH 6.0), heated in a microwave oven until they were boiled, and then de-energized; the process was repeated 5 min later. After a wash with PBS, the sections were blocked with 5% bovine serum albumin (BSA) confining liquid (AR0004, BOSTER, Wuhan, China) for 10 min, at room temperature, and then excess liquids were removed. Sections were incubated overnight at 4 ℃ with primary antibody against NF-κB p65 (sc-8008, Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:200 dilution) or TGF-β (sc-146, Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:100 dilution). After washing with PBS, the sections were incubated with biotinylated goat-anti-mouse or goat-anti-rabbit IgG (SA2010, BOSTER, Wuhan, China) at 37 ℃ for 30 min and then incubated with a SABC-POD Kit (SA2010, BOSTER, Wuhan, China) for 30 min at 37 ℃. Finally, the sections were stained with a DAB-kit (AR1022, BOSTER, Wuhan, China) for 20 min. The sections were rinsed in tap water and counterstained with haematoxylin. Immunohistochemistry sections were observed and scored in a blinded manner by specialists, using the scoring system described by Xu *et al*[23]. Briefly, the evaluation of the nuclear or cytoplasmic staining reaction was performed in accordance with the immunoreactive score (IRS): IRS = staining intensity (SI) × percentage of positive cells (PP). SI was determined as follows: 0, colourless; 1, light yellow; 2, brownish yellow; and 3, brown. PP was defined as follows: 0, negative; 1, 10% positive cells; 2, 11%-50% positive cells; 3, 51%-75% positive cells; and 4, 75% positive cells. Ten visual fields from different areas of each pancreatic section were used for the IRS evaluation, using the average for statistical analysis.

TUNEL assay for apoptotic cells in pancreatic tissues

The levels of apoptotic cells in pancreatic tissue samples were analysed using a TUNEL detection kit (14590900, Roche, San Francisco, CA, United States), following the manufacturer's instructions. Briefly, the pancreatic tissue sections were covered with proteinase K solution (20 μg/mL proteinase K + 0.01 mol/L Tris/HCL, pH 7-8.0) at room temperature for 15 min before the addition of 50 μL of the TUNEL reaction mixture. After incubation in a humid chamber in the dark for 1 h, the sections were incubated with 50 μL of converter-POD solution at 37 ℃ for 30 min, followed by a final PBS wash. Next, 100 μL of DAB solution (5 μL 20 × DAB + 1 μL 300 g/L H_2O_2 + 94 μL PBS) was added for 10 min at room temperature to develop the slides, followed by three washes with PBS and haematoxylin counterstaining for 2 min. Images were captured using a fluorescence microscope (AX10 imager A2/AX10 cam HRC, Carl Zeiss Jena, Oberkochen, Germany), and the apoptotic index was calculated as the number of apoptotic cells/total number of cells × 100%.

Cell culture

Rat pancreatic acinar AR42J cells (CRL-1492, ATCC, Manassas, VA, United States) were maintained at 37 ℃ in DMEM/F12 medium (SH30023.01B, HyClone, Logan, UT, United States) supplemented with 10% foetal bovine serum (FBS; 16000044, Gibco, Waltham, MA United States), 100 IU penicillin, and 100 μg/mL streptomycin (SV30010, HyClone, Logan, UT, United States) in a 50 mL/L CO_2 atmosphere. Prior to stimulation, cells in the logarithmic growth phase were seeded at 1×10^6 cells/well in 6-well plates and incubated until completely adherent.

Rat pancreatic stellate cells (PSCs; RAT-iCell-g003, Shanghai Deyu Bio-tech Co., Ltd, Shanghai, China) were cultured under the same conditions described above, but the culture medium was changed to 90% RPMI 1640 (SH30809.01B, HyClone, Logan, UT, United

States) supplemented with 10% FBS, 100 IU penicillin, and 100 μg/mL streptomycin. PSCs in the logarithmic growth phase were seeded on polylysine-treated slides to perform cell-climbing. After the PSCs covered the slides, they were treated according to the following experimental design.

Induction of a cell model and stimulation

A high-fat AR42J acinar cell injury model was established by stimulation with 0.06 mg/mL of very low-density lipoprotein (VLDL; LP1, Merck-Millipore, Billerica, MA, United States)[24]. AR42J cells were divided into five groups: normal group (AR42J cells + culture medium), model group (AR42J cells + VLDL), SJP group (AR42J cells + VLDL + SJP), VLDL + Compound C group (AR42J cells + VLDL + Compound C), and SJP + Compound C group (AR42J cells + VLDL + SJP + Compound C). After the AR42J cells were completely adherent, 0.5 mL of medium (80% DMEM/F12 + 20% FBS + 100 IU penicillin + 100 μg/mL streptomycin) was added to the normal group and the model group; 0.25 mL of medium and 0.25 mL of SJP were added to the SJP group; 0.25 mL of medium and 0.25 mL of Compound C were added to the VLDL + Compound C group; and 0.25 mL of SJP and 0.25 mL of Compound C were added to the SJP + Compound C group. Thirty minutes later, 30 μL of culture medium was added to the normal group, and 30 μL of VLDL (5 mg/mL) was added to the other groups for model induction. Culture supernatants were collected 24 h after treatment administration to treat the PSCs, according to the following experimental design.

PSCs that covered the slides were divided into seven groups: A, normal PSCs (VLDL-, culture supernatants-); B, PSCs stimulated directly with VLDL (VLDL+, culture supernatant-); C, PSCs stimulated with normal acinar cell culture supernatant (VLDL-, culture supernatant+); D, PSCs stimulated with acinar cell culture supernatant treated with VLDL (VLDL+, culture supernatant+); E, PSCs stimulated with acinar cell culture supernatant treated with SJP (VLDL+, culture supernatant+, SJP+); F, PSCs stimulated with acinar cell culture supernatant treated with Compound C (VLDL+, culture supernatant+, Compound C+); G, PSCs stimulated with acinar cell culture supernatant treated with Compound C and SJP (VLDL+, culture supernatant+, SJP+, Compound C+). The culture medium was added to group A, diluted VLDL (30 μL VLDL + 2.5 mL medium) was added to group B, and the appropriate acinar cell culture supernatants, as described above, were added to each of the remaining five groups for 6 h, according to a ratio of 100 μl/mL. Then, the slides were collected for immunofluorescence analysis of the expression of fibronectin and type I collagenase.

Immunofluorescence

Slides were fixed in 40 g/L paraformaldehyde for 30 min and rinsed with PBS three times (10 min each time). Slides were permeabilized with 1% Triton X-100 (Sigma, Saint Louis, MO, United States) for 30 min and rinsed with PBS three times (10 min each time). Slides were blocked with 10% goat serum (AR0009, BOSTER, Wuhan, China) at 37 ℃ for 2 h. Anti-collagen I antibody (ab34710, Abcam, Cambridge, MA, United States) and anti-fibronectin antibody (ab6328, Abcam, Cambridge, MA, United States) were added separately and incubated overnight at 4 ℃. After washing with PBS, the secondary antibodies, goat anti-rabbit IgG H&L (Alexa Fluor® 488) (ab150077, Abcam, Cambridge, MA, United States) and goat anti-mouse IgG H&L (Alexa Fluor® 488) (ab150113, Abcam, Cambridge, MA, United States), were added separately and protected against light for 2 h at room temperature. The nucleus was stained with DAPI, and the sections were sealed with glycerine. A fluorescence microscope (AX10 imager A2/AX10 cam HRC, Carl Zeiss Jena, Oberkochen, Germany) was used for observation.

Statistical analysis

The statistical methods of this study were reviewed by Dr. Hai Niu from College of Mathematics, Sichuan University. All values are expressed as the mean ± standard deviation. GraphPad Prism 6.01 software (GraphPad Prism 6.01 software Inc., San Diego, CA, United States) was used for statistical analyses. For each test, the experimental unit was an individual animal. Normality was assessed by the Shapiro-Wilk normality test, and homogeneity of variance was assessed by the Bartlett's test. If data were normally distributed and the variances of three experimental groups were equal, one-way analysis of variance was used for multi-group comparisons, and Dunnett-*t* test was used for comparisons of two groups. Statistical significance is expressed as $^{a}P < 0.05$ *vs* NG or $^{b}P < 0.05$ *vs* HLG.

RESULTS

SJP reduces BW, Lee's index, and serum triglyceride levels of obese rats

After 12 wk of experimental diet consumption, BW, Lee's index, which is a rapid means of determining obesity, and the levels of serum triglyceride of the rats in the HLG were significantly higher than those of the rats in the NG ($P < 0.05$; Table 1). Conversely, the above three parameters of the HSG were significantly lower than those of the HLG ($P < 0.05$; Table 1). However, food intake did not differ significantly among the experimental groups.

SJP relieves the pathological damage to pancreatic tissues in obese rats

The histopathological evaluation results showed significantly higher pathological scores for pancreatic tissues from rats in the HLG than for those in the NG ($P < 0.05$; Figure 1A). Conversely, SJP treatment distinctly lowered the pathological scores of the pancreas, with reduced inflammatory cell infiltration, mild tissue oedema, and

Table 1 Body weight, Lee's index, serum triglyceride levels, and dairy food intake of rats in the three experimental groups

Parameter	NG	HLG	HSG
Initial body weight (g)	69 ± 4	70 ± 5	69 ± 8
Final body weight (g)	461 ± 56	537 ± 46[a]	467 ± 49[b]
Lee's index	3.13 ± 0.07	3.43 ± 0.16[a]	3.12 ± 0.13[b]
Triglyceride (mmol/L)	1.57 ± 0.46	3.24 ± 1.48[a]	1.39 ± 0.41[b]
Food intake (g/d)	19.57 ± 0.87	18.91 ± 1.12	18.01 ± 0.77

The results are presented as the mean ± SD, n = 8 for each group. [a]P < 0.05 *vs* NG; [b]P < 0.05 *vs* HLG. NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder.

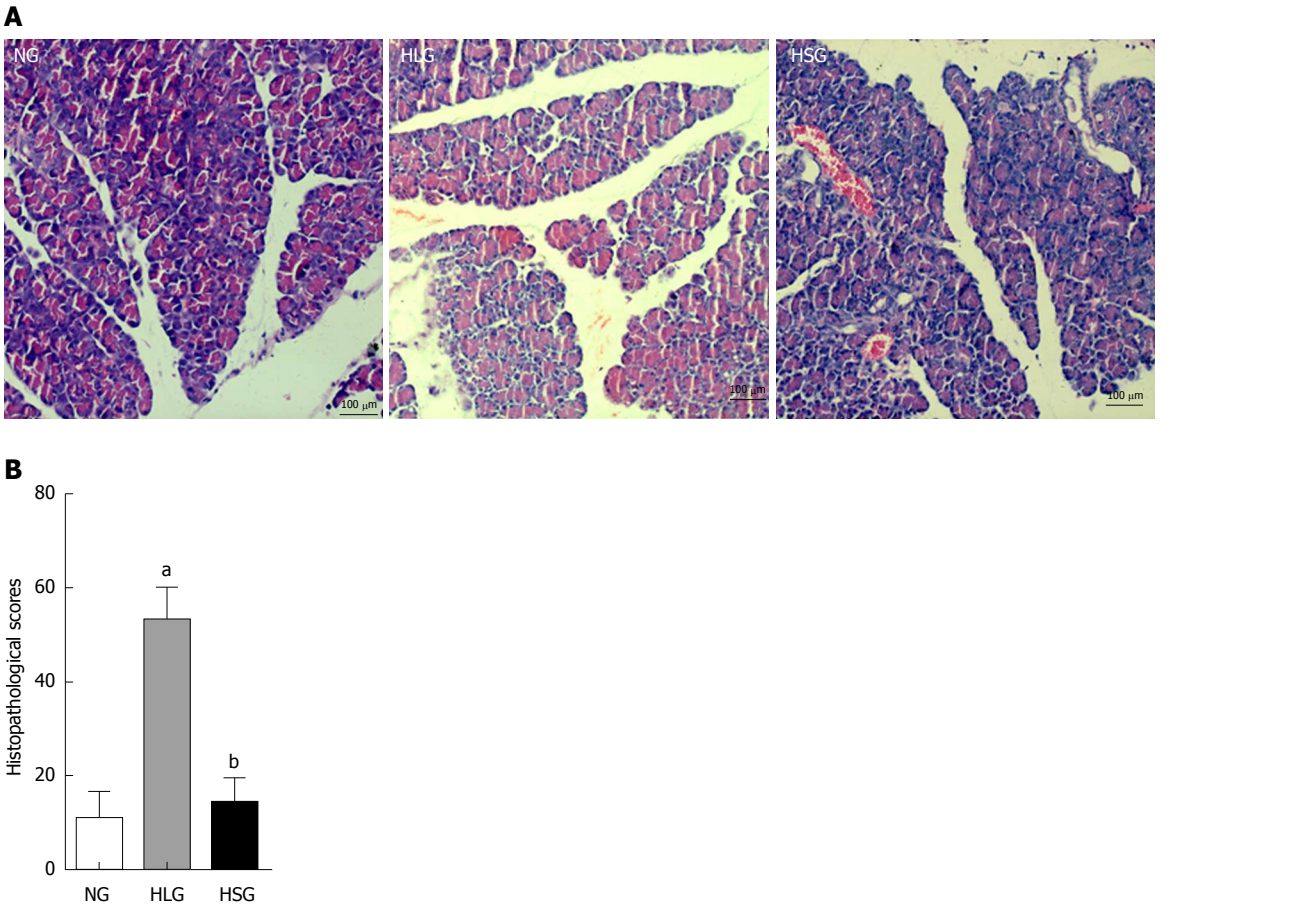


Figure 1 Histological images and pathologic scores of pancreatic tissues from the three experimental groups. A: Pathological images of the pancreatic tissues (× 200); B: Histological scores of the pancreatic tissues. The results are presented as the mean ± SD. [a]P < 0.05 *vs* NG; [b]P < 0.05 *vs* HLG. NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder.

reduced cell necrosis (Figure 1A and B).

SJP stimulates the expression of serum adiponectin in obese rats

Adiponectin is an adipokine with anti-inflammatory, anti-oxidant, anti-atherogenic, pro-angiogenic, vasoprotective, and insulin-sensitizing properties, which is markedly decreased in obesity[25]. Thus, we determined the levels of adiponectin in serum after SJP administration. Adiponectin levels were significantly reduced in the HLG (P < 0.05; Figure 2), whereas they were absent in the NG. After SJP administration, adiponectin levels were much higher in rats in the HSG than in rats in the HLG (P < 0.05; Figure 2).

Effect of SJP on the expression levels of NF-κB and TGF-β in pancreatic acinar cells and inflammatory cells from obese rats

As shown in Figure 3A, the expression levels of NF-κB in both pancreatic acinar cells and inflammatory cells from rats were higher in the HLG than in the NG (P < 0.05). After SJP administration, the expression of NF-κB in both types of cells was inhibited. Although no differences were found in TGF-β expression levels among the groups in the pancreatic acinar cells, an expression pattern similar to that of NF-κB was observed in inflammatory cells,

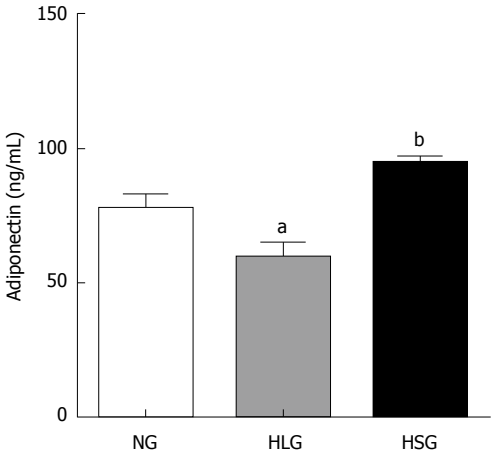


Figure 2 Levels of serum adiponectin. The results are presented as the mean ± SD. [a]*P* < 0.05 *vs* NG; [b]*P* < 0.05 *vs* HLG. NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder.

where TGF-β expression was stimulated by HFD and inhibited by SJP administration (Figure 3A).

Effects of SJP on apoptosis of pancreatic acinar cells in obese rats

Apoptosis of pancreatic acinar cells was significantly higher in the HLG than in the NG ($P < 0.05$; Figure 4). Although we found no significant differences in apoptosis following treatment with SJP, we found a downward trend in the HSG (Figure 4).

SJP promotes PSC activation

To explore whether the repair effects of SJP on pancreatic acinar cell damage occur through adenosine 5'-monophosphate-activated protein kinase (AMPK) signalling, we performed cellular tests of AR42J cells and PSCs. The results showed that the expression of fibronectin and type I collagenase was weak in normal PSCs (Figure 5A). After stimulation with VLDL or normal acinar cell culture supernatant, the expression of fibronectin and type I collagenase increased (Figure 5B and C). The supernatant of VLDL-treated AR42J cells stimulated PSCs to trigger increased expression levels of fibronectin and type I collagenase (Figure 5D), and the expression of these two proteins was enhanced after stimulation with acinar cell culture supernatant treated with SJP (Figure 5E). The fibronectin and type I collagenase expression levels were reduced (Figure 5F and G) in the two groups of PSCs treated with the AMPK inhibitor.

DISCUSSION

In the present study, HFD successfully induced an obese rat model, as in our previous study[7]. Our results showed significantly higher BW, Lee's index scores, and serum triglyceride levels, lower serum adiponectin levels, higher expression levels of NF-κB in pancreatic tissues, and increased apoptosis of pancreatic acinar cells in obese rats, while SJP effectively reduced BW, Lee's index scores, and serum triglyceride levels, stimulated the expression of serum adiponectin, and inhibited the expression of NF-κB in pancreatic tissues. In addition, the expression levels of TGF-β in inflammatory cells of the pancreas were significantly higher in obese rats, while SJP could reduce the expression of TGF-β in inflammatory cells but had no influence in acinar cells. The *in vitro* studies have shown that the culture supernatant from AR42J acinar cells that were incubated with VLDL stimulated the proliferation and matrix synthesis of PSCs. After SJP treatment, PSC activation was enhanced, and the expression of fibronectin and type I collagenase was further increased. Interestingly, AMPK inhibitors inhibited the PSC activation process described above.

Adipose tissue is considered to be an endocrine organ with an important role in local and systemic homeostasis. It has been demonstrated that adipose is responsible for the production and release of many potent signalling molecules, including adipokines, lipokines, and inflammatory mediators[25]. Adiponectin is a well-known adipokine that promotes insulin sensitivity and has an anti-inflammatory effect, and its production becomes blunted as adiposity increases[26]. Generally, obesity in humans is a symptom of energy imbalance, where energy intake exceeds energy output, while AMPK plays a key role in controlling energy homeostasis[27]. Importantly, adiponectin can regulate energy intake and consumption by stimulating the phosphorylation of AMPK; adiponectin phosphorylates and subsequently inhibits acetyl-CoA carboxylase and inhibits malonyl-CoA synthesis, thereby decreasing the inhibitory effect of carnitine acyltransferase 1 (the key enzyme required for activated fatty acid entry into the mitochondria) and leading to increased fatty acid oxidation and glucose uptake[28]. Therefore, with the long-term intake of HFD, the decrease in adiponectin observed in obese rats may affect the energy imbalance and promote fat infiltration or accumulation; in turn, fat accumulation may affect the expression of adiponectin, which leads to a vicious cycle.

In addition to its effect on controlling glucose and lipid metabolism, adiponectin can also inhibit lipopolysaccharide (LPS)-primed inflammasome activation in macrophages via AMPK signalling-dependent mechanisms[29], while adiponectin-AMPK signalling can be inhibited during chronic low-grade inflammatory responses, including obesity, non-alcoholic fatty liver disease, atherosclerosis, IR, and T2DM[30]. Therefore, the decrease in adiponectin observed in obese rats may reduce its inhibitory effect on inflammasome activation and promote the inflammatory response. In our study, SJP ameliorated the expression of adiponectin in rats with obesity induced with an HFD. Similarly, some studies showed that SJP could significantly increase serum adiponectin levels in obesity-related glomerulopathy patients and T2DM patients with dyslipidaemia[18,31]. As a

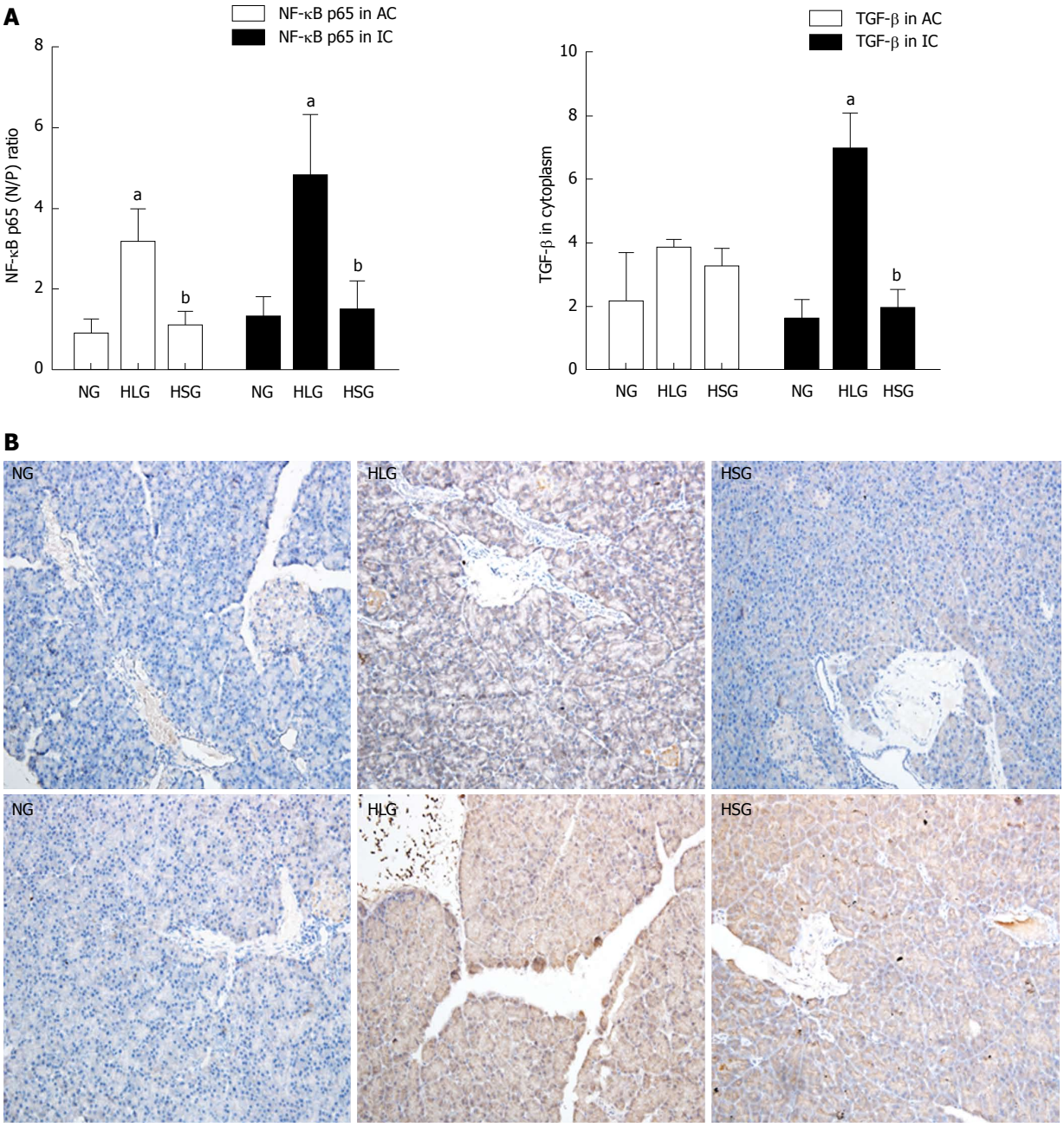


Figure 3 Nuclear factor kappa-light-chain-enhancer of activated B cells and transforming growth factor beta expression in pancreatic tissues. A: Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transforming growth factor beta (TGF-β) expression in pancreatic acinar cells and inflammatory cells; B: Immunohistochemistry assay for NF-κB expression; C: Immunohistochemistry assay for TGF-β expression. Negative: Blue; Positive: Yellow-brown. The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* NG; $^{b}P < 0.05$ *vs* HLG. NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; TGF-β: Transforming growth factor beta; NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder; N/P ratio: Nuclear/cytoplasmic ratio; AC: Acinar cells; IC: Inflammatory cells.

component of *Curcuma longa*, curcumin could attenuate HFD-induced hepatic steatosis by regulating hepatic lipid metabolism via AMPK activation[32]. Thus, combined with the anti-inflammatory effect of SJP, we speculate that SJP may reduce the suppressive effect of obesity on the adiponectin-AMPK signalling pathway, which may contribute to energy consumption and further inhibit the inflammatory response, eventually regulating lipid metabolism in obese rats.

In addition to the aforementioned decrease in adiponectin levels associated with the inflammatory response in obese rats, the NF-κB signalling pathway and ER stress are two other important mechanisms of the obesity-induced inflammatory response. As adiposity increases, the balance between pro-inflammatory and anti-inflammatory cytokines secreted by adipocytes gradually becomes deregulated. In addition, those unbalanced inflammatory cytokines can activate macrophages via the Toll-like receptor 4 signalling pathways, whereas the binding of TNF-α released by macrophages to TNF-α receptors on adipocytes activates the NF-κB signalling pathway[33] and promotes the

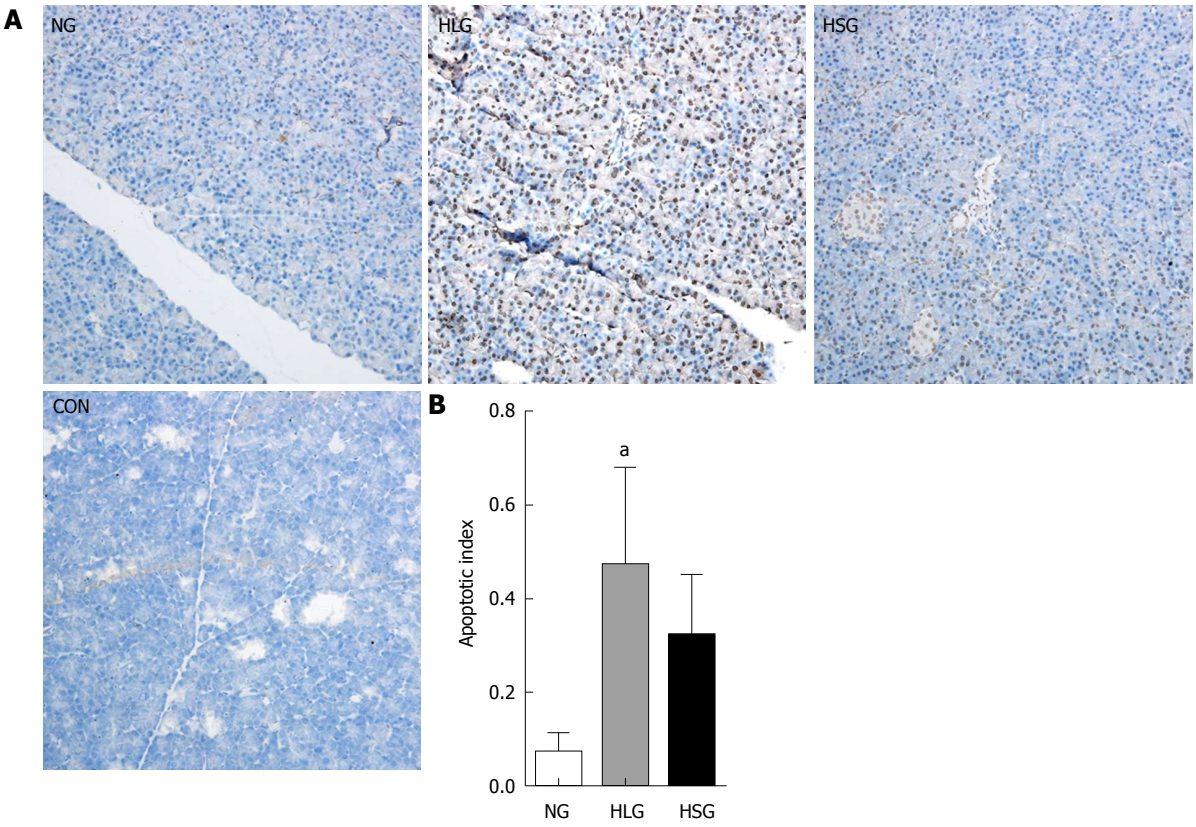


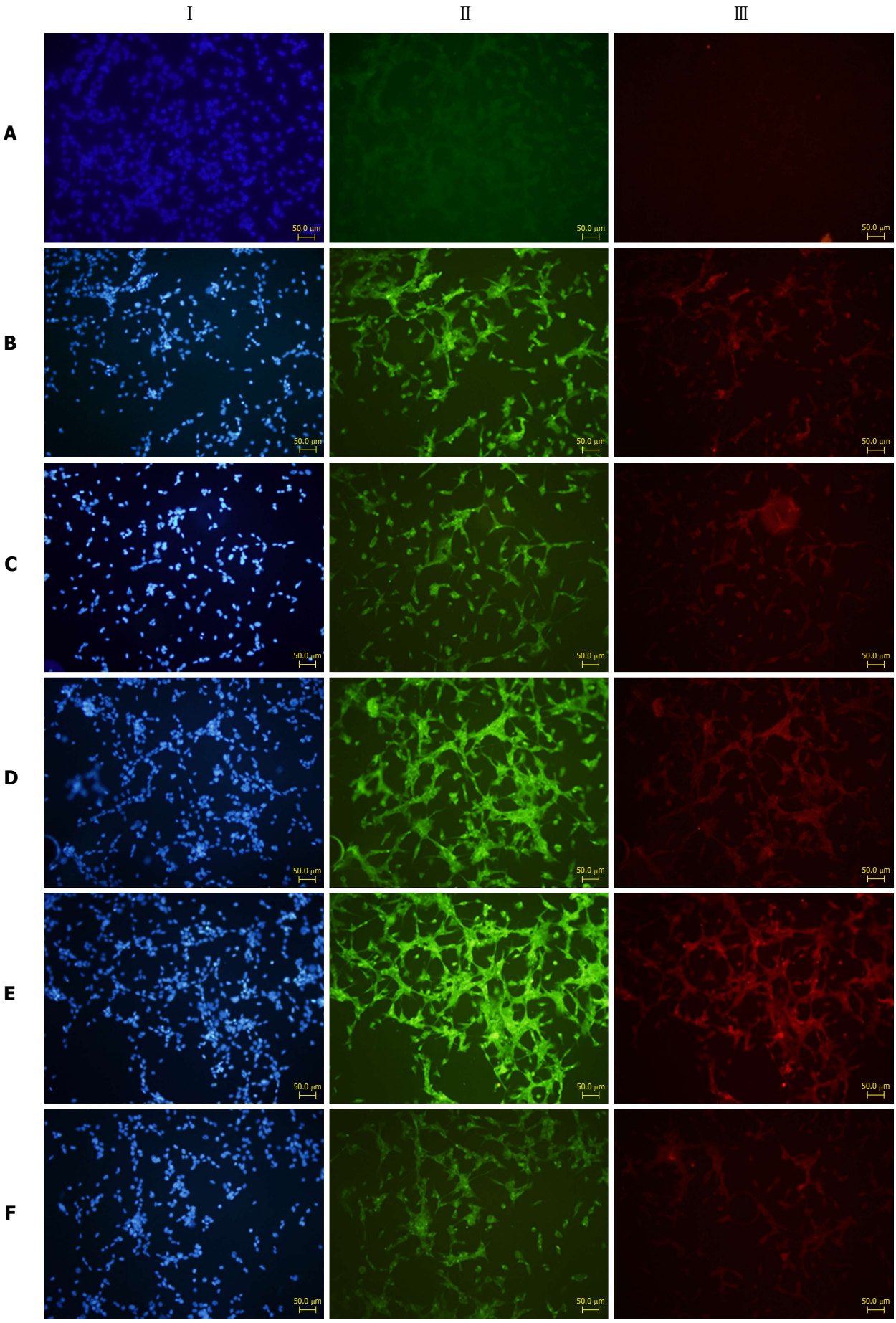
Figure 4 Results of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling staining of pancreatic acinar cells. A: TUNEL staining images of pancreatic acinar cells; B: Apoptotic index of pancreatic acinar cells. Negative: blue; positive: yellow-brown. The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* NG. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder group; CON: Negative staining control group.

amplification of inflammatory responses. In addition, ER stress is currently recognized to be a mechanism of the obesity-induced inflammatory response. Obesity-induced ER stress primarily manifests itself in the activation of two classic signalling pathways: The nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor (IκB)/NF-κB signalling pathway and the c-Jun N-terminal kinase (JNK) signalling pathway[34,35]. The phosphorylation of IκB and JNK triggers the activation of transcription factors, such as NF-κB, that are closely related to the inflammatory response downstream, thereby promoting the development of inflammatory responses[36]. We know that SJP was effective for anti-inflammation and could significantly downregulate the expression of NF-κB in inflammatory diseases, such as acute lung injury and glomerulonephritis[37,38]. This study also confirmed that SJP reduced the expression of NF-κB in pancreatic tissues. Therefore, SJP may reduce the inflammatory response in the pancreas of obese rats via the NF-κB signalling pathway.

TGF-β is a regulatory molecule with pleiotropic effects on cell proliferation, differentiation, migration, and survival and affects multiple biological processes, including development, carcinogenesis, fibrosis, wound healing, and immune responses[39]. Although TGF-β is an important cytokine that regulates tissue inflammation and repair, its overexpression induces fibrosis, and the inhibition of TGF-β improves fibrotic disorder[40,41]. Matsuda *et al*[42] found that, in Zucker diabetic fatty rats fed a chronic HFD, fat could accumulate in pancreatic acinar cells, which was related to subsequent pancreatic fibrosis and acinar cell injury. Similarly, Yoshikawa *et al*[43] demonstrated that TGF-β1 could extend from peri-islets to the exocrine pancreas to become involved in pancreatic fibrosis in Otsuka Long-Evans Tokushima fatty rats, a model of naturally occurring obesity-related diabetes.

In our study, the expression of TGF-β in pancreatic acinar cells was rarely increased after 12 wk of HFD intake, but it was highly expressed in pancreatic inflammatory cells. Therefore, we speculate that obesity, a persistent chronic injury with an accompanying inflammatory response, may result in pancreatic fibrosis. Interestingly, after SJP treatment, the expression of TGF-β in pancreatic inflammatory cells was significantly decreased with a dramatic reduction in NF-κB and an increase in adiponectin. Thus, given the common use of anti-inflammatory drugs in the treatment of fibrosis[40] and the anti-inflammatory effect of SJP, we speculate that SJP may prevent pancreatic fibrosis by inhibiting the inflammatory response in the pancreas of obese rats.

It has been well established that brain, liver, and heart cells undergo apoptosis under obesity conditions[44-46]. To confirm whether obesity induces pancreatic acinar cell


I
II
III
A
B
C
D
E
F
50.0 μm

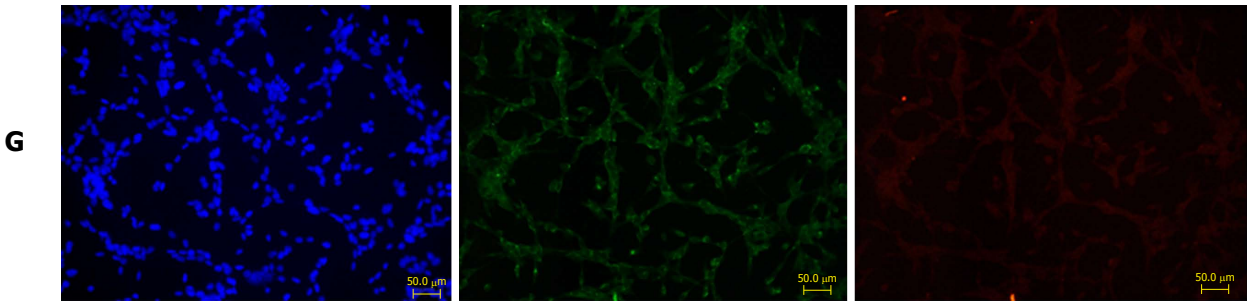


Figure 5 Immunofluorescence results of fibronectin and type I collagenase expression in cells growing on glass coverslips from each group. I: Nuclear staining of rat pancreatic stellate cells (blue fluorescence); II: Fibronectin staining (green fluorescence); III: Type I collagenase staining (red fluorescence). A: Normal PSC (VLDL-, culture supernatant-); B: PSC stimulated directly with VLDL (VLDL+, culture supernatant-); C: PSC stimulated with normal acinar cell culture supernatants (VLDL-, culture supernatant+); D: PSC stimulated with acinar cell culture supernatant treated with VLDL (VLDL+, culture supernatant+); E: PSC stimulated with acinar cell culture supernatant treated with SJP (VLDL+, culture supernatant+, SJP+); F: PSC stimulated with acinar cell culture supernatant treated with Compound C (VLDL+, culture supernatant+, Compound C+); G: PSC stimulated with acinar cell culture supernatant treated with Compound C and SJP (VLDL+, culture supernatant+, SJP+, Compound C+). PSC: Pancreatic stellate cell; VLDL: Very low-density lipoprotein; SJP: Sheng-jiang powder.

apoptosis, we performed TUNEL staining. The results showed that obesity could induce apoptosis of pancreatic acinar cells. Moreover, one study has shown that the phosphorylation of AMPK induced by adiponectin could block interleukin 8-mediated endothelial cell death and exert an anti-apoptotic effect[47]. Considering the fact that SJP increased the levels of serum adiponectin in our study and that curcumin effectively reduced apoptosis of pancreatic acinar cells caused by the long-term intake of alcohol and different amounts of proteins[48], we speculated that SJP might play a role in the obesity-induced apoptosis of pancreatic acinar cells. However, there was no significant difference in the amount of apoptosis in acinar cells after SJP treatment in this study. A possible explanation for this finding might be that a single dose or the dosing concentration was insufficient. To the best of our knowledge, only one study has demonstrated that SJP inhibited the apoptosis of brain cells in rats with vascular dementia, and its administration method was intravenous drip at 10 mL/kg of BW SJP[49].

Almost immediately, from the start of injury, multiple types of cells participate in the process of exocrine pancreas repair and regeneration. These cells include not only acinar cells, which are both villains and victims in pancreatic injury, but also ductal epithelial cells, inflammatory cells of the immune system, and PSCs[50]. Given the importance of epithelial-mesenchymal interactions during pancreas development[51], interactions between parenchymal cells and PSCs are almost certain to be important for proper pancreatic repair. Under physiological conditions, PSCs are at rest. In the presence of profibrogenic mediators, such as inflammatory cytokines and oxidative stress, PSCs are activated[52]. Activated PSCs produce large amounts of a-smooth muscle actin and extracellular matrix proteins, particularly fibronectin and type I collagenase, to achieve the replacement of inflammatory infiltrates and the repair or regeneration of tissue injuries[53]. On the basis of the *in vivo* data, under obesity conditions, inflammation occurred in pancreatic tissues, and subsequently, acinar cell injury arose. Furthermore, culture media treated with fat could stimulate acinar cells to produced profibrogenic mediators. In addition, the supernatants collected from acinar cells stimulated the proliferation of PSCs and the synthesis of extracellular matrix proteins, particularly fibronectin and type I collagenase. After treatment with the AMPK inhibitor, the expression levels of fibronectin and type I collagenase were reduced. For the first time, we found that the inhibition of the AMPK signalling pathway could impair the therapeutic effects of SJP by diminishing the expression of fibronectin and type I collagenase. Therefore, we boldly speculate that SJP may promote acinar cell injury repair through the activation of the AMPK signalling pathway.

This study expanded on the research from our previous study[7]. However, some limitations exist. First, in the *in vivo* experiment, no critical upstream or downstream factors were detected in the adiponectin-AMPK pathway in pancreatic tissue other than serum adiponectin. Second, the relationship between dose or dose frequency and the concentration effect requires further study. Finally, the specific effective monomer components of SJP should be taken under consideration.

In conclusion, we demonstrated that obesity exacerbates pancreatic inflammatory injury in rats and promotes the apoptosis of pancreatic acinar cells. SJP can inhibit the inflammatory response, prevent pancreatic fibrosis, and promote pancreatic acinar cell repair, through the regulation of key molecules of the adiponectin-AMPK signalling pathway, and eventually ameliorate obesity-induced pancreatic inflammatory injury in rats.

ARTICLE HIGHLIGHTS

Research background

Obesity is a risk factor for non-alcoholic fatty pancreas disease and induces pancreatic inflammatory injury. Sheng-jiang powder (SJP) can ameliorate obesity-induced pancreatic inflammatory injury, but the specific mechanisms remain unclear. Therefore, the investigation of the specific mechanisms underlying the SJP amelioration of obesity-induced pancreatic inflammatory

injury is urgently required.

Research motivation

Our previous studies have demonstrated that SJP can ameliorate the inflammatory response and histopathological lesions in the pancreas of obese rats. However, the specific mechanisms underlying ameliorating effects of SJP on obesity-induced pancreatic inflammatory injury are far from sufficiently understood. Therefore, this study aimed to further explore the specific mechanisms of SJP on obesity-induced pancreatic inflammatory injury, to provide evidence for its clinical application in the future.

Research objectives

This study aimed to investigate the specific mechanisms by which SJP can ameliorate obesity-induced pancreatic inflammatory injury.

Research methods

In the *in vivo* study, an obese rat model was induced by high-fat diet feeding, which is widely accepted and used for the induction of obesity in rats. The serum adiponectin levels were measured by enzyme-linked immunosorbent assay (ELISA), which is a simple, rapid, accurate, and sensitive method. The expression levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transforming growth factor beta (TGF-β) in pancreatic tissues were measured by immunohistochemistry. The levels of apoptotic cells in pancreatic tissue samples were analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay.

In the *in vitro* study, a high-fat AR42J acinar cell injury model was established with very low-density lipoprotein (VLDL), and the AR42J acinar cell culture supernatants, treated with different interventions, were applied to pancreatic stellate cells (PSCs). The proliferation of PSCs and the expression of fibronectin and type I collagenase were measured by immunofluorescence analysis.

All statistical analyses were performed with GraphPad Prism 6.01 software. Quantitative data are expressed as the mean ± standard deviation when normally distributed. One-way analysis of variance followed by multiple pair-wise comparisons using Dunnett-*t* test was used to detect differences among the above parameters.

Research results

In the *in vivo* study, compared to the obese group (HLG), we found reduced body weight, Lee's index scores, serum triglyceride levels, and pathological scores of pancreatic tissues; higher serum adiponectin levels; and lower expression levels of NF-κB in pancreatic tissue and TGF-β in the inflammatory cells of the pancreas in the SJP treatment group (HSG) ($P < 0.05$). In the *in vitro* study, PSC activation was enhanced after SJP treatment, and the expression levels of fibronectin and type I collagenase were increased after SJP treatment. An adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibitor inhibited the PSC activation process described above.

What remains to be determined is the relationship between dose or dose frequency and the concentration effect. Furthermore, the specific effective monomer components of SJP should be taken under consideration to provide more systematic and comprehensive evidence for the clinical application of this Chinese decoction.

Research conclusions

This study demonstrates, for the first time, that obesity exacerbates pancreatic inflammatory injury in rats and promotes apoptosis in pancreatic acinar cells. In addition, SJP can inhibit the inflammatory response, prevent pancreatic fibrosis, promote pancreatic acinar cell repair, through the regulation of key molecules of the adiponectin-AMPK signalling pathway, and eventually ameliorate obesity-induced pancreatic inflammatory injury in rats. Therefore, our study provides molecular mechanisms as evidence for the clinical application of SJP.

Research perspectives

As we have found that SJP may ameliorate obesity-induced pancreatic inflammatory injury in rats by regulating key molecules of the adiponectin-AMPK signalling pathway, further investigation regarding the potential active components of SJP and the interactions among these components is urgently required to provide evidence for wider clinical usage and to optimize and simplify the formula.

REFERENCES

1 **World Health Organization**. Obesity and overweight. October 18, 2017. Accessed July 2, 2018 Available from: URL: http://www.who.int/mediacentre/factsheets/fs311/en/

2 **Ng M**, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, Abraham JP, Abu-Rmeileh NM, Achoki T, AlBuhairan FS, Alemu ZA, Alfonso R, Ali MK, Ali R, Guzman NA, Ammar W, Anwari P, Banerjee A, Barquera S, Basu S, Bennett DA, Bhutta Z, Blore J, Cabral N, Nonato IC, Chang JC, Chowdhury R, Courville KJ, Criqui MH, Cundiff DK, Dabhadkar KC, Dandona L, Davis A, Dayama A, Dharmaratne SD, Ding EL, Durrani AM, Esteghamati A, Farzadfar F, Fay DF, Feigin VL, Flaxman A, Forouzanfar MH, Goto A, Green MA, Gupta R, Hafezi-Nejad N, Hankey GJ, Harewood HC, Havmoeller R, Hay S, Hernandez L, Husseini A, Idrisov BT, Ikeda N, Islami F, Jahangir E, Jassal SK, Jee SH, Jeffreys M, Jonas JB, Kabagambe EK, Khalifa SE, Kengne AP, Khader YS, Khang YH, Kim D, Kimokoti RW, Kinge JM, Kokubo Y, Kosen S, Kwan G, Lai T, Leinsalu M, Li Y, Liang X, Liu S, Logroscino G, Lotufo PA, Lu Y, Ma J, Mainoo NK, Mensah GA, Merriman TR, Mokdad AH, Moschandreas J, Naghavi M, Naheed A, Nand D, Narayan KM, Nelson EL, Neuhouser ML, Nisar MI, Ohkubo T, Oti SO, Pedroza A, Prabhakaran D, Roy N, Sampson U, Seo H, Sepanlou SG, Shibuya K, Shiri R, Shiue I, Singh GM, Singh JA, Skirbekk V, Stapelberg NJ, Sturua L, Sykes BL, Tobias M, Tran BX, Trasande L, Toyoshima H, van de Vijver S, Vasankari TJ, Veerman JL, Velasquez-Melendez G, Vlassov VV, Vollset SE, Vos T, Wang C, Wang X, Weiderpass E, Werdecker A, Wright JL, Yang YC, Yatsuya H, Yoon J, Yoon SJ, Zhao Y, Zhou M, Zhu S, Lopez AD, Murray CJ, Gakidou E. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2014; **384**: 766-781 [PMID: 24880830 DOI: 10.1016/S0140-6736(14)60460-8]

3 **Cefalu WT**, Bray GA, Home PD, Garvey WT, Klein S, Pi-Sunyer FX, Hu FB, Raz I, Van Gaal L, Wolfe BM, Ryan DH. Advances in the Science, Treatment, and Prevention of the Disease of Obesity: Reflections From a Diabetes Care Editors' Expert Forum. *Diabetes Care* 2015; **38**: 1567-1582 [PMID: 26421334 DOI: 10.2337/dc15-1081]

4 **Fraulob JC**, Ogg-Diamantino R, Fernandes-Santos C, Aguila MB, Mandarim-de-Lacerda CA. A Mouse Model of Metabolic Syndrome: Insulin Resistance, Fatty Liver and Non-Alcoholic Fatty Pancreas Disease (NAFPD) in C57BL/6 Mice Fed a High Fat Diet. *J Clin Biochem Nutr* 2010; **46**: 212-223 [PMID: 20490316 DOI: 10.3164/jcbn.09-83]

5 **Catanzaro R**, Cuffari B, Italia A, Marotta F. Exploring the metabolic syndrome: Nonalcoholic fatty pancreas disease. *World J Gastroenterol* 2016; **22**: 7660-7675 [PMID: 27678349 DOI: 10.3748/wjg.v22.i34.7660]

6 **Withrow D**, Alter DA. The economic burden of obesity worldwide: a systematic review of the direct costs of obesity. *Obes Rev* 2011; **12**: 131-141 [PMID: 20122135 DOI: 10.1111/j.1467-789X.2009.00712.x]

7 **Li J**, Zhang YM, Li JY, Zhu L, Kang HX, Ren HY, Chen H, Yuan L, Miao YF, Wan MH, Tang WF. Effect of Sheng-Jiang Powder on Obesity-Induced Multiple Organ Injuries in Rats. *Evid Based Complement Alternat Med* 2017; **2017**: 6575276 [PMID: 29234419 DOI: 10.1155/2017/6575276]

8 **Di Ciaula A**, Portincasa P. Fat, epigenome and pancreatic diseases. Interplay and common pathways from a toxic and obesogenic environment. *Eur J Intern Med* 2014; **25**: 865-873 [PMID: 25457435 DOI: 10.1016/j.ejim.2014.10.012]

9 **Soeda J**, Mouralidarane A, Cordero P, Li J, Nguyen V, Carter R, Kapur SR, Pombo J, Poston L, Taylor PD, Vinciguerra M, Oben

JA. Maternal obesity alters endoplasmic reticulum homeostasis in offspring pancreas. *J Physiol Biochem*2016; **72**: 281-291 [PMID: 26979740 DOI: 10.1007/s13105-016-0476-6]
10 **Debnath M**, Agrawal S, Agrawal A, Dubey GP. Metaflammatory responses during obesity: Pathomechanism and treatment. *Obes Res Clin Pract* 2016; **10**: 103-113 [PMID: 26614484 DOI: 10.1016/j.orcp.2015.10.012]
11 **Gotoh K**, Inoue M, Shiraishi K, Masaki T, Chiba S, Mitsutomi K, Shimasaki T, Ando H, Fujiwara K, Katsuragi I, Kakuma T, Seike M, Sakata T, Yoshimatsu H. Spleen-derived interleukin-10 downregulates the severity of high-fat diet-induced non-alcoholic fatty pancreas disease. *PLoS One* 2012; **7**: e53154 [PMID: 23285260 DOI: 10.1371/journal.pone.0053154]
12 **Yu TY**, Wang CY. Impact of non-alcoholic fatty pancreas disease on glucose metabolism. *J Diabetes Investig* 2017; **8**: 735-747 [PMID: 28371475 DOI: 10.1111/jdi.12665]
13 **Poitout V**, Amyot J, Semache M, Zarrouki B, Hagman D, Fontés G. Glucolipotoxicity of the pancreatic beta cell. *Biochim Biophys Acta* 2010; **1801**: 289-298 [PMID: 19715772 DOI: 10.1016/j.bbalip.2009.08.006]
14 **Liu XM,** Tong XL, Wang PQ. Discussion on the theory of turbidity pathopoiesis. *Shijie Zhongxiyi Jiehe Zazhi* 2009; **4**: 839-842 [DOI: 10.13935/j.cnki.sjzx.2009.12.002]
15 **Tian SX,** Li SM. Clinical application on the effect of Sheng-jiang powder. *Hebei Zhongxiyi Xuebao* 1994; **9**: 40-44 [DOI: 10.16370/j.cnki.13-1214/r.1994.01.012]
16 **Zheng WL,** Su HS. Treatment of 60 cases of hyperlipidemia by modified Sheng-jiang powder. *Shaanxi Zhongyi* 2010; **31**: 1486-1487
17 **Han JH,** Su HS. Clinical observation on the effect of Sheng-jiang powder combined with metformin on nonalcoholic fatty liver disease with metabolic syndrome. *Shaanxi Zhongyi* 2013; **34**: 989-991
18 **Yang J,** Ni HG, Guo XY, Li L. Effect of modified Sheng-jiang powder on the pancreatic function of patients with T2DM and dyslipidemia. *Zhongxiyi JIehe Yanjiu* 2016; **8**: 1-4 [DOI: 10.3969/j.issn.1674-4616.2016.01.001]
19 **Zhu L**, Li JY, Zhang YM, Kang HX, Chen H, Su H, Li J, Tang WF. Pharmacokinetics and pharmacodynamics of Shengjiang decoction in rats with acute pancreatitis for protecting against multiple organ injury. *World J Gastroenterol* 2017; **23**: 8169-8181 [PMID: 29290653 DOI: 10.3748/wjg.v23.i46.8169]
20 **Qi MM,** Ma J, He LW. Simultaneous determination of four components in sheng-jiang powder by HPLC. *Zhongyaocai* 2015; **38**: 2418-2420 [DOI: 10.13863/j.issn1001-4454.2015.11.044]
21 **Li M**, Ye T, Wang XX, Li X, Qiang O, Yu T, Tang CW, Liu R. Effect of Octreotide on Hepatic Steatosis in Diet-Induced Obesity in Rats. *PLoS One* 2016; **11**: e0152085 [PMID: 27002331 DOI: 10.1371/journal.pone.0152085]
22 **Kusske AM**, Rongione AJ, Ashley SW, McFadden DW, Reber HA. Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. *Surgery* 1996; **120**: 284-288; discussion 289 [PMID: 8751594 DOI: 10.1016/S0039-6060(96)80299-6]
23 **Xu LZ,** Yang WT. Judging criteria for the results of immunohistochemical reactions. *Zhongguo Aizheng Zazhi* 1996; **6**: 229-231 [DOI: 10.19401/j.cnki.1007-3639.1996.04.001]
24 **Siech M,** Zhou ZF, Zhou SX, Bair B, Alt A, Hamm S, Gross H, Mayer J, Beger HG, Tian XD, Kornmann M, Bachem MG. Stimulation of stellate cells by injured acinar cells: A model of acute pancreatitis induced by alcohol and fat (VLDL). *Am J Physiol-Gastr L* 2009; **297**: G1163-G1171 [PMID: 19779015 DOI: 10.1152/ajpgi.90468.2008]
25 **Johnson AR**, Milner JJ, Makowski L. The inflammation highway: metabolism accelerates inflammatory traffic in obesity. *Immunol Rev* 2012; **249**: 218-238 [PMID: 22889225 DOI: 10.1111/j.1600-065X.2012.01151.x]
26 **Arita Y**, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. 1999. *Biochem Biophys Res Commun* 2012; **425**: 560-564 [PMID: 22925674 DOI: 10.1016/j.bbrc.2012.08.024]
27 **Carling D**. AMPK signalling in health and disease. *Curr Opin Cell Biol* 2017; **45**: 31-37 [PMID: 28232179 DOI: 10.1016/j.ceb.2017.01.005]
28 **Lafontan M**, Viguerie N. Role of adipokines in the control of energy metabolism: focus on adiponectin. *Curr Opin Pharmacol* 2006; **6**: 580-585 [PMID: 16973420 DOI: 10.1016/j.coph.2006.08.002]
29 **Kim MJ**, Kim EH, Pun NT, Chang JH, Kim JA, Jeong JH, Choi DY, Kim SH, Park PH. Globular Adiponectin Inhibits Lipopolysaccharide-Primed Inflammasomes Activation in Macrophages via Autophagy Induction: The Critical Role of AMPK Signaling. *Int J Mol Sci* 2017; **18**: pii: E1275 [PMID: 28617316 DOI: 10.3390/ijms18061275]
30 **Day EA**, Ford RJ, Steinberg GR. AMPK as a Therapeutic Target for Treating Metabolic Diseases. *Trends Endocrinol Metab* 2017; **28**: 545-560 [PMID: 28647324 DOI: 10.1016/j.tem.2017.05.004]
31 **Hao YJ,** Dong YF, Wang GY, Tian LM, Yin XW, Wang YH, Zhang PX. Effect of Sheng-jiang powder combined acupuncture on serum leptin and adiponectin in patients with obesity-related glomerulopathy. *Jichu Zhangyi Zazhi* 2015; **21**: 1438-1440
32 **Um MY**, Hwang KH, Ahn J, Ha TY. Curcumin attenuates diet-induced hepatic steatosis by activating AMP-activated protein kinase. *Basic Clin Pharmacol Toxicol* 2013; **113**: 152-157 [PMID: 23574662 DOI: 10.1111/bcpt.12076]
33 **Razolli DS**, Moraes JC, Morari J, Moura RF, Vinolo MA, Velloso LA. TLR4 expression in bone marrow-derived cells is both necessary and sufficient to produce the insulin resistance phenotype in diet-induced obesity. *Endocrinology* 2015; **156**: 103-113 [PMID: 25375037 DOI: 10.1210/en.2014-1552]
34 **Arkan MC**, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* 2005; **11**: 191-198 [PMID: 15685170 DOI: 10.1038/nm1185]
35 **Hirosumi J**, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature* 2002; **420**: 333-336 [PMID: 12447443 DOI: 10.1038/nature01137]
36 **Yin J**, Gu L, Wang Y, Fan N, Ma Y, Peng Y. Rapamycin improves palmitate-induced ER stress/NF κB pathways associated with stimulating autophagy in adipocytes. *Mediators Inflamm* 2015; **2015**: 272313 [PMID: 25653476 DOI: 10.1155/2015/272313]
37 **Wei KF,** Geng YH, Chang JS, Pan Y, Zhang QH, Zhang MY. Effects of Sheng-jiang powder on expression of NF-κB in pulmonary microvascular endothelial cells of rats with acute lung injury. *Nanjing Zhongyiyao Daxue Xuebao* 2008; **24**: 341-342 [DOI: 10.14148/j.issn.1672-0482.2008.05.019]
38 **Yu JS,** Wang Q, Yu HQ. Effects of Shengjiangsan on expression of NF-κB in rats with mesangial proliferative glomerulonephritis. *Zhongguo Yanfangxue Xuebao* 2011; **17**: 190-193 [DOI: 10.13422/j.cnki.syfjx.2011.10.066]
39 **Blobe GC**, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000; **342**: 1350-1358 [PMID: 10793168 DOI: 10.1056/NEJM200005043421807]
40 **Gerarduzzi C**, Di Battista JA. Myofibroblast repair mechanisms post-inflammatory response: a fibrotic perspective. *Inflamm Res* 2017; **66**: 451-465 [PMID: 28040859 DOI: 10.1007/s00011-016-1019-x]
41 **Nagashio Y**, Ueno H, Imamura M, Asaumi H, Watanabe S, Yamaguchi T, Taguchi M, Tashiro M, Otsuki M. Inhibition of transforming growth factor beta decreases pancreatic fibrosis and protects the pancreas against chronic injury in mice. *Lab Invest* 2004; **84**: 1610-1618 [PMID: 15502860 DOI: 10.1038/labinvest.3700191]
42 **Matsuda A**, Makino N, Tozawa T, Shirahata N, Honda T, Ikeda Y, Sato H, Ito M, Kakizaki Y, Akamatsu M, Ueno Y, Kawata S. Pancreatic fat accumulation, fibrosis, and acinar cell injury in the Zucker diabetic fatty rat fed a chronic high-fat diet. *Pancreas* 2014; **43**: 735-743 [PMID: 24717823 DOI: 10.1097/

MPA.0000000000000129]
43 **Yoshikawa H**, Kihara Y, Taguchi M, Yamaguchi T, Nakamura H, Otsuki M. Role of TGF-beta1 in the development of pancreatic fibrosis in Otsuka Long-Evans Tokushima Fatty rats. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G549-G558 [PMID: 11842006 DOI: 10.1152/ajpgi.00323.2001]
44 **Kang DH**, Heo RW, Yi CO, Kim H, Choi CH, Roh GS. High-fat diet-induced obesity exacerbates kainic acid-induced hippocampal cell death. *BMC Neurosci* 2015; **16**: 72 [PMID: 26518260 DOI: 10.1186/s12868-015-0202-2]
45 **Long W**, Hui Ju Z, Fan Z, Jing W, Qiong L. The effect of recombinant adeno-associated virus-adiponectin (rAAV2/1-Acrp30) on glycolipid dysmetabolism and liver morphology in diabetic rats. *Gen Comp Endocrinol* 2014; **206**: 1-7 [PMID: 25019654 DOI: 10.1016/j.ygcen.2014.07.003]
46 **Lekli I**, Szabo G, Juhasz B, Das S, Das M, Varga E, Szendrei L, Gesztelyi R, Varadi J, Bak I, Das DK, Tosaki A. Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: the role of GLUT-4 and endothelin. *Am J Physiol Heart Circ Physiol* 2008; **294**: H859-H866 [PMID: 18065527 DOI: 10.1152/ajpheart.01048.2007]
47 **Chandrasekar B**, Boylston WH, Venkatachalam K, Webster NJ, Prabhu SD, Valente AJ. Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-kappaB/PTEN suppression. *J Biol Chem* 2008; **283**: 24889-24898 [PMID: 18632660 DOI: 10.1074/jbc.M804236200]
48 **Zhou XC**. Effects of Curcumin on Pancreatic Acinar Cell Injury in Rats with Long-term Alcohol Intake and Different Amount of Protein. *Zhongguo Yaofang* 2011; **22**: 4041-4043
49 **Shi JF,** Ma J, Guo CH. Efficacy and mechanism of Sheng-jiang powder on vascular dementia in rats. *Zhongguo Laonianxue Zazhi* 2017; **37**: 1341-1344 [DOI: 10.3969/j.issn.1005-9202.2017.06.016]
50 **Murtaugh LC**, Keefe MD. Regeneration and repair of the exocrine pancreas. *Annu Rev Physiol* 2015; **77**: 229-249 [PMID: 25386992 DOI: 10.1146/annurev-physiol-021014-071727]
51 **Landsman L**, Nijagal A, Whitchurch TJ, Vanderlaan RL, Zimmer WE, Mackenzie TC, Hebrok M. Pancreatic mesenchyme regulates epithelial organogenesis throughout development. *PLoS Biol* 2011; **9**: e1001143 [PMID: 21909240 DOI: 10.1371/journal.pbio.1001143]
52 **Apte MV**, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut* 1998; **43**: 128-133 [PMID: 9771417 DOI: 10.1136/gut.43.1.128]
53 **Wehr AY**, Furth EE, Sangar V, Blair IA, Yu KH. Analysis of the human pancreatic stellate cell secreted proteome. *Pancreas* 2011; **40**: 557-566 [PMID: 21499210 DOI: 10.1097/MPA.0b013e318214efaf]

P- Reviewer: Bramhall S, Neri V **S- Editor**: Ma RY
L- Editor: Wang TQ **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4462-4471

DOI: 10.3748/wjg.v24.i39.4462

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

ORIGINAL ARTICLE

Case Control Study

Molecular evaluation of glutathione S transferase family genes in patients with sporadic colorectal cancer

Gabriela Helena Rodrigues-Fleming, Glaucia Maria de Mendonça Fernandes, Anelise Russo, Patrícia Matos Biselli-Chicote, João Gomes Netinho, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo

Gabriela Helena Rodrigues-Fleming, Glaucia Maria de Mendonça Fernandes, Anelise Russo, Patrícia Matos Biselli-Chicote, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo, Genetics and Molecular Biology Research Unit - UPGEM, São José do Rio Preto Medical School, FAMERP, São José do Rio Preto, SP 15090-000, Brazil

João Gomes Netinho, Department of Surgery and Coloproctology, São José do Rio Preto Medical School, FAMERP, São José do Rio Preto, SP 15090-000, Brazil

ORCID number: Gabriela Helena Rodrigues-Fleming (0000-0002-6714-6931); Glaucia Maria de Mendonça Fernandes (0000-0002-8113-3598); Anelise Russo (0000-0003-1963-2043); Patrícia Matos Biselli-Chicote (0000-0001-6936-4716); João Gomes Netinho (0000-0003-0264-1883); Érika Cristina Pavarino (0000-0003-0959-0695); Eny Maria Goloni-Bertollo (0000-0002-2622-4673).

Author contributions: Rodrigues-Fleming GH planned and conducted the study, collected and interpreted data, and drafted and wrote the manuscript; Fernandes GMM participated in the collection of the genetic material, performed the analytical assessments, and revised the manuscript; Russo A participated in the collection of the genetic material; Biselli-Chicote PM critically revised the analytical tools and the manuscript; Netinho JG collected data on sporadic colorectal cancer patients; Pavarino ÉC served as scientific advisor; Goloni-Bertollo EM was the guarantor, planned the study, and critically revised the manuscript.

Supported by the São Paulo Research Foundation (FAPESP), No. 2011/23969-1 and No. 2012/02473-0; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 (Master Grant); and National Council of Technological and Scientific Development (CNPq) No. 310582/2014-8.

Institutional review board statement: The study was reviewed and approved by the FAMERP Institutional Review Board, and it was approved by the Ethics in Research Committee CEP/FAMERP, protocol no. 012/2012.

Informed consent statement: All patients gave informed consent.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: Participants gave written informed consent for data sharing.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement.

Manuscript source: Unsolicited manuscript

Correspondence to: Eny Maria Goloni-Bertollo, PhD, Adjunct Professor, Postdoc, Genetics and Molecular Biology Research Unit - UPGEM, Department of Molecular Biology, São José do Rio Preto Medical School (FAMERP), Av. Brigadeiro Faria Lima, - 5416 - Vila São Pedro, São José do Rio Preto, SP 15090-000, Brazil. eny.goloni@famerp.br
Telephone: +55-17-32015720
Fax: +55-17-32015708

Received: June 29, 2018
Peer-review started: July 2, 2018
First decision: July 17, 2018
Revised: July 27, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 21, 2018

Abstract

AIM

To evaluate the association between polymorphisms

in glutathione S transferases (GSTs) and the risk of sporadic colorectal cancer (SCRC), tumor progression and the survival of patients.

METHODS

A case-control study of 970 individuals from the Brazilian population was conducted (232 individuals from the case group with colorectal cancer and 738 individuals from the control group without a history of cancer). PCR multiplex and PCR-RFLP techniques were used to genotype the GST polymorphisms. The tumors were categorized according to the TNM classification: tumor extension (T), affected lymph nodes (N), and presence of metastasis (M). Logistic regression, multiple logistic regression and survival analysis were used to analyze the data. The results are presented in terms of odds ratio (OR) and 95% confidence interval (CI). The level of significance was set at 5% ($P \leq 0.05$).

RESULTS

Age equal to or over 62 years (OR = 8.79; 95%CI: 5.90-13.09, $P < 0.01$) and female gender (OR = 2.91; 95%CI: 1.74-4.37; $P < 0.01$) were associated with increased risk of SCRC. Analysis of the polymorphisms revealed an association between the *GSTM1* polymorphisms and a risk of SCRC (OR = 1.45; 95%CI: 1.06-2.00; $P = 0.02$), as well as between *GSTT1* and a reduced risk of the disease (OR = 0.65; 95%CI: 0.43-0.98; $P = 0.04$). An interaction between the presence of the wild-type allele of *GSTP1* Ile105Val polymorphism and tobacco consumption on risk of SCRC (OR = 2.33; 95%CI: 1.34-4.05; $P = 0.05$) was observed. There was an association between the *GSTM1* null genotype and the presence of advanced tumors (OR = 2.33; 95%CI: 1.23-4.41; $P = 0.009$), as well as increased risk of SCRC in the presence of a combination of *GSTT1* non-null/*GSTM1* null genotypes (OR = 1.50; 95%CI: 1.03-2.19; $P = 0.03$) and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (OR = 1.85; 95%CI: 1.01-3.36, $P = 0.04$). Combined *GSTT1* non-null/*GSTM1* null genotypes (OR = 2.40; 95%CI: 1.19-4.85; $P = 0.01$) and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (OR = 2.92; 95%CI: 1.05-8.12; $P = 0.04$) were associated with tumor progression. Polymorphisms were not associated with the survival of patients with SCRC.

CONCLUSION

Females aged 62 years or older are more susceptible to SCRC. Polymorphisms of *GSTT1* and *GSTM1* null genotypes modulated the susceptibility to SCRC in the population studied.

Key words: Colorectal neoplasms; Smoking; Alcohol; Glutathione S transferase; Genetic polymorphisms

Core tip: Sporadic colorectal cancer (SCRC) is the third most common cancer worldwide and includes malignancies that occur in the colon and rectum. Age greater than 60 years, smoking, and alcohol habits are some of the risk factors for SCRC. Detoxification and elimination of carcinogens contained in tobacco and alcohol require metabolic activation mediated by enzymes that metabolize the xenobiotics (XME). Polymorphisms in genes such as *GSTP1*, *GSTT1*, and *GSTM1* that encode enzymes involved in XMEs may be related to important processes in colorectal carcinogenesis.

Rodrigues-Fleming GH, Fernandes GMM, Russo A, Biselli-Chicote PM, Netinho JG, Pavarino ÉC, Goloni-Bertollo EM. Molecular evaluation of glutathione S transferase family genes in patients with sporadic colorectal cancer. *World J Gastroenterol* 2018; 24(39): 4462-4471 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4462.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4462

INTRODUCTION

Colorectal cancer is the third most frequent cancer worldwide[1] and the fifth most frequent type of cancer in Brazil[2]. Estimates for the year 2018 in Brazil are 17380 new cases for men and 18980 new cases for women[2].

Sporadic colorectal cancer (SCRC) develops from polyps (adenomas) in the colon and rectum walls, of varying sizes, and can change to dysplasia, triggering the development of cancer[2-4].

SCRC is a multifactorial disease, influenced by genetic factors, such as mutations or polymorphisms in genes that participate in pathways responsible for regulating cell growth, including tumor suppressor genes and proto-oncogenes[5,6]. Other related factors are age, gender, environmental factors, and lifestyle habits such as smoking and alcohol consumption[7]. Genetic factors may influence the effect of the environment on predisposition to the disease. Therefore, the incidence of SCRC varies among populations[1,8,9].

There are many genes encoding enzymes responsible for the metabolism of xenobiotics, in which detoxification occurs. Some of the major genes involved in phase II are the cytosolic glutathione S transferase (GST) superfamily, including GST mi (*GSTM1*), theta (*GSTT1*), and pi (*GSTP1*)[10,11]. These catalyze the conjugation of structurally different by-products of oxidative stress and xenobiotics to glutathione (GSH), which leads to the elimination of toxic substances from the cells and the protection of important cellular components such as nucleic acids and proteins[12]. GST gene expression varies between different tissues and cell types[13].

In addition to being very common in the general population, the complete absence of GSTT1 and/or GSTM1 may alter their expression or the activity of the protein itself[14]. In general, GSTP1 appears to be highly expressed in proliferating cells compared with differentiated cells. In addition, many of the GSTs are overexpressed in various neoplastic cells and higher

levels are observed in aggressive cancer cells[15]. The change in the GSTP1 gene also significantly alters the enzymatic activity[16,17], influencing the detoxification of carcinogens, causing DNA damage, and exerting an indirect effect on the risk of cancer development[18].

Therefore, the objectives of this study were to evaluate the association of epidemiological risk factors and these polymorphisms with the development of SCRC, the interaction between these polymorphisms and both smoking and alcohol habits, and the association between the polymorphisms and clinical-histopathological parameters and survival among patients with SCRC.

MATERIALS AND METHODS

Approval and consent

The study was approved by the Ethics Committee-Medical School of Sao Jose do Rio Preto - FAMERP (No. 012/2012). The 970 individuals who agreed to participate in the study signed a consent form. The variables analyzed included gender, age, ethnicity, profession, smoking, alcohol consumption, and personal and familial history of cancer.

Study populations

The case group consisted of 232 (112 men and 120 women) patients from the Department of Coloproctology of the Base Hospital of Sao Jose do Rio Preto who received the clinical and/or histopathological diagnosis of SCRC between 2010 and 2016. The exclusion criterion was previous treatment with chemotherapy and/or radiotherapy. The control group included 738 (370 men and 368 women) blood donors from the Blood Center of Sao Jose do Rio Preto. The exclusion criterion for controls was personal and family history of cancer in at least three previous generations. Individuals who had smoked at least 100 cigarettes throughout their lives were considered smokers, and those who drank more than four servings of alcohol per week (one serving corresponded to 30 mL of liquor, a 102-mL glass of wine containing 12% alcohol, or a 340-mL can of beer) were considered alcohol consumers[19,20]. SCRC was categorized according to TNM classification: tumor extension (T), affected lymph nodes (N), and presence of metastasis (M)[21].

Molecular analysis

Analysis of the *GSTT1* and *GSTM1* polymorphisms was performed using the polymerase chain reaction (PCR) multiplex technique, with the *CYP1A1* gene as the internal positive control of amplification[22]. PCR products were analyzed on 1.5% agarose gel stained with red gel.

Analysis of the *GSTP1* A313G polymorphism was performed using the polymerase chain reaction-polymorphism restriction fragment chain reaction (PCR-RFLP) technique with primers described by Harries *et al*[23]. The 176 base pair (bp) PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with red gel. The restriction enzyme digestion was performed using B*sma*I. The results and genotyping were performed after 2.0% agarose gel electrophoresis stained with red gel. The presence of 91 and 85 bp bands corresponded to the GG polymorphic genotype; the 176, 91, and 85 bp bands corresponded to the heterozygous genotype AG; and the 176 bp band corresponded to the wild-type AA genotype.

Statistical analysis

Descriptive statistics included mean values, standard deviation for continuous data, and percentage for categorical data. The Hardy-Weinberg equilibrium (HWE) was evaluated using the chi-square test through the BioEstat Program version 5.0. The binary logistic regression model, using the Minitab/Windows-Version 12.22 program, was used to evaluate the association of age, gender, smoking, and drinking habits with SCRC, and to evaluate the association between SCRC and clinical-histopathological parameters. Binary multiple logistic regression, adjusted for age, gender, and smoking and drinking habits, was used to evaluate the association between the genetic models of the polymorphisms and the development of SCRC using the SNPStats program (available at: http://bioinfo.iconcologia.net/SNPstats_web). The effect of the polymorphisms was evaluated in the models as (1) codominant (heterozygous *vs* wild-type homozygous and polymorphic homozygous *vs* wild-type homozygous); (2) dominant (heterozygous + polymorphic homozygous *vs* wild-type homozygous); (3) recessive (homozygous polymorphic *vs* wild- type homozygous + heterozygous); (4) overdominant (heterozygous *vs* wild-type homozygous + polymorphic homozygous); or (5) additive (polymorphic homozygous with 2 + heterozygous *vs* wild-type homozygous). The SNPStats program was used to evaluate the interaction between the polymorphisms and smoking habit, adjusted for age, gender, and alcohol consumption, and to evaluate the interaction between polymorphisms and alcohol consumption, adjusted for age, gender, and smoking, in SCRC risk. The effect of the polymorphisms on the overall survival time of SCRC patients was analyzed by the Kaplan-Meier curve and log rank test using the StatsDirect version 2.7.2 program. The results are presented in terms of odds ratio (OR) and 95% confidence interval (CI). For all statistical analyses the level of significance was set at 5% ($P < 0.05$).

RESULTS

Sociodemographic data

Table 1 presents the demographic data of SCRC patients and controls. Age equal to or above 62 years (OR = 8.79; 95%CI: 5.90-13.09; $P < 0.01$) and female gender (OR = 2.91; 95%CI: 1.74-4.37; $P < 0.01$) were associated with a risk of SCRC. The genotypic frequencies of *GSTP1* Ile105Val polymorphism were observed in the HWE in both groups (Case: $P = 1$, Control: $P = 0.29$).

Table 1 Sociodemographic characteristics, risk factors, and polymorphisms *GSTT1*, *GSTM1*, *GSTP1* A313G in patients with colorectal cancer and controls *n* (%)

Variables		Case (*n* = 232)	Control (*n* = 738)	OR[1] (95%CI)
Gender				
Male		112 (48)	370 (50)	1.00
Female		120 (52)	368 (50)	2.91 (1.94-4.37)[a]
Age [yr (mean) ± SD]		(62) ± 12	(48) ± 12	
< 62		112 (49)	621 (84)	1.00
≥ 62		120 (51)	117 (16)	8.79 (5.90-13.09)[a]
Smoking Habit				
Non-smoker		130 (56)	465 (63)	1.00
Smoker		102 (44)	273 (37)	1.45 (0.98-2.14)
Alcohol Consumption				
Non-drinker		132 (57)	395 (54)	1.00
Drinker		100 (43)	343 (46)	1.28 (0.85-1.91)
GSTP1				
Codominant	A/A	227 (43.7)	107 (46.1)	1.00
	A/G	224 (43.2)	102 (44)	1.06 (0.73-1.54)
	G/G	68 (13.1)	23 (9.9)	0.88 (0.48-1.59)
Dominant	A/A	227 (43.7)	107 (46.1)	1.00
	A/G-G/G	292 (56.3)	125 (53.9)	1.02 (0.71-1.45)
Recessive	A/A-A/G	451 (86.9)	209 (90.1)	1
	G/G	68 (13.1)	23 (9.9)	0.85 (0.48-1.50)
Overdominant	A/A-G/G	295 (56.8)	130 (56)	1.00
	A/G	224 (43.2)	102 (44)	1.09 (0.76-1.55)
Additive		-	-	0.97 (0.75-1.27)
GSTT1				
	+/+	573 (77.6)	192 (82.8)	1.00
	0/0	165 (22.4)	40 (17.2)	0.65 (0.43-0.98)[a]
GSTM1				
	+/+	385 (52.2)	100 (43.1)	1.00
	0/0	353 (47.8)	132 (56.9)	1.45 (1.06-2.00)[a]

[1]OR adjusted for age, gender, and alcohol and smoking habits and polymorphisms; [a]$P < 0.05$ *vs* control. OR: Odds ratio.

Individual polymorphism analysis

GSTM1 null genotype carriers had a higher risk of developing the disease (OR = 1.45; 95%CI: 1.06-2.00; *P* = 0.022). On the other hand, the *GSTT1* polymorphism was associated with a reduced risk of SCRC (OR = 0.65; 95%CI: 0.43-0.98; *P* = 0.037; Table 1).

In the present study, there was a significant interaction between the presence of the wild-type allele of the *GSTP1* Ile105Val polymorphism and smoking habit on the risk of SCRC (OR = 2.33; 95%CI: 1.34-4.05; *P* = 0.049). However, there was no interaction between the other polymorphisms and smoking or drinking habits on the risk of the disease (Table 2).

With regard to the clinical-histopathological parameters of the SCRC samples, the rectum was the most frequent primary site (60%), in addition to aggressive tumors (69.65%; Table 3). There was an association between the *GSTM1* null genotype and the presence of aggressive tumors (OR = 2.33, 95%CI: 1.23-4.41; *P* = 0.0087).

Analysis of the combined polymorphisms

An increased risk of SCRC was observed in the presence of the combination of the *GSTT1* non-null/*GSTM1* null genotypes (OR = 1.50; 95%CI: 1.03-2.19; *P* = 0.033) and the *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (*with the presence of at least one polymorphic allele) (OR = 1.85; 95%CI: 1.01-3.36; *P* = 0.045). The combined *GSTT1* non-null/*GSTM1* null genotypes (OR = 2.40; 95%CI: 1.19-4.85; *P* = 0.015) and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (OR = 2.92; 95%CI: 1.05-8.12; *P* = 0.040) were associated with tumor progression (Table 4).

Survival analysis

Kaplan-Meier curve analysis showed that the survival time of carriers of the polymorphic allele *GSTP1* Ile105Val, and the *GSTM1* and *GSTT1* null genotypes, were not significantly different from the survival time of non- carriers of these polymorphisms (Table 5).

DISCUSSION

In the present study, it was observed that individuals with advanced age (≥ 62 years) were more susceptible to SCRC, which is consistent with previous reports where old age was considered to be an etiological factor for this tumor type[2,24]. In terms of gender, women are more susceptible to SCRC. Other studies have observed a similar trend in gender among patients with SCRC and the control group[25-27]. An increase in the number of cases among women due to an increase in cigarette smoking and alcohol consumption has been observed[28,29]. It is important to note that the group

Table 2 Interaction between polymorphisms in the genes *GSTP1*, *GSTT1*, and *GSTM1* and smoking or alcohol habits on the risk of sporadic colorectal cancer

	Tobacco consumption						Alcohol consumption					
	Non-smoker			Smoker			Non-smoker			Smoker		
	Case	Control	OR[1] (95%CI)	Case	Control	OR[1] (95%CI)	Case	Control	OR[1] (95%CI)	Case	Control	OR[1] (95%CI)
GSTP1												
A/A	50	136	1.00	57	91	2.33 (1.34-4.05)[a]	59	116	1.00	48	111	1.31 (0.74-2.31)
A/G-G/G	80	177	1.40 (0.87-2.27)	45	115	1.59 (0.91-2.77)	73	147	1.12 (0.69-1.81)	52	145	1.19 (0.70-2.04)
GSTT1												
+/+	110	362	1.00	82	211	1.42 (0.98-2.08)	108	300	1.00	84	273	0.76 (0.52-1.12)
0/0	20	103	0.60 (0.34-1.04)	20	62	1.03 (0.57-1.88)	24	95	0.63 (0.37-1.07)	16	70	0.53 (0.28-1.01)
GSTM1												
+/+	52	231	1.00	48	154	1.40 (0.86-2.28)	56	206	1.00	44	179	0.76 (0.46-1.26)
0/0	78	234	1.38 (0.90-2.10)	54	119	2.19 (1.34-3.57)	76	189	1.42 (0.93-2.18)	56	164	1.14 (0.72-1.82)

[1]OR adjusted for age, gender, alcohol and smoking habits and polymorphisms; [a]$P < 0.05$ *vs* control. OR: Odds ratio.

of women with SCRC evaluated in this study had a mean age of 62 ± 13 years, which may suggest that hormonal factors might contribute to SCRC. Some studies have associated postmenopausal state with the incidence of colorectal cancer in women[30-32]. In addition, hormone replacement therapy has been proved to be a protective factor for SCRC[33-36]. A meta-analysis demonstrated an association between the protective effect of soy estrogen in women with SCRC who were postmenopausal[37].

Smoking and drinking habits were not associated with SCRC in the present study. On the other hand, Koh *et al*[38] observed a threefold increased risk of colorectal cancer among smokers compared to those who had never smoked. Some data on the risk of SCRC due to alcohol consumption are inconsistent, which can be explained by the variation in the amount of alcohol consumption analyzed in the different studies[39,40]. Analysis of the HWE revealed that the *GSTP1* Ile105Val polymorphism was in equilibrium in both the case and control groups. This result was similar to that observed by other studies in SCRC[26,41]. With regard to the *GSTT1* and *GSTM1* polymorphisms, the HWE test was not possible because the molecular analysis did not distinguish wild-type homozygous and heterozygous individuals[25].

In the present study, the *GSTP1* gene polymorphism showed no association with SCRC, corroborating other investigations in Bulgarian and Chinese populations[3,25,27,42]. However, one study in a Tunisian population observed a significant difference in the frequency of polymorphisms between the case and control groups and was associated with the risk of SCRC[26]. A single study observed a reduced risk of SCRC in the presence of the *GSTP1* Ile105Val polymorphism; however, there are no consistent data to explain the biological relevance of this finding[16].

The *GSTP1* gene polymorphism results in an alteration of the amino acid sequence of the protein and a consequent reduction in enzymatic activity and inefficient detoxification[43]. However, although the *GSTP1* Ile105Val polymorphism was not associated with SCRC in this study, the level of expression of this gene may be an important factor, which is not dependent on this genetic change. A hepatocellular carcinoma (HCC) study found that increased *GSTP1* gene expression *in vivo* and *in vitro* resulted in reduced cell proliferation in tumor cells, inhibition of Akt phosphorylation, and cell cycle disruption in G1/S by increasing p21 and p27 cell cycle inhibitors[44]. High *GSTP1* expression was also associated with better prognosis in patients with HCC[44]. In addition, hypermethylation of *GSTP1* has been observed in several types of cancers, such as prostate, breast, lung, and liver cancers[45].

In relation to the *GSTT1* and *GSTM1* gene polymorphisms, the *GSTT1* null genotype was associated with a reduced risk of the development of SCRC, whereas the presence of the *GSTM1* null genotype was associated with increased risk of SCRC. The absence of some of the GST isoenzymes in normal colorectal mucosa resulting from null genotypes such as the presence of the *GSTM1* polymorphism may alter the major detoxification function of GSTs in the metabolism of xenobiotics[4]. In Chinese and Iranian populations, an increased risk of SCRC in the presence of *GSTT1* and *GSTM1* null genotypes was determined[25,46]. On the other hand, other studies did not find an association between *GSTT1* and *GSTM1* null genotypes with SCRC[3,16,26,46-49]. In a case-control study, Vlaykova *et al*[4] found no association between *GSTM1* null genotype and the risk of SCRC, but the *GSTT1* null genotype was associated with an increased risk of SCRC. These different results may be related to the time of

Table 3 Distribution of the clinical-histopathological parameters in relation to the polymorphisms in the genes *GSTP1*, *GSTT1*, and *GSTM1* in patients with colorectal cancer *n* (%)

Models	Genotypes	Tumor progression (TNM) (*n* = 201)				Primary site			
		Non-advanced 61 (31)	Advanced 140 (69)	OR[1]	95%CI	Colon	Rectum	OR[1]	95%CI
GSTP1									
Codominant	A/A	31 (51)	62 (44)	1.00		42 (46)	65 (46)	1.00	
	A/G	23 (38)	65 (47)	1.37	(0.70-2.66)	38 (41)	64 (45)	0.96	(0.54-1.72)
	G/G	6 (10)	11 (8)	1.14	(0.37-3.50)	11 (12)	12 (8)	0.73	(0.29-1.85)
Dominant	A/A	31 (51)	62 (44)	1.00		42 (46)	65 (46)	1.00	
	A/G-G/G	29 (48)	76 (55)	1.32	(0.71-2.48)	49 (53)	76 (53)	0.91	(0.53-1.57)
Recessive	A/A-A/G	54 (90)	127 (92)	1.00		80 (87)	129 (91)	1.00	
	G/G	6 (10)	11 (8)	1.00	(0.34-2.95)	11 (12)	12 (8)	0.74	(0.31-1.81)
Overdominant	A/A-G/G	37 (61)	73 (52)	1.00		53 (58)	77 (54)	1.00	
	A/G	23 (38)	65 (47)	1.34	(0.70-2.56)	38 (41)	64 (45)	1.02	(0.59-1.77)
Aditivo	-	-	-	1.18	(0.73-1.92)	-	-	0.89	(0.59-1.34)
GSTT1									
	+/+	47 (78)	47 (78)	1.00		78 (85)	114 (80)	1.00	
	0/0	13 (22)	20 (14)	0.57	(0.26-1.27)	13 (14)	27 (19)	1.47	(0.71-3.06)
GSTM1									
	+/+	34 (56)	53 (38)	1.00		45 (49.5)	55 (39)	1.00	
	0/0	26 (43)	85 (61)	2.33	(1.23-4.41)[a]	46 (50.5)	86 (61)	1.49	(0.87-2.57)

[1]OR adjusted for age, gender, alcohol and smoking habits and polymorphisms; [a]$P < 0.05$ *vs* control. OR: Odds ratio.

exposure to environmental factors and the population heterogeneity.

It has been observed that the effect of GST polymorphisms, when combined, may increase the risk of SCRC two- or threefold[41]. The present study demonstrated that combinations of *GSTT1* non-null/*GSTM1* null genotypes and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (presence of at least one polymorphic allele) are associated with an increased risk of SCRC and tumor progression. These findings corroborate the results of individual analyses of polymorphisms, which indicate the influence of the *GSTT1* non-null genotype on SCRC because the null genotype was associated with a reduced risk of the disease.

In the Indian population, an association between the *GSTM1* null/*GSTT1* null genotypes and the combination of *GSTM1* null/*GSTT1* null/*GSTP1* Val* and the risk of SCRC was observed[41]. This result was also observed in a study by Vlaykova *et al*[4] in the Bulgarian population. A study in the Turkish population found an association between the *GSTT1* null/*GSTM1* non-null genotypes and *GSTT1* null/*GSTM1* non-null/*GSTP1* Ile (wild-type homozygote) and SCRC[3]. Cong *et al*[25] observed an increased risk in the presence of *GSTT1*/*GSTM1* genotypes, whereas the combination of *GSTT1* non-null/*GSTM1* null genotypes resulted in a significant reduced risk of SCRC, differing from the findings of this and other studies. On the other hand, other studies that analyzed the effect of the combined genotypes *GSTT1*/*GSTM1* did not find an association with the risk of SCRC[26,47,48]. Several studies have evaluated the potential association between SCRC and the combined genotypes of these polymorphisms. The observed results vary, indicating the importance of studying the effects of the genotypic combination in SCRC.

In the present study, a significant interaction between the presence of the wild-type allele of *GSTP1* Ile105Val polymorphism and smoking habit on the risk of SCRC was demonstrated. Differing from the results of the present study, a study in the Chinese population found no interaction between the *GSTP1* Ile105Val and smoking habit or drinking habit on the risk of SCRC[38]. The literature is sparse in terms of studies evaluating the interaction between risk factors and the *GSTP1* Ile105Val polymorphism in the development of SCRC. The biological relevance of this finding is unclear as the presence of at least one polymorphic allele of the *GSTP1* gene combined with the nullity of *GSTM1* and the presence of the *GSTT1* allele were associated with increased risk of SCRC. In addition, smoking habit was not associated with this tumor type in the present study.

With regard to the *GSTT1* and *GSTM1* polymorphisms, this study did not find an association between smoking or drinking habits and the risk of SCRC. These results are in accordance with two other studies in a Korean and Japanese population[46,48]. The study by Piao *et al*[49] did not show a relationship between drinking habit and the *GSTT1* and *GSTM1* null genotypes on the risk of SCRC. However, a study in Singapore found an increased risk for smokers carrying at least two null genotypes that caused low enzyme activity[38].

The controversial results regarding these polymorphisms may suggest that other genes involved in the metabolism of xenobiotics may be more relevant in the development of SCRC, such as polymorphisms in genes acting on phase I xenobiotic metabolism[27,50]. Although the polymorphisms studied change in order to reduce or eliminate the enzymatic activity, other genes can also act, compensating for the detoxification of the

Table 4 Association between the double combined *GSTT1/GSTM* genotypes and triple combined *GSTT1/GSTM/GSTP1*, colorectal cancer, tumor progression, and primary site, adjusted for gender, age, smoking, and alcohol consumption

			Colorectal cancer				Tumor progression (TNM) (*n* = 198)				Primary site			
			Case (*n* = 198)	Control	OR[1]	95%CI	Non- advanced (*n* = 60)	Advanced (*n* = 138)	OR[1]	95%CI	Colon (*n* = 81)	Rectum (*n* = 117)	OR[1]	95%CI
Double combination of genotypes				*n* = 738										
GSTT1	*GTM1*													
(+)	(+)		68	303	1.00		26	42	1.00		34	34	1.00	
(+)	(-)		97	270	1.50	(1.03-2.19)[a]	21	76	2.40	(1.19-4.85)[a]	36	61	1.67	(0.88-3.18)
(-)	(+)		19	82	1.00	(0.55-1.84)	8	11	0.74	(0.26-2.15)	7	12	1.70	(0.59-4.94)
(-)	(-)		14	83	0.61	(0.32-1.19)	5	9	1.20	(0.35-4.10)	4	10	2.60	(0.72-9.46)
Triple combination of genotypes				*n* = 519										
GSTT1	*GSTM1*	*GSTP1*												
(+)	(+)	Ile/Ile	32	96	1.00		12	20	1.00		16	10	1.00	
(+)	(+)	Val*	36	126	1.13	(0.61-2.10)	14	22	0.93	(0.34-2.56)	12	17	1.81	(0.67-4.92)
(-)	(+)	Ile/Ile	10	22	1.45	(0.56-3.77)	5	5	0.50	(0.12-2.18)	18	22	1.41	(0.33-5.98)
(-)	(+)	Val*	9	34	0.90	(0.36-2.25)	3	6	1.07	(0.21-5.31)	2	7	4.57	(0.80-26.24)
(+)	(-)	Ile/Ile	42	86	1.52	(0.81-2.83)	8	22	1.78	(0.65-4.86)	11	19	2.58	(0.99-6.75)
(+)	(-)	Val*	55	98	1.85	(1.01-3.36)[a]	10	42	2.92	(1.05-8.12)[a]	19	33	2.06	(0.83-5.15)
(-)	(-)	Ile/Ile	9	23	1.27	(0.48-3.40)	3	5	1.25	(0.25-6.19)	1	7	5.47	(0.92-32.60)
(-)	(-)	Val*	5	34	1.27	(0.11-1.08)	1	3	1.01	(0.14-7.42)	2	2	1.88	(0.27-13.33)

[1]OR adjusted for age, gender, alcohol and smoking habits and polymorphisms; [a]$P < 0.05$ *vs* control; *Ile/Val ou Val / Ile. OR: Odds Ratio.

substances present in tobacco and alcohol.

With regard to the clinical-histopathological parameters of SCRC, some studies have shown that low activity GST genotypes can be associated with more aggressive tumors and survival in colorectal cancer patients[51,52]. An association between the *GSTM1* null genotype and the presence of advanced tumors has been observed. One study demonstrated an association between aggressive tumors and the presence of the *GSTT1* null genotype[47]. However, other studies that evaluated the same polymorphisms did not find an association between the polymorphic genotypes and the clinical- histopathological parameters of SCRC[3,27,42,49].

This biological relationship between GST and progression is still not well described. However, a possible explanation for this is that GSTs play important roles in the regulation of genes related to activation of cellular maintenance, proliferation and apoptosis evasion. Thus, GSTs interact with tumor necrosis factor (TNF) receptor associated factor 2 (TRAF2) and decrease signal transduction from receptors in the TNF alpha-like (TNF-α) and c-Jun NH2-terminal kinase (JNK kinase) pathways[12,53,54].

No association between polymorphisms and the primary sites of SCRC were identified in the present study. In accordance with these findings, the study by Vlaykova *et al*[4] did not find an association between polymorphisms of *GSTT1* and *GSTM1* null genotypes and the primary site. However, Wang *et al*[41], observed an increased risk of rectal cancer in the presence of the *GSTM1* null genotype, while the *GSTT1* null genotype was associated with a risk of colon cancer.

It is worth noting that predisposition to SCRC is multifactorial and results from the interaction between allelic variants of low-penetrance genes and environmental factors such as advanced age, eating habits, and smoking and drinking habits[3,55,56]. Therefore, the findings regarding the modulation of susceptibility to SCRC in the presence of the polymorphisms analyzed, regardless of smoking or drinking habits, reinforce the influence of these polymorphisms on the etiology of SCRC, even though they do not influence patient survival. These results may contribute to the understanding of the mechanisms involved in colorectal carcinogenesis.

In conclusion, females with advanced age are more susceptible to SCRC. The presence of the *GSTM1* null genotype is associated with an increased risk of SCRC. The *GSTM1* null genotype is associated with tumor progression. The combination of *GSTT1/GSTM1* and *GSTT1/GSTM1/GSTP1* genotypes are associated with

Table 5 Polymorphisms *GSTT1*, *GSTM1*, and *GSTP1* in relation to overall survival of colorectal cancer patients

Polymorphisms	Survival (5 yr)
GSTT1	
Positive	64
Negative	68
GSTM1	
Positive	67
Negative	63
GSTP1 A313G	
AA	61
AG	70
GG	63

[a]$P < 0.05$ *vs* control.

an increased risk of SCRC and tumor progression. Polymorphisms are not associated with the overall survival rate of SCRC patients.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer is the third most common cancer worldwide and develops on the inner walls of the colon and rectum. Genetic and environmental factors may increase the risk of colorectal cancer *via* the metabolism of carcinogens. Therefore, the evaluation of polymorphisms in genes involved in this process may help to modulate the development of colorectal cancer. Polymorphisms in genes encoding GSTP1, GSTT1, and GSTM1 may alter enzymatic activity. This change can lead to DNA damage and deregulation of the mechanisms involved in colorectal cancer.

Research motivation

Polymorphisms in the coding genes *GSTP1*, *GSTT1*, and *GSTM1* have been studied in terms of susceptibility to diseases such as cancer. However, the literature presents conflicting results. Therefore, several studies are needed to assess and confirm the role of factors that influence changes in metabolic processes related to colorectal cancer.

Research objectives

The main objective of this study was to evaluate the influence of polymorphisms in the *GSTP1*, *GSTT1* and *GSTM1* genes on the risk of colorectal cancer. The data showed that carriers of polymorphisms in the *GSTM1* genes and the combination of *GSTT1* non-null/*GSTM1* null genotypes and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (*with the presence of at least one polymorphic allele) constitute a risk group for sporadic colorectal cancer (SCRC), and polymorphisms in the *GSTM1* gene and the *GSTT1* non-null/*GSTM1* null combinations, *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* increase the aggressiveness of the tumor. Thus, considering the high incidence of this cancer, it is important to understand the factors that lead to carcinogenesis in order to develop preventive and therapeutic strategies for the management of cancer.

Research methods

This case-control study evaluated 970 individuals, 232 cases and 738 controls, using multiplex polymerase chain reaction (PCR) and polymerase chain reaction-restriction fragment chain reaction (PCR-RFLP) polymorphism, Demographics are tabulated by percentage. The binary logistic regression model was used to evaluate the association of age, gender, smoking and eating habits with SCRC, and to evaluate the association of the Hardy-Weinberg equilibrium (HWE) with the Chi-square test. Multiple binary logistic regression, adjusted for age, gender and smoking and alcohol habits, was also used to evaluate the association between the genetic models of the polymorphisms and the development of SCRC. The dominant genotypic model was used to assess the interaction between polymorphisms and smoking habits, adjusted for age, gender, and ethnicity, and to evaluate the interaction of polymorphisms and alcohol consumption, adjusted for age, gender and smoking, on the risk of SCRC. In addition, the Kapla-Meier curve was used to assess the overall survival of patients with SCRC.

Research results

The data showed that carriers of polymorphisms in the *GSTM1* genes and the combination of *GSTT1* non-null/*GSTM1* null genotypes and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (*with the presence of at least one polymorphic allele) constitute a risk group for SCRC, and polymorphisms in the *GSTM1* gene and the *GSTT1* non-null/*GSTM1* null combinations, *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* increase the aggressiveness of the tumor. Thus, considering the high incidence of this cancer, it is important to understand the factors that lead to carcinogenesis in order to develop preventive and therapeutic strategies for the management of cancer.

Research conclusions

Similar studies have not previously been performed in the Brazilian population. Therefore, this work is unprecedented in this population. In addition, we emphasize the importance of the association between female gender and susceptibility to SCRC as well as the survival analysis associated with the polymorphisms studied, which have not been extensively studied in the literature. Polymorphisms in the *GSTP1*, *GSTT1* and *GSTM1* genes were involved in carcinogenesis and the poor prognosis of SCRC. In the Brazilian population it was observed that females with advanced age were more susceptible to SCRC. The presence of the *GSTM1* null genotype, and the combination of *GSTT1*/*GSTM1* and *GSTT1*/*GSTM1*/*GSTP1* genotypes are associated with an increased risk of SCRC and tumor progression.

This study provides a perspective on biomarkers of GSTs related to the prognosis of SCRC that has not been extensively studied in other populations, especially the Brazilian population. These data may contribute to clinical practice. Another interesting fact was the association between females, age over 60 years and the risk of SCRC. Menopausal women (estrogen reduction) were also shown to be more susceptible to SCRC. Polymorphisms in the genes involved in the xenobiotic metabolism pathway are associated with the development and poor prognosis of SCRC.

In this study, statistical analyses were widely used, and unlike other studies, multiple logistic regression was performed to evaluate the interactions between the polymorphisms studied and variables. In addition, survival was assessed by Kaplan Meier analysis. These analyses are extremely relevant in studies involving population genetic polymorphisms.

An association between some polymorphisms of xenobiotic metabolism and the development and progression of SCRC was observed. Advanced age and female gender were associated with the development of SCRC and polymorphisms in the genes involved in the xenobiotic metabolism pathway were associated with the development and poor prognosis of SCRC. This study may contribute to GSTs being used as diagnostic and prognostic biomarkers for SCRC. These data together with the findings of other studies may contribute to the development of treatment strategies for SCRC.

Research perspectives

This study demonstrated the importance of population studies with a large sample size in research on polymorphisms. Thus, we intend to expand the sample size to validate the results and include more polymorphisms related to the xenobiotic pathways in order to better understand the roles of these pathways in SCRC carcinogenesis. Research methods such as real-time PCR, are important in order to accurately quantify the presence of polymorphisms.

REFERENCES

1 **Ferlay J**, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Lyon, France: International Agency for Research on Cancer; 2013. Available from: URL: http://globocan.iarc.fr

2 **Instituto Nacional do Câncer**. Ministério da Saúde; 2018. Accessed February 20, 2018 Available from: URL: http://www.inca.gov.br

3 **Øines M**, Helsingen LM, Bretthauer M, Emilsson L. Epidemiology and risk factors of colorectal polyps. *Best Pract Res Clin Gastroenterol* 2017; **31**: 419-424 [PMID: 28842051 DOI: 10.1016/j.bpg.2017.06.004]
4 **Jass JR**. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007; **50**: 113-130 [PMID: 17204026 DOI: 10.1111/j.1365-2559.2006.02549.x]
5 **Bhalla A**, Zulfiqar M, Bluth MH. Molecular Diagnostics in Colorectal Carcinoma: Advances and Applications for 2018. *Clin Lab Med* 2018; **38**: 311-342 [PMID: 29776633 DOI: 10.1016/j.cll.2018.02.008]
6 **Huang D**, Sun W, Zhou Y, Li P, Chen F, Chen H, Xia D, Xu E, Lai M, Wu Y, Zhang H. Mutations of key driver genes in colorectal cancer progression and metastasis. *Cancer Metastasis Rev* 2018; **37**: 173-187 [PMID: 29322354 DOI: 10.1007/s10555-017-9726-5]
7 **Marley AR**, Nan H. Epidemiology of colorectal cancer. *Int J Mol Epidemiol Genet* 2016; **7**: 105-114 [PMID: 27766137]
8 **Gorukmez O**, Yakut T, Gorukmez O, Sag SO, Topak A, Sahinturk S, Kanat O. Glutathione S-transferase T1, M1 and P1 Genetic Polymorphisms and Susceptibility to Colorectal Cancer in Turkey. *Asian Pac J Cancer Prev* 2016; **17**: 3855-3859 [PMID: 27644629]
9 **Nascimento H**, Coy CS, Teori MT, Boin IF, Góes JR, Costa FF, Lima CS. Possible influence of glutathione S-transferase GSTT1 null genotype on age of onset of sporadic colorectal adenocarcinoma. *Dis Colon Rectum* 2003; **46**: 510-515 [PMID: 12682546 DOI: 10.1080/13102818.2009.10817617]
10 **Shen X**, Wang J, Yan X, Ren X, Wang F, Chen X, Xu Y. Predictive value of GSTP1 Ile105Val polymorphism in clinical outcomes of chemotherapy in gastric and colorectal cancers: a systematic review and meta-analysis. *Cancer Chemother Pharmacol* 2016; **77**: 1285-1302 [PMID: 27154175 DOI: 10.1007/s00280-016-3047-1]
11 **Ramsay EE**, Dilda PJ. Glutathione S-conjugates as prodrugs to target drug-resistant tumors. *Front Pharmacol* 2014; **5**: 181 [PMID: 25157234 DOI: 10.3389/fphar.2014.00181]
12 **Hayes JD**, Flanagan JU, Jowsey IR. Glutathione transferases. *Annu Rev Pharmacol Toxicol* 2005; **45**: 51-88 [PMID: 15822171 DOI: 10.1146/annurev.pharmtox.45.120403.095857]
13 **Rowe JD**, Nieves E, Listowsky I. Subunit diversity and tissue distribution of human glutathione S-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. *Biochem J* 1997; **325** (Pt 2): 481-486 [PMID: 9230131]
14 **Economopoulos KP**, Sergentanis TN. GSTM1, GSTT1, GSTP1, GSTA1 and colorectal cancer risk: a comprehensive meta-analysis. *Eur J Cancer* 2010; **46**: 1617-1631 [PMID: 20207535 DOI: 10.1016/j.ejca.2010.02.009]
15 **Oguztuzun S**, Abu-Hijleh A, Coban T, Bulbul D, Kilic M, Iscan M, Iscan M. GST isoenzymes in matched normal and neoplastic breast tissue. *Neoplasma* 2011; **58**: 304-310 [PMID: 21520986]
16 **Hezova R**, Bienertova-Vasku J, Sachlova M, Brezkova V, Vasku A, Svoboda M, Radová L, Kiss I, Vyzula R, Slaby O. Common polymorphisms in GSTM1, GSTT1, GSTP1, GSTA1 and susceptibility to colorectal cancer in the Central European population. *Eur J Med Res* 2012; **17**: 17 [PMID: 22697302 DOI: 10.1186/2047-783X-17-17]
17 **Tew KD**, Manevich Y, Grek C, Xiong Y, Uys J, Townsend DM. The role of glutathione S-transferase P in signaling pathways and S-glutathionylation in cancer. *Free Radic Biol Med* 2011; **51**: 299-313 [PMID: 21558000 DOI: 10.1016/j.freeradbiomed.2011.04.013]
18 **Gong M**, Dong W, Shi Z, Xu Y, Ni W, An R. Genetic polymorphisms of GSTM1, GSTT1, and GSTP1 with prostate cancer risk: a meta-analysis of 57 studies. *PLoS One* 2012; **7**: e50587 [PMID: 23189206 DOI: 10.1371/journal.pone.0050587]
19 **Ahrendt SA**, Chow JT, Yang SC, Wu L, Zhang MJ, Jen J, Sidransky D. Alcohol consumption and cigarette smoking increase the frequency of p53 mutations in non-small cell lung cancer. *Cancer Res* 2000; **60**: 3155-3159 [PMID: 10866304]
20 **Kjaerheim K**, Gaard M, Andersen A. The role of alcohol, tobacco, and dietary factors in upper aerogastric tract cancers: a prospective study of 10,900 Norwegian men. *Cancer Causes Control* 1998; **9**: 99-108 [PMID: 9486469 DOI: 10.1023/A:1008809706062]
21 **American Joint Committe on Cancer.** In: Edge SB, Compton CC, Fritz AG, Greene FL, Trotti A, editors. Cancer Staging Manual. New York: Springer; 2009
22 **Abdel-Rahman SZ**, el-Zein RA, Anwar WA, Au WW. A multiplex PCR procedure for polymorphic analysis of GSTM1 and GSTT1 genes in population studies. *Cancer Lett* 1996; **107**: 229-233 [PMID: 8947518]
23 **Harries LW**, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis* 1997; **18**: 641-644 [PMID: 9111193]
24 **Fernandes GM**, Russo A, Proença MA, Gazola NF, Rodrigues GH, Biselli-Chicote PM, Silva AE, Netinho JG, Pavarino ÉC, Goloni-Bertollo EM. *CYP1A1*, *CYP2E1* and *EPHX1* polymorphisms in sporadic colorectal neoplasms. *World J Gastroenterol* 2016; **22**: 9974-9983 [PMID: 28018104 DOI: 10.3748/wjg.v22.i45.9974]
25 **Cong N**, Liu L, Xie Y, Shao W, Song J. Association between glutathione S-transferase T1, M1, and P1 genotypes and the risk of colorectal cancer. *J Korean Med Sci* 2014; **29**: 1488-1492 [PMID: 25408579 DOI: 10.3346/jkms.2014.29.11.1488]
26 **Kassab A**, Msolly A, Lakhdar R, Gharbi O, Miled A. Polymorphisms of glutathione-S-transferases M1, T1, P1 and susceptibility to colorectal cancer in a sample of the Tunisian population. *Med Oncol* 2014; **31**: 760 [PMID: 24254297 DOI: 10.1007/s12032-013-0760-z]
27 **Vlaykova T**, Miteva L, Gulubova M, Stanilova S. Ile105Val GSTP1 polymorphism and susceptibility to colorectal carcinoma in Bulgarian population. *Int J Colorectal Dis* 2007; **22**: 1209-1215 [PMID: 17404745 DOI: 10.1007/s00384-007-0305-z]
28 **Osazuwa-Peters N**, Massa ST, Christopher KM, Walker RJ, Varvares MA. Race and sex disparities in long-term survival of oral and oropharyngeal cancer in the United States. *J Cancer Res Clin Oncol* 2016; **142**: 521-528 [PMID: 26507889 DOI: 10.1007/s00432-015-2061-8]
29 **Turati F**, Rossi M, Pelucchi C, Levi F, La Vecchia C. Fruit and vegetables and cancer risk: a review of southern European studies. *Br J Nutr* 2015; **113** Suppl 2: S102-S110 [PMID: 26148912 DOI: 10.1017/S0007114515000148]
30 **Hendifar A**, Yang D, Lenz F, Lurje G, Pohl A, Lenz C, Ning Y, Zhang W, Lenz HJ. Gender disparities in metastatic colorectal cancer survival. *Clin Cancer Res* 2009; **15**: 6391-6397 [PMID: 19789331 DOI: 10.1158/1078-0432.CCR-09-0877]
31 **Iida Y**, Kawai K, Tsuno NH, Ishihara S, Yamaguchi H, Sunami E, Kitayama J, Watanabe T. Proximal shift of colorectal cancer along with aging. *Clin Colorectal Cancer* 2014; **13**: 213-218 [PMID: 25245544 DOI: 10.1016/j.clcc.2014.06.005]
32 **Rossouw JE**, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J; Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA* 2002; **288**: 321-333 [PMID: 12117397]
33 **Calle EE**, Miracle-McMahill HL, Thun MJ, Heath CW Jr. Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. *J Natl Cancer Inst* 1995; **87**: 517-523 [PMID: 7707438]
34 **Chan JA**, Meyerhardt JA, Chan AT, Giovannucci EL, Colditz GA, Fuchs CS. Hormone replacement therapy and survival after colorectal cancer diagnosis. *J Clin Oncol* 2006; **24**: 5680-5686 [PMID: 17179103 DOI: 10.1200/JCO.2006.08.0580]
35 **Mandelson MT**, Miglioretti D, Newcomb PA, Harrison R, Potter JD. Hormone replacement therapy in relation to survival in women diagnosed with colon cancer. *Cancer Causes Control* 2003; **14**: 979-984 [PMID: 14750537]
36 **Slattery ML**, Anderson K, Samowitz W, Edwards SL, Curtin K, Caan B, Potter JD. Hormone replacement therapy and improved survival among postmenopausal women diagnosed with colon cancer (USA).

Cancer Causes Control 1999; **10**: 467-473 [PMID: 10530618]
37 **Yan L**, Spitznagel EL, Bosland MC. Soy consumption and colorectal cancer risk in humans: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 148-158 [PMID: 20056634 DOI: 10.1158/1055-9965.EPI-09-0856]
38 **Koh WP**, Nelson HH, Yuan JM, Van den Berg D, Jin A, Wang R, Yu MC. Glutathione S-transferase (GST) gene polymorphisms, cigarette smoking and colorectal cancer risk among Chinese in Singapore. *Carcinogenesis* 2011; **32**: 1507-1511 [PMID: 21803734 DOI: 10.1093/carcin/bgr175]
39 **Rossi M**, Jahanzaib Anwar M, Usman A, Keshavarzian A, Bishehsari F. Colorectal Cancer and Alcohol Consumption-Populations to Molecules. *Cancers* (Basel) 2018; **10**: [PMID: 29385712 DOI: 10.3390/cancers10020038]
40 **Cai S**, Li Y, Ding Y, Chen K, Jin M. Alcohol drinking and the risk of colorectal cancer death: a meta-analysis. *Eur J Cancer Prev* 2014; **23**: 532-539 [PMID: 25170915 DOI: 10.1097/CEJ.0000000000000076]
41 **Wang J**, Jiang J, Zhao Y, Gajalakshmi V, Kuriki K, Suzuki S, Nagaya T, Nakamura S, Akasaka S, Ishikawa H, Tokudome S. Genetic polymorphisms of glutathione S-transferase genes and susceptibility to colorectal cancer: a case-control study in an Indian population. *Cancer Epidemiol* 2011; **35**: 66-72 [PMID: 20688591 DOI: 10.1016/j.canep.2010.07.003]
42 **Khabaz MN**. The GSTP1 Ile105Val polymorphism is not associated with susceptibility to colorectal cancer. *Asian Pac J Cancer Prev* 2012; **13**: 2949-2953 [PMID: 22938488]
43 **Gonzales FJ,** Coughtrie M, Tukey RH. Metabolismo dos fármacos. In: As bases Farmacológicas da Terapêutica de Goodman & Gilman. 12th ed. Rio de Janeiro: AMGH, **2012**:135-136
44 **Liu X**, Tan N, Liao H, Pan G, Xu Q, Zhu R, Zou L, He S, Zhu H. High GSTP1 inhibits cell proliferation by reducing Akt phosphorylation and is associated with a better prognosis in hepatocellular carcinoma. *Oncotarget* 2017; **9**: 8957-8971 [PMID: 29507666 DOI: 10.18632/oncotarget.23420]
45 **Gurioli G**, Martignano F, Salvi S, Costantini M, Gunelli R, Casadio V. GSTP1 methylation in cancer: a liquid biopsy biomarker? *Clin Chem Lab Med* 2018; **56**: 702-717 [PMID: 29305565 DOI: 10.1515/cclm-2017-0703]
46 **Saadat I**, Saadat M. Glutathione S-transferase M1 and T1 null genotypes and the risk of gastric and colorectal cancers. *Cancer Lett* 2001; **169**: 21-26 [PMID: 11410321 DOI: 10.1016/S0304-3835(01)00550-X]
47 **Nissar S**, Sameer AS, Rasool R, Chowdri NA, Rashid F. Evaluation of deletion polymorphisms of glutathione S-transferase genes and colorectal cancer risk in ethnic Kashmiri population: A case-control study. *Indian J Cancer* 2016; **53**: 524-528 [PMID: 28485343 DOI: 10.4103/ijc.IJC_17_17]
48 **Nisa H**, Kono S, Yin G, Toyomura K, Nagano J, Mibu R, Tanaka M, Kakeji Y, Maehara Y, Okamura T, Ikejiri K, Futami K, Maekawa T, Yasunami Y, Takenaka K, Ichimiya H, Terasaka R. Cigarette smoking, genetic polymorphisms and colorectal cancer risk: the Fukuoka Colorectal Cancer Study. *BMC Cancer* 2010; **10**: 274 [PMID: 20534171 DOI: 10.1186/1471-2407-10-274]
49 **Piao JM**, Shin MH, Kweon SS, Kim HN, Choi JS, Bae WK, Shim HJ, Kim HR, Park YK, Choi YD, Kim SH. Glutathione-S-transferase (GSTM1, GSTT1) and the risk of gastrointestinal cancer in a Korean population. *World J Gastroenterol* 2009; **15**: 5716-5721 [PMID: 19960570 DOI: 10.3748/wjg.15.5716]
50 **Zhu X**, Wang Z, He J, Wang W, Xue W, Wang Y, Zheng L, Zhu ML. Associations between CYP1A1 rs1048943 A > G and rs4646903 T > C genetic variations and colorectal cancer risk: Proof from 26 case-control studies. *Oncotarget* 2016; **7**: 51365-51374 [PMID: 27384991 DOI: 10.18632/oncotarget.10331]
51 **Hunter DJ**, Riboli E, Haiman CA, Albanes D, Altshuler D, Chanock SJ, Haynes RB, Henderson BE, Kaaks R, Stram DO, Thomas G, Thun MJ, Blanché H, Buring JE, Burtt NP, Calle EE, Cann H, Canzian F, Chen YC, Colditz GA, Cox DG, Dunning AM, Feigelson HS, Freedman ML, Gaziano JM, Giovannucci E, Hankinson SE, Hirschhorn JN, Hoover RN, Key T, Kolonel LN, Kraft P, Le Marchand L, Liu S, Ma J, Melnick S, Pharaoh P, Pike MC, Rodriguez C, Setiawan VW, Stampfer MJ, Trapido E, Travis R, Virtamo J, Wacholder S, Willett WC; National Cancer Institute Breast and Prostate Cancer Cohort Consortium. A candidate gene approach to searching for low-penetrance breast and prostate cancer genes. *Nat Rev Cancer* 2005; **5**: 977-985 [PMID: 16341085 DOI: 10.1038/nrc1754]
52 **Mitsudomi T**, Hamajima N, Ogawa M, Takahashi T. Prognostic significance of p53 alterations in patients with non-small cell lung cancer: a meta-analysis. *Clin Cancer Res* 2000; **6**: 4055-4063 [PMID: 11051256]
53 **Holley SL**, Rajagopal R, Hoban PR, Deakin M, Fawole AS, Elder JB, Elder J, Smith V, Strange RC, Fryer AA. Polymorphisms in the glutathione S-transferase mu cluster are associated with tumour progression and patient outcome in colorectal cancer. *Int J Oncol* 2006; **28**: 231-236 [PMID: 16328000]
54 **Wu Y**, Fan Y, Xue B, Luo L, Shen J, Zhang S, Jiang Y, Yin Z. Human glutathione S-transferase P1-1 interacts with TRAF2 and regulates TRAF2-ASK1 signals. *Oncogene* 2006; **25**: 5787-5800 [PMID: 16636664 DOI: 10.1038/sj.onc.1209576]
55 **Adler V**, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z. Regulation of JNK signaling by GSTp. *EMBO J* 1999; **18**: 1321-1334 [PMID: 10064598 DOI: 10.1093/emboj/18.5.1321]
56 **de la Chapelle A**. Genetic predisposition to colorectal cancer. *Nat Rev Cancer* 2004; **4**: 769-780 [PMID: 15510158 DOI: 10.1038/nrc1453]

P- Reviewer: Chirila DN, Kai K **S- Editor**: Gong ZM
L- Editor: Webster JR **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4472-4481

DOI: 10.3748/wjg.v24.i39.4472

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Barrett's esophagus with high grade dysplasia is associated with non-esophageal cancer

Nir Bar, Naama Schwartz, Michal Nissim, Naomi Fliss-Isacov, Shira Zelber-Sagi, Revital Kariv

Nir Bar, Naama Schwartz, Michal Nissim, Naomi Fliss-Isacov, Shira Zelber-Sagi, Revital Kariv, Department of Gastroenterology, Tel Aviv Medical Center, Tel Aviv 6423906, Israel

Nir Bar, Naama Schwartz, Michal Nissim, Naomi Fliss-Isacov, Revital Kariv, Faculty of Medicine, Tel Aviv University, Tel Aviv 6423906, Israel

Shira Zelber-Sagi, School for Public Health, University of Haifa, Haifa 31905, Israel

ORCID number: Nir Bar (0000-0002-6148-5668); Naama Schwartz (0000-0002-5238-4080); Michal Nissim (0000-0001-9423-7583); Naomi Fliss-Isacov (0000-0003-4849-0291); Shira Zelber-Sagi (0000-0002-1324-7497); Revital Kariv (0000-0002-3831-9020).

Author contributions: Kariv R designed the study; Nissim M and Bar N collected the data; Schwartz N, Fliss-Isakov N and Bar N did the statistical analysis; Bar N, Kariv R, and Zelber-Sagi S prepared the manuscript; Bar N, Kariv R, Zelber-Sagi S, Fliss-Isacov N and Schwartz N critically reviewed the manuscript.

Institutional review board statement: The study was reviewed and approved for publication by our Institutional Review board (protocol number 0022-09).

Informed consent statement: Informed consent was waivered by the Institutional review board.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at revitalk@tlvmc.gov.il.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Manuscript source: Unsolicited manuscript

Correspondence to: Revital Kariv, MD, Doctor, Department of Gastroenterology, Tel Aviv Medical Center, Weitzman 6 street, Tel Aviv 6423906, Israel. revitalk@tlvmc.gov.il
Telephone: +972-3-6974458
Fax: +972-3-6974868

Received: July 30, 2018
Peer-review started: July 30, 2018
First decision: August 27, 2018
Revised: September 3, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

AIM

To study factors associated with esophageal and non-esophageal cancer morbidity among Barrett's esophagus (BE) patients.

METHODS

A cohort study within a single tertiary center included 386 consecutive patients with biopsy proven BE, who were recruited between 2004-2014. Endoscopic and histologic data were prospectively recorded. Cancer morbidity was obtained from the national cancer registry. Main outcomes were BE related (defined as esophagus and cardia) and non-BE related cancers (all other cancers). Cancer incidence and all-cause

mortality were compared between patients with high-grade dysplasia (HGD) and with low-grade or no dysplasia (non-HGD) using Kaplan-Meier curves and cox regression models.

RESULTS

Of the 386 patients, 12 had HGD, 7 had a BE related cancer. There were 75 (19.4%) patients with 86 cases of lifetime cancers, 76 of these cases were non-BE cancers. Seven (1.8%) and 18 (4.7%) patients had BE and non-BE incident cancers, respectively. Twelve (3.1%) patients had HGD as worst histologic result. Two (16.7%) and 16 (4.4%) incident non-BE cancers occurred in the HGD and non-HGD group, respectively. Ten-year any cancer and non-BE cancer free survival was 63% and 82% in the HGD group compared to 93% and 95% at the non-HGD group, respectively. Log-rank test for patients with more than one endoscopy, assuring longer follow up, showed a significant difference ($P < 0.001$ and $P = 0.017$ respectively). All-cause mortality was not significantly associated with BE HGD.

CONCLUSION

Patients with BE and HGD, may have a higher risk for all-cause cancer morbidity. The implications on cancer prevention recommendations should be further studied.

Key words: Barrett's esophagus; High grade dysplasia; Esophageal cancer; Upper endoscopy; Cancer morbidity

Core tip: Barrett's esophagus (BE) is known to be associated with esophageal carcinoma (EAC) and increased all cause and cancer specific mortality, but EAC is responsible only for a minority of BE mortality cases. We found patients with high-grade dysplasia to be more prone to non-BE related cancers, on top of BE related cancers. Such information can affect the recommended extraesophageal surveillance, and contribute the debate about the cost-effectiveness of endoscopic surveillance and to health systems decision making.

Bar N, Schwartz N, Nissim M, Fliss-Isacov N, Zelber-Sagi S, Kariv R. Barrett's esophagus with high grade dysplasia is associated with non-esophageal cancer. *World J Gastroenterol* 2018; 24(39): 4472-4481 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4472.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4472

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition in which intestinal metaplasia replaces normal squamous epithelium at the distal esophagus[1,2]. BE predisposes for esophageal adenocarcinoma (EAC), and current guidelines recommend endoscopic surveillance for early detection[1,2] and endoscopic treatment of early esophageal neoplasia[3-7]. BE surveillance is associated with earlier stage EAC and increased survival[8,9]. In addition, endoscopic treatment can result in complete eradication of both dysplasia and intestinal metaplasia and a reduced risk of disease progression[7,10-12].

Mortality in the overwhelming majority of BE patients is not related to EAC but is rather due to non-esophageal malignancies and cardiovascular disorders[11,13,14]. All-cause mortality is higher in patients with advanced grades of BE dysplasia compared to matched controls[11,14]. Non-esophageal cancer mortality in Danish patients with high grade dysplasia (HGD) was higher than non-dysplastic BE and matched controls, though comparing HGD to non-dysplastic BE was not an endpoint, and was not analyzed for significance[14]. Another population based cohort study conducted in Israel showed increased prevalence of colorectal, prostate, kidney, bladder and thyroid cancer in BE patients occurring at a younger age compared to matched controls[15]. To the best of our knowledge, no other publications examined the potential association between histologic features of BE and non-EAC cancer morbidity. Better characterization of cancer morbidity among patients with BE may identify risk factors and enable better surveillance, cancer prevention and optimal resource use[16]. Therefore, the primary aim of the current study was to study cancer morbidity and overall mortality within a prospectively followed cohort of BE patients according to grade of dysplasia.

MATERIALS AND METHODS

Patients and definitions

All consecutive BE patients undergoing upper endoscopy at the Tel-Aviv Sourasky medical center between 2009-2014 were included, thus determining sample size. Clinical, endoscopic, and histologic data were collected from patient files in a prospective manner between 2009 and 2014. Pre-study data was retrospectively collected, as far back as 2004.

BE was defined as having a characteristic endoscopic appearance of any length, and histologic diagnosis of intestinal metaplasia with goblet cells on biopsies taken from the columnar esophageal mucosa[1].

Study design

This is a retrospective cohort study.

Study setting

Tel-Aviv Sourasky medical center - a tertiary referral center for BE.

Data retrieval and databases used

Data collection included the following parameters for each endoscopy: BE segment length-circumferential and maximal lengths were calculated and recorded according to the Prague classification[17]. We categorized

the BE segment length as long (BE segment measuring 3 cm and above), short (1-2.9 cm), and ultra-short (< 1 cm). Presence of endoscopic abnormalities was also recorded. Histologic results for each endoscopy were classified as no dysplasia, low grade dysplasia (LGD), HGD, intramucosal adenocarcinoma (IMC), and EAC[18]. All biopsies with suspected dysplasia were reviewed by 2 expert GI-pathologists. If a patient had more than one dysplasia result or endoscopic report, the most severe dysplasia as well as the longest BE segment during follow up were chosen for analysis, respectively. Individual follow up was censored either by a diagnosis of cancer, at the end of the follow up period (December 2014), or death. Patient information collected included age, gender, cancer history (including type of cancer), individual number of endoscopies during the study period, and date of death.

The primary outcome of this study was non-BE cancer incidence, and secondary aims included BE related cancer and overall mortality. In order to determine the difference in cancer morbidity in patients with higher degrees of dysplasia, we compared patients who had HGD and patients with LGD or non-dysplastic BE (non-HGD group).

Cancer morbidity data was retrieved from the Israeli national cancer registry (NCR). The NCR records all incident cases of malignant neoplasms other than basal or squamous cell skin cancers. Trained registrars review available documents from hospitals, pathology labs, and death certificates from local health authorities. Upon retrieval of data from the NCR, its records were updated until December 2014.

Cancers were categorized as BE related or non-BE related. As diagnostic inaccuracies between EAC and gastric cardia adenocarcinoma are known to occur[19], we classified them both as BE cancers. All other malignancies were recorded as non-BE cancer. For all cancer free analysis, we used cancer cases which occurred within the follow up period. We also recorded cases occurring before the first available endoscopy as pre-study cancer, and reported the total life-time cancers retrieved from the NCR.

The date of death information was retrieved from the Central Bureau of Statistics.

This study was approved by our center's institutional review board - approval number 0022-09. As data were collected from medical records throughout the study, informed consent was waivered by the institutional review board.

Statistical analysis

Categorical variables were described as frequency and percentage and continuous variables were presented with mean (standard deviation) median, (range) as needed. Comparisons between patients with HGD and patients with lower degrees of dysplasia (non-HGD) were performed using Chi-square test (or Fisher's exact test) for categorical variables. For continuous variables, the independent-sample *t*-tests (or Mann-Whitney test) were used.

For each patient, the cancer free survival time was calculated based on the first endoscopy date (*i.e.*, start date) and the first cancer date or the end of the follow-up date (December 2014) for patients who were cancer free. For non-BE cancer, we used the first non-BE cancer date for the calculation. Kaplan-Meier curve was utilized to compare survival trends, using the Log-rank test. All Kaplan-Meier analysis was done for patients with multiple endoscopies to avoid confounding. In addition, the Cox regression was used to perform univariate and multivariable regression in all patients (adjusting for potential confounders, found to be associated with HGD in the univariate analysis), displaying the hazard ratios (HRs) and adjusted HR (Adj.HR) with 95% confidence intervals (95%CI).

The statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, United States). Significance was set at $P < 0.05$.

RESULTS

A total of 387 patients with biopsy proven BE were included with endoscopic data starting at May 2004 until December 2014. One foreign patient with missing NCR data was excluded, leaving 386 for analysis, see Figure 1. The analysis for non-BE cancer among BE patients with or without dysplasia, included 379 patients who did not develop IMC or BE-cancer during the study follow-up. The total cohort number of endoscopies was 963, with a mean of 2.5 ± 2.0 endoscopies per patient. Two hundred sixty-eight (69.4%) were males with an overall mean age of 60.0 ± 13.1 years (Table 1). Long segment BE was found in 225 (59.7%) of patients. The worst degrees of dysplasia/neoplasia were LGD, HGD, IMC, and EAC in 19 (4.9%), 12 (3.1%), 1 (0.3%), and 6 (1.6%), respectively. Study inclusion per year is shown in Supplementary Table 1 in the supplementary section.

Seventy-five patients (19.4%) had invasive lifetime cancers, reported by the NCR database, of whom 10 (2.6%) had lifetime BE cancers. There were pre-study cancers in 50 (13%) patients, of which 3 were BE cancers (0.8%) and 47 were non-BE cancers (12.2%). Incident cancers occurred in 7 BE cancers (1.8%) patients and non-BE cancers in 18 (4.7%) patients. The pre-study cancers and incident cancers are detailed in Table 2. Of note, one of the 2 esophageal pre-study cancers was a squamous cell carcinoma and not EAC. Subjects pre-study cancers were not included in cancer outcome analysis as they occurred outside of follow up period as explained in the materials and methods section.

The HGD group included 12 patients, and the non HGD group (LGD and non-dysplastic BE) 367 patients. Comparison of these 2 groups is presented in Table 2 for demographic and endoscopic characteristics. In the HGD group 2 patients (16.7%) had non-BE incident

Table 1 Demographic, endoscopic and histologic characteristics of the study population

Patient data	All sample (*n* = 386)	HGD (*n* = 12)	Non-HGD (*n* = 367)
Age at study inclusion	60.0 (13.1) [61.1, 18.4-89.6]	66.3 (12.8) [66.6, 42.4-85.8]	59.8 (13.2) [60.6, 18.4-89.6]
Gender - male	268 (69.43)	9 (75)	253 (68.94)
Number of endoscopies per patient	2.5 (2.0) [2, 1-17]	6.6 (2.6) [7, 3-11]	2.4 (1.7) [2, 1-10][b]
Patients with multiple endoscopies	245 (63.47%)	12 (100)	227 (61.9)[a]
Circumferential extent of BE (cm)	3.3 (3.3) [2, 0-19]	4.5 (2.92) [4.5, 1-11]	3.2 (3.23) [2, 0-19]
Maximal extent of BE (cm)	4.4 (3.2) [3, 0.2-20]	6.0 (3.22) [6, 2-14]	4.25 (3.17) [3, 0.2-20][a]
Presence of endoscopic abnormalities	56 (14.51)	8 (66.67)	44 (11.99)[b]
Ultra-short BE segment (BE < 1 cm)	28 (7.43)	0 (0)	28 (7.8)
Short BE segment (1 cm ≤ BE < 3 cm)	124 (32.89)	2 (18.18)	122 (33.98)
Long BE segment (BE ≥ 3 cm)	225 (59.68)	9 (81.82)	209 (58.22)
Worst degree of dysplasia			
Esophageal adenocarcinoma	6 (1.6)		
Intramucosal carcinoma	1 (0.3)		
High grade dysplasia	12 (3.1)		
Low grade dysplasia	19 (4.9)		

Continuous variables were presented as mean (SD) and [median, range]. Categorical variables are presented as *n* (%). Statistical difference refers to the HGD and non-HGD groups. [a]$P < 0.01$; [b]$P < 0.001$. BE: Barrett's esophagus; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia.

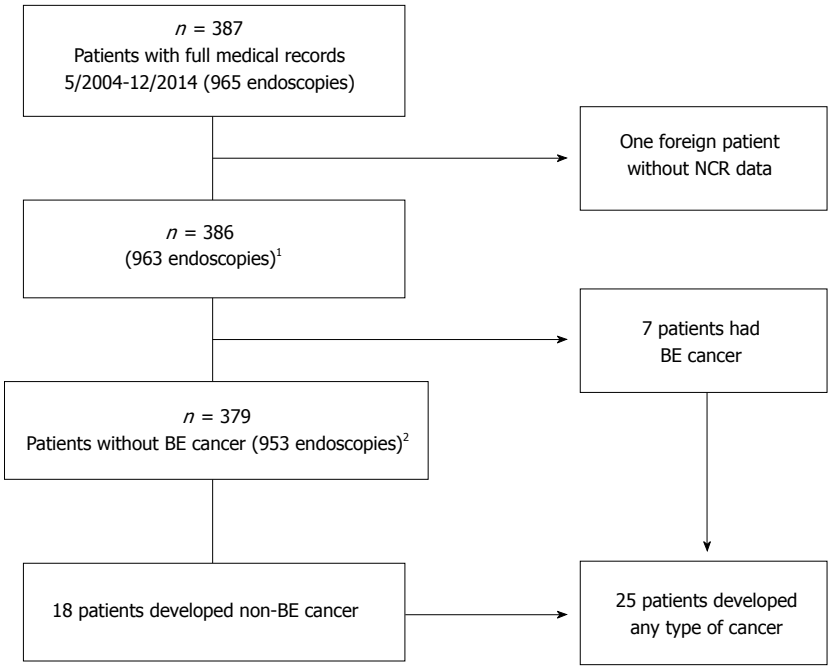


Figure 1 Study flow-chart. [1]Out of the 386 patients, 245 (63%) had more than one endoscopy; [2]Out of the 379 patients, 239 (63%) had more than one endoscopy. BE: Barrett's esophagus; NCR: National Cancer Registry.

cancers (lung and pancreatic cancers), compared with 16 (4.4%) patients in the non HGD group who had 18 cancers (in patients with 2 incident cancers the earliest one was included for analysis). As expected, patients with HGD, had a higher frequency of endoscopies, higher rate of endoscopic abnormalities ($P < 0.001$), and a longer maximal extent of BE compared with non-HGD patients ($P < 0.01$).

Kaplan-Meier survival curves for cancer free and all-cause survival are presented in Figure 2. To avoid the confounding effect of the number of endoscopies and assuring longer follow-up for all patients, Kaplan Meier analysis was done on patients with at least 2 endoscopies.

For the primary endpoint, non-BE cancer free survival was worse in patients with HGD compared with non-HGD, as shown in the Kaplan-Meier curves, Figure 2A. Log-rank test *P*-value was 0.0166 ($n = 239$), The 2-year and 10-year non-BE cancer free survival rates were 91% and 82% and 98% and 95% for the in HGD group and non-HGD group, respectively, in patients with multiple endoscopies. Univariate Cox regression analysis, Table 3, showed that HGD was not a significant predictor non-BE cancer (HR = 3.40, 95%CI: 0.78-14.84, $P = 0.104$), but in multivariable Cox regression adjusting for age, cancer history and number of endoscopies, HGD was significantly associated with increased risk for non-BE cancer (Adj.HR = 8.32, 95%CI: 1.35-51.33, $P = 0.022$).

Table 2 Overall cancer cases, stratified to pre-study and incident cases *n* (%)

	Pre-study cancers	Incident cancers	Lifetime cancers
Esophagus	2 (0.5)[2]	4 (1)	6 (1.6)
Cardia	2 (0.5)	3 (0.8)	5 (1.3)
Stomach	3 (0.8)	0 (0)	3 (0.8)
Colorectal cancer	9 (2.3)	1 (0.3)	10 (2.6)
Small intestine	1 (0.3)	0 (0)	1 (0.3)
Cholangiocarcinoma	1 (0.3)	0 (0)	1 (0.3)
Pancreas	0 (0)	2 (0.5)	2 (0.5)
Bladder	5 (1.3)	4 (1)	9 (2.3)
Prostate	11 (2.8)	0 (0)	11 (2.8)
Kidney	2 (0.5)	2 (0.5)	4 (1)
Hematologic cancer	8 (2.07)	4 (1)	12 (3.1)
Skin	4 (1)	2 (0.5)	6 (1.6)
Breast	4 (1)	2 (0.5)	6 (1.6)
Thyroid	3 (0.8)	0 (0)	3 (0.8)
Lung	0 (0)	2 (0.5)	2 (0.5)
Kaposi	0 (0)	1 (0.3)	1 (0.3)
Laryngeal	3 (0.8)	0 (0)	3 (0.8)
Cervical	1 (0.3)	0 (0)	1 (0.3)
Any type of cancer	59 (15.3)	27 (7)	86 in 75 (19.4) patients[1]
BE cancer	3 (0.8)	7 (1.8)	10 in 10 (2.6) patients
Non-BE cancers	56 (14.5)	20 (5.2)	76 in 65 (16.8) patients

[1]There were 9 patients with 2 lifetime cancers, and one with 3 cancer cases; [2]One patient had a pre-study diagnosis of esophageal squamous cell carcinoma (thus, not counted as BE related and not included in the outcome analysis for non-BE cancer free survival). BE: Barrett's esophagus.

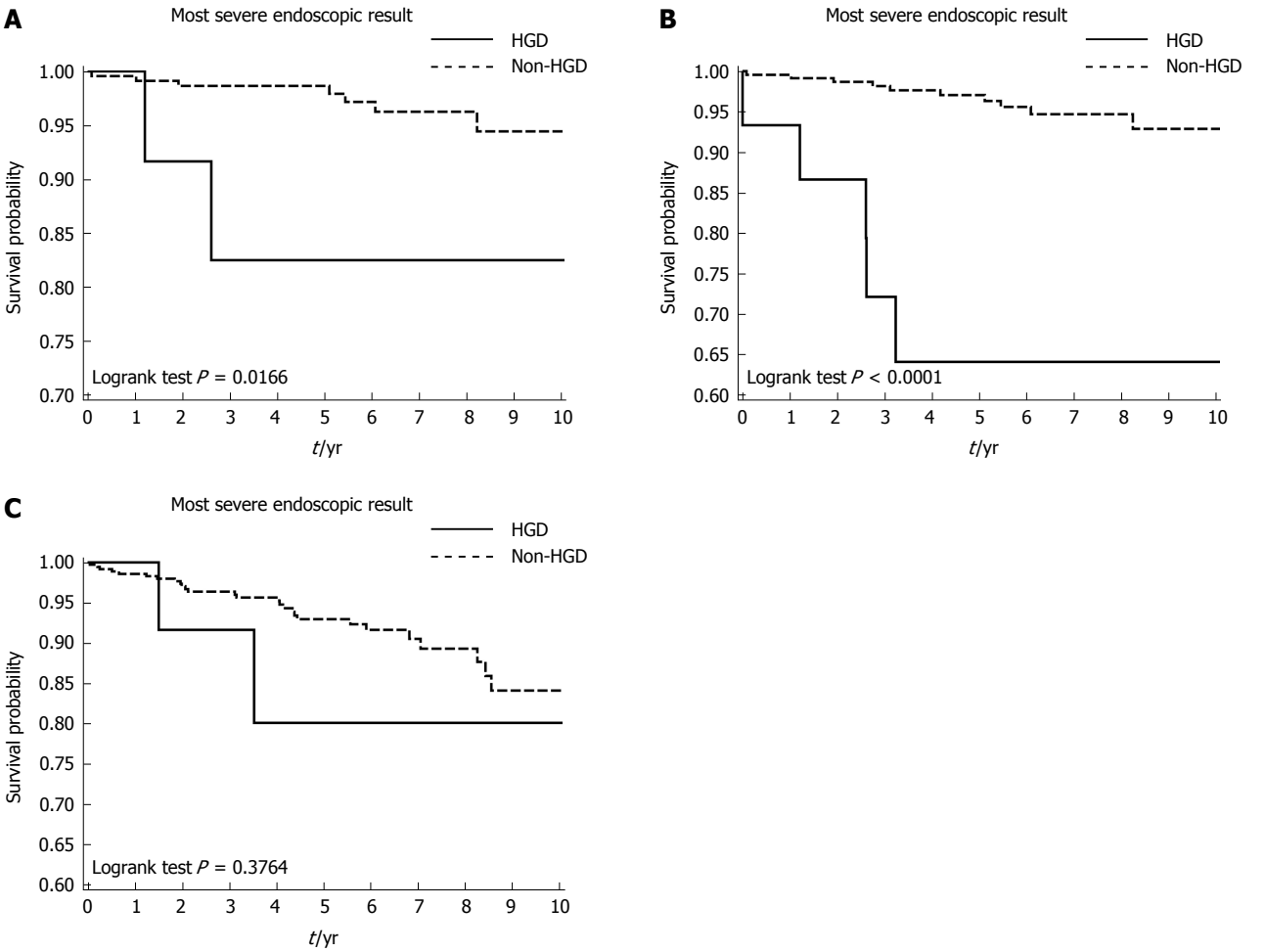


Figure 2 Kaplan Meier curves by high-grade dysplasia *vs* low grade or without dysplasia groups. A: Non-Barrett's esophagus (BE) cancer free survival, *n* = 239; B: Any cancer (BE and non-BE cancers) free survival, *n* = 245; C: All-cause mortality, *n* = 245. BE: Barrett's esophagus; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia.

Table 3 Univariate and multivariable Cox regression for the prediction of non- Barrett's esophagus cancers

n = 379 (excluding EAC and IMC)	HR (95%CI)	Adjusted HR (95%CI)
Age at study inclusion[1]	1.11 (0.99-1.24)	1.07 (0.95-1.21)
Gender - male	2.19 (0.63-7.55)	
Number of endoscopies per patient	0.82 (0.59-1.12)	0.72 (0.50-1.03)
Presence of endoscopic abnormalities	1.19 (0.34-4.09)	
Circumferential extent of BE (cm)	0.99 (0.79-1.26)	
Maximal extent of BE (cm)	1.06 (0.92-1.23)	
Ultra-short segment (BE < 1 cm)	1	
Short segment (1 cm ≤ BE < 3 cm)	0.96 (0.11-8.24)	
Long segment (BE ≥ 3 cm)	0.99 (0.13-7.85)	
Pre-study cancer history	2.58 (0.92-7.25)	2.12 (0.73-6.17)
HGD *vs* non-HGD	3.40 (0.78-14.84)	8.32 (1.35-51.33)[a]

[a]$P < 0.05$; [1]For a 3-year increase. BE: Barrett's esophagus; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia; EAC: Esophageal adenocarcinoma; IMC: Intramucosal carcinoma.

Table 4 Univariate and multivariable Cox regression for the prediction of any cancer

n = 386 (including EAC and IMC)	HR (95%CI)	Adjusted HR (95%CI)
Age at study inclusion[1]	1.09 (0.99-1.20)	1.08 (0.97-1.21)
Gender - male	2.29 (0.79-6.67)	
Number of endoscopies per patient	1.11 (0.96-1.29)	0.99 (0.82-1.21)
Presence of endoscopic abnormalities	2.27 (0.95-5.44)	
Circumferential extent of BE (cm)	1.10 (0.96-1.26)	
Maximal extent of BE (cm)	1.12 (1.008-1.24)[a]	1.13 (1.000-1.27)
Ultra-short segment (BE < 1cm)	1	
Short segment (1 cm ≤ BE < 3 cm)	0.97 (0.11-8.30)	
Long segment (BE ≥ 3 cm)	1.77 (0.23-13.34)	
Pre-study cancer history	2.15 (0.86-5.39)	
HGD *vs* non-HGD	6.33 (2.37-16.91)[b]	4.28 (1.17-15.76)[a]

[a]$P < 0.05$, [b]$P < 0.01$. [1]For a 3-year increase. BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; IMC: Intramucosal carcinoma; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia.

Examining the secondary endpoints, we saw a significant difference in the any-cancer free survival time among patients with HGD and patients with non-HGD, Figure 2B. Again, the worse outcome was in the HGD group, log rank test $P < 0.001$ ($n = 245$). For all-cause cancer, the 2-year and 10-year cancer free survival were 86% and 64% in the HGD group compared to 98% and 93% at the non HGD group, respectively.

Occurrence of all-cause cancer was associated with the maximal extent of the BE segment and HGD at the univariate analysis, see Table 4. After adjusting for age, number of endoscopies and pre-study cancer history, HGD was independently associated with all-cause cancer occurrence (Adj.HR = 4.28, 95%CI: 1.17-15.76, $P = 0.029$) whereas the maximal BE segment length was borderline significant (Adj.HR = 1.13 95%CI: 1-1.27, $P = 0.050$).

As BE cancer outcome was uncommon, we compared it with the Fisher exact test and not by statistical modeling. Among the 7 incident BE-cancer cases, 3 (42%) had HGD previously documented, while among the other 379 BE patients, only 12 (3.2%) had HGD (RR = 18.6, 95%CI: 4.6-75.6, $P = 0.002$)

There were 31 (8%) patients who died out of the entire cohort during the study period. Two (22.2%) of patients with IMC/EAC, 2 (16.7%), and 27 (7.0%) in the HGD and non-HGD groups respectively. Kaplan-Meier curve for mortality presented no association between HGD and all-cause mortality, Figure 2C. Log rank test $P = 0.376$ ($n = 245$). Cox regression analysis maintained this conclusion, after adjusting for age, cancer history and number of endoscopies for HGD (Adj. HR = 3.19, 95%CI: 0.66-15.46, $P = 0.149$).

The Kaplan-Meier curves for cancer free survival of the total cohort, including patients with a single endoscopy are shown in the online supplementary section, Supplementary Figure 1.

DISCUSSION

Our study reveals an association between BE with HGD and cancer outcome which, to the best of our knowledge, has not been reported before. Our main finding is that BE patients with HGD had a significantly higher risk of having non-BE cancer compared to patients with lower grades of dysplasia. This association was found in the group of patients who underwent more than a single endoscopy, which decreases the chance of dysplasia grade misclassification.

As expected, the known association between BE with HGD and adenocarcinoma of the esophagus or gastro-esophageal junction was also demonstrated

in this study. Since this was an uncommon event, we did not use survival analysis models to investigate the association. We did not find HGD to be associated with all-cause mortality compared to lower levels of dysplasia.

The association between BE and extra-esophageal cancers mortality has long been studied. Most studies established increased cancer mortality risk in BE compared to normal population, even after matching for other risk factors[14,15,20-23]. In a large Danish registry study[14], patients with BE had a 71% increased all-cause mortality compared to matched controls, while the non-esophageal cancer mortality incidence rate was increased by 77% (14.7 cases per 1000 patient years) and was the leading cause for mortality. Moreover, patients with HGD had higher non-esophageal cancer mortality rates than patients with LGD or non-dysplastic BE: HR (95%CI) were 2.47 (1.98-3.07), 1.62 (1.31-2.01), and 1.44 (1.34-1.56), respectively. However, statistical significance of the difference between groups was not reported.

Wolf *et al*[11] looked at patients following radiofrequency ablation, in a United States based registry. A dose response effect for all-cause mortality to baseline BE degree of dysplasia with HGD having an adjusted odds ratio (95%CI) of 2.7 (1.7-4.4) *vs* 1.3 (0.7-2.2) for LGD and 1.6 (0.8-3.3) for indefinite dysplasia compared with non-dysplastic BE.

Solaymani-Dodaran *et al*[13] showed increased cancer specific mortality rates in patients with BE, but did not stratify the population according to dysplasia grade.

The above data described mortality, and not morbidity. Due to the lack of data about non-BE cancer morbidity we aimed to correlate it with BE degree of dysplasia. Assuming patients with dysplastic BE have higher mortality rate, our findings imply that the above association may be related, at least in part, to increased cancer incidence.

The role of gastro-esophageal reflux disease in BE is clear, but what predisposes certain patients to develop BE and neoplasia is still under debate. Studies have linked various factors such as smoking[24,25], abdominal obesity[26,27] genetics[28], and nutrition[29,30] to BE and dysplasia/EAC. Most of these factors are also associated with other non-BE malignancies[31].

The molecular basis of BE and EAC has been studied avidly, P53 and SMAD4 somatic mutations play a role in dysplastic BE and EAC development[32-35]. P53 is also a key player in many non-esophageal neoplasia, such as colorectal cancer, prostate cancer, and melanoma[36]. SMAD4 somatic mutations are prevalent in pancreatic cancer and colorectal cancer[37]. This complex association of molecular and environmental factors with BE dysplasia/neoplasia and other cancers may indicate similar cancer pathways induced by similar exposures.

Our findings imply that HGD in BE may be a marker of increased risk for cancer morbidity and therefore may require extraesophageal surveillance and lifestyle modification to prevent and decrease cancer risk. As for now, it may be prudent to stringently perform routine cancer screening tests among patients with BE and those with HGD in particular, according to age and gender and to recommend adherence to cancer protective lifestyle.

Given the low incidence of EAC mortality rates in BE patients, the risk-benefit and cost effectiveness of surveillance has been a matter of discussion, with conflicting evidence concerning EAC and mortality prevention and cost effectiveness[9,38-40]. We show another potential motivation for BE surveillance to better define overall cancer risk.

Our study carries some limitations. Investigator initiated studies done in teaching hospitals are prone to referral bias and are also smaller in size than population-based studies, limiting generalizability, and perhaps overestimating associations. On the other hand, patients with dysplasia are usually managed in a tertiary center.

We could not ascertain how long patients had BE before study inclusion, which may have influenced the outcomes. However, these estimates are approximate at best, as BE itself may be asymptomatic and this limitation is shared by other studies.

In our study, we adjusted for age and a past history of cancer, but we were not able to adjust for other risk factors such as lifestyle parameters. Our study did not include a population with no BE as a control, but we assume based on previous studies[14,15,20-23], that cancer rates are even lower in subjects with no BE. Death as an outcome was determined in this study according to ministry of health database, but cancer specific mortality was not available and cannot be associated with cancer morbidity. Our major limitation is the low statistical power due to the small number of patients with HGD and incident cancers, which reflects real life clinical data of an uncommon condition. In addition, the majority of patients were male, as prevalent in other studies[41]. Gender may act as a confounder when addressing cancer morbidity (as with prostate and breast cancer). Again, this reflects reality in many centers treating patients with BE.

The advantages of our study include a prospective comprehensive 6 year follow up of a relatively large number of consecutive BE patients within a single referral center, enabling a uniform data collection and fully verified clinical, endoscopic and revised histologic data.

In conclusion, in our cohort we found an endoscopic and histologic profile comparable to other Western world data. Non-BE related malignancies were more prevalent, and significantly associated with HGD as well as BE related malignancies in comparison with non HGD BE. Our findings suggest BE patients with HGD may have a significantly higher overall risk for cancer morbidity. This may imply endoscopic surveillance for BE patients could aid in prediction of all-cause cancer risk and encourage current cancer prevention measures such as lifestyle modification and appropriate cancer screening among patients. Further characterization of cancer morbidity and mortality profile among patients with BE should follow

with large population-based studies.

ARTICLE HIGHLIGHTS

Research background

Patients with Barrett's esophagus (BE) are at risk for esophageal adenocarcinoma, and surveillance is recommended. However, non-esophageal cancer is the leading cause of death in this population. This raises questions about the focus we give to surveillance for esophageal cancer, and the need for broader cancer surveillance.

Research motivation

We wanted to better describe the non-esophageal cancer morbidity in patients with BE, and specifically in patients with high grade dysplasia (HGD). Finding that patients with HGD carry a higher non-esophageal cancer risk can direct efforts and resources for cancer prevention.

Research objectives

We aimed to describe the non-esophageal cancer morbidity in patients with BE, and to test whether patients with HGD have a higher risk as compared to low grade dysplasia. Indeed, in this study we have shown that compared to non-HGD, patients with HGD have a lower all cancer and non-BE cancer free survival. The significance of these findings is in the recognition of the importance of total cancer surveillance in these patients. In addition, by comparing non-esophageal cancer morbidity in HGD and less dysplastic BE, we show the added value of information received in surveillance endoscopies. These findings put the foundations for larger cohort studies, preferably multi-center for reaching a significant number of patients.

Research methods

Endoscopic and histologic data were collected, and cancer morbidity data were retrieved from the national cancer registry. We compared non-esophageal cancers, all cancers and mortality between patients with HGD and less dysplastic BE. Cancer free survival analysis was done.

Research results

We found patients with HGD had a worse non-BE cancer free survival and all cancer free survival. The higher frequency of non-esophageal cancer in patients with HGD raises the question as to the reason for this association. Further population based and mechanistic studies are required to further investigate these reasons.

Research conclusions

Our study shows that HGD may act as a marker for all cause cancer outcome, not just esophageal cancer. Perhaps it reflects a behavioral, environmental and genetic inclination towards malignancy. After endoscopic treatment for the dysplasia, we should focus our efforts to teach these patients about healthier lifestyle, and modifiable cancer risk factors such as smoking cessation and weight reduction. Perhaps in this population, screening for other malignancies may hold a different cost-effective profile.

Research perspectives

Patients with BE and HGD have a higher non-esophageal cancer risk, on top of esophageal cancer risk. This should be confirmed in more prospective studies and population-based studies. This may shift the focus of esophageal based surveillance to a more holistic cancer prevention program for certain patients. Future research should include larger cohorts of patients from multiple centers, with detailed endoscopic and histologic data as well as other cancer risk factors including obesity measures and lifestyle behaviors as smoking, physical activity and dietary intake to better encompass risk stratification and prevention potential.

REFERENCES

1 **Shaheen NJ**, Falk GW, Iyer PG, Gerson LB; American College of Gastroenterology. ACG Clinical Guideline: Diagnosis and Management of Barrett's Esophagus. *Am J Gastroenterol* 2016; **111**: 30-50; quiz 51 [PMID: 26526079 DOI: 10.1038/ajg.2015.322]

2 **Fitzgerald RC**, di Pietro M, Ragunath K, Ang Y, Kang JY, Watson P, Trudgill N, Patel P, Kaye PV, Sanders S, O'Donovan M, Bird-Lieberman E, Bhandari P, Jankowski JA, Attwood S, Parsons SL, Loft D, Lagergren J, Moayyedi P, Lyratzopoulos G, de Caestecker J; British Society of Gastroenterology. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. *Gut* 2014; **63**: 7-42 [PMID: 24165758 DOI: 10.1136/gutjnl-2013-305372]

3 **Ell C**, May A, Gossner L, Pech O, Günter E, Mayer G, Henrich R, Vieth M, Müller H, Seitz G, Stolte M. Endoscopic mucosal resection of early cancer and high-grade dysplasia in Barrett's esophagus. *Gastroenterology* 2000; **118**: 670-677 [PMID: 10734018]

4 **Pech O**, Behrens A, May A, Nachbar L, Gossner L, Rabenstein T, Manner H, Guenter E, Huijsmans J, Vieth M, Stolte M, Ell C. Long-term results and risk factor analysis for recurrence after curative endoscopic therapy in 349 patients with high-grade intraepithelial neoplasia and mucosal adenocarcinoma in Barrett's oesophagus. *Gut* 2008; **57**: 1200-1206 [PMID: 18460553 DOI: 10.1136/gut.2007.142539]

5 **Pouw RE**, Wirths K, Eisendrath P, Sondermeijer CM, Ten Kate FJ, Fockens P, Devière J, Neuhaus H, Bergman JJ. Efficacy of radiofrequency ablation combined with endoscopic resection for barrett's esophagus with early neoplasia. *Clin Gastroenterol Hepatol* 2010; **8**: 23-29 [PMID: 19602454 DOI: 10.1016/j.cgh.2009.07.003]

6 **Prasad GA**, Wang KK, Buttar NS, Wongkeesong LM, Krishnadath KK, Nichols FC 3rd, Lutzke LS, Borkenhagen LS. Long-term survival following endoscopic and surgical treatment of high-grade dysplasia in Barrett's esophagus. *Gastroenterology* 2007; **132**: 1226-1233 [PMID: 17408660 DOI: 10.1053/j.gastro.2007.02.017]

7 **Shaheen NJ**, Sharma P, Overholt BF, Wolfsen HC, Sampliner RE, Wang KK, Galanko JA, Bronner MP, Goldblum JR, Bennett AE, Jobe BA, Eisen GM, Fennerty MB, Hunter JG, Fleischer DE, Sharma VK, Hawes RH, Hoffman BJ, Rothstein RI, Gordon SR, Mashimo H, Chang KJ, Muthusamy VR, Edmundowicz SA, Spechler SJ, Siddiqui AA, Souza RF, Infantolino A, Falk GW, Kimmey MB, Madanick RD, Chak A, Lightdale CJ. Radiofrequency ablation in Barrett's esophagus with dysplasia. *N Engl J Med* 2009; **360**: 2277-2288 [PMID: 19474425 DOI: 10.1056/NEJMoa0808145]

8 **Incarbone R**, Bonavina L, Saino G, Bona D, Peracchia A. Outcome of esophageal adenocarcinoma detected during endoscopic biopsy surveillance for Barrett's esophagus. *Surg Endosc* 2002; **16**: 263-266 [PMID: 11967675 DOI: 10.1007/s00464-001-8161-3]

9 **Corley DA**, Levin TR, Habel LA, Weiss NS, Buffler PA. Surveillance and survival in Barrett's adenocarcinomas: a population-based study. *Gastroenterology* 2002; **122**: 633-640 [PMID: 11874995 DOI: 10.1053/gast.2002.31879]

10 **Orman ES**, Li N, Shaheen NJ. Efficacy and durability of radiofrequency ablation for Barrett's Esophagus: systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2013; **11**: 1245-1255 [PMID: 23644385 DOI: 10.1016/j.cgh.2013.03.039]

11 **Wolf WA**, Pasricha S, Cotton C, Li N, Triadafilopoulos G, Muthusamy VR, Chmielewski GW, Corbett FS, Camara DS, Lightdale CJ, Wolfsen H, Chang KJ, Overholt BF, Pruitt RE, Ertan A, Komanduri S, Infantolino A, Rothstein RI, Shaheen NJ. Incidence of Esophageal Adenocarcinoma and Causes of Mortality After Radiofrequency Ablation of Barrett's Esophagus. *Gastroenterology* 2015; **149**: 1752-1761.e1 [PMID: 26327132 DOI: 10.1053/j.gastro.2015.08.048]

12 **Brimhall B**, Wani S. Current Endoscopic Approaches for the Treatment of Barrett Esophagus. *J Clin Gastroenterol* 2017; **51**: 2-11 [PMID: 27941583 DOI: 10.1097/MCG.0000000000000742]

13 **Solaymani-Dodaran M**, Card TR, West J. Cause-specific mortality of people with Barrett's esophagus compared with the general population: a population-based cohort study. *Gastroenterology* 2013; **144**: 1375-1383, 1383.e1 [PMID: 23583429 DOI: 10.1053/

j.gastro.2013.02.050]
14 **Erichsen R**, Horvath-Puho E, Lund JL, Dellon ES, Shaheen NJ, Pedersen L, Davey Smith G, Sørensen HT. Mortality and cardiovascular diseases risk in patients with Barrett's oesophagus: a population-based nationwide cohort study. *Aliment Pharmacol Ther* 2017; **45**: 973-982 [PMID: 28139003 DOI: 10.1111/apt.13962]
15 **Pines G**, Dickman R, Niv Y, Kashtan H, Birkenfeld S. Extraesophageal malignancies among patients with Barrett esophagus. *J Clin Gastroenterol* 2014; **48**: e8-e11 [PMID: 23507770 DOI: 10.1097/MCG.0b013e31828bf26f]
16 **Richter JE**. Editorial: maybe our 'Barrett's recommendation speech' should change? *Aliment Pharmacol Ther* 2017; **45**: 1266-1267 [PMID: 28370045 DOI: 10.1111/apt.14001]
17 **Sharma P**, Dent J, Armstrong D, Bergman JJ, Gossner L, Hoshihara Y, Jankowski JA, Junghard O, Lundell L, Tytgat GN, Vieth M. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. *Gastroenterology* 2006; **131**: 1392-1399 [PMID: 17101315 DOI: 10.1053/j.gastro.2006.08.032]
18 **Reid BJ**, Haggitt RC, Rubin CE, Roth G, Surawicz CM, Van Belle G, Lewin K, Weinstein WM, Antonioli DA, Goldman H. Observer variation in the diagnosis of dysplasia in Barrett's esophagus. *Hum Pathol* 1988; **19**: 166-178 [PMID: 3343032 DOI: 10.1016/S0046-8177(88)80344-7]
19 **Lindblad M**, Ye W, Lindgren A, Lagergren J. Disparities in the classification of esophageal and cardia adenocarcinomas and their influence on reported incidence rates. *Ann Surg* 2006; **243**: 479-485 [PMID: 16552198 DOI: 10.1097/01.sla.0000205825.34452.43]
20 **Sonnenberg A**, Genta RM. Barrett's metaplasia and colonic neoplasms: a significant association in a 203,534-patient study. *Dig Dis Sci* 2013; **58**: 2046-2051 [PMID: 23371013 DOI: 10.1007/s10620-013-2565-5]
21 **Andrici J**, Tio M, Cox MR, Eslick GD. Meta-analysis: Barrett's oesophagus and the risk of colonic tumours. *Aliment Pharmacol Ther* 2013; **37**: 401-410 [PMID: 23163592 DOI: 10.1111/apt.12146]
22 **Khalaf N**, Ramsey D, Kramer JR, El-Serag HB. Personal and family history of cancer and the risk of Barrett's esophagus in men. *Dis Esophagus* 2015; **28**: 283-290 [PMID: 24529029 DOI: 10.1111/dote.12185]
23 **Solaymani-Dodaran M**, Logan RF, West J, Card T, Coupland C. Risk of extra-oesophageal malignancies and colorectal cancer in Barrett's oesophagus and gastro-oesophageal reflux. *Scand J Gastroenterol* 2004; **39**: 680-685 [PMID: 15370691 DOI: 10.1080/00365520410004802]
24 **Smith KJ**, O'Brien SM, Smithers BM, Gotley DC, Webb PM, Green AC, Whiteman DC. Interactions among smoking, obesity, and symptoms of acid reflux in Barrett's esophagus. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2481-2486 [PMID: 16284367 DOI: 10.1158/1055-9965.EPI-05-0370]
25 **Coleman HG**, Bhat S, Johnston BT, McManus D, Gavin AT, Murray LJ. Tobacco smoking increases the risk of high-grade dysplasia and cancer among patients with Barrett's esophagus. *Gastroenterology* 2012; **142**: 233-240 [PMID: 22062359 DOI: 10.1053/j.gastro.2011.10.034]
26 **Corley DA**, Kubo A, Levin TR, Block G, Habel L, Zhao W, Leighton P, Quesenberry C, Rumore GJ, Buffler PA. Abdominal obesity and body mass index as risk factors for Barrett's esophagus. *Gastroenterology* 2007; **133**: 34-41; quiz 311 [PMID: 17631128 DOI: 10.1053/j.gastro.2007.04.046]
27 **Nelsen EM**, Kirihara Y, Takahashi N, Shi Q, Lewis JT, Namasivayam V, Buttar NS, Dunagan KT, Prasad GA. Distribution of body fat and its influence on esophageal inflammation and dysplasia in patients with Barrett's esophagus. *Clin Gastroenterol Hepatol* 2012; **10**: 728-734; quiz e61- e62 [PMID: 22433923 DOI: 10.1016/j.cgh.2012.03.007]
28 **Su Z**, Gay LJ, Strange A, Palles C, Band G, Whiteman DC, Lescai F, Langford C, Nanji M, Edkins S, van der Winkel A, Levine D, Sasieni P, Bellenguez C, Howarth K, Freeman C, Trudgill N, Tucker AT, Pirinen M, Peppelenbosch MP, van der Laan LJ, Kuipers EJ, Drenth JP, Peters WH, Reynolds JV, Kelleher DP, McManus R, Grabsch H, Prenen H, Bisschops R, Krishnadath K, Siersema PD, van Baal JW, Middleton M, Petty R, Gillies R, Burch N, Bhandari P, Paterson S, Edwards C, Penman I, Vaidya K, Ang Y, Murray I, Patel P, Ye W, Mullins P, Wu AH, Bird NC, Dallal H, Shaheen NJ, Murray LJ, Koss K, Bernstein L, Romero Y, Hardie LJ, Zhang R, Winter H, Corley DA, Panter S, Risch HA, Reid BJ, Sargeant I, Gammon MD, Smart H, Dhar A, McMurtry H, Ali H, Liu G, Casson AG, Chow WH, Rutter M, Tawil A, Morris D, Nwokolo C, Isaacs P, Rodgers C, Ragunath K, MacDonald C, Haigh C, Monk D, Davies G, Wajed S, Johnston D, Gibbons M, Cullen S, Church N, Langley R, Griffin M, Alderson D, Deloukas P, Hunt SE, Gray E, Dronov S, Potter SC, Tashakkori-Ghanbaria A, Anderson M, Brooks C, Blackwell JM, Bramon E, Brown MA, Casas JP, Corvin A, Duncanson A, Markus HS, Mathew CG, Palmer CN, Plomin R, Rautanen A, Sawcer SJ, Trembath RC, Viswanathan AC, Wood N, Trynka G, Wijmenga C, Cazier JB, Atherfold P, Nicholson AM, Gellatly NL, Glancy D, Cooper SC, Cunningham D, Lind T, Hapeshi J, Ferry D, Rathbone B, Brown J, Love S, Attwood S, MacGregor S, Watson P, Sanders S, Ek W, Harrison RF, Moayyedi P, de Caestecker J, Barr H, Stupka E, Vaughan TL, Peltonen L, Spencer CC, Tomlinson I, Donnelly P, Jankowski JA; Esophageal Adenocarcinoma Genetics Consortium; Wellcome Trust Case Control Consortium 2. Common variants at the MHC locus and at chromosome 16q24.1 predispose to Barrett's esophagus. *Nat Genet* 2012; **44**: 1131-1136 [PMID: 22961001 DOI: 10.1038/ng.2408]
29 **Kubo A**, Levin TR, Block G, Rumore GJ, Quesenberry CP Jr, Buffler P, Corley DA. Dietary antioxidants, fruits, and vegetables and the risk of Barrett's esophagus. *Am J Gastroenterol* 2008; **103**: 1614-1623; quiz 1624 [PMID: 18494834 DOI: 10.1111/j.1572-0241.2008.01838.x]
30 **Kubo A**, Corley DA, Jensen CD, Kaur R. Dietary factors and the risks of oesophageal adenocarcinoma and Barrett's oesophagus. *Nutr Res Rev* 2010; **23**: 230-246 [PMID: 20624335 DOI: 10.1017/S0954422410000132]
31 **Danaei G**, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M; Comparative Risk Assessment collaborating group (Cancers). Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet* 2005; **366**: 1784-1793 [PMID: 16298215 DOI: 10.1016/S0140-6736(05)67725-2]
32 **Fléjou JF**. Barrett's oesophagus: from metaplasia to dysplasia and cancer. *Gut* 2005; **54** Suppl 1: i6- i12 [PMID: 15711008 DOI: 10.1136/gut.2004.041525]
33 **Gregson EM**, Bornschein J, Fitzgerald RC. Genetic progression of Barrett's oesophagus to oesophageal adenocarcinoma. *Br J Cancer* 2016; **115**: 403-410 [PMID: 27441494 DOI: 10.1038/bjc.2016.219]
34 **Weaver JMJ**, Ross-Innes CS, Shannon N, Lynch AG, Forshew T, Barbera M, Murtaza M, Ong CJ, Lao-Sirieix P, Dunning MJ, Smith L, Smith ML, Anderson CL, Carvalho B, O'Donovan M, Underwood TJ, May AP, Grehan N, Hardwick R, Davies J, Oloumi A, Aparicio S, Caldas C, Eldridge MD, Edwards PAW, Rosenfeld N, Tavaré S, Fitzgerald RC; OCCAMS consortium. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. *Nat Genet* 2014; **46**: 837-843 [PMID: 24952744 DOI: 10.1038/ng.3013]
35 **Dulak AM**, Stojanov P, Peng S, Lawrence MS, Fox C, Stewart C, Bandla S, Imamura Y, Schumacher SE, Shefler E, McKenna A, Carter SL, Cibulskis K, Sivachenko A, Saksena G, Voet D, Ramos AH, Auclair D, Thompson K, Sougnez C, Onofrio RC, Guiducci C, Beroukhim R, Zhou Z, Lin L, Lin J, Reddy R, Chang A, Landrenau R, Pennathur A, Ogino S, Luketich JD, Golub TR, Gabriel SB, Lander ES, Beer DG, Godfrey TE, Getz G, Bass AJ. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. *Nat Genet* 2013; **45**: 478-486 [PMID: 23525077 DOI: 10.1038/ng.2591]
36 **Whibley C**, Pharoah PD, Hollstein M. p53 polymorphisms: cancer implications. *Nat Rev Cancer* 2009; **9**: 95-107 [PMID: 19165225

DOI: 10.1038/nrc2584]
37 **Xia X**, Wu W, Huang C, Cen G, Jiang T, Cao J, Huang K, Qiu Z. SMAD4 and its role in pancreatic cancer. *Tumour Biol* 2015; **36**: 111-119 [PMID: 25464861 DOI: 10.1007/s13277-014-2883-z]
38 **Bhat SK**, McManus DT, Coleman HG, Johnston BT, Cardwell CR, McMenamin U, Bannon F, Hicks B, Kennedy G, Gavin AT, Murray LJ. Oesophageal adenocarcinoma and prior diagnosis of Barrett's oesophagus: a population-based study. *Gut* 2015; **64**: 20-25 [PMID: 24700439 DOI: 10.1136/gutjnl-2013-305506]
39 **Corley DA**, Mehtani K, Quesenberry C, Zhao W, de Boer J, Weiss NS. Impact of endoscopic surveillance on mortality from Barrett's esophagus-associated esophageal adenocarcinomas. *Gastroenterology* 2013; **145**: 312-319.e1 [PMID: 23673354 DOI: 10.1053/j.gastro.2013.05.004]
40 **Provenzale D**, Schmitt C, Wong JB. Barrett's esophagus: a new look at surveillance based on emerging estimates of cancer risk. *Am J Gastroenterol* 1999; **94**: 2043-2053 [PMID: 10445526 DOI: 10.1111/j.1572-0241.1999.01276.x]
41 **Cook MB**, Wild CP, Forman D. A systematic review and meta-analysis of the sex ratio for Barrett's esophagus, erosive reflux disease, and nonerosive reflux disease. *Am J Epidemiol* 2005; **162**: 1050-1061 [PMID: 16221805 DOI: 10.1093/aje/kwi325]

P- Reviewer: Hillman LC, Hirahara N, Sanaei O **S- Editor**: Wang XJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4482-4488

DOI: 10.3748/wjg.v24.i39.4482

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

ORIGINAL ARTICLE

Retrospective Study

Agitation thrombolysis combined with catheter-directed thrombolysis for the treatment of non-cirrhotic acute portal vein thrombosis

Chao-Yang Wang, Le-Qun Wei, Huan-Zhang Niu, Wan-Qin Gao, Tong Wang, Shun-Jun Chen

Chao-Yang Wang, Le-Qun Wei, Huan-Zhang Niu, Wan-Qin Gao, Tong Wang, Shun-Jun Chen, Department of Interventional Radiology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technolgy, Luoyang 471000, Henan Province, China

ORCID number: Chao-Yang Wang (0000-0002-9740-2158); Le-Qun Wei (0000-0002-0213-3216); Huan-Zhang Niu (0000-0001-9844-9778); Wan-Qin Gao (0000-0002-7450-2914); Tong Wang (0000-0003-3573-1148); Shun-Jun Chen (0000-0002-4597-9131).

Author contributions: All authors helped to performed the research; Wang CY contributed to manuscript writing, performing procedures and data analysis; Wei LQ contributed to drafting conception and design, and data analysis; Niu HZ contributed to writing the manuscript, drafting conception and design; Gao WQ, Wang T and Chen SJ contributed to drafting conception and design.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technolgy.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no conflict-of interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Huan-Zhang Niu, MD, Chief Doctor, Department of Interventional Radiology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technolgy, No. 24 Jing Hua Road, Luoyang 471000, Henan Province, China. niuhuanzhangsci@sina.com
Telephone: +86-379-64830771

Received: July 23, 2018
Peer-review started: July 23, 2018
First decision: August 30, 2018
Revised: September 13, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

AIM

To evaluate the safety and efficacy of agitation thrombolysis (AT) combined with catheter-directed thrombolysis (CDT) for the treatment of non-cirrhotic acute portal vein thrombosis (PVT).

METHODS

Nine patients with non-cirrhotic acute PVT who underwent AT combined with CDT were analyzed retrospectively. Portography was carried out *via* the transjugular intrahepatic portosystemic (commonly known as TIP) or percutaneous transhepatic (commonly known as PT) route, followed by AT combined with CDT. Complications of the procedure, and the changes in clinical symptoms, hemodynamics of the portal vein and liver function were recorded. Follow-up was scheduled at

1, 3 and 6 mo after treatment, and every 6 mo thereafter, or when the patients developed clinical symptoms related to PVT. Color Doppler ultrasound and contrast-enhanced computed tomography/magnetic resonance imaging were performed during the follow-up period to determine the condition of the portal vein.

RESULTS

AT combined with CDT was successfully performed. The portal vein was reached *via* the TIP route in 6 patients, and *via* the PT route in 3 patients. All clinical symptoms were relieved or disappeared, with the exception of 1 patient who died of intestinal necrosis 9 d after treatment. Significant differences in the changes in portal vein hemodynamics were observed, including the maximum lumen occupancy of PVT, portal vein pressure and flow velocity between pre- and post-treatment ($P < 0.05$). During the follow-up period, recurrence was observed in 1 patient at 19 mo after the procedure, and the portal vein was patent in the remaining patients.

CONCLUSION

AT combined with CDT is a safe and effective method for the treatment of non-cirrhotic acute PVT.

Key words: Agitation thrombolysis; Catheter-directed thrombolysis; Portal vein thrombosis

Core tip: Agitation is a common phenomenon in daily life, and can accelerate the rate of solute dissolution in a solvent. As the thrombus is porous in the acute stage, it can easily be broken into smaller fragments by agitation, which increases the contact area between the thrombus and thrombolytics, and increases the speed of thrombus dissolution. According to our research, agitation thrombolysis combined with catheter-directed thrombolysis is a safe and effective method for the treatment of non-cirrhotic acute portal vein thrombosis, with a good short- to middle-term efficacy. However, the long-term efficacy of agitation thrombolysis combined with catheter-directed thrombolysis in a large population requires further investigation.

Wang CY, Wei LQ, Niu HZ, Gao WQ, Wang T, Chen SJ. Agitation thrombolysis combined with catheter-directed thrombolysis for the treatment of non-cirrhotic acute portal vein thrombosis. *World J Gastroenterol* 2018; 24(39): 4482-4488 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4482.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4482

INTRODUCTION

Non-cirrhotic portal vein thrombosis (PVT) has a low incidence with a prevalence of approximately 1%, and partial obstruction of the portal vein is often clinically imperceptible[1]. However, complete obstruction due to acute thrombus can lead to a series of clinical symptoms, such as abdominal pain, ascites, and even intestinal necrosis. Common treatment options, including anticoagulation or indirect thrombolysis, are useful for relieving symptoms, but the outcome is unsatisfactory as most patients developed cavernous transformation of the portal vein due to incomplete recanalization[2,3]. Catheter-directed thrombolysis (CDT) *via* the transjugular intrahepatic portosystemic (TIP) or percutaneous transhepatic (PT) route is a minimally invasive method, which can dissolve the thrombus and relieve symptoms rapidly with a lower dose of thrombolytics[4]. In addition, agitation thrombolysis (AT) can break the thrombus into smaller fragments and accelerate the speed of thrombolysis[5]. The objective of this study was to evaluate the safety and efficacy of AT combined with CDT for the treatment of non-cirrhotic acute PVT.

MATERIALS AND METHODS

Patients

The data from 9 patients with non-cirrhotic acute PVT who underwent AT combined with CDT in our hospital between September 2015 and December 2017 were analyzed retrospectively. The patients consisted of 3 men and 6 women, with a mean age of 47.9 ± 10.6 years. All patients met the following eligibility criteria: (1) the PVT was diagnosed definitively by color Doppler ultrasound (CDUS) and enhanced-contrast computed tomography (CT) or magnetic resonance imaging (MRI) (Figures 1A and 2A); (2) absence of massive periportal collaterals and features of liver cirrhosis in the imaging findings; and (3) excluded malignant tumor. In addition, the following information was collected for each patient before treatment: clinical symptoms, days from onset to operation, routine blood examination, liver function, etiology, the maximum lumen occupancy of PVT and the flow velocity of the portal vein measured by CDUS (Table 1).

Treatment

The procedures were performed under digital subtraction angiographic guidance (Artis zeego; Siemens, Munich, Germany). The choice of TIP or PT route depended on if the patients had ascites.

The portal vein was reached *via* the TIP route in 6 patients with ascites. A Rosch-Uchida set (Cook, Bloomington, IN, United States) was used to gain access to the portal vein branch from the hepatic vein under fluoroscopic guidance. When a 0.035-in hydrophilic guidewire (Terumo, Tokyo, Japan) reached the superior mesenteric vein or splenic vein in cooperation with a Cobra catheter (Cook), a pigtail catheter (Cook) was exchanged to perform portography (Figure 1B) and measure the portal pressure. The Rosch-Uchida sheath

Table 1 Characteristics of the included patients

Patient No.	Age (yr)	Sex	Etiology	Symptoms	Time from onset to treatment (d)
1	29	M	Myelodysplastic syndromes	Abdominal pain, abdominal distension	4
2	39	F	Protein S deficiency	Abdominal pain, abdominal distension, vomiting	11
3	43	M	Myelodysplastic syndromes	Abdominal pain, vomiting	14
4	48	F	Protein C deficiency	Abdominal pain, abdominal distension	5
5	40	F	Nephrotic syndrome	Abdominal pain, vomiting	8
6	53	M	Protein S deficiency	Abdominal pain, diarrhea	2
7	61	F	Splenectomy after trauma	Abdominal pain, abdominal distension, vomiting	10
8	36	F	Myelodysplastic syndromes	Abdominal pain, abdominal distension	5
9	57	F	Unknown	Abdominal pain	16

F: Female; M: Male.

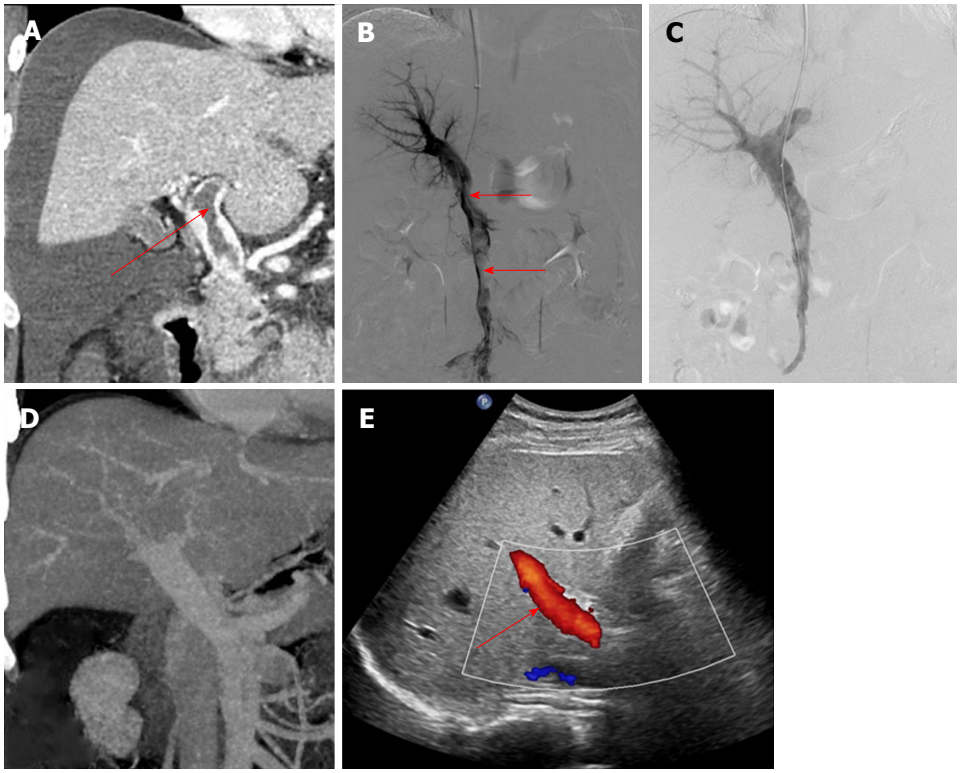


Figure 1 A 29-year-old man had acute abdominal pain and distension for 4 d. A and B: Contrast-enhanced computed tomography (CT) and portography *via* the transjugular intrahepatic portosystemic route showed thrombus formation in the portal vein and superior mesenteric vein (arrow) with massive ascites; C: Portography after agitation thrombolysis and catheter-directed thrombolysis showed that the filling defect in the portal vein had decreased; D: Contrast-enhanced CT after surgery showed that the thrombus had disappeared completely; E: At 6 mo after treatment, color Doppler ultrasound showed smooth blood flow in the portal vein (arrow).

was then introduced into the portal vein following intrahepatic dilatation by a 6-mm balloon catheter (Boston Scientific, Natick, MA, United States). A pigtail catheter was inserted into the thrombus through the sheath, and a guidewire with a helical tip molded *in vitro* was advanced through the catheter (Figure 3). The guidewire and pigtail catheter were pushed and drawn together, and then rotated clockwise and anticlockwise alternatively, to agitate the thrombus into smaller fragments. In addition, 2×10^5 IU urokinase (Tianjin Biochemical Pharmaceutical Co., Ltd., Tianjin, China) was injected through the catheter by intermittent pulsatile delivery. The AT procedure was continued for approximately 10 min. The Rosch-Uchida sheath was then removed and the indwelling pigtail catheter was left in the PVT.

The portal vein was reached *via* the PT route in 3 patients without ascites. After successful puncture of a portal vein branch using a 22-gauge Chiba needle (Cook), a 6F coaxial dilator (Cook) was inserted into the portal vein. Then, a 5F sheath (Cook) and a pigtail catheter were exchanged. AT was performed after

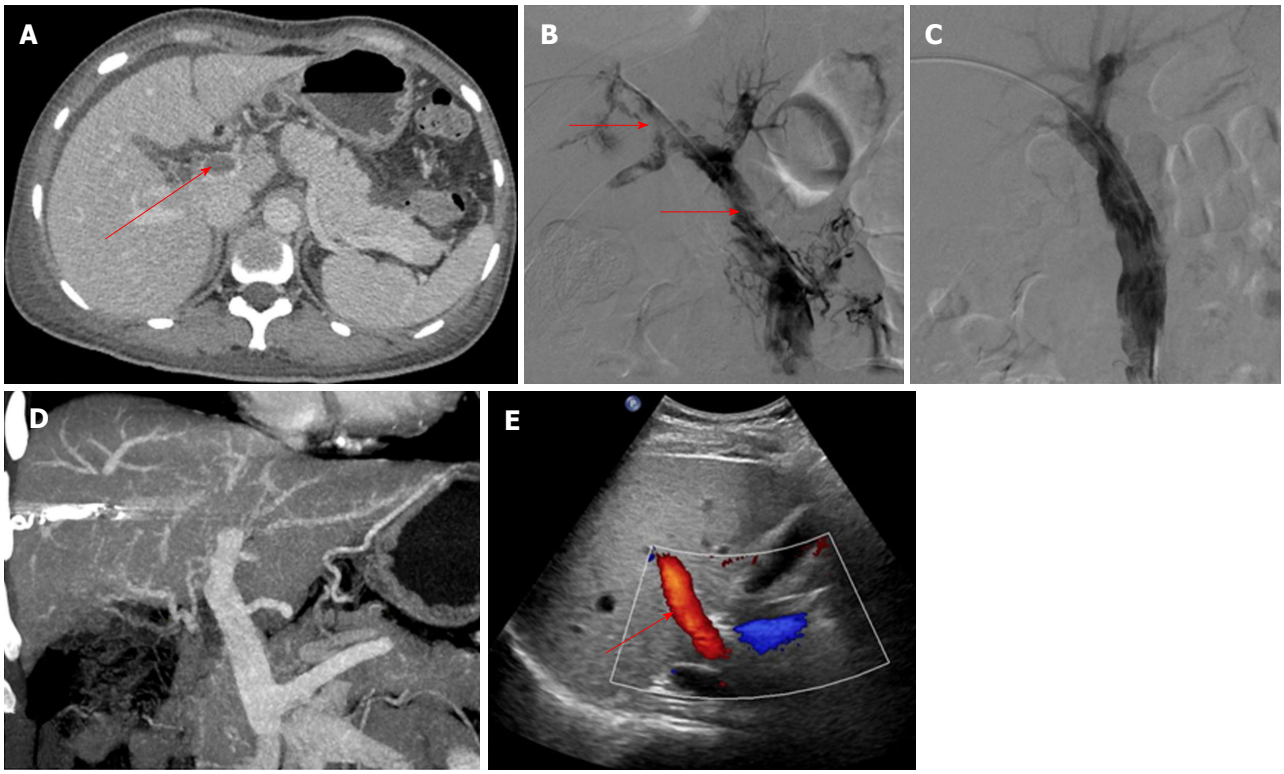


Figure 2 A 57-year-old woman had acute abdominal pain for 16 d. A and B: Contrast-enhanced computed tomography (CT) and portography *via* the percutaneous transhepatic route showed thrombus formation in the portal vein and superior mesenteric vein (arrow); C: Portography after agitation thrombolysis and catheter-directed thrombolysis showed that the filling defect in the portal vein had decreased; D: Contrast-enhanced CT after treatment showed that the thrombus had disappeared completely; E: At 12 mo after treatment, color Doppler ultrasound showed smooth blood flow in the portal vein (arrow).

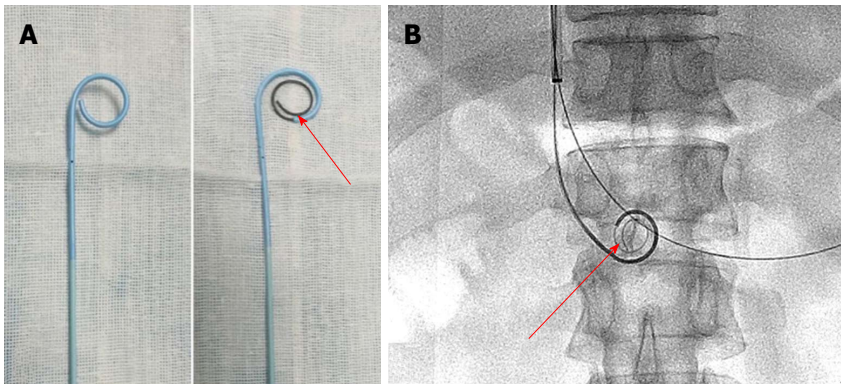


Figure 3 Pigtail catheter was inserted into the thrombus through the sheath, and a guidewire with a helical tip molded *in vitro* was advanced through the catheter. A: A pigtail catheter, and a guidewire with a helical tip (arrow) through the catheter *in vitro*; B: The catheter and guidewire in the portal vein (arrow) during agitation thrombolysis *via* the transjugular intrahepatic portosystemic route.

portography (Figure 2B), and the AT procedures were similar to those carried out *via* the TIP route. The indwelling pigtail catheter was left in the PVT.

Six hundred thousand IU of urokinase was infused continuously *via* the pigtail catheter each day, unless complications developed. In addition, 5000 IU of low molecular weight heparin was injected twice a day, and warfarin was administered after treatment at an initial dose of 5 mg. The low molecular weight heparin was discontinued when the international normalized ratio was maintained at 2.0-3.0, and warfarin was continued for at least 12 mo. Portography was repeated every 24 h, and termination of CDT was based on the patency of the portal vein assessed by imaging and an obvious improvement in clinical symptoms. The portal vein pressure was measured again before the pigtail catheter was removed.

Follow-up study

Follow-up was scheduled at 1, 3 and 6 mo after treatment, and every 6 mo thereafter, or when the patients developed clinical symptoms related to PVT.

Table 2 Results of treatment and follow-up

Patient No.	Treatment route	Duration of CDT (d)	Dose of urokinase ($\times 10^6$ IU)	Complications	Follow-up time (mo) and results
1	TIP	2	1.0	Hematuresis	27/patent
2	PT	2	1.4	None	23/patent
3	TIP	-	-	Death	Death
4	TIP	1	0.8	None	31/patent
5	PT	3	2.0	Hemorrhage from the puncture tract	5/patent
6	TIP	4	2.6	Subcapsular hematoma	22/reappearance
7	TIP	1	0.8	None	9/patent
8	TIP	1	0.8	None	12/patent
9	PT	3	2.0	Hemorrhage from the puncture tract	18/patent

PT: Percutaneous transhepatic; TIP: Transjugular intrahepatic portosystemic.

Table 3 Changes in liver function and portal vein hemodynamics

	Pre-treatment	Post-treatment	*P* value
Albumin, g/L	34.9 min	36.7 min	0.872
Alanine aminotransferase, U/L	38.2 inea	33.6 inea	0.934
Total bilirubin, sferase	17.9 lbi	17.4 lbi	0.991
Flow velocity of the portal vein, cm/s	4.2 tal	16.3 alv	< 0.05
Maximum lumen occupancy of PVT, %	78.2 occ	14.1 occ	< 0.01
Portal pressure, mmHg	31.0 alp	14.1 alp	< 0.05

PVT: Portal vein thrombosis.

Observations during follow-up mainly included clinical symptoms, routine blood examination, liver function and the condition of the portal vein evaluated by CDUS and contrast-enhanced CT/MRI. The end of follow-up was April 30, 2018.

Statistical analysis

Data are shown as mean ± standard deviation (SD). Paired Student's *t*-test was used to determine statistically significant differences between pre- and post-treatment values. Significance was set at $P < 0.05$. Statistical analysis was performed using SPSS (version 19.0; IBM, Armonk, NY, United States) software.

RESULTS

Treatment outcome

AT combined with CDT was successful in all patients. Immediate portography after AT showed greater blood flow than pre-AT. One patient treated *via* the TIP route underwent intestinal resection as a result of congestive necrosis 5 d after the procedure; however, this patient died 9 d later. In the remaining patients, CDT was continued for 2.1 ± 1.1 d with a total dose of urokinase of 0.8-2.6 × 10^6 IU (Table 2). All clinical symptoms were relieved or disappeared, except in 1 patient who continued to experience abdominal distension after meals. There was a significant difference in the maximum lumen occupancy of PVT (Figures 1C and 2C), portal vein pressure and flow velocity between pre- and post-treatment ($P < 0.05$). No significant differences in the changes in liver function, such as alanine aminotransferase, albumin and bilirubin were observed ($P > 0.05$) (Table 3).

Complications

One patient developed subcapsular hemorrhage during puncture of the portal vein *via* the TIP route and experienced a rapid heartbeat. The patient's vital signs were stable after erythrocyte transfusion, and CDT was started 3 d later. One patient *via* PT route had hematuresis during CDT. These symptoms disappeared when urokinase was discontinued (Table 2).

Follow-up

The follow-up period ranged from 5 mo to 31 mo. During this period, 1 patient who had been taking warfarin for 12 mo had an irregular poor appetite at 19 months after treatment, and contrast-enhanced CT showed that cavernous transformation of the portal vein had developed in the right portal vein. In the remaining patients, the portal vein was patent and none of the clinical symptoms related to PVT reappeared (Table 2). Liver function and routine blood examination were normal in all patients during follow-up (Figures 1D and E, 2D and E).

DISCUSSION

PVT is mostly seen in liver cirrhosis, with a prevalence

ranging from 10% to 23%[6]. Non-cirrhotic PVT rarely occurs, with a prevalence of approximately 1%, and has an intimate relationship with inherited or acquired coagulation diseases, such as primary myeloproliferative disorders, Protein C/S deficiency, and abdominal surgery[7]. In addition to the etiology of PVT, it is particularly important to distinguish acute PVT from chronic PVT as the treatment is different. The American Association for the Study of Liver Diseases (commonly known as AASLD) describes acute PVT as the sudden formation of thrombus in the portal vein, and chronic PVT occurs when the obstructed portal vein is replaced by periportal collaterals bypassing the PVT[8]. However, the time boundary is not mentioned. In our study, acute PVT was diagnosed by clinical symptoms and the absence of massive periportal collaterals on imaging.

For non-cirrhotic acute PVT with obvious symptoms, the aim of treatment is recanalization of the obstructed portal vein and the prevention of complications. Common treatment methods including anticoagulation or indirect thrombolysis can be useful for relieving symptoms and recanalizing the portal vein. A meta-analysis involving 353 patients showed a significantly higher rate of recanalization following anticoagulation compared with the control group, which did not receive anticoagulation (71% *vs* 42%; $P < 0.01$)[2]. However, less than 20% of the patients achieved complete recanalization, and the others developed cavernous transformation of the portal vein due to incomplete recanalization, which can lead to chronic portal hypertension. Indirect thrombolysis *via* a peripheral vein or the superior mesenteric artery is less technically demanding, whereas the effect is limited as the thrombolytics are diverted from the patent branches or collaterals[3]. In addition, a high dose of thrombolytics may increase the risk of gastrointestinal hemorrhage.

CDT is an effective treatment for acute thrombus, which can enhance the efficacy of thrombolytics at a lower dose compared to indirect thrombolysis[4]. CDT *via* the PT route is relatively simple to perform, but it is not suitable for patients with massive ascites as it may cause hemorrhage through the PT tract during subsequent anticoagulation or thrombolysis. In addition, patients often felt pain when breathing as the indwelling catheter traversed the hepatic capsule. CDT *via* the TIP route can avoid these complications, but puncturing the portal vein from the hepatic vein is more difficult than using the PT route. Chen *et al*[9] reported that direct portography *via* the PT route, then a balloon catheter inflated with contrast medium to 80% of its volume positioned at the site of the bifurcation of the right and left portal veins can improve the success rate. Wang *et al*[10] reported the successful treatment of 12 patients with acute PVT treated with aspiration combined with CDT *via* the TIP route. The CDT time was 7.6 h. Eight patients achieved complete recanalization and four patients had partial recanalization, and AT combined with CDT was more effective than anticoagulation or indirect thrombolysis.

Agitation is a common phenomenon in daily life, and can accelerate the rate of solute dissolution in a solvent. AT was first reported by Ding *et al*[5] following the successful treatment of acute inferior vein thrombus in Budd-Chiari syndrome, which showed a good long-term efficacy[11], but has not yet been reported in the treatment of PVT. As the thrombus is porous in the acute stage, it can easily be broken into smaller fragments by agitation, which increases the contact area between the thrombus and thrombolytics, and increases the speed of thrombus dissolution. The shape of the catheter tip is important. In this study, the pigtail catheter was chosen as an agitator, as it did not injure the vessel wall and resulted in greater fragmentation of the thrombus during rotation. In addition, a guidewire with a helical tip can enhance the fracture resistance of the pigtail catheter, and increase the range of agitation. Compared with other methods, such as endovascular aspiration or mechanical thrombectomy[12], AT is simpler and safer, with less blood loss. In our study, all the patients had a smoother blood flow after treatment than before treatment, and the duration of CDT was shorter and the rate of recanalization was higher than those reported by Wang *et al*[10].

There are some limitations in this study. Firstly, the study was retrospective, with a limited number of cases; therefore, the data may have been affected by various potential biases. Secondly, puncture of the portal vein is difficult to perform under fluoroscopic guidance, and required extensive experience and a good understanding of imaging data including contrast-enhanced CT/MRI; thus, the treatment is restricted to University Hospitals.

In conclusion, AT combined with CDT is a safe and effective method for the treatment of non-cirrhotic acute PVT, with a good short- to middle-term efficacy. However, the long-term efficacy of AT combined with CDT in a large population requires further investigation.

ARTICLE HIGHLIGHTS

Research background

Non-cirrhotic portal vein thrombosis (PVT) has a low incidence, with a prevalence of approximately 1%, and partial obstruction of the portal vein is often clinically imperceptible. However, complete obstruction due to acute thrombus can lead to a series of clinical symptoms, such as abdominal pain, ascites, and even intestinal necrosis. Common treatment opinions including anticoagulation or indirect thrombolysis are useful for relieving symptoms, but the outcome is unsatisfactory as most patients develop into cavernous transformation of the portal vein due to incomplete recanalization.

Research motivation

Catheter-directed thrombolysis (CDT) *via* the transjugular intrahepatic portosystemic (commonly known as TIP) or percutaneous transhepatic (PT) route is a minimally invasive method, which can dissolve the thrombus and relieve symptoms rapidly with a lower dose of thrombolytics. In addition, agitation thrombolysis (AT) can break the thrombus into smaller fragments and accelerate the speed of thrombolysis.

Research objectives

The objective of this study was to evaluate the safety and efficacy of AT combined with CDT for the treatment of non-cirrhotic acute PVT.

Research methods

Nine patients with non-cirrhotic acute PVT who underwent AT combined with CDT *via* TIP or percutaneous transhepatic route were analyzed retrospectively. AT had not been reported for the treatment of PVT so far. The changes in clinical symptoms, hemodynamics of the portal vein and liver function were recorded and followed up to evaluate the safety and efficacy.

Research results

According to our research, AT combined with CDT is a safe and effective method for the treatment of non-cirrhotic acute PVT, with a good short- to middle-term efficacy. However, the long-term efficacy of AT combined with CDT in a large population requires further investigation.

Research conclusions

Agitation is a common phenomenon in daily life, and can accelerate the rate of solute dissolution in a solvent. AT was first reported for the treatment of acute inferior vein thrombus in Budd-Chiari syndrome, which showed good long-term efficacy, but has not yet been reported in the treatment of PVT. As the thrombus is porous in the acute stage, it can easily be broken into smaller fragments by agitation, which increases the contact area between the thrombus and thrombolytics, and increases the speed of thrombus dissolution. Compared with other methods, such as endovascular aspiration or mechanical thrombectomy, AT is simpler and safer, with less blood loss.

Research perspectives

The shape of the catheter tip is important. In this study, the pigtail catheter was chosen as an agitator, as it did not injure the vessel wall and resulted in greater fragmentation of the thrombus during rotation. In addition, a guidewire with a helical tip can enhance the fracture resistance of the pigtail catheter, and increase the range of agitation. The future research we will focus on involves the long-term efficacy of AT combined with CDT in a large population.

REFERENCES

1 **Basit SA,** Stone CD, Gish R. Portal vein thrombosis. *Clin Liver Dis* 2015; **19**: 199-221 [PMID: 25454305 DOI: 10.1016/j.cld.2014.09.012]

2 **Loffredo L**, Pastori D, Farcomeni A, Violi F. Effects of Anticoagulants in Patients With Cirrhosis and Portal Vein Thrombosis: A Systematic Review and Meta-analysis. *Gastroenterology* 2017; **153**: 480-487.e1 [PMID: 28479379 DOI: 10.1053/j.gastro.2017.04.042]

3 **Liu K**, Li WD, Du XL, Li CL, Li XQ. Comparison of Systemic Thrombolysis Versus Indirect Thrombolysis via the Superior Mesenteric Artery in Patients with Acute Portal Vein Thrombosis. *Ann Vasc Surg* 2017; **39**: 264-269 [PMID: 27671456 DOI: 10.1016/j.avsg.2016.06.029]

4 **Avgerinos ED**, Chaer RA. The ATTRACTiveness of catheter-directed thrombolysis. *J Vasc Surg Venous Lymphat Disord* 2018; **6**: 303 [PMID: 29661361 DOI: 10.1016/j.jvsv.2018.02.002]

5 **Ding PX**, Li YD, Han XW, Wu G. Agitation thrombolysis for fresh iatrogenic IVC thrombosis in patients with Budd-Chiari syndrome. *J Vasc Surg* 2010; **52**: 782-784 [PMID: 20471769 DOI: 10.1016/j.jvs.2010.02.016]

6 **Harding DJ**, Perera MT, Chen F, Olliff S, Tripathi D. Portal vein thrombosis in cirrhosis: Controversies and latest developments. *World J Gastroenterol* 2015; **21**: 6769-6784 [PMID: 26078553 DOI: 10.3748/wjg.v21.i22.6769]

7 **Cruz-Ramón V**, Chinchilla-López P, Ramírez-Pérez O, Aguilar-Olivos NE, Alva-López LF, Fajardo-Ordoñez E, Ponciano-Rodríguez G, Northup PG, Intagliata N, Caldwell SH, Qi X, Méndez-Sánchez N. Thrombosis of the Portal Venous System in Cirrhotic vs. Non-Cirrhotic Patients. *Ann Hepatol* 2018; **17**: 476-481 [PMID: 29735798 DOI: 10.5604/01.3001.0011.7392]

8 **DeLeve LD**, Valla DC, Garcia-Tsao G; American Association for the Study Liver Diseases. Vascular disorders of the liver. *Hepatology* 2009; **49**: 1729-1764 [PMID: 19399912 DOI: 10.1002/hep.22772]

9 **Chen Y**, Ye P, Li Y, Ma S, Zhao J, Zeng Q. Percutaneous transhepatic balloon-assisted transjugular intrahepatic portosystemic shunt for chronic, totally occluded, portal vein thrombosis with symptomatic portal hypertension: procedure technique, safety, and clinical applications. *Eur Radiol* 2015; **25**: 3431-3437 [PMID: 25903717 DOI: 10.1007/s00330-015-3777-1]

10 **Wang MQ**, Liu FY, Duan F, Wang ZJ, Song P, Fan QS. Acute symptomatic mesenteric venous thrombosis: treatment by catheter-directed thrombolysis with transjugular intrahepatic route. *Abdom Imaging* 2011; **36**: 390-398 [PMID: 20652243 DOI: 10.1007/s00261-010-9637-1]

11 **Ding PX**, He X, Han XW, Zhang Y, Wu Y, Liang XX, Liu C. An Individualised Strategy and Long-Term Outcomes of Endovascular Treatment of Budd-Chiari Syndrome Complicated by Inferior Vena Cava Thrombosis. *Eur J Vasc Endovasc Surg* 2018; **55**: 545-553 [PMID: 29478911 DOI: 10.1016/j.ejvs.2017.12.016]

12 **Jun KW**, Kim MH, Park KM, Chun HJ, Hong KC, Jeon YS, Cho SG, Kim JY. Mechanical thrombectomy-assisted thrombolysis for acute symptomatic portal and superior mesenteric venous thrombosis. *Ann Surg Treat Res* 2014; **86**: 334-341 [PMID: 24949327 DOI: 10.4174/astr.2014.86.6.334]

P- Reviewer: Eleftheriadis NP **S- Editor**: Ma RY
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4489-4498

DOI: 10.3748/wjg.v24.i39.4489

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

ORIGINAL ARTICLE

Retrospective Study

Ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of common bile duct stones

Hai-Yang Chang, Chang-Jun Wang, Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Dong Li, Yu-Liang Li

Hai-Yang Chang, Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Dong Li, Yu-Liang Li, Department of Intervention Medicine, the Second Hospital of Shandong University, Jinan 250033, Shandong Province, China

Hai-Yang Chang, Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Dong Li, Yu-Liang Li, Interventional Oncology Institute, Shandong University, Jinan 250033, Shandong Province, China

Chang-Jun Wang, Department of Radiology, Jiyang People's Hospital, Jinan 251400, Shandong Province, China

ORCID number: Hai-Yang Chang (0000-0001-8684-4877); Chang-Jun Wang (0000-0002-9390-832X); Bin Liu (0000-0003-1686-1553); Yong-Zheng Wang (0000-0001-5889-2118); Wu-Jie Wang (0000-0001-9431-7153); Wei Wang (0000-0002-2124-5328); Dong Li (0000-0002-3944-3680); Yu-Liang Li (0000-0001-8117-4317).

Author contributions: All authors helped to perform the research; Chang HY contributed to manuscript writing, statistical analysis, and manuscript critical revision; Li YL contributed to study conception and design, manuscript writing, statistical analysis, and manuscript critical revision; Wang CJ, Liu B, Wang YZ, Wang WJ, Wang W, and Li D contributed to manuscript writing.

Supported by the Natural Science Foundation of Shandong Province, No. ZR2014HM050; the Youth Foundation of the Second Hospital of Shandong University, No. Y2014010047; the Nature Science Foundation of Shandong Province, No. ZR2018PH032 and No. ZR2018PH033; and the National Natural Science Foundation of China, No. 61671276.

Institutional review board statement: This study was reviewed and approved by the ethics committee of the Second Hospital of Shandong University.

Informed consent statement: All participants provided written informed consent.

Conflict-of-interest statement: All authors declare no conflicts of interest related to this article.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Yu-Liang Li, MD, PhD, Chief Doctor, Doctor, Professor, Department of Intervention Medicine, The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, Shangdong Province, China. lyl.pro@sdu.edu.cn
Telephone: +86-531-85875927

Received: July 25, 2018
Peer-review started: July 25, 2018
First decision: August 27, 2018
Revised: August 29, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

AIM

To evaluate the effectiveness and safety of combined ursodeoxycholic acid and percutaneous transhepatic balloon dilation for management of gallstones after expulsion of common bile duct (CBD) stones.

METHODS

From April 2014 to May 2016, 15 consecutive patients (6 men and 9 women) aged 45-86 (mean, 69.07 ± 9.91) years suffering from CBD stones associated with gallstones were evaluated. Good gallbladder contraction function was confirmed by type B ultrasonography. Dilation of the CBD and cystic duct was detected. Percutaneous transhepatic balloon dilation of the papilla was performed, ursodeoxycholic acid was administered, and all patients had a high-fat diet. All subjects underwent repeated cholangiography, and percutaneous transhepatic removal was carried out in patients with secondary CBD stones originating from the gallbladder.

RESULTS

All patients underwent percutaneous transhepatic balloon dilation with a primary success rate of 100%. The combined therapy was successful in 86.7% of patients with concomitant CBD stones and gallstones. No remaining stones were detected in the gallbladder. Transient adverse events include abdominal pain (*n* = 1), abdominal distension (*n* = 1), and fever (*n* = 1). Complications were treated successfully *via* nonsurgical management without long-term complications. No procedure-related mortality occurred.

CONCLUSION

For patients with concomitant CBD stones and gallstones, after percutaneous transhepatic removal of primary CBD stones, oral ursodeoxycholic acid and a high-fat diet followed by percutaneous transhepatic removal of secondary CBD stones appear to be a feasible and effective option for management of gallstones.

Key words: Common bile duct stones; Gallstones; Percutaneous transhepatic removal; Ursodeoxycholic acid

Core tip: Percutaneous transhepatic removal combined with oral ursodeoxycholic acid and a high-fat diet appears to be a feasible and safe alternative to surgery or endoscopic procedure for elimination of gallstones, especially for patients with good gallbladder contraction function, diameter of gallstones no greater than 12 mm, and dilation of the cystic duct. It also provides an alternative when operative management is not available for patients in poor condition.

Chang HY, Wang CJ, Liu B, Wang YZ, Wang WJ, Wang W, Li D, Li YL. Ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of common bile duct stones. *World J Gastroenterol* 2018; 24(39): 4489-4498 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4489.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4489

INTRODUCTION

Bile duct stones are the major cause of benign biliary diseases[1]. Surgical exploration or endoscopic intervention can be managed successfully in most common bile duct (CBD) stones[2]. However, open surgery is contraindicated in cases with severe comorbidities, and endoscopic sphincterotomy (EST) for extraction of CBD stones in patients with prior surgically modified gastrointestinal tract may result in failure due to invisibility of the papilla of Vater[3]. Hence, percutaneous transhepatic intervention appears to be an alternative for these patients. To prevent the recurrence of CBD stones for patients with concomitant CBD stones and gallstones, subsequent cholecystectomy is the first choice after the elimination of CBD stones within 48 h[4]. Herein, we present our experience in percutaneous transhepatic removal of stones for patients with CBD stones associated with gallstones *via* an innovative nonsurgical treatment including percutaneous transhepatic balloon dilation (PTBD) combined with oral ursodeoxycholic acid. Moreover, this study aimed to assess the efficacy and safety of this combined therapy.

MATERIALS AND METHODS

This was a retrospective study to assess the efficacy and safety of PTBD combined with ursodeoxycholic acid for removal of CBD stones associated with gallstones. The procedure was approved by the ethics committee of our institution. Written informed consent was obtained from all patients.

Patients

Fifteen consecutive patients (6 men and 9 women) aged 45-86 (mean, 69.07 ± 9.91) years, diagnosed with concomitant CBD stones and gallstones, admitted to our institution from April 2014 to May 2016 were evaluated.

Overall, 2-5 CBD stones and gallstones were detected in 15 patients, with diameters ranging from 2 to 25 mm. Eleven patients were confirmed to have concomitant CBD stones and gallstones before procedure using type B ultrasonography, enhanced computed tomography, or magnetic resonance cholangiopancreatography (MRCP), and the remaining 4 were detected by cholangiography during the removal of CBD stones. All patients suffered from fever, jaundice, abdominal discomfort, poor appetite, or vomiting.

Ultrasonography, enhanced CT, MRCP, or cholangiography were carried out to determine the diagnosis of stones (Figure 1A and B). Pancreatitis was not detected. For patients with poor condition, multiple

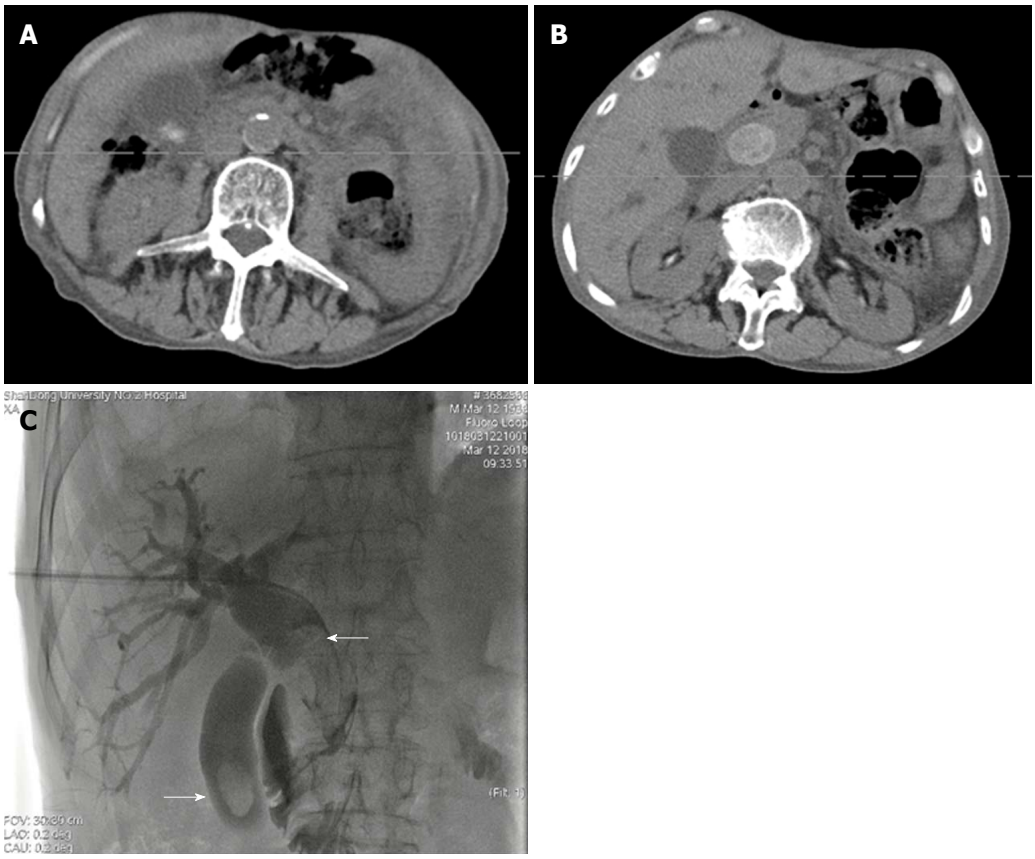


Figure 1 Computed tomography scan and cholangiography showing filling defect in the common bile duct and gallbladder (white arrow). Dilation of the common bile duct and cystic duct was detected. A and B: Ultrasonography, enhanced computed tomography, magnetic resonance cholangiopancreatography, or cholangiography was carried out to determine the diagnosis of stones; C: Advancing cholangiography was performed to detect the number, size, and location of stones.

disciplinary consultations were carried out as pre-procedure assessment.

Follow-up of patients included clinical assessment, physical examination, laboratory test and imaging evaluation for 1 year at a 3-mo interval. Technical success was defined as complete absence of CBD stones. The absence of symptoms was regarded as medical success regardless of the presence or absence of residual stones.

Procedure

After pretreatment with antibiotics (levofloxacin or cephalosporin), all patients were positioned under intravenous sedation and fluoroscopic monitoring, and a 21G Chiba needle (Neff Percutaneous Access Set, Cook Medical LLC, Bloomington, IN, United States) was used to puncture the right hepatic duct. The biliary tree was shown by injecting a contrast agent *via* the needle. A tiny guidewire (Wire Guide Diameter inch. 018, Cook Medical LLC, Bloomington, IN, United States) was introduced into the biliary system, and a sheath was inserted into the bile duct over the tiny guidewire. Advancing cholangiography was performed to detect the number, size, and location of stones (Figure 1C). A hydrophilic guidewire [150 cm in length, Terumo (China) Holding Co., Ltd. China] was deployed in the CBD *via* the transhepatic route. A 6F to 10F sheath [Terumo (China) Holding Co., Ltd. China] was introduced into the right hepatic duct according to the balloon size to dilate the papilla of Vater. A Vert catheter (Cook Medical LLC, Bloomington, IN, United States) was introduced into the duodenum or jejunum. A stiff guidewire [260 cm in length, Terumo (China) Holding Co., Ltd. China] was passed through the catheter and papilla of Vater. An angiographic catheter balloon was inserted through the stiff guidewire and was placed across the papilla. The diameter of the balloon varied from 12 mm to 24 mm and its length was 40 mm or 60 mm depending on the size of the stones (Figure 2). The papilla was inflated gradually until the maximal pressure reached 6-8 atm. Stone-crushing device such as a basket was used in some cases with large stones. Larger balloon was inserted to dilate the papilla in patients with primary failure, and stone expulsion was performed repeatedly. Intraoperative cholangiography was performed to confirm residual stones in CBD. An 8.5F external drainage tube (Biliary Drainage Catheter, Cook Medical LLC, Bloomington, IN, United States) was deployed in the CBD for postoperative drainage and assessment of efficacy of the procedure (Figure 3).

Oral ursodeoxycholic acid (250 mg, Losan Pharma

Table 1 Baseline characteristics of patients

No.	Gender /age	CBD/gallbladder		
		Number of stones	Diameter of the largest stone (mm)	Diameter of the largest balloon (mm)
1	F/58	2/2	10/6	12/8
2	M/45	3/1	15/7	16/8
3	M/75	1/2	25/10	24/10
4	F/67	1/1	20/8	20/8
5	F/64	3/2	20/9	20/10
6	M/68	2/2	21/10	20/10
7	F/73	3/1	22/14	20/-
8	F/76	3/2	20/11	20/12
9	M/86	2/1	21/10	20/10
10	F/81	3/1	19/8	18/8
11	F/67	1/2	20/10	20/10
12	F/72	2/1	21/12	20/12
13	M/76	2/3	18/15	18/-
14	F/65	3/1	18/12	18/12
15	M/63	1/1	20/12	20/12

CBD: Common bile duct.

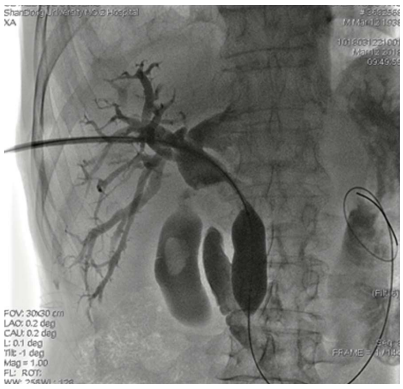


Figure 2 Dilatation of the sphincter of Oddi with a balloon catheter was performed.

GmbH) was initiated in all patients after procedure. The prescribed dose was 250 mg three times a day. After 7-10 d, repeated cholangiography *via* external drainage catheter was performed, and balloon dilation of the sphincter of Oddi and elimination of stones were carried out in patients with secondary CBD stones (Figures 4 and 5). Intraoperative cholangiography confirmed the absence of all stones and the external drainage tube was left (Figure 6A). Furthermore, 3-5 d after the procedure, cholangiography was performed again to confirm no residual of stones, and the catheter was retrieved (Figure 6B).

Statistics analysis

Data are reported as mean ± standard deviation. Comparison of means was analyzed by the paired *t*-test. All statistical analyses were performed using IBM SPSS Statistics 24.0. *P*-values < 0.05 were defined as statistical difference for all data.

RESULTS

Table 1 shows baseline characteristics of the patients. Table 2 shows that complete clearance of CBD stones was obtained in one session for all patients. No plastic or bare mental stent was inserted in any patients. All patients were administered subsequently with oral ursodeoxycholic acid after undergoing PTBD. Secondary CBD stones originating in the gallbladder were detected in 13 of these patients with concomitant CBD stones and gallstones. The stones were eliminated in one session in all these patients. Gallstones with reduced size still existed *in situ* in the remaining two patients. One patient with residual gallstones with symptoms was transferred to department of general surgery for laparoscopic cholecystectomy. One asymptomatic patient was discharged. Intensive long-term follow-up was essential for them. No further treatment except for observation was carried out for this patient. No evidence of retained CBD stones was detected in any patients. The technical success rate was 86.7%, and the overall medical successful rate was 93.3%.

Table 3 demonstrates the result of laboratory tests pre and postintervention. Serum alanine transaminase and total bilirubin (TBIL) levels became normal in patients with jaundice after the procedure. White blood cell (WBC) levels decreased significantly on day 14 postoperatively. However, there was no statistical difference between preoperative and postoperative values for hemoglobin and amylase.

Transient adverse effects including vomiting, chills, fever, and abdominal distension were found in a few patients after the procedure. They were cured with analgesic and antiemetic argents. No severe complications such as bile peritonitis, hemobilia, and cholangitis occurred. TBIL and WBC values of one patient complicated with fever were 63 μmol/L and 12.21 × 10^9/L, respectively, after the procedure. One patient suffered from abdominal distention and decreased hemoglobin levels from 122 g/L to 82 g/L. The WBC count increased slightly for the patient complicated with abdominal

Table 2 Operative parameters

No.	Primary technical success	Secondary technical success	Adverse events	Treatment
1	Yes	Yes	No	
2	Yes	Yes	Fever	Medication
3	Yes	Yes	No	
4	Yes	Yes	No	
5	Yes	Yes	Abdominal distension	Medication
6	Yes	Yes	No	
7	Yes	No	No	
8	Yes	Yes	No	
9	Yes	Yes	No	
10	Yes	Yes	No	
11	Yes	Yes	Abdominal pain	Medication
12	Yes	Yes	No	
13	Yes	No	No	
14	Yes	Yes	No	
15	Yes	Yes	No	

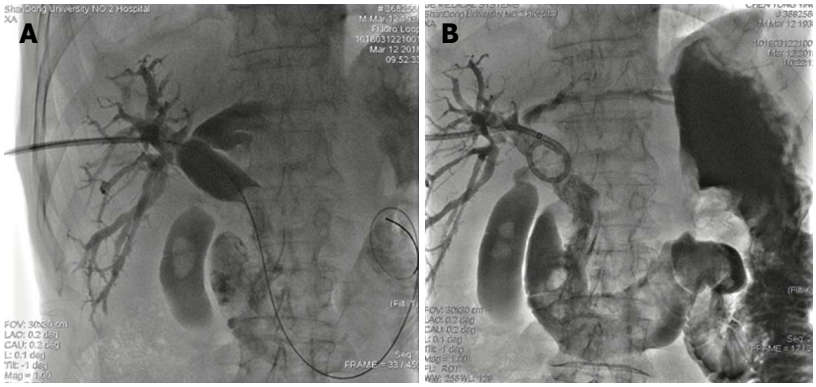


Figure 3 Common bile duct stones were expelled into the duodenum through the dilated sphincter. A and B: An 8.5F external drainage tube was deployed in the common bile duct for postoperative drainage and assessment of efficacy of the procedure.

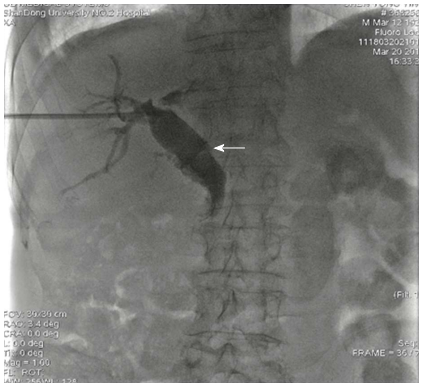


Figure 4 Ursodeoxycholic acid was given and repeated cholangiography was performed. The secondary common bile duct stones originating from the gallbladder (white arrow) and shrunk gallbladder were detected by cholangiography.

pain. All complications were treated successfully *via* nonsurgical management without remote complications. Antibiotics (Ceftriaxone) and somatostatin were injected until the symptoms vanished. No procedure-related mortality occurred. During 1-year follow-up, no obstruction of bile ducts or recurrence of symptoms was detected.

DISCUSSION

Bile duct stones, one of the most common digestive problems needing admission to hospital, are the major cause of benign diseases of the biliary tract, such as obstructive jaundice and cholangitis[1,5]. It includes intrahepatic and extrahepatic bile duct stones, CBD stones and gallstones. CBD stones comprise primary and secondary stones. Secondary stones from the gallbladder and migrating into the ductal system are different from primary stones that form in the biliary tract. Primary stones may be the consequence of bacterial infection and biliary stasis. The majority of the secondary stones are cholesterol gallstones, while primary stones are mainly pigment stones[6]. Compared to the Western population, primary stones are more prevalent in Asia[7]. The prevalence of CBD stones in patients with symptomatic gallstones varies from 10% to 20%[8]. In this study, 15 patients with CBD stones suffered from gallstones, of which 11 were confirmed before the procedure, while 4 patients who underwent PTBD had gallstones detected by cholangiography.

Table 3 Laboratory tests pre and post-intervention

	Pre-intervention	2 wk after intervention	*t*	*P* value
ALT (U/L)	98.93 ± 24.47	36.13 ± 8.99	10.41	< 0.001
TBIL (μmol/L)	39.40 ± 7.76	21.47 ± 12.09	6.52	< 0.001
Amylase (U/L)	80.73 ± 14.94	82.07 ± 17.77	0.34	0.741
WBC (× 10^9/L)	11.58 ± 1.45	7.65 ± 2.11	5.90	< 0.001
HGB (g/L)	122.93 ± 8.66	118.80 ± 13.39	1.52	0.150

ALT: Alanine transaminase; HGB: Hemoglobin; TBIL: Total bilirubin; WBC: White blood cell.

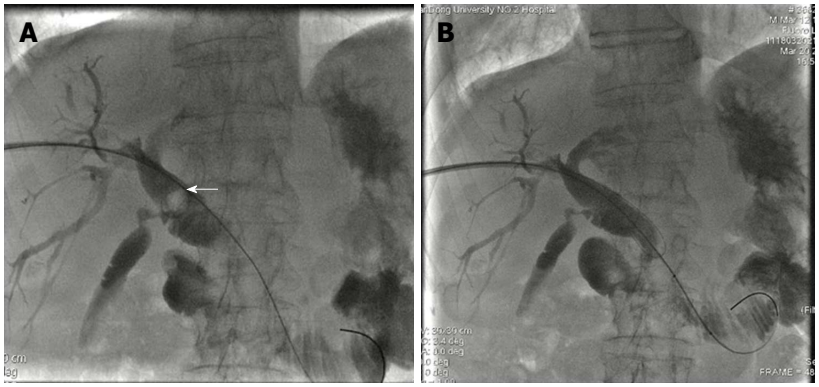


Figure 5 Secondary common bile duct stones (white arrow) were expulsed into the duodenum without gallstone residual. A and B: After 7-10 d, repeated cholangiography *via* external drainage catheter was performed, and balloon dilation of the sphincter of Oddi and elimination of stones were carried out in patients with secondary common bile duct stones.

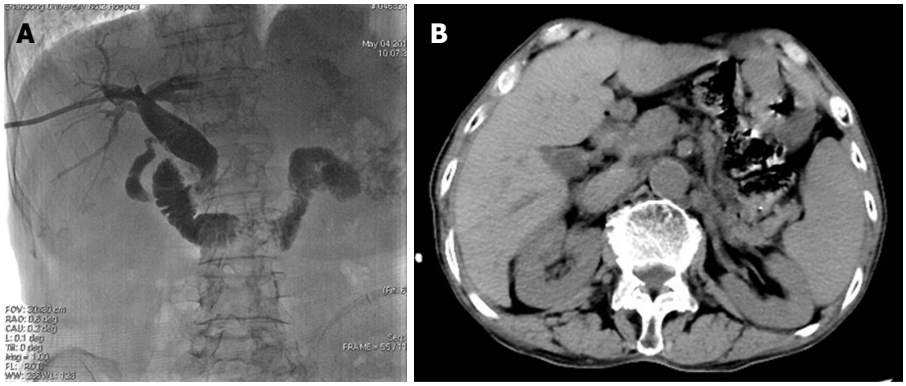


Figure 6 Computed tomography and postoperative cholangiography demonstrating that there was no residual of common bile duct stones or gallstones. A: Intraoperative cholangiography confirmed the absence of all stones and external drainage tube was left; B: Furthermore, 3-5 d after the procedure, cholangiography was performed again with no residual of stones, and the catheter was retrieved.

Many people are hospitalized for acute pancreatitis due to CBD stones that occlude the ampulla. In addition, bile duct obstruction caused by stones result in septic cholangitis. Chronic occlusion could induce secondary biliary cirrhosis. All types of CBD stones should be cured aggressively. Many management options, including open surgery, laparoscopic surgery, endoscopic and percutaneous procedure, are available for removal of CBD stones[1,2,9-11]. Abdominal exploration with incision of the CBD and stone removal was the predominant choice a few decades ago. With technological advances and improvement of skills, various alternatives could be employed in the extraction of bile duct stones. However, open surgery still retains its important role in the management of complicated stone disease. Laparoscopic procedure has comparable morbidity and mortality rates to open surgery. Hence, both open and laparoscopic surgery should be considered in cases unsuitable to be treated by nonsurgical options.

Endoscopic retrograde cholangiopancreatography (ERCP) was first introduced in 1968[12]. It was accepted quickly as a feasible diagnostic and therapeutic technique for CBD stones[13,14]. In the 1990s, EST was considered a feasible alternative for patients with serious comorbidity contraindicated to open surgery[15,16]. It appears to be a better choice for elder patients with benign biliary

tract diseases. CBD stones could be eliminated by ERCP *via* sphincterotomy or balloon dilation[17]. For patients requiring maintenance of papillary function, balloon dilation may be an effective and safe alternative to EST in the management of bile duct stones[18-20]. However, open surgery is superior to ERCP for clearance of CBD stones. Compared to open surgery, ERCP necessitates increased number of procedures for each patient[2]. Complications of ERCP with sphincterotomy include hemorrhage, papillary stenosis, pancreatitis, duodenal perforation, and recurrent stones[21], and the complication rate ranges from 0.5%-5.4%[22].

In the past decades, percutaneous intervention has been reported as an effective alternative to open or laparoscopic surgery and endoscopic intervention for elimination of CBD stones[9,23,24]. Several reports indicated that transhepatic balloon dilation of papilla could be an alternative to extraction of biliary stones[23-25]. Numerous devices, such as Dormia basket, occlusive, or cutting balloon, were introduced to improve the success rate of the technique[25-27]. The technique success rate varies from 94.7% to 100%[28,29]. Papillary dilation was performed using balloons with a diameter ranging from 8 mm to 20 mm[9,28,30]. Transient adverse events, including nausea, vomiting, and abdominal pain, were observed in some cases which resolved with medication composed with analgesic and antiemetic drugs. A study by Nevzat Ozcan revealed 18 complications, including cholangitis (2.7%), subcapsular biloma (1.5%), subcapsular hemotoma (0.38%), subcapsular abscess (0.38%), bile peritonitis (0.38%), duodenal perforation (0.38%), and CBD perforation (0.38%)[9]. Only 2 of 38 main complications were observed by Santiago Gil with complete expulsion of stones in 36 of the 38 patients. No procedure-related deaths occurred[29]. Although a few cases were reported, ERCP for patients with prior Billroth II gastrectomy may be challenging[26,31,32]. EST for extraction of CBD stones may lead to failure, even in experienced surgeons[33]. For these cases, percutaneous transhepatic intervention appears to be an available and safe management for expulsion of stones[34].

Several other methods for percutaneous expulsion of stones to the duodenum were reported. Extraction from the T-tube or existing gallbladder drain for access has been published as an effective percutaneous technique for stone expulsion[30,35]. A novel technique of combined percutaneous transhepatic and endoscopic or laparoscopic approach also acts an important role in patients unsuitable to be treated with routine ERCP[36-38].

Gallstones with a higher prevalence in adults may occur in all societies and races. Its increasing prevalence associates with age in both sexes, and women are involved more commonly than men[6]. Gallstones are composed of cholesterol, calcium bilirubinate, protein, lipid, and less water. Occlusion of the gallbladder duct can cause abdominal pain, chills, fever, and jaundice. Treatment is indicated in patients with symptomatic gallstones. Cholecystectomy is the most effective procedure for symptomatic patients[39]. Laparoscopic, small-incision, or open cholecystectomy could be a feasible treatment in the management of gallstones. These three techniques can resolve symptoms caused by gallstones. No statistically significant differences in the outcome have been found. Although laparoscopic cholecystectomy is the most popular method, small-incision cholecystectomy has shorter operative time and appears to be less costly[40]. However, the increased incidence of colon cancer is associated with cholecystectomy[41]. Several nonsurgical treatments have been developed for treatment of gallstones with recurrence. Percutaneous cholecytostomy serves a role with few complications in management acute calculous cholecystitis[42,43]. Medical treatment also plays an important role in management of gallstones. Gallstone dissolution may be achieved by oral administration of ursodeoxycholic acid which decreases biliary cholesterol secretion, increases solubility of cholesterol by formation of liquid crystals, and reduces intestinal cholesterol absorption[39].

To prevent the recurrence of stones, for CBD stones associated with gallstones, subsequent cholecystectomy is the first choice after the elimination of the CBD stones within 48 h[4]. Patients with suspected or proven CBD stones undergoing cholecystectomy can anticipate benefit from the perioperative management of CBD stones[11]. Nowadays, several procedures depending on the experience of surgeons are available for treatment of combined cholecystocholedocholithiasis, such as laparoscopic treatment, simultaneous laparo-endoscopic treatment, and combined ERCP and EST with cholecystectomy[44]. Concurrent transhepatic percutaneous balloon dilation combined with laparoscopic cholecystectomy is introduced for treatment of gallstones associated with CBD stones[38]. Fifteen patients with concomitant CBD stones and gallstones were enrolled in our study, and the primary technical success rate was 100%. Subsequently, PTBD was performed repeatedly to expel secondary CBD stones originating in the gallbladder. Immediate complications including bile peritonitis, bile pleura effusion, hemobilia, acute pancreatitis, and duodenum perforation, were not observed in our study. All slight complications were treated successfully *via* nonsurgical management.

In our series, 15 patients with CBD stones and gallstones were enrolled and 13 of them were treated successfully *via* an innovative technique. For these patients, the strategy of treatment was as follows: First, routine PTBD was performed to eliminate the CBD stones without any difficulties. Then, all patients with good gallbladder contraction function were confirmed. Second, ursodeoxycholic acid, a kind of oral dissolution agent, was administered to patients with 250 mg for three times per day. A high-fat diet was initiated similar to that in gallbladder contraction test. Third, repeated

cholangiography was performed 7-10 d later, and 13 cases showed secondary CBD stones originating in the gallbladder retaining in the CBD. Gallstones with reduced size still existed *in situ* in the remaining two patients. For patients with secondary CBD stones, subsequently PTBD was carried out repeatedly with great care, and the stones were expulsed into the duodenum. One asymptomatic patient with reduced gallstones was discharged directly with intending long-term follow-up. The remaining patient underwent cholecystectomy. Three to five days later, cholangiography demonstrated no residual stones in all patients with secondary CBD stones, and the drainage tubes were removed.

In conclusion, PTBD is an option for patients with CBD stones. Percutaneous transhepatic removal combined with oral ursodeoxycholic acid and a high-fat diet appears to be a feasible and safe alternative to surgery or endoscopic procedure for elimination of gallstones, especially for patients with good gallbladder contraction function, diameter of gallstones no greater than 12 mm, and dilation of the cystic duct. It also provides an alternative when operative management is not available for patients in poor condition.

ARTICLE HIGHLIGHTS

Research background

Bile duct stones are the most frequent cause of benign bile duct disease. The choice of management of common bile duct (CBD) stones includes surgical exploration, endoscopic intervention and percutaneous transhepatic intervention. Subsequent cholecystectomy is the first choice to prevent the recurrence of stones for patients with concomitant CBD stones and gallstones. This retrospective study aimed to evaluate the clinical efficacy and safety of ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of CBD Stones.

Research motivation

Percutaneous transhepatic intervention served as an effective option for management of CBD stones in the past decades. The preferable choice of management for patients with concomitant CBD stones and gallstones is controversial.

Research objectives

The retrospective study evaluated the effectiveness and safety of a novel technique for management of gallstones after expulsion of CBD stones in terms of technical success and postoperative complications.

Research methods

Fifteen consecutive patients diagnosed with concomitant CBD stones and gallstones were evaluated. All patients underwent application of ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of CBD stones. Clinical assessment, physical examination, laboratory tests and imaging were assessed in all patients. All statistics analyses were performed using SPSS 24.0. *P*-values < 0.05 were defined as statistically difference for all data.

Research results

The novel technique was successful in 86.7% of patients with concomitant CBD stones and gallstones with few postoperative complications treated successfully *via* nonsurgical management. It seems to be an alternative to open or laparoscopic surgery and endoscopic intervention.

Research conclusions

The present study showed that ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation was secure and feasible for management of gallstones after elimination of CBD stones, especially for patients with good gallbladder contraction function, diameter of gallstone no greater than 12 mm, and dilation of the cystic duct. It also provides an alternative when operative management is not available for patients in poor condition.

Research perspectives

In case of therapeutic failure, good gallbladder contraction function or dilation of the cystic duct was not observed. However, the diameters of stones in failed cases were much greater than those of successful cases. This novel technique provides a feasible option for patients with concomitant gallstones and CBD stones. Prospective studies are needed for further confirmation.

ACKNOWLEDGMENTS

The statistical methods of this study were reviewed by Yuan Zhang from Center of Evidence-based Medicine, Second Hospital of Shandong University.

REFERENCES

1 **Buxbaum J**. Modern management of common bile duct stones. *Gastrointest Endosc Clin N Am* 2013; **23**: 251-275 [PMID: 23540960 DOI: 10.1016/j.giec.2012.12.003]
2 **Dasari BV**, Tan CJ, Gurusamy KS, Martin DJ, Kirk G, McKie L, Diamond T, Taylor MA. Surgical versus endoscopic treatment of bile duct stones. *Cochrane Database Syst Rev* 2013; (**12**): CD003327 [PMID: 24338858 DOI: 10.1002/14651858.CD003327.pub4]
3 **Ross AS**. Endoscopic retrograde cholangiopancreatography in the surgically modified gastrointestinal tract. *Gastrointest Endosc Clin N Am* 2009; **19**: 497-507 [PMID: 19647654 DOI: 10.1016/j.giec.2009.04.009]
4 **European Association for the Study of the Liver (EASL)**. EASL Clinical Practice Guidelines on the prevention, diagnosis and treatment of gallstones. *J Hepatol* 2016; **65**: 146-181 [PMID: 27085810 DOI: 10.1016/j.jhep.2016.03.005]
5 **ASGE Standards of Practice Committee**, Chathadi KV, Chandrasekhara V, Acosta RD, Decker GA, Early DS, Eloubeidi MA, Evans JA, Faulx AL, Fanelli RD, Fisher DA, Foley K, Fonkalsrud L, Hwang JH, Jue TL, Khashab MA, Lightdale JR, Muthusamy VR, Pasha SF, Saltzman JR, Sharaf R, Shaukat A, Shergill AK, Wang A, Cash BD, DeWitt JM. The role of ERCP in benign diseases of the biliary tract. *Gastrointest Endosc* 2015; **81**: 795-803 [PMID: 25665931 DOI: 10.1016/j.gie.2014.11.019]
6 **Portincasa P**, Moschetta A, Palasciano G. Cholesterol gallstone disease. *Lancet* 2006; **368**: 230-239 [PMID: 16844493 DOI: 10.1016/S0140-6736(06)69044-2]
7 **Williams EJ**, Green J, Beckingham I, Parks R, Martin D, Lombard M; British Society of Gastroenterology. Guidelines on the management of common bile duct stones (CBDS). *Gut* 2008; **57**: 1004-1021 [PMID: 18321943 DOI: 10.1136/gut.2007.121657]
8 **Frossard JL**, Morel PM. Detection and management of bile duct stones. *Gastrointest Endosc* 2010; **72**: 808-816 [PMID: 20883860 DOI: 10.1016/j.gie.2010.06.033]
9 **Ozcan N**, Kahriman G, Mavili E. Percutaneous transhepatic removal of bile duct stones: results of 261 patients. *Cardiovasc Intervent Radiol* 2012; **35**: 621-627 [PMID: 21647808 DOI: 10.1007/s00270-011-0190-2]
10 **Ponsky JL**. Laparoscopic therapy of cholelithiasis. *Annu Rev Med* 1993; **44**: 317-322 [PMID: 8476253 DOI: 10.1146/annurev.me.44.020193.001533]
11 **Graham SM**, Flowers JL, Scott TR, Bailey RW, Scovill WA, Zucker KA, Imbembo AL. Laparoscopic cholecystectomy and

common bile duct stones. The utility of planned perioperative endoscopic retrograde cholangiography and sphincterotomy: experience with 63 patients. *Ann Surg* 1993; **218**: 61-67 [PMID: 8328830]
12 **McCune WS**, Shorb PE, Moscovitz H. Endoscopic cannulation of the ampulla of vater: a preliminary report. *Ann Surg* 1968; **167**: 752-756 [PMID: 5646296]
13 **Law R**, Baron TH. ERCP. *Gastrointest Endosc* 2013; **78**: 428-433 [PMID: 23948191 DOI: 10.1016/j.gie.2013.06.018]
14 **Moon JH**, Choi HJ, Lee YN. Endoscopic retrograde cholangiopancreatography. *Endoscopy* 2014; **46**: 775-778 [PMID: 25133479 DOI: 10.1055/s-0034-1377506]
15 **Tham TC**, Carr-Locke DL, Collins JS. Endoscopic sphincterotomy in the young patient: is there cause for concern? *Gut* 1997; **40**: 697-700 [PMID: 9245919]
16 **Wojtun S**, Gil J, Gietka W, Gil M. Endoscopic sphincterotomy for choledocholithiasis: a prospective single-center study on the short-term and long-term treatment results in 483 patients. *Endoscopy* 1997; **29**: 258-265 [PMID: 9255528 DOI: 10.1055/s-2007-1004186]
17 **Minami A**, Nakatsu T, Uchida N, Hirabayashi S, Fukuma H, Morshed SA, Nishioka M. Papillary dilation vs sphincterotomy in endoscopic removal of bile duct stones. A randomized trial with manometric function. *Dig Dis Sci* 1995; **40**: 2550-2554 [PMID: 8536511]
18 **Yasuda I**, Tomita E, Enya M, Kato T, Moriwaki H. Can endoscopic papillary balloon dilation really preserve sphincter of Oddi function? *Gut* 2001; **49**: 686-691 [PMID: 11600473]
19 **Bergman JJ**, van Berkel AM, Bruno MJ, Fockens P, Rauws EA, Tijssen JG, Tytgat GN, Huibregtse K. A randomized trial of endoscopic balloon dilation and endoscopic sphincterotomy for removal of bile duct stones in patients with a prior Billroth II gastrectomy. *Gastrointest Endosc* 2001; **53**: 19-26 [PMID: 11154484]
20 **Liao WC**, Tu YK, Wu MS, Wang HP, Lin JT, Leung JW, Chien KL. Balloon dilation with adequate duration is safer than sphincterotomy for extracting bile duct stones: a systematic review and meta-analyses. *Clin Gastroenterol Hepatol* 2012; **10**: 1101-1109 [PMID: 22642953 DOI: 10.1016/j.cgh.2012.05.017]
21 **Tse F**, Yuan Y. Early routine endoscopic retrograde cholangiopancreatography strategy versus early conservative management strategy in acute gallstone pancreatitis. *Cochrane Database Syst Rev* 2012; CD009779 [PMID: 22592743 DOI: 10.1002/14651858.CD009779.pub2]
22 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918 [PMID: 8782497 DOI: 10.1056/NEJM199609263351301]
23 **Perez MR**, Oleaga JA, Freiman DB, McLean GL, Ring EJ. Removal of a distal common bile duct stone through percutaneous transhepatic catheterization. *Arch Surg* 1979; **114**: 107-109 [PMID: 758870]
24 **Haskin PH**, Teplick SK, Gambescia RA, Zitomer N, Pavlides CA. Percutaneous transhepatic removal of a common bile duct stone after mono-octanoin infusion. *Radiology* 1984; **151**: 247-248 [PMID: 6701322 DOI: 10.1148/radiology.151.1.6701322]
25 **Clouse ME**. Dormia basket modification for percutaneous transhepatic common bile duct stone removal. *AJR Am J Roentgenol* 1983; **140**: 395-397 [PMID: 6600361 DOI: 10.2214/ajr.140.2.395]
26 **Itoi T**, Ishii K, Itokawa F, Kurihara T, Sofuni A. Large balloon papillary dilation for removal of bile duct stones in patients who have undergone a billroth ii gastrectomy. *Dig Endosc* 2010; **22** Suppl 1: S98-S102 [PMID: 20590782 DOI: 10.1111/j.1443-1661.2010.00955.x]
27 **Oguzkurt L**, Ozkan U, Gumus B. Percutaneous transhepatic cutting balloon papillotomy for removal of common bile duct stones. *Cardiovasc Intervent Radiol* 2009; **32**: 1117-1119 [PMID: 19093146 DOI: 10.1007/s00270-008-9487-1]
28 **Han JY**, Jeong S, Lee DH. Percutaneous papillary large balloon dilation during percutaneous cholangioscopic lithotripsy for the treatment of large bile-duct stones: a feasibility study. *J Korean Med Sci* 2015; **30**: 278-282 [PMID: 25729250 DOI: 10.3346/jkms.2015.30.3.278]
29 **Gil S**, de la Iglesia P, Verdú JF, de España F, Arenas J, Irurzun J. Effectiveness and safety of balloon dilation of the papilla and the use of an occlusion balloon for clearance of bile duct calculi. *AJR Am J Roentgenol* 2000; **174**: 1455-1460 [PMID: 10789811 DOI: 10.2214/ajr.174.5.1741455]
30 **Atar E**, Neiman C, Ram E, Almog M, Gadiel I, Belenky A. Percutaneous trans-papillary elimination of common bile duct stones using an existing gallbladder drain for access. *Isr Med Assoc J* 2012; **14**: 354-358 [PMID: 22891395]
31 **Dolay K**, Soylu A. Easy sphincterotomy in patients with Billroth II gastrectomy: a new technique. *Turk J Gastroenterol* 2008; **19**: 109-113 [PMID: 19110666]
32 **Kim KH**, Kim TN. Endoscopic papillary large balloon dilation for the retrieval of bile duct stones after prior Billroth II gastrectomy. *Saudi J Gastroenterol* 2014; **20**: 128-133 [PMID: 24705151 DOI: 10.4103/1319-3767.129478]
33 **Arnold JC**, Benz C, Martin WR, Adamek HE, Riemann JF. Endoscopic papillary balloon dilation vs. sphincterotomy for removal of common bile duct stones: a prospective randomized pilot study. *Endoscopy* 2001; **33**: 563-567 [PMID: 11473325 DOI: 10.1055/s-2001-15307]
34 **Shirai N**, Hanai H, Kajimura M, Kataoka H, Yoshida K, Nakagawara M, Nemoto M, Nagasawa M, Kaneko E. Successful treatment of percutaneous transhepatic papillary dilation in patients with obstructive jaundice due to common bile duct stones after Billroth II gastrectomy: report of two emergent cases. *J Clin Gastroenterol* 2000; **30**: 91-93 [PMID: 10636221]
35 **Baban C**, Rajendran S, O'Hanlon D, Murphy M. Utilisation of cholecystostomy and cystic duct as a route for percutaneous cutting balloon papillotomy and expulsion of common bile duct stones. *BMJ Case Rep* 2012; **2012** [PMID: 22802565 DOI: 10.1136/bcr.11.2011.5256]
36 **Itoi T**, Shinohara Y, Takeda K, Nakamura K, Sofuni A, Itokawa F, Moriyasu F, Tsuchida A. A novel technique for endoscopic sphincterotomy when using a percutaneous transhepatic cholangioscope in patients with an endoscopically inaccessible papilla. *Gastrointest Endosc* 2004; **59**: 708-711 [PMID: 15114320]
37 **Matsumoto S**, Ikeda S, Maeshiro K, Okamoto K, Miyazaki R. Management of giant common bile duct stones in high-risk patients using a combined transhepatic and endoscopic approach. *Am J Surg* 1997; **173**: 115-116 [PMID: 9074375 DOI: 10.1016/S0002-9610(96)00406-0]
38 **Li S**, Li Y, Geng J, Liu B, Gao R, Zhou Z, Hu S. Concurrent Percutaneous Transhepatic Papillary Balloon Dilatation Combined with Laparoscopic Cholecystectomy for the Treatment of Gallstones with Common Bile Duct Stones. *J Laparoendosc Adv Surg Tech A* 2015; **25**: 886-891 [PMID: 26575245 DOI: 10.1089/lap.2015.0220]
39 **Lammert F**, Miquel JF. Gallstone disease: from genes to evidence-based therapy. *J Hepatol* 2008; **48** Suppl 1: S124-S135 [PMID: 18308417 DOI: 10.1016/j.jhep.2008.01.012]
40 **Keus F**, Gooszen HG, van Laarhoven CJ. Open, small-incision, or laparoscopic cholecystectomy for patients with symptomatic cholecystolithiasis. An overview of Cochrane Hepato-Biliary Group reviews. *Cochrane Database Syst Rev* 2010; CD008318 [PMID: 20091665 DOI: 10.1002/14651858.CD008318]
41 **Shao T**, Yang YX. Cholecystectomy and the risk of colorectal cancer. *Am J Gastroenterol* 2005; **100**: 1813-1820 [PMID: 16086719 DOI: 10.1111/j.1572-0241.2005.41610.x]
42 **Bortoff GA**, Chen MY, Ott DJ, Wolfman NT, Routh WD. Gallbladder stones: imaging and intervention. *Radiographics* 2000; **20**: 751-766 [PMID: 10835126 DOI: 10.1148/radiographics.20.3.g00ma16751]

43 **Gurusamy KS**, Rossi M, Davidson BR. Percutaneous cholecystostomy for high-risk surgical patients with acute calculous cholecystitis. *Cochrane Database Syst Rev* 2013; CD007088 [PMID: 23939652 DOI: 10.1002/14651858.CD007088.pub2]

44 **La Greca G**, Barbagallo F, Sofia M, Latteri S, Russello D. Simultaneous laparoendoscopic rendezvous for the treatment of cholecystocholedocholithiasis. *Surg Endosc* 2009; **24**: 769-780 [PMID: 19730946 DOI: 10.1007/s00464-009-0680-3]

P- Reviewer: Kazuya S, Morling JR, Schievenbusch S
S- Editor: Wang XJ **L- Editor**: Wang TQ **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4499-4509

DOI: 10.3748/wjg.v24.i39.4499

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

ORIGINAL ARTICLE

Retrospective Study

Postoperative survival analysis and prognostic nomogram model for patients with portal hypertension

Ya-Fei Zhang, Hong Ji, Hong-Wei Lu, Le Lu, Lei Wang, Jin-Long Wang, Yi-Ming Li

Ya-Fei Zhang, Hong Ji, Hong-Wei Lu, Le Lu, Lei Wang, Jin-Long Wang, Yi-Ming Li, Department of General Surgery, the Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

ORCID number: Ya-Fei Zhang (0000-0001-8223-4406); Hong Ji (0000-0002-4800-679X); Hong-Wei Lu (0000-0003-2904-9978); Le Lu (0000-0002-0529-1391); Lei Wang (0000-0001-7384-7913); Jin-Long Wang (0000-0002-3380-5425); Yi-Ming Li (0000-0002-4122-7020).

Author contributions: Li YM conceived the research and critically reviewed and revised the paper; Zhang YF, Ji H, Lu HW and Lu L designed the research and analysed the data; Zhang YF and Li YM performed the research; all authors participated in the operations; Zhang YF drafted the paper.

Supported by the National Natural Science Foundation of China, No. 81170454, No. 30772049 and No. 30571765.

Institutional review board statement: The study was reviewed and approved by the Second Affiliated Hospital of Xi'an Jiaotong University Institutional Review Board.

Informed consent statement: The requirement for written informed patient consent was waived due to the retrospective and anonymous nature of this study; all data were used only for statistical analysis.

Conflict-of-interest statement: All authors declare no conflicts of interest related to this study.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Yi-Ming Li, MD, Professor, Department of General Surgery, the Second Affiliated Hospital of Xi'an Jiaotong University, No. 157, Xiwu Road, Xi'an 710004, Shaanxi Province, China. liyiming@xjtu.edu.cn
Telephone: +86-29-87679746
Fax: +86-29-87679746

Received: August 8, 2018
Peer-review started: August 8, 2018
First decision: August 30, 2018
Revised: September 6, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

AIM

To analyse the postoperative survival of patients with portal hypertension and determine the factors that influence survival and construct nomograms.

METHODS

We retrospectively followed 1045 patients who underwent splenectomy plus pericardial devascularisation (SPD) between January 2002 and December 2017. Two SPD types are used in our department: splenectomy plus simplified pericardial devascularisation (SSPD) and splenectomy plus traditional pericardial devascularisation (STPD). The Kaplan-Meier method and Cox regression analysis were used to evaluate the prognostic effects of multiple parameters on overall survival (OS), disease-specific survival (DSS) and bleeding-free survival (BFS). Significant prognostic factors were combined to build nomograms to predict the survival rate of individual patients.

RESULTS

Five hundred and fifty-seven (53.30%) patients were

successfully followed with 192 in the SSPD group and 365 in the STPD group; 93 (16.70%) patients died, of whom 42 (7.54%) died due to bleeding. Postoperative bleeding was observed in 84 (15.10%) patients. The 5- and 10-year OS, DSS and BFS rates in the group of patients who underwent SSPD were not significantly different from those in patients who underwent STPD. Independent prognostic factors for OS were age, operative time, alanine transaminase level and albumin-bilirubin score. Independent prognostic factors for BFS were male sex, age, intraoperative blood loss and time to first flatus. Independent prognostic factors for DSS were the Comprehensive Complication Index and age. These characteristics were used to establish nomograms, which showed good accuracy in predicting 1-, 3- and 5-year OS and BFS.

CONCLUSION

SSPD achieves or surpasses the long-term survival effect of traditional pericardial devascularisation and is worthy of clinical promotion and application. Nomograms are effective at predicting prognosis.

Key words: Nomogram; Portal hypertension; Pericardial devascularisation; Survival analysis

Core tip: The mortality and re-bleeding rate are still extremely high among patients with portal hypertension after splenectomy plus pericardial devascularisation. This study aimed to analyse the postoperative survival, identify risk factors, construct nomograms, and explore the clinical effect of splenectomy plus simplified pericardial devascularisation (SSPD). Five hundred and fifty-seven (53.30%) patients were successfully followed, and the results suggested that the 5- and 10-year overall survival, disease-specific survival and bleeding-free survival rates were not significantly different between patients who underwent SSPD and patients who underwent splenectomy plus traditional pericardial devascularisation. Age, operative time, alanine transaminase level and albumin-bilirubin score were independent prognostic factors influencing overall survival. Male sex, age, intraoperative blood loss and time to first flatus were independent prognostic factors influencing bleeding-free survival. Comprehensive Complication Index and age were independent prognostic factors influencing disease-specific survival.

Zhang YF, Ji H, Lu HW, Lu L, Wang L, Wang JL, Li YM. Postoperative survival analysis and prognostic nomogram model for patients with portal hypertension. *World J Gastroenterol* 2018; 24(39): 4499-4509 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4499.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4499

INTRODUCTION

Portal hypertension (PH) is mainly caused by cirrhosis, of which most cases are posthepatitic cirrhosis in China and alcoholic cirrhosis in Western countries. Its clinical manifestations are splenomegaly and hypersplenism, and eventually oesophageal and gastric varices. The most important complication of PH is acute variceal bleeding, with a high mortality rate of more than 50%. And there is also a high risk of re-bleeding in surviving cases[1,2]. Due to a difference in aetiology, the therapy of PH is different. Shunts are the main method in the West. However, in oriental countries, such as China, where patients who develop PH after hepatitis cirrhosis have poor liver function, treatment mainly involves devascularisation. Splenectomy plus pericardial devascularisation (SPD) is the main operative method used to prevent and treat PH[3,4]. This operation does not reduce portal vein blood flow to the liver, does not damage liver function, can dislodge hypersplenism, and can effectively reduce the incidence of hepatic encephalopathy. However, splenectomy plus traditional pericardial devascularisation (STPD) is difficult and complicated, which will still cause greater tissue damage and increase the operative time, thereby increasing the damage of the liver and kidneys. By simplifying STPD, we put forward splenectomy plus simplified pericardial devascularisation (SSPD). The short-term curative effect of SSPD has been verified, but its effects on long-term survival are not clear[5,6].

Patients with PH still face high risks of re-bleeding and death after SPD. Therefore, the best postoperative treatment must be identified to improve the prognosis of these patients, and a determination of the parameters impacting survival by examining factors related to the disease course in patients with PH is useful. Moreover, the identification of prognostic factors in patients with PH is important for estimating outcomes and determining the appropriate treatments. Nomograms have been used to integrate a variety of prognostic factors, quantify the impact of different factors on survival and visualise the results to predict the survival rate of individual patients. Nomograms have been widely used to assess patient prognosis[7-9].

In the present study, 1045 patients with PH who underwent SPD were followed retrospectively to explore the long-term survival effect of SSPD and the prognostic factors for patients with PH, as well as construct a survival nomogram to predict the overall survival rate of patients with PH.

MATERIALS AND METHODS

Study subjects

Patients with PH presenting with oesophagogastric varices and hypersplenism who were treated in our department from January 2002 to February 2017 were

screened for this single-centre retrospective cohort study. Two SPD types are used in our department: SSPD and STPD. The surgical details are described in a previous publication[5,6]. The inclusion criteria were: (1) patients with PH who were diagnosed with oesophagogastric varices and hypersplenism based on clinical symptoms combined with laboratory, digestive endoscopy or image examinations; (2) patients with PH classified as grade A or B according to the Child-Pugh grading criteria or Child-Pugh grade C at admission and assigned a reduced classification to preoperative Child-Pugh grade A or B after liver preservation therapy to attain appropriate surgical indications; and (3) patients who were able to tolerate general anaesthesia and had no surgical contraindications. The exclusion criteria were: (1) patients with hepatocellular carcinoma, acute heart failure, shock, or other vital organ diseases; (2) patients in an acute haemorrhagic state with unstable vital signs; and (3) patients with poor heart, lung, liver or kidney function. This research was approved by the Ethical Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. All procedures were conducted in accordance with the Declaration of Helsinki from the World Medical Association and with the ethical standards of the committees responsible for human experimentation (institutional and national). The requirement for written informed patient consent was waived due to the retrospective and anonymous nature of this study; all data were used only for statistical analysis.

Data collection

Survival and postoperative bleeding conditions of the patients were monitored and recorded. The following primary data were collected: age, gender, aetiology, Charlson score, blood type, history of variceal ligation, history of abdominal surgery, smoking, drinking, history of variceal bleeding, body mass index (BMI), Child-Pugh score at admission, model for end-stage liver disease (MELD) score at admission, albumin-bilirubin (ALBI) score at admission, and Comprehensive Complication Index (CCI) score. The following laboratory data were collected at admission and during the perioperative period: White blood cell (WBC) count, haemoglobin (Hb) level, platelet count, prothrombin time (PT), international normalised ratio (INR), total bilirubin (TBIL) level, direct bilirubin (DBIL) level, alanine transaminase (ALT) level, aspartate transaminase (AST) level, albumin (ALB) level, globulin (GLB) level, serum creatinine (Scr) level, cystatin C (Cys C) level, duration of the preoperative hospital stay, duration of the postoperative hospital stay, total hospital stay, operative time, intraoperative blood loss, time to first flatus, and type of surgery. Information about the therapies used to correct a specific complication was also collected to calculate the CCI.

Calculation of CCI and ALBI, CP and MELD scores

The ALBI, CP, and MELD scores were calculated using the relevant formulas[10-12]. The ALBI score was calculated as follows: ALBI score = 0.66 × $\log_{10}$ [total bilirubin (μmol/L)] - 0.085 × [albumin (g/L)][10]. The CP score included five parameters: presence or absence of encephalopathy and ascites, serum total bilirubin level, albumin level and prothrombin time[13]. The MELD score was calculated using the following formula: 11.2 × ln (international normalised ratio) + 9.57 × ln (creatinine, mg/dL) + 3.78 × ln (bilirubin, mg/dL) + 6.43 × (aetiology: 0 if cholestatic or alcoholic, 1 otherwise)[14]. Complications that occurred within 30 d after the operation were considered surgical complications and the severity of all complications was graded using the Centers for Disease Control (CDC) criteria[15]. The CCI was calculated as the severity-weighted sum of all postoperative complications (available at http://www.assessurgery.com). The CCI ranges from 0 (no complications) to 100 points (death)[16-18].

Follow-up and survival endpoints

All included patients underwent routine follow-up examinations. Follow-up methods were mainly telephone calls or inpatient or outpatient re-examinations, and the last follow-up occurred on January 31, 2018. One of our primary endpoints of interest was overall survival (OS), which was defined as the time from surgery to death from any cause. In the analysis of OS, patients who were alive at the last follow-up date were counted as censored observations. The other primary endpoint of interest was disease-specific survival (DSS), which was defined as the time from surgery to death attributed to PH. In the DSS analysis, patients who died of other causes or were alive at the last follow-up date were counted as censored observations. Another primary endpoint was bleeding-free survival (BFS), which was defined as the time from surgery to the first appearance of initial oesophagogastric variceal bleeding. In the BFS analysis, patients who died of other causes or were alive at the last follow-up date were counted as censored observations. Univariate and multivariate Cox regression models were used to determine survival-related factors. Factors that were observed to have significant associations with survival in multivariate analyses ($P < 0.05$) were used to build the nomograms for OS and BFS.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 22 software (SPSS Inc., Chicago, IL, United States) and R version 3.2.2 software (Institute for Statistics and Mathematics, Vienna, Austria; http://www.r-project.org/). Continuous variables are presented as means ± SD, and categorical variables are presented as frequencies and percentages. Survival curves were generated using the Kaplan-Meier method, and the significance of difference in survival among selected variables was verified using the log-rank test. A univariate Cox regression analysis with an Enter method was used to estimate the relative risk (RR). A multivariate Cox regression analysis with a Forward Condition method

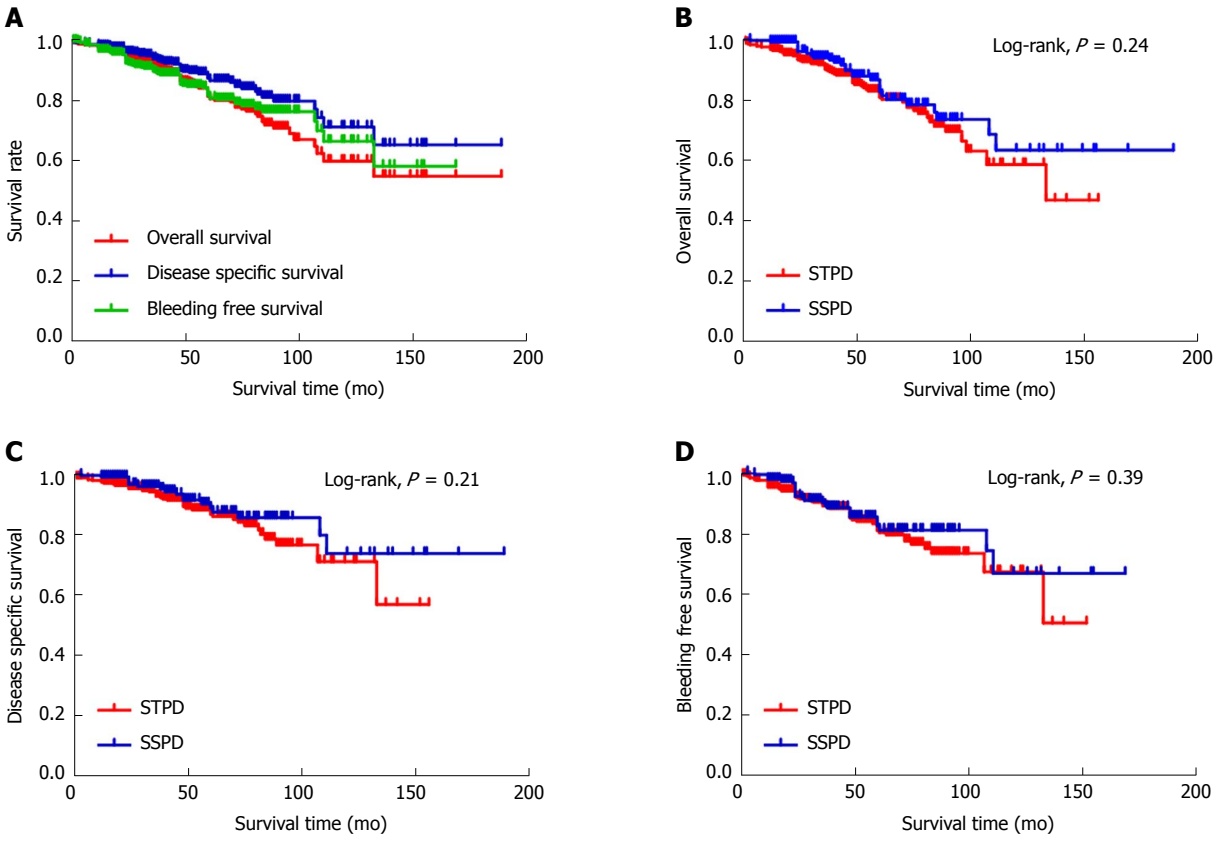


Figure 1 Kaplan-Meier survival curves for overall survival, disease-specific survival and bleeding-free survival. A: Kaplan-Meier survival curves for OS, DSS and BFS; B: Kaplan-Meier survival curves for OS stratified by the type of operation; C: Kaplan-Meier survival curves for DSS stratified by the type of operation. D: Kaplan-Meier survival curves for BFS stratified by the type of operation. OS: Overall survival; DSS: Disease-specific survival; BFS: Bleeding-free survival.

was used to estimate the RR and identify independent prognostic factors. The "rms" R library (cran.r-project.org/web/packages/ rms) was used to construct nomogram models. All *P*-values were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Follow-up

Five hundred and fifty-seven (53.30%) patients were successfully followed, with 192 (34.47%) in the SSPD group and 283 (50.81%) males. These included 48 patients who had been followed in 2011 and were alive at that time[5,6]. However, we were unable to reach these 48 patients now because of a change of contact or address, so we used this data as a censored data. The mean age of the patients was 48.79 ± 11.53 years. Ninety-three (16.70%) patients died, of whom 42 (7.54%), 21 (3.77%), 18 (3.23%) and 12 (2.15%) died of bleeding, liver failure, liver cancer, and cardiovascular and circulatory diseases, respectively. Postoperative bleeding was observed in 84 (15.10%) patients, and the bleeding mortality rate was 50.00%.

Kaplan-Meier survival curves for OS, DSS and BFS

Figure 1 shows the Kaplan-Meier survival curves for the OS, DSS and BFS of patients with PH. The 5-year OS rate was 81.3% and the 10-year OS rate was 59.7%. The 5-year DSS rate was 87.3% and 10-year DSS rate was 71.0%. The 5-year BFS rate was 81.6% and 10-year BFS rate was 66.3%. Figure 1B shows the Kaplan-Meier survival curves for OS stratified by the type of operation. For the STPD group, the 5-year OS rate was 80.7% and 10-year OS rate was 58.4%. For the SSPD group, the 5-year OS rate was 82.5% and 10-year OS rate was 63.2%. The 5-year and 10-year OS rates in the SSPD group were higher than those in the STPD group, but the *P*-value of the log-rank analysis was 0.24 ($P > 0.05$), indicating that the difference was not statistically significant. Figure 1C shows the Kaplan-Meier survival curves for DSS stratified by the type of operation. For the STPD group, the 5-year DSS rate was 86.6% and 10-year DSS rate was 71.0%. For the SSPD group, the 5-year DSS rate was 88.7% and 10-year DSS rate was 73.6%. The 5-year and 10-year DSS rates in the SSPD group were higher than those in the STPD group, but the *P*-value of the log-rank analysis was 0.21 ($P > 0.05$), indicating that the difference was not statistically significant. Figure 1D shows the Kaplan-Meier survival curves for BFS stratified by the type of operation. For the STPD group, the 5-year BFS rate was 81.1% and 10-year BFS rate was 67.4%. The 5-year and 10-year BFS rates in the SSPD group were 82.6% and 66.9%, respectively. The *P*-value of the log-

Table 1 Patient demographics, laboratory information and perioperative characteristics (n = 319) n (%)

Parameter	STPD (n = 200)	SSPD (n = 119)	P value
Age (yr)	49.79 ± 11.14	48.92 ± 10.09	0.49
Gender (male)	93 (46.50)	54 (45.38)	0.85
Aetiology: Hepatitis B/hepatitis C/others	131 (65.50)/23 (11.50)/46 (23.00)	88 (73.95)/17 (14.29)/14 (11.76)	0.04
Charlson score: 0/1/2/3/≥ 3	105 (52.50)/54 (27.00)/21 (10.50)/ 20 (10.00)	62 (52.10)/42 (35.29)/12 (10.08)/3 (2.52)	0.06
Blood type: A/B/O/AB	57 (28.50)/62 (31.00)/59 (29.50)/22 (11.00)	36 (30.25)/33 (27.73)/36 (30.25)/14 (11.76)	0.94
History of variceal ligation	27 (13.50)	9 (7.56)	0.11
History of abdominal surgery	35(17.50)	21(17.65)	0.97
Smoking	57 (28.50)	28 (23.53)	0.33
Drinking	38 (19.00)	25 (21.01)	0.66
History of variceal bleeding	105 (52.50)	43 (36.13)	0.01
BMI	21.97 ± 3.04	21.62 ± 2.54	0.29
Child-Pugh score	6.56 ± 1.27	6.92 ± 1.29	0.02
MELD score	5.92 ± 0.40	5.97 ± 0.47	0.32
ALBI score	-2.26 ± 0.50	-2.10 ± 0.54	0.01
CCI score	18.64 ± 11.78	17.53 ± 9.53	0.38
WBC count (10^9/L)	2.79 ± 1.78	2.40 ± 1.38	0.04
Hb (g/L)	93.35 ± 24.77	94.74 ± 25.82	0.63
Platelet count (10^9/L)	49.32 ± 28.63	43.28 ± 21.20	0.05
PT (s)	13.85 ± 1.96	14.29 ± 1.82	0.04
INR	1.19 ± 0.18	1.32 ± 1.10	0.10
TBIL (mmol/L)	27.24 ± 16.22	29.37 ± 16.81	0.26
DBIL (mmol/L)	11.87 ± 7.67	12.33 ± 6.87	0.59
ALT (IU/L)	35.79 ± 55.76	38.20 ± 31.58	0.67
AST (IU/L)	43.72 ± 52.72	44.55 ± 33.76	0.88
ALB (g/L)	37.24 ± 5.50	35.61 ± 5.66	0.01
GLB (g/L)	28.36 ± 5.90	29.03 ± 5.91	0.32
Scr (mmol/L)	62.44 ± 18.02	58.71 ± 13.68	0.05
Cys C (mg/L)	1.12 ± 0.32	1.11 ± 0.25	0.71
Duration of preoperative hospital stay (d)	14.56 ± 9.43	16.08 ± 9.29	0.16
Duration of postoperative hospital stay (d)	15.75 ± 6.46	14.97 ± 5.30	0.27
Total hospital stay (d)	30.61 ± 12.20	31.77 ± 11.75	0.40
Operative time, min	139.76 ± 50.73	124.55 ± 42.49	0.00
Intraoperative blood loss (mL)	869.51 ± 692.77	591.34 ± 477.54	0.00
Time to first flatus (d)	4.69 ± 1.70	3.67 ± 1.18	0.00

BMI: Body mass index; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; CCI: Comprehensive Complication Index; WBC: White blood cell; Hb: Haemoglobin; PT: Prothrombin time; INR: International normalised ratio; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase; ALB: Albumin; GLB: Globulin; Scr: Serum creatinine; Cys C: Cystatin C; SSPD: Splenectomy plus simplified pericardial devascularisation.

rank analysis was 0.39 (P > 0.05), indicating that the difference was not statistically significant.

Independent prognostic factors for OS, DSS and BFS

In the follow-up analysis, we selected 319 patients with complete data to perform Cox regression analyses and to explore the prognostic factors for OS. Table 1 shows the demographics, laboratory information and perioperative characteristics of patients with PH.

As shown in Table 2, CCI; age; operative time; intraoperative blood loss; WBC, Hb, ALT, AST, and ALB levels; and Child-Pugh and ALBI scores at admission were remarkably correlated with OS in univariate Cox regression analyses (P < 0.05). CCI, age, intraoperative blood loss, platelet count, Scr level, Cys C level and MELD score were remarkably correlated with DSS in univariate Cox regression analyses (P < 0.05). Gender, age, history of variceal ligation, history of variceal bleeding, operative time, intraoperative blood loss, time to first flatus, Hb level, platelet count and GLB level at admission were remarkably correlated with BFS in univariate Cox regression analyses (P < 0.05).

A multivariate Cox regression analysis was used to further explore the influences of all variables that were significant in the univariate analysis. The multivariate analyses of OS showed that age, operative time, ALT levels and ALBI score were independent positive risk factors (P < 0.05); CCI and age were independent prognostic factors influencing DSS (P < 0.05), and male sex, age, intraoperative blood loss and time to first flatus were independent positive risk factors (P < 0.05) (Table 3).

Nomograms for predicting OS and BFS

We recruited all independent prognostic factors identified in Cox regression analysis of OS and BFS to construct the nomograms. The prognostic nomograms for 1-, 3- and 5-year OS rates are shown in Figure 2A, and nomograms for 1-, 3- and 5-year BFS rates are shown in Figure 2B. Each variable is projected upward to the value of the small ruler (Points) to get the score of each parameter. The higher the score, the worse the prognosis of survival. The sum of all small rulers is the total score (Total Points). The 1-, 3- and 5-year OS and

Table 2 Univariate Cox regression analysis of overall survival, bleeding-free survival and disease-specific survival

Parameter	OS				BFS				DSS			
	B	*P* value	*RR*	95%CI	*B*	*P* value	*RR*	95%CI	*B*	*P* value	*RR*	95%CI
Type of operation	0.16	0.62	1.18	0.62-2.23	-0.39	0.33	0.68	0.31-1.48	-0.45	0.17	0.64	0.34-1.21
CCI	0.04	0.00	1.04	1.01-1.07	0.02	0.36	1.02	0.98-1.05	0.06	0.00	1.06	1.02-1.09
Gender	0.01	0.99	1.01	0.54-1.89	0.94	0.02	2.56	1.20-5.43	0.06	0.91	1.06	0.43-2.60
Age	0.06	0.00	1.06	1.03-1.09	0.04	0.01	1.05	1.01-1.08	0.05	0.00	1.05	1.02-1.09
Aetiology	0.07	0.74	1.07	0.73-1.56	0.16	0.44	1.18	0.78-1.78	-0.16	0.43	0.86	0.58-1.26
History of variceal ligation	0.75	0.10	2.12	0.87-5.19	1.07	0.01	2.92	1.24-6.88	0.54	0.21	1.72	0.73-4.02
History of abdominal surgery	0.33	0.42	1.39	0.63-3.03	0.16	0.73	1.17	0.48-2.87	-1.28	0.20	0.28	0.04-1.96
Smoking	0.45	0.19	1.56	0.80-3.04	0.54	0.15	1.72	0.82-3.60	0.27	0.55	1.31	0.55-3.11
Drinking	0.17	0.65	1.19	0.56-2.51	0.71	0.07	2.04	0.96-4.33	-0.12	0.81	0.89	0.34-2.35
Charlson Score	0.11	0.53	1.12	0.80-1.56	0.02	0.93	1.02	0.69-1.51	0.06	0.77	1.06	0.72-1.58
History of variceal bleeding	0.07	0.82	1.08	0.57-2.03	1.02	0.01	2.77	1.30-5.90	-0.30	0.43	0.74	0.36-1.55
Blood type	-0.20	0.25	0.82	0.58-1.15	-0.15	0.41	0.86	0.60-1.23	-0.16	0.29	0.85	0.63-1.15
BMI	-0.06	0.34	0.94	0.83-1.07	0.00	0.97	1.00	0.88-1.14	-0.03	0.57	0.97	0.86-1.09
Total hospital stay	0.01	0.36	1.01	0.99-1.04	0.00	0.79	1.00	0.98-1.03	-0.03	0.26	0.97	0.93-1.02
Duration of preoperative hospital stay	0.01	0.43	1.01	0.98-1.05	0.01	0.59	1.01	0.97-1.05	0.04	0.20	1.04	0.98-1.09
Duration of postoperative hospital stay	0.03	0.20	1.03	0.98-1.09	-0.01	0.71	0.99	0.92-1.06	0.00	0.91	1.00	0.94-1.08
Operative time	0.01	0.00	1.01	1.00-1.02	0.01	0.04	1.01	1.00-1.02	0.00	0.33	1.00	1.00-1.01
Intraoperative blood loss	0.00	0.01	1.00	1.00-1.01	0.00	0.00	1.00	1.00-1.01	0.00	0.09	1.00	1.00-1.00
Time to first flatus	-0.01	0.94	0.99	0.81-1.21	0.23	0.03	1.26	1.03-1.53	-0.06	0.57	0.94	0.78-1.15
WBC count	0.20	0.03	1.22	1.02-1.45	0.15	0.13	1.16	0.96-1.41	-0.02	0.88	0.98	0.80-1.21
Hb	-0.01	0.04	0.99	0.97-1.00	-0.02	0.04	0.99	0.97-1	-0.01	0.31	0.99	0.97-1.01
Platelet count	0.01	0.08	1.01	1.00-1.02	0.01	0.01	1.01	1.00-1.02	0.01	0.05	1.01	1.00-1.03
PT	0.07	0.46	1.07	0.90-1.27	-0.08	0.44	0.92	0.74-1.14	-0.26	0.75	0.77	0.16-3.66
INR	-0.03	0.93	0.97	0.51-1.83	-0.93	0.44	0.39	0.04-4.29	-1.80	0.84	0.17	0.00-9821579.13
TBIL	0.01	0.22	1.01	0.99-1.03	-0.01	0.45	0.99	0.97-1.02	0.00	0.97	1.00	0.96-1.04
DBIL	0.02	0.20	1.02	0.99-1.06	-0.03	0.34	0.97	0.92-1.03	0.02	0.72	1.02	0.93-1.12
ALT	0.00	0.00	1.00	1.00-1.01	-0.01	0.35	0.99	0.98-1.01	0.01	0.12	1.02	1.00-1.03
AST	0.00	0.01	1.00	1.00-1.01	-0.02	0.10	0.98	0.96-1.00	-0.01	0.27	0.99	0.97-1.01
ALB	-0.11	0.00	0.90	0.84-0.96	-0.03	0.45	0.98	0.91-1.04	-0.39	0.25	0.68	0.35-1.31
GLB	0.02	0.53	1.02	0.96-1.07	-0.07	0.04	0.93	0.87-1.00	0.03	0.28	1.03	0.97-1.10
Scr	0.01	0.32	1.01	0.99-1.03	0.02	0.07	1.02	1.00-1.03	-0.08	0.01	0.93	0.87-0.98
Cys C	0.71	0.09	2.03	0.91-4.57	0.23	0.67	1.26	0.44-3.66	1.17	0.02	3.21	1.20-8.62
Child-Pugh score	0.29	0.01	1.33	1.08-1.65	0.13	0.36	1.13	0.87-1.48	0.14	0.54	1.15	0.74-1.81
MELD score	0.50	0.18	1.64	0.80-3.40	0.02	0.97	1.02	0.44-2.36	4.18	0.05	65.31	0.94-4536.32
ALBI score	1.07	0.00	2.92	1.49-5.76	0.19	0.59	1.21	0.60-2.46	-3.86	0.33	0.02	0.00-48.67

OS: Overall survival; BFS: Bleeding-free survival; CCI: Comprehensive Complication Index; BMI: Body mass index; WBC: White blood cell; Hb: Haemoglobin; PT: Prothrombin time; INR: International normalised ratio; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase; ALB: Albumin; GLB: Globulin; Scr: Serum creatinine; Cys C: Cystatin C; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; B: Regression coefficient; SE: Standard error; RR: Relative risk; CI: Confidence interval.

BFS rates can be obtained from the downward projection of the Total Points. This nomogram can predict the survival rate individually according to the different conditions of different patients, so as to improve the prediction efficiency and accuracy. The nomograms showed that the OS rates were higher for patients with younger age, patients with shorter operative time, patients with lower alanine transaminase levels and patients with lower albumin-bilirubin scores. The BFS rates were better for females,

Table 3 Multivariate Cox regression analysis of overall survival and bleeding free survival

Parameter	*B*	*SE*	*P* value	*RR*	95%CI
OS					
Age	0.06	0.02	0.00	1.06	1.03-1.09
Operative time	0.01	0.00	0.01	1.01	1.00-1.02
ALT	0.01	0.00	0.00	1.01	1.00-1.01
ALBI score	1.03	0.37	0.01	2.79	1.36-5.72
BFS					
Male	1.17	0.40	0.00	3.22	1.48-7.01
Age	0.05	0.02	0.01	1.05	1.01-1.09
Intraoperative blood loss	0.00	0.00	0.05	1.00	1.00-1.01
Time to first flatus	0.23	0.10	0.02	1.26	1.04-1.53
DSS					
CCI	0.06	0.01	0.00	1.06	1.05-1.08
Age	0.04	0.01	0.00	1.04	1.02-1.06

ALT: Alanine transaminase; ALBI: Albumin-bilirubin; B: Regression coefficient; SE: Standard error; RR: Relative risk; CI: Confidence interval.

younger patients, patients with less intraoperative blood loss and patients with less time to the first flatus. Guided by nomograms, we can better predict the prognosis based on the different characteristics of each patient.

DISCUSSION

We simplified STPD and introduced SSPD in 2002, which includes cutting and ligating of the posterior gastric vessels, suturing left gastric vessels and suturing from the lesser curvature to the lower esophageal vessels. Holding the paraoesophageal vein and cutting off the perforating vein only can effectively block the reflux of esophageal vessels, lower the portal vein pressure and ensure thorough haemostasis. On the other hand, SSPD can reduce gastric mucosal congestion, so as to reduce the incidence of PHG and prevent postoperative re-bleeding. Changing to suture and refraining from incision of seromuscular layer minimises wound injuries and reduces intraoperative blood loss. In addition, the operative time is significantly shortened, which can reduce liver injury to some extent. Higher 5- and 10-year OS and DSS rates were reported for the SSPD group than the STPD group, but the difference was not statistically significant. Moreover, the 5- and 10-year BFS rates for the SSPD group were not statistically significant compared with the STPD group. Based on these results, SSPD achieved or surpassed the long-term survival effect of STPD. However, the procedure of SSPD is simple and prone to master, with less tissue injury and inflammatory reactions. Therefore, SSPD is a good method of treating PH patients, and it can and should be promoted and applied to hospitals at different levels.

Five hundred and fifty-seven (53.30%) patients were successfully followed; 93 (16.70%) patients died, of whom 42 (7.54%) died due to bleeding. Postoperative bleeding was observed in 84 (15.10%) patients, and the bleeding mortality rate was 50.00%. Acute variceal bleeding is the most life-threatening complication of PH, and despite the recent progress in management, this complication still occurs in approximately 20% of patients at 6 wk[19,20]. Therefore, the risk factors for OS, DSS and BFS must be determined to accurately predict the OS and BFS of patients with PH and to conduct individualised prevention and treatment as early as possible. The multivariate analyses of OS showed that age, operative time, ALT levels and ALBI score were independent positive risk factors. Not surprisingly, age and operative time were independent positive risk factors for OS, as older age and longer operative time are always accompanied by underlying disease and more severe conditions, respectively. ALT is a sensitive marker of acute hepatocyte damage[21], which may dramatically influence the OS. The ALBI score was recently established as an evidence-based model to assess the liver function of patients with hepatocellular carcinoma[10] and has already been validated and proven to be more objective and precise than CP and MELD scores in predicting postoperative efficacy and survival[22-28]. In clinical practice, we should pay more attention to older patients and patients with longer operative time and higher ALT levels and ALBI scores at admission to improve the preventative treatments for these patients during the perioperative period. According to the multivariate analyses of DSS, CCI and age were independent positive risk factors. The CCI is a novel method that mathematically integrates all complications graded by the conventional CDC criteria into one number, regardless of the number and severity of the complications, to capture the overall burden of an operation. Additionally, CCI is a continuous variable ranging from 0 (no complications) to 100 (death) points that easily quantifies complications and can be included in multifactor analyses. Thus, the CCI is the most attractive method for evaluating postoperative complications[29]. The CCI has been applied in abdominal surgery and in the context of randomised controlled trials for patients undergoing oesophagectomy and has achieved better results[30-32]. The multivariate analyses of

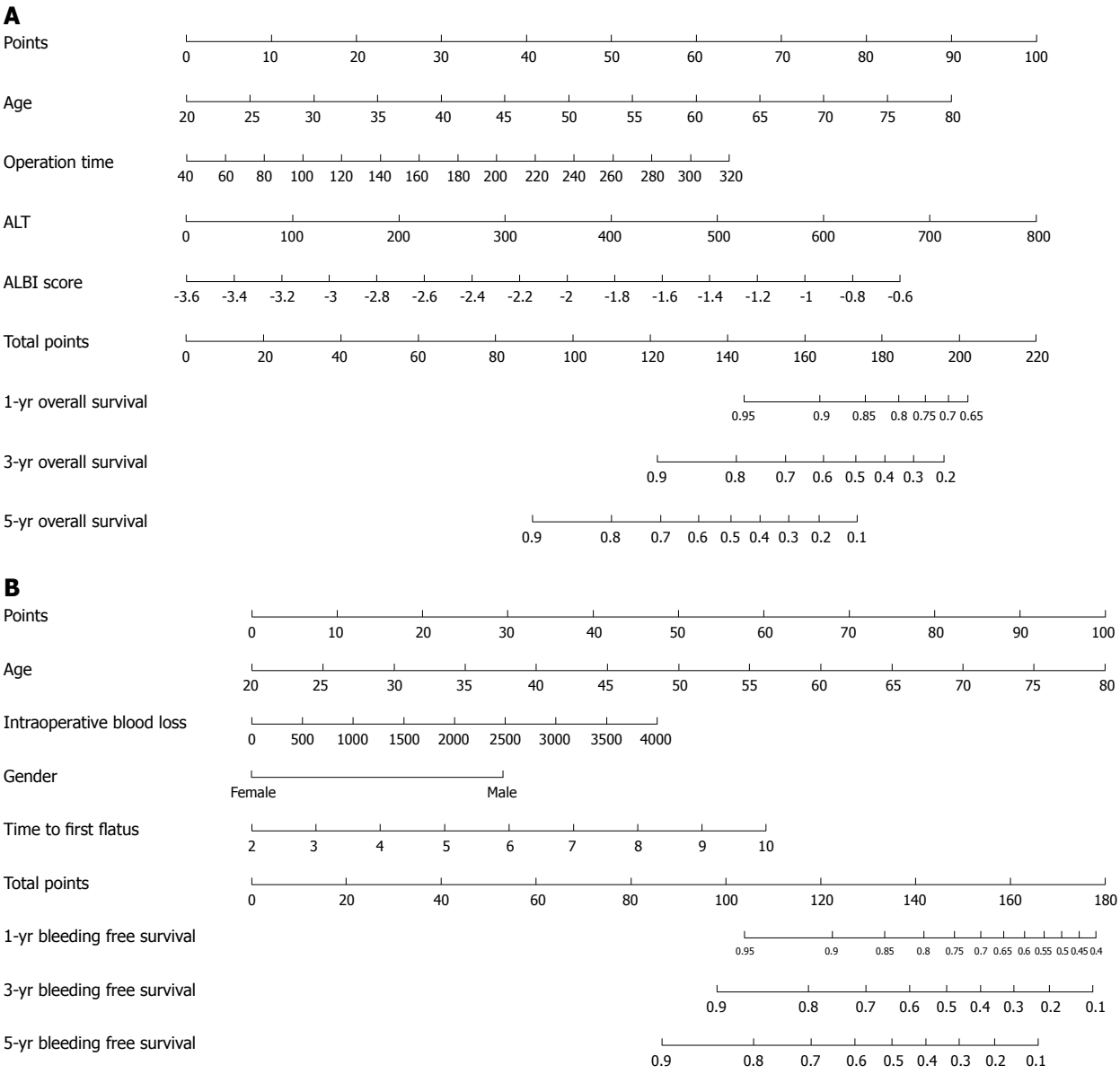


Figure 2 Nomograms for predicting 1-, 3-, and 5-year overall survival (A) and bleeding-free survival (B). ALT: Alanine transaminase; ALBI: Albumin-bilirubin score.

BFS showed that male sex, age, intraoperative blood loss and time to first flatus were independent positive risk factors. Intraoperative blood loss and time to first flatus were common factors in the perioperative evaluation index. However, male sex was an independent positive risk factor that has not been reported in previous studies. This discrepancy may be more related to the smoking and drinking history of male patients. In clinical practice, we should pay more attention to older male patients and patients with larger amounts of intraoperative bleeding and longer postoperative exhaust time. We should strengthen the measures to protect these patients during the perioperative period, pay attention to the postoperative re-examination, and perform ligation in a timely manner to prevent bleeding. In addition, a precise survival and re-bleeding prognostic tool is urgently needed to guide therapy selection for high-risk patients.

Integrating independent prognostic parameters, a nomogram can provide individualised evaluation of the clinical event incidence, such as survival rate[7-9]. Compared with traditional methods, nomograms make predictions more quickly, conveniently and accurately. Its predictive value is better than other evaluation systems and is very important in clinical decision-making processes[33,34]. However, the application of nomogram in PH patients has rarely been reported. In the present report, the prognostic nomograms included all significant independent factors in the Cox regression analyses of OS and BFS. According to the nomograms, the OS rates were better for younger patients, patients with shorter operative time, patients with lower ALT levels and patients with lower ALBI scores. In addition, the BFS rates were better for females, younger patients, patients with less intraoperative blood loss and patients

with less time to first flatus. Guided by nomograms, we can better predict the prognosis based on the different characteristics of each patient. To the best of our knowledge, our study is the first to construct nomograms to predict OS and BFS rates in patients with PH.

There are several limitations in the present study. First, potential bias may exist for the retrospective nature of our study[35]. However, a randomised clinical trial may be not realizable for the reason of ethics. Second, for the research included only Chinese patients, our results may not be directly applicable to other races. In some cases, it may need to be verified. Third, this study was just conducted in a single hospital. Our results may not be fully applicable to other hospitals for the difference in treatment modalities and medical conditions. In addition, the nomograms constructed in our study lacked external validation due to the limited number of cases, which may reduce the credibility of the nomograms. Despite the aforementioned limitations, the present study has identified the prognostic factors for patients with PH after SPD and is the first to construct a nomogram to forecast the postoperative survival and re-bleeding rates of PH patients.

In summary, SSPD achieves or surpasses the long-term survival effect of STPD and is worthy of clinical promotion and application, particularly in primary hospitals. In clinical practice, we should pay more attention to males, older patients, and patients with longer operative time, patients with higher CCI scores, ALT levels and ALBI scores at admission, and patients with larger amounts of intraoperative bleeding and longer postoperative exhaust time. Nomograms are effective in predicting prognosis according to individual patient characteristics. Further large-scale prospective studies are needed to confirm our findings.

ARTICLE HIGHLIGHTS

Research background

Patients with portal hypertension (PH) still have higher re-bleeding rates and mortality after splenectomy plus pericardial devascularisation. We simplified splenectomy plus traditional pericardial devascularisation (STPD) and put forward splenectomy plus simplified pericardial devascularisation (SSPD), whose initial curative effects have been verified, but its long-term survival effects are not clear. Therefore, we need to identify the best postoperative treatment to improve the prognosis of these patients, and a determination of the underlying influencing factors is useful for estimating outcomes and determining the appropriate treatments.

Research motivation

SSPD achieves or surpasses the long-term survival outcome of STPD and is worthy of clinical promotion and application. In clinical practice, males and older patients, patients with longer operative time, patients with higher Comprehensive Complication Index (CCI), alanine transaminase (ALT) and albumin-bilirubin (ALBI) scores at admission, patients with larger amounts of intraoperative bleeding and patients with longer postoperative exhaust time should receive more attention.

Research objectives

The main aim of the retrospective research was to assess the postoperative survival rates of PH patients and identify the clinical efficacy of SSPD. Factors influencing survival and nomograms were also identified.

Research methods

Five hundred fifty-seven (53.30%) patients were successfully followed. We performed a Kaplan-Meier analysis to construct survival curves. We also applied log-rank test to verify the significance of difference in survival rates. The risk factors were estimated using a univariate Cox regression analysis. A multivariate Cox regression analysis was used to estimate the relative risk and to identify independent prognostic factors. The "rms" R library was used to construct nomograms.

Research results

Five hundred and fifty-seven (53.30%) patients were successfully followed; 93 (16.70%) patients died, of whom 42 (7.54%) patients died due to bleeding. Postoperative bleeding was observed in 84 (15.10%) patients. There was no significant difference between SSPD and STPD in 5- and 10-year overall survival (OS), disease-specific survival (DSS) and bleeding-free survival (BFS) rates. Age, operative time, ALT level and the ALBI score were independent prognostic factors for OS. Male sex, age, intraoperative blood loss and time to the first flatus were independent prognostic factors for BFS. CCI and age were independent prognostic factors for DSS. Nomograms were established and were better at predicting 1-, 3-, and 5-year OS and BFS rates.

Research conclusions

SSPD achieves or surpasses the long-term survival outcomes of STPD, which is worthy of clinical promotion and application. In clinical practice, males, older patients, patients with longer operative time, patients with higher CCI scores, ALT levels and ALBI scores at admission, and patients with larger amounts of intraoperative bleeding and longer postoperative exhaust time should receive more attention. Nomograms are better in predicting prognosis according to individual patient characteristics.

Research perspectives

In the future, the long-term survival of patients with PH undergoing SSPD should be assessed in large-scale prospective studies.

REFERENCES

1 **Bosch J**, Abraldes JG, Berzigotti A, Garcia-Pagan JC. Portal hypertension and gastrointestinal bleeding. *Semin Liver Dis* 2008; **28**: 3-25 [PMID: 18293274 DOI: 10.1055/s-2008-1040318]

2 **GBD 2013 Mortality and Causes of Death Collaborators.** Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015; **385**: 117-171 [PMID: 25530442 DOI: 10.1016/S0140-6736(14)61682-2]

3 **Yao HS**, Wang WJ, Wang Q, Gao WC, Xiang HG, Hu ZQ, Gao JD, Chen XY, Wang WM. Randomized clinical trial of vessel sealing system (LigaSure) in esophagogastric devascularization and splenectomy in patients with portal hypertension. *Am J Surg* 2011; **202**: 82-90 [PMID: 21741519 DOI: 10.1016/j.amjsurg.2010.10.016]

4 **Zheng X**, Liu Q, Yao Y. Laparoscopic splenectomy and esophagogastric devascularization is a safe, effective, minimally invasive alternative for the treatment of portal hypertension with refractory variceal bleeding. *Surg Innov* 2013; **20**: 32-39 [PMID: 22461108 DOI: 10.1177/1553350612441863]

5 **Zhang YF**, Ji H, Lu HW, Lu L, Wang L, Wang JL, Li YM. Comparison of simplified and traditional pericardial devascularisation combined with splenectomy for the treatment of portal hypertension. *World J Clin Cases* 2018; **6**: 99-109 [PMID: 29988874 DOI: 10.12998/wjcc.v6.i6.99]

6 **Lu H**, Liu S, Zhang Y, Shang H, Ji H, Li Y. Therapeutic effects and complications of simplified pericardial devascularization for patients with portal hypertension. *Int J Clin Exp Med* 2015; **8**: 14036-14041 [PMID: 26550364]

7 **Balachandran VP**, Gonen M, Smith JJ, DeMatteo RP. Nomograms in oncology: more than meets the eye. *Lancet Oncol* 2015; **16**: e173-e180 [PMID: 25846097 DOI: 10.1016/S1470-2045(14)71116-7]

8 **Liang W**, Zhang L, Jiang G, Wang Q, Liu L, Liu D, Wang Z, Zhu Z, Deng Q, Xiong X, Shao W, Shi X, He J. Development and validation of a nomogram for predicting survival in patients with resected non-small-cell lung cancer. *J Clin Oncol* 2015; **33**: 861-869 [PMID: 25624438 DOI: 10.1200/JCO.2014.56.6661]

9 **Tang LQ**, Li CF, Li J, Chen WH, Chen QY, Yuan LX, Lai XP, He Y, Xu YX, Hu DP, Wen SH, Peng YT, Zhang L, Guo SS, Liu LT, Guo L, Wu YS, Luo DH, Huang PY, Mo HY, Xiang YQ, Sun R, Chen MY, Hua YJ, Lv X, Wang L, Zhao C, Cao KJ, Qian CN, Guo X, Zeng YX, Mai HQ, Zeng MS. Establishment and Validation of Prognostic Nomograms for Endemic Nasopharyngeal Carcinoma. *J Natl Cancer Inst* 2015; **108**: [PMID: 26467665 DOI: 10.1093/jnci/djv291]

10 **Johnson PJ**, Berhane S, Kagebayashi C, Satomura S, Teng M, Reeves HL, O'Beirne J, Fox R, Skowronska A, Palmer D, Yeo W, Mo F, Lai P, Iñarrairaegui M, Chan SL, Sangro B, Miksad R, Tada T, Kumada T, Toyoda H. Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach-the ALBI grade. *J Clin Oncol* 2015; **33**: 550-558 [PMID: 25512453 DOI: 10.1200/JCO.2014.57.9151]

11 **Child CG**, Turcotte JG. Surgery and portal hypertension. *Major Probl Clin Surg* 1964; **1**: 1-85 [PMID: 4950264]

12 **Kamath PS**, Kim WR; Advanced Liver Disease Study Group. The model for end-stage liver disease (MELD). *Hepatology* 2007; **45**: 797-805 [PMID: 17326206 DOI: 10.1002/hep.21563]

13 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649 [PMID: 4541913]

14 **Kamath PS**, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464-470 [PMID: 11172350 DOI: 10.1053/jhep.2001.22172]

15 **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]

16 **Slankamenac K**, Nederlof N, Pessaux P, de Jonge J, Wijnhoven BP, Breitenstein S, Oberkofler CE, Graf R, Puhan MA, Clavien PA. The comprehensive complication index: a novel and more sensitive endpoint for assessing outcome and reducing sample size in randomized controlled trials. *Ann Surg* 2014; **260**: 757-762; discussion 762-763 [PMID: 25379846 DOI: 10.1097/SLA.0000000000000948]

17 **Yamashita S**, Sheth RA, Niekamp AS, Aloia TA, Chun YS, Lee JE, Vauthey JN, Conrad C. Comprehensive Complication Index Predicts Cancer-specific Survival After Resection of Colorectal Metastases Independent of RAS Mutational Status. *Ann Surg* 2017; **266**: 1045-1054 [PMID: 27735824 DOI: 10.1097/SLA.0000000000002018]

18 **Tahiri M**, Sikder T, Maimon G, Teasdale D, Hamadani F, Sourial N, Feldman LS, Guralnick J, Fraser SA, Demyttenaere S, Bergman S. The impact of postoperative complications on the recovery of elderly surgical patients. *Surg Endosc* 2016; **30**: 1762-1770 [PMID: 26194260 DOI: 10.1007/s00464-015-4440-2]

19 **Sarin SK**, Kumar A, Angus PW, Baijal SS, Baik SK, Bayraktar Y, Chawla YK, Choudhuri G, Chung JW, de Franchis R, de Silva HJ, Garg H, Garg PK, Helmy A, Hou MC, Jafri W, Jia JD, Lau GK, Li CZ, Lui HF, Maruyama H, Pandey CM, Puri AS, Rerknimitr R, Sahni P, Saraya A, Sharma BC, Sharma P, Shiha G, Sollano JD, Wu J, Xu RY, Yachha SK, Zhang C; Asian Pacific Association for the Study of the Liver (APASL) Working Party on Portal Hypertension. Diagnosis and management of acute variceal bleeding: Asian Pacific Association for Study of the Liver recommendations. *Hepatol Int* 2011; **5**: 607-624 [PMID: 21484145 DOI: 10.1007/s12072-010-9236-9]

20 **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey W; Practice Guidelines Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Hepatology* 2007; **46**: 922-938 [PMID: 17879356 DOI: 10.1002/hep.21907]

21 **Mangus RS**, Fridell JA, Kubal CA, Davis JP, Tector AJ. Elevated alanine aminotransferase (ALT) in the deceased donor: impact on early post-transplant liver allograft function. *Liver Int*2015; **35**: 524-531 [PMID: 25756162]

22 **Liu PH**, Hsu CY, Hsia CY, Lee YH, Chiou YY, Huang YH, Lee FY, Lin HC, Hou MC, Huo TI. ALBI and PALBI grade predict survival for HCC across treatment modalities and BCLC stages in the MELD Era. *J Gastroenterol Hepatol* 2017; **32**: 879-886 [PMID: 27696519 DOI: 10.1111/jgh.13608]

23 **Hiraoka A**, Kumada T, Michitaka K, Toyoda H, Tada T, Ueki H, Kaneto M, Aibiki T, Okudaira T, Kawakami T, Kawamura T, Yamago H, Suga Y, Miyamoto Y, Tomida H, Azemoto N, Mori K, Miyata H, Ninomiya T, Kawasaki H. Usefulness of albumin-bilirubin grade for evaluation of prognosis of 2584 Japanese patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 2016; **31**: 1031-1036 [PMID: 26647219 DOI: 10.1111/jgh.13250]

24 **Wang YY**, Zhong JH, Su ZY, Huang JF, Lu SD, Xiang BD, Ma L, Qi LN, Ou BN, Li LQ. Albumin-bilirubin versus Child-Pugh score as a predictor of outcome after liver resection for hepatocellular carcinoma. *Br J Surg* 2016; **103**: 725-734 [PMID: 27005482 DOI: 10.1002/bjs.10095]

25 **Guan CT**, Zhao H, Li XQ, Qu CX, Cai JQ, Wei WW, Qiao YL. [Basic characteristics and survival analysis of patients with hepatocellular carcinoma]. *Zhonghua Zhong Liu Za Zhi* 2017; **39**: 231-235 [PMID: 28316226 DOI: 10.3760/cma.j.issn.0253-3766.2017.03.015]

26 **Toyoda H**, Lai PB, O'Beirne J, Chong CC, Berhane S, Reeves H, Manas D, Fox RP, Yeo W, Mo F, Chan AW, Tada T, Iñarrairaegui M, Vogel A, Schweitzer N, Chan SL, Sangro B, Kumada T, Johnson PJ. Long-term impact of liver function on curative therapy for hepatocellular carcinoma: application of the ALBI grade. *Br J Cancer* 2016; **114**: 744-750 [PMID: 27022825 DOI: 10.1038/bjc.2016.33]

27 **Chan AW**, Chan RC, Wong GL, Wong VW, Choi PC, Chan HL, To KF. New simple prognostic score for primary biliary cirrhosis: Albumin-bilirubin score. *J Gastroenterol Hepatol* 2015; **30**: 1391-1396 [PMID: 25753927 DOI: 10.1111/jgh.12938]

28 **Li MX**, Zhao H, Bi XY, Li ZY, Huang Z, Han Y, Zhou JG, Zhao JJ, Zhang YF, Cai JQ. Prognostic value of the albumin-bilirubin grade in patients with hepatocellular carcinoma: Validation in a Chinese cohort. *Hepatol Res* 2017; **47**: 731-741 [PMID: 27558521 DOI: 10.1111/hepr.12796]

29 **Kodali P**, Wu P, Lahiji PA, Brown EJ, Maher JJ. ANIT toxicity toward mouse hepatocytes in vivo is mediated primarily by neutrophils via CD18. *Am J Physiol Gastrointest Liver Physiol*2006; **291**: G355-G363 [PMID: 16614373 DOI: 10.1152/ajpgi.00458.2005]

30 **Kim TH**, Suh YS, Huh YJ, Son YG, Park JH, Yang JY, Kong SH, Ahn HS, Lee HJ, Slankamenac K, Clavien PA, Yang HK. The comprehensive complication index (CCI) is a more sensitive complication index than the conventional Clavien-Dindo classification in radical gastric cancer surgery. *Gastric Cancer* 2018; **21**: 171-181 [PMID: 28597328 DOI: 10.1007/s10120-017-0728-3]

31 **Kalisvaart M**, de Haan JE, Polak WG, Metselaar HJ, Wijnhoven BPL, IJzermans JNM, de Jonge J. Comparison of Postoperative Outcomes Between Donation After Circulatory Death and Donation After Brain Death Liver Transplantation Using the Comprehensive Complication Index. *Ann Surg* 2017; **266**: 772-778 [PMID: 28742700 DOI: 10.1097/SLA.0000000000002419]

32 **Nederlof N**, Slaman AE, van Hagen P, van der Gaast A, Slankamenac K, Gisbertz SS, van Lanschot JJ, Wijnhoven BP, van Berge Henegouwen MI; CROSS-Study Group. Using the Comprehensive Complication Index to Assess the Impact of Neoadjuvant Chemoradiotherapy on Complication Severity After

Esophagectomy for Cancer. *Ann Surg Oncol* 2016; **23**: 3964-3971 [PMID: 27301849 DOI: 10.1245/s10434-016-5291-3]
33 **Wang Y**, Li J, Xia Y, Gong R, Wang K, Yan Z, Wan X, Liu G, Wu D, Shi L, Lau W, Wu M, Shen F. Prognostic nomogram for intrahepatic cholangiocarcinoma after partial hepatectomy. *J Clin Oncol* 2013; **31**: 1188-1195 [PMID: 23358969 DOI: 10.1200/JCO.2012.41.5984]
34 **Wang ZX**, Qiu MZ, Jiang YM, Zhou ZW, Li GX, Xu RH. Comparison of prognostic nomograms based on different nodal staging systems in patients with resected gastric cancer. *J Cancer*2017; **8**: 950-958 [PMID: 28529606 DOI: 10.7150/jca.17370]
35 **Kammerman LA**, Grosser S. Statistical considerations in the design, analysis and interpretation of clinical studies that use patient-reported outcomes. *Stat Methods Med Res* 2014; **23**: 393-397 [PMID: 25258383 DOI: 10.1177/0962280213498013]

P- Reviewer: Garcia-Compean D, Li LQ **S- Editor**: Ma RY
L- Editor: Wang TQ **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4510-4516

DOI: 10.3748/wjg.v24.i39.4510

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

ORIGINAL ARTICLE

Observational Study

Fungal dysbiosis predicts the diagnosis of pediatric Crohn's disease

Mohammad I El Mouzan, Kirill S Korolev, Mohammad A Al Mofarreh, Rajita Menon, Harland S Winter, Ahmad A Al Sarkhy, Scot E Dowd, Ahmad M Al Barrag, Asaad A Assiri

Mohammad I El Mouzan, Ahmad A Al Sarkhy, Department of Pediatrics, King Saud University, Riyadh 11461, Saudi Arabia

Asaad A Assiri, Department of Pediatrics, Supervisor of Prince Abdullah Bin Khalid Celiac Disease Research Chair, King Saud University, Riyadh 11461, Saudi Arabia

Kirill S Korolev, Rajita Menon, Bioinformatics Program, Boston University, Boston, MA 02215, United States

Mohammad A Al Mofarreh, Al Mofarreh PolyClinic, Riyadh 11423, Saudi Arabia

Harland S Winter, MassGeneral Hospital for Children, Boston, MA 02114, United States

Scot E Dowd, MRDNA, Shallowater, TX 79363, United States

Ahmad M Al Barrag, Department of Microbiology, King Saud University, Riyadh 11461, Saudi Arabia

ORCID number: Mohammad I El Mouzan (0000-0001-8699-3143); Kirill S Korolev (0000-0003-1988-0645); Mohammad A Al Mofarreh (0000-0001-5426-9425); Rajita Menon (0000-0002-4767-0971); Harland S Winter (0000-0003-1122-4811); Ahmad A Al Sarkhy (0000-0002-1424-5784); Scot E Dowd (0000-0002-6296-1427); Ahmad M Al Barrag (0000-0002-8829-9371); Asaad A Assiri (0000-0003-3357-5794).

Author contributions: El Mouzan MI and Winter HS contributed to the conception and design of the study; Al Mofarreh MA, Al Sarkhy AA, Assiri AA and Al Barrag AM contributed to data acquisition and samples' storage; Dowd SE performed DNA extraction and fungal sequencing; Korolev KS and Menon R performed the biostatistics and bioinformatics; El Mouzan MI drafted the manuscript and all co-authors contributed to reviewing, editing and giving approval of the final manuscript.

Institutional review board statement: The manuscript is part of the study of the characteristics of inflammatory bowel disease in Saudi children approved by the IRB of the College of Medicine, King Saud University (No: 10/2647/IRB dated 29/6/2010).

Informed consent statement: Parents and/or children gave consent and/or assent to participate in the study before enrollment.

Conflict-of-interest statement: All authors declare no conflict of interest related to this study.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement - checklist items

Manuscript source: Unsolicited manuscript

Correspondence to: Mohammad I El Mouzan, MD, Professor and Consultant Pediatrician, Department of Pediatrics, Gastroenterology Unit, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia. drmouzan@gmail.com
Telephone: +966-55-5479281
Fax: +966-11-4679364

Received: June 29, 2018
Peer-review started: July 2, 2018
First decision: July 12, 2018
Revised: September 3, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 21, 2018

Abstract

AIM

To investigate the accuracy of fungal dysbiosis in

mucosa and stool for predicting the diagnosis of Crohn's disease (CD).

METHODS

Children were prospectively enrolled in two medical centers: one university hospital and one private gastroenterology clinic in the city of Riyadh, Kingdom of Saudi Arabia. The children with confirmed diagnosis of CD by standard guidelines were considered cases, and the others were considered non-inflammatory bowel disease controls. Mucosal and stool samples were sequenced utilizing Illumina MiSeq chemistry following the manufacturer's protocols, and abundance and diversity of fungal taxa in mucosa and stool were analyzed. Sparse logistic regression was used to predict the diagnosis of CD. The accuracy of the classifier was tested by computing the receiver operating characteristic curves with 5-fold stratified cross-validation under 100 permutations of the training data partition and the mean area under the curve (AUC) was calculated.

RESULTS

All the children were Saudi nationals. There were 15 children with CD and 20 controls. The mean age was 13.9 (range: 6.7-17.8) years for CD children and 13.9 (3.25-18.6) years for controls, and 10/15 (67%) of the CD and 13/20 (65%) of the control subjects were boys. CD locations at diagnosis were ileal (L1) in 4 and colonic (L3) in 11 children, while CD behavior was non-stricturing and non-penetrating (B1) in 12 and stricturing (B2) in 3 children. The mean AUC for the fungal dysbiosis classifier was significantly higher in stools (AUC = 0.85 ± 0.057) than in mucosa (AUC = 0.71 ± 0.067) ($P < 0.001$). Most fungal species were significantly more depleted in stools than mucosal samples, except for *Saccharomyces cerevisiae* and *S. bayanus*, which were significantly more abundant. Diversity was significantly more reduced in stools than in mucosa.

CONCLUSION

We found high AUC of fungal dysbiosis in fecal samples of children with CD, suggesting high accuracy in predicting diagnosis of CD.

Key words: Fungiome; Mycobiome; Crohn's disease; Inflammation; Saudi children

Core tip: We found high accuracy of fungal dysbiosis in predicting diagnosis of Crohn's disease (CD), a finding similar to bacterial dysbiosis. However, the higher area under the curve for the fungal dysbiosis classifier in stool (0.85 ± 0.057) than in mucosa (0.71 ± 0.067) ($P < 0.001$), contrasts with bacterial studies, suggesting higher accuracy of stool samples. Although the clinical application of this finding is limited at present by the high cost of fungal analysis, such information is important from a scientific viewpoint, to increase the understanding of the role of fungal flora in CD and to stimulate further studies.

El Mouzan MI, Korolev KS, Al Mofarreh MA, Menon R, Winter HS, Al Sarkhy AA, Dowd SE, Al Barrag AM, Assiri AA. Fungal dysbiosis predicts the diagnosis of pediatric Crohn's disease. *World J Gastroenterol* 2018; 24(39): 4510-4516 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4510.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4510

INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, are chronic conditions. Their incidence is highest, with an increasing trend, in Western populations[1,2]. However, their incidence is increasing in non-Western populations as well[3,4]. The cause of CD remains unknown despite extensive research, and a multifactorial etiology has been suggested. In genetically-susceptible individuals, environmental triggering factors play a major role and diet and microbiota are the most relevant causative factors for children[5]. Dietary components may act directly or through alteration of gut microbiota to initiate and maintain inflammation in susceptible subjects[6,7]. Significant fungal dysbiosis has been demonstrated in adults and children with CD[8-10]. Recent reports found high accuracy of bacterial dysbiosis in predicting the diagnosis of IBD in general and CD in particular[11-13].

Despite the demonstration of fungal dysbiosis in adults and children with CD, there are no similar reports on the potential role of fungal dysbiosis in the diagnosis of CD. The objective of this report was, therefore, to evaluate the accuracy of fungal dysbiosis in stool and mucosal samples, for the diagnosis of CD in a cohort of non-Western children with new onset disease.

MATERIALS AND METHODS

Ethical considerations

This report is a portion of the main study project titled "Characteristics of inflammatory bowel disease in Saudi children". The study was reviewed and approved by the Institutional Review Board of the College of Medicine, King Saud University. Riyadh, Kingdom of Saudi Arabia (Approval number: 10/2647/IRB).

Study population, sample collection, storage, and processing

Children were prospectively enrolled in two medical centers: One university hospital and one private gastroenterology clinic in the same city of Riyadh, Kingdom of Saudi Arabia. The children were referred to these clinics for investigation of suspected IBD. The

children with confirmed diagnosis of CD by standard guidelines[14] were considered cases and those in whom the diagnosis of CD was excluded were considered non-IBD controls. The most common final diagnoses in non-IBD controls were functional abdominal pain and polyps. Mucosal and fecal samples were collected from 15 children with confirmed CD and 20 controls without inflammation or infection. A total of 78 samples (58 from CD children and 20 from non-IBD controls) were obtained. Stool samples were collected before bowel preparation, and none afterward. Mucosal forceps biopsies were taken from various parts of the colon and ileum. All samples were put into cryovials without preservatives and transported immediately in ice to the laboratory and stored at -80 ℃. At the end of the study, all samples were shipped by express mail in dry ice to MRDNA Laboratories (Shallowater, TX, United States) for microbiome analysis.

Fungal DNA extraction and sequencing

Fungal DNA was extracted using the Mobio PowerSoil kit as per the manufacturer's instructions (Mobio, Carlsbad, CA, United States). Amplicon sequencing service (bTEFAP®) was performed at MRDNA Laboratories and used for the fungal analysis[15]. The internal transcribed spacer primers, ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC), were used. A single-step 30-cycle polymerase chain reaction (PCR with HotStart *Taq* Plus Master Mix kit (Qiagen, Valencia, CA, United States) was employed. Samples were sequenced utilizing Illumina MiSeq chemistry following the manufacturer's protocols.

The Q25 sequence data derived from the sequencing process was processed using the MRDNA ribosomal and functional gene analysis pipeline (www.mrdnalab.com). Sequences were depleted of barcodes and primers, short sequences of < 150 bp were removed, and sequences with ambiguous base calls were removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity), followed by removal of singleton sequences and chimeras. Final operational taxonomic units were taxonomically classified using BLASTn top hit analysis against a curated database derived from RDPII and NCBI (http://rdp.cme.msu.edu and www.ncbi.nlm.nih.gov, respectively) and compiled into each taxonomic level as both "counts" and "percentage" files.

Statistical analysis

All analyses were performed using Python and scikit-learn[16]. Custom functions implementing the permutation test were written to detect the taxa with abundances significantly different between CD and control samples. When more than one sample was available from the same patient for analysis, the log relative abundances from these samples were averaged.

It has been shown that variations in species abundance are better captured by a log-transformed than a linear scale, improving the statistical power[13]. Therefore, we followed this approach. In addition, rare taxa (< 1% abundance or absent from > 50% of the samples) were removed to improve the statistical power. Statistical significance was assessed *via* a permutation test (Fisher's exact test), which yielded raw, uncorrected *P*-values. These were transformed into q-values (corrected *P*-value) that measure the probability of false discovery following the Benjamini Hochberg procedure[17]. We considered associations statistically significant only when the corrected *P*-values were less than 0.05.

A linear logistic regression classifier (linearmodel. LogisticRegression) in scikit-learn, Machine Learning in Python[16], was used to predict CD based on the subject's microbiota. The accuracy of the classifier was tested by computing the receiver operating characteristic (ROC) curve with 5-fold stratified cross-validation under 100 permutations of the training data partition. We partitioned the data into randomly assigned training and test sets 100 times; in each case, the classifier was trained on 4/5 of the data and tested on 1/5 of the data (*i.e.* 5-fold cross-validation).

Alpha diversity, a measure of genera richness (number of genera), was evaluated using the Shannon index. We used Fisher's *t*-test to determine *P*-values for alpha diversity.

The difference in community composition (Beta diversity) was quantified by the Bray-Curtis distance, which accounts for both patterns of presence-absence of taxa and changes in their relative abundances. Nonparametric multidimensional scaling (NMDS) was applied to visualize the distance between mucosa and stool samples taken from CD and control subjects. NMDS quantifies the dissimilarity in community composition between samples *via* a combination of presence-absence and absolute abundance of taxa. For the data shown, the separations were analyzed by the ANOSIM or analysis of (dis)similarity. The ANOSIM statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups.

The statistical analyses in this article were performed and reviewed by the coauthors Kirill S Korolev, PhD and Rajita Menon, PhD from the Bioinformatics Program, Boston University, Boston, MA, United States.

RESULTS

Demographic data

All children were Saudi nationals. There were 15 CD children and 20 controls; mean age was 13.9 (range: 6.7-17.8) years for CD children and 13.9 (3.25-18.6) years for controls, and 10/15 (67%) of the CD and 13/20 (65%) of the control subjects were boys. At diagnosis, CD locations were ileal (L1) in 4 and ileocolonic (L3) in 11 children, while CD behavior was non-stricturing and non-penetrating (B1) in 12 and stricturing (B2) in 3 children. Controls included all patients with no evidence of IBD

Table 1 Fungal species abundance in mucosa and stool of controls

Fungal species	Mucosa abundance, %	Stool abundance, %	Ratio	*P* value
Volvariella dunensis	0.027	0.0013	0.047	0.036
Lepraria humida	0.015	0.0010	0.063	0.042

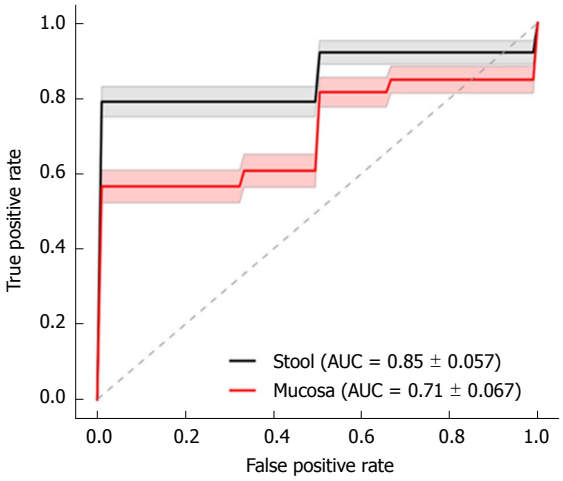


Figure 1 Dysbiosis score classification curve to distinguish between children with Crohn's disease and controls. The receiver operating characteristic (ROC) curve demonstrated the logistic regression dysbiosis classifier. The mean ROC curve for stool (solid black line) and mucosal (solid red line) dysbiosis scores in fungi cohorts is shown. The standard deviation from 100 permutations is shown in gray and light red shading. The area under the curve for stool dysbiosis is significantly higher than in mucosal dysbiosis, using a Fisher's *t*-test computed $P < 0.001$.

or other causes of inflammation. The final diagnoses in control subjects were juvenile polyps, recurrent abdominal pain, recurrent cyclic vomiting or other functional gastrointestinal disorders.

Fungal dysbiosis

In a previous report, description of fungal community structure in mucosa and stool in children with CD relative to controls indicated significantly-abundant CD-associated taxa included Psathyrellaceae ($P = 0.01$), Cortinariaceae ($P = 0.04$), Psathyrella ($P = 0.003$), and Gymnopilus ($P = 0.03$). Monilinia was significantly depleted ($P = 0.03$), whereas other depleted taxa, although not statistically significant, included Leotiomycetes ($P = 0.06$), Helotiales ($P = 0.08$), and Sclerotiniaceae ($P = 0.07$)[10].

Prediction analysis

The mean area under the ROC curve (AUC) for the fungal dysbiosis classifier is illustrated in Figure 1, indicating a significantly-higher AUC in stools (0.85 ± 0.057) than in mucosa (0.71 ± 0.067) ($P < 0.001$).

This analysis was further expanded to demonstrate the difference in abundance and diversity between mucosa and stool in controls and children with CD separately. Table 1 shows a comparison of fungal abundance between mucosa and stools in controls, indicating that only two species, *Volvariella dunensis* ($P = 0.03$) and *Lepraria humida* ($P = 0.04$), were significantly less abundant in stool than in mucosal samples. In contrast, about 50 species were significantly less abundant in stools of children with CD ($P < 0.05$) and only two species, *Saccharomyces cerevisiae* ($P = 0.02$) and *S. bayanus* ($P = 0.001$), were significantly more abundant in stools than in mucosal samples (Table 2). Alpha diversity, as measured by the Shannon Index and illustrated in Figure 2, was different in mucosa and stool. The stool community for children with CD was more than 5 times less diverse than that of mucosa ($P = 0.0001$), whereas in controls the reduction in stool diversity was statistically not significant ($P = 0.35$). Beta diversity, as measured by the Bray-Curtis distance and visualized by the NMDS in Figure 3, shows a significant difference in fungal community separation between mucosal and stool samples ($P = 0.005$).

DISCUSSION

Microbial dysbiosis in the form of depletion of beneficial organisms, expansion of harmful organisms, and reduced microbial diversity may occur independently or concurrently and result in significant effects on immune responses[18]. Fungal dysbiosis demonstrated in Saudi children with CD is in line with previous studies[19,20].

Key findings

This is the first report on the accuracy of fungal dysbiosis in mucosa and stools in predicting the diagnosis of CD in children. The main finding in this study is the high AUC for the fungal dysbiosis classifier, suggesting high accuracy in predicting the diagnosis of CD, which is in line with the high AUC for bacterial dysbiosis[11-13]. However, the higher AUC for the fungal dysbiosis classifier is in stool (0.85 ± 0.057) rather than in mucosa (0.71 ± 0.067) ($P < 0.001$), in contrast with the higher AUC for bacterial dysbiosis in mucosal samples[12-13]. Since this is the first report on fungal dysbiosis accuracy in predicting the diagnosis of CD, further studies are needed to clarify this result.

The finding of significant differences in a large number of species between stool and mucosa in CD children compared to much smaller numbers of species in controls reflects the degree of disturbance of the fungal community in children with CD. In this study, except for *S. cerevisiae* and *S. bayanus*, which were significantly more abundant in stool samples ($P = 0.02$ and $P = 0.003$, respectively), the significantly lower abundance of most fungal species in stool than in mucosal samples indicates

Table 2 Fungal species abundance in mucosa and stool of children with Crohn's disease

Fungal species	Mucosa abundance, %	Stool abundance, %	Ratio	*P* value
Volvariella dunensis	0.06	0.0014	0.025	< 0.001
Malassezia restricta	0.09	0.0044	0.049	< 0.001
Ceriporia lacerate	0.03	0.0022	0.065	< 0.001
Cl. Cladosporioides	0.11	0.004	0.035	< 0.001
Trametes hirsute	0.048	0.0043	0.089	< 0.001
Psathyrella artemisiae	0.44	0.028	0.065	< 0.001
Amyloporia sp	0.027	0.0012	0.046	< 0.001
Irpex sp	0.095	0.0019	0.02	< 0.001
Bjerkandera adusta	0.022	0.0011	0.049	< 0.001
Lepista sordida	0.041	0.0015	0.037	< 0.001
Cerrena sp	0.022	0.0011	0.05	< 0.001
Coprinellus radians	0.034	0.0011	0.032	< 0.001
Phlebia acanthocystis	0.022	0.0011	0.048	< 0.001
Leptosphaerulina sp	0.037	0.0013	0.036	< 0.001
Coprinus sp	0.085	0.0019	0.022	< 0.001
Malassezia globose	0.028	0.0026	0.095	< 0.001
Alternaria alternate	0.054	0.0037	0.069	< 0.001
Ramalinopsis mannii	0.066	0.0018	0.027	< 0.001
Saccharomyces bayanus	2.5	53	21	< 0.001
Trichoderma hypocrea	0.022	0.0014	0.067	< 0.001
Aspergillus penicillioides	0.11	0.0066	0.062	< 0.001
Psathyrella candolleana	0.063	0.0059	0.093	< 0.001
Cladosporium sp	0.072	0.0053	0.074	< 0.001
Aspergillus sp	0.064	0.0049	0.076	< 0.001
Galactomyces geotrichum	0.079	0.0063	0.08	< 0.001
Peniophora incarnate	0.011	0.0027	0.25	< 0.001
Eutypella sp	0.038	0.0044	0.12	< 0.001
Ophiocordyceps sinensis	0.059	0.004	0.068	< 0.001
Nakaseomyces candida	0.12	0.0058	0.049	0.019
Hypocrea ceramic	0.02	0.0037	0.18	0.019
Saccharomyces cerevisiae	0.043	0.23	5.3	0.024

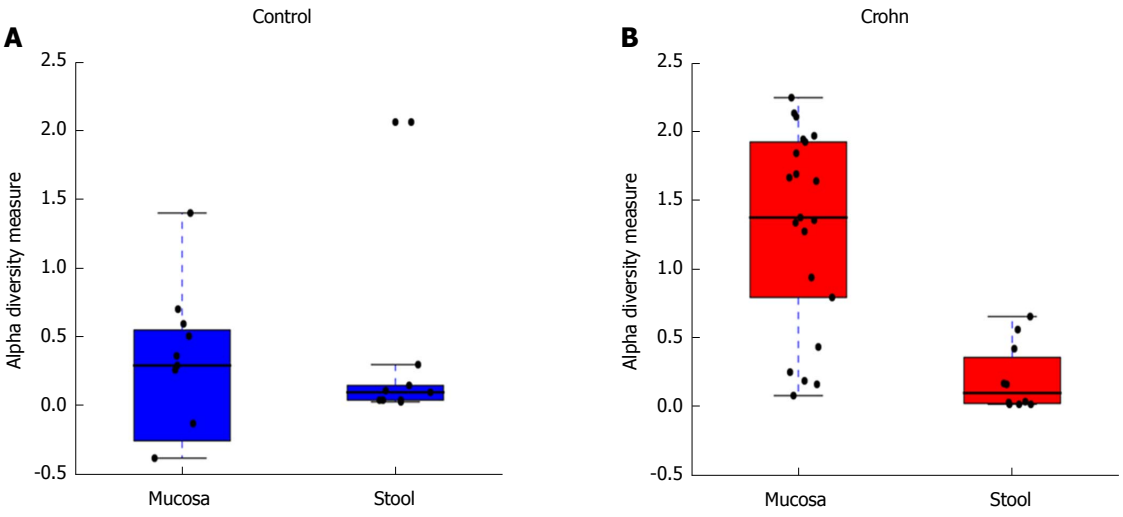


Figure 2 Alpha diversity evaluated by the Shannon Index. Comparison between mucosa and stool in children with Crohn's disease (CD) and controls. Alpha diversity is significantly lower in stool than in mucosa in CD samples ($P = 0.0001$), while the difference is not significant in control samples ($P = 0.35$).

variation in dysbiosis between stools and mucosa and supports the variable accuracy in predicting the diagnosis of CD.

It has been suggested that the presence of some fungi in the gut may reflect environmental exposure, rather than colonization of the mucosa[21]. However, reports indicate that many fungi, such as *Candida, Cryptococcus, Malassezia, Saccharomyces* and *Trichosporon* spp, have been isolated from the stool of humans[22,23]. Furthermore, the role of fungi in the pathogenesis of IBD has been suggested based on animal models of colitis. Pattern recognition receptors in the innate immune system cells include dectin-1, dectin-2, DC-SIGN, mannose receptor, and mannose receptor lecithin[24], and mice lacking dectin-1 had increased susceptibility to experimental colitis[25]. In addition, treatment with antifungal drugs may

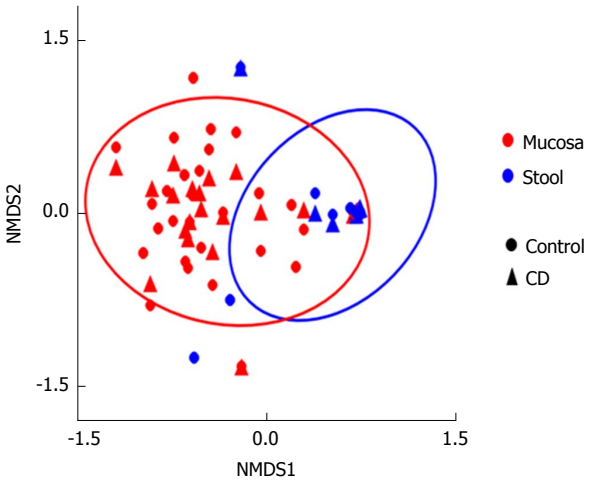


Figure 3 Beta diversity evaluated by Bray-Curtis distance and illustrated by the NMDS plot. This is a measure of beta diversity, and quantifies the similarity in abundance of taxa between samples. The ovals contain 95% of the probabilities for mucosa and stool. This plot shows a clear separation of mucosa and stool samples (P = 0.005). NMDS: Nonparametric multidimensional scaling; CD: Crohn's disease.

reduce the inflammation[26].

Strengths and weaknesses of this study

The strengths of this study comprise the facts that it is the first to investigate the subject and provides inclusion of newly-diagnosed, treatment-naïve children with CD and controls from a well-defined population. Weaknesses include the relatively small number of cases which may be adequate for a first report. The controls were not completely healthy. Obviously, performing endoscopy and biopsies to exclude IBD in healthy children is unethical. Therefore, children who are free of IBD, infection and inflammation have been considered appropriate non-IBD controls.

In conclusion, the most important finding in this study is the high AUC in fecal samples of children with CD, suggesting high accuracy in predicting the diagnosis. Although, the clinical application of this finding is limited at present by the high cost of fungal analysis, such information is important from a scientific viewpoint, to increase the understanding of the role of fungal flora in CD and to stimulate further research, possibly leading to a "dysbiosis test" as a noninvasive screening tool for CD.

ARTICLE HIGHLIGHTS

Research background

Bacterial dysbiosis has been reported to predict the diagnosis of Crohn's disease (CD), but no similar reports for fungal dysbiosis exist. The study is of scientific significance to stimulate further research important for further clarification of the role of fungi in CD.

Research motivation

The role of microbiota in CD, bacterial or fungal, is of worldwide research interest. However, a key problem to be solved is whether dysbiosis is the cause or the result of inflammation. Solving this problem may facilitate discovery of new microbiota-based treatment options. Regarding the accuracy of fungal dysbiosis in predicting the diagnosis, the main problem would be the current high cost of fungal analysis. Solving this problem could lead to development of a dysbiosis screening test for CD.

Research objectives

The objective of this study was to evaluate the accuracy of intestinal fungal dysbiosis as a predictor of CD. High accuracy was found. Future research is needed to confirm this finding and to develop low-cost fungal dysbiosis tests.

Research methods

Mucosal and stool samples were collected from children with CD at presentation and controls. Fungal DNA was extracted from theses samples and sequencing was performed. Fungal abundance and diversities were determined. Fungal dysbiosis in children with CD was demonstrated. This is the first study of the accuracy of fungal dysbiosis in predicting the diagnosis of CD in children.

Research results

The main finding was the high accuracy of fungal dysbiosis in predicting diagnosis of CD. This should stimulate further research to confirm our findings and to develop a low-cost dysbiosis test.

Research conclusions

The high accuracy of fungal dysbiosis in predicting the diagnosis of CD is a new finding. The finding could lead to further research in the role of fungal dysbiosis in CD. A new theory suggests the possibility of design of a noninvasive fungal dysbiosis screening test for CD.

Research perspectives

The role of microbiota in CD may include development of a noninvasive screening test. Further research is needed to confirm the findings and to develop low-cost fungal analysis.

ACKNOWLEDGMENTS

The authors extend their appreciations to the Deanship of Scientific Research at King Saud University in Riyadh, Kingdom of Saudi Arabia for funding this work through Research Group No [RGP-1436-007]. This work was also supported by a grant from the Simons Foundation [No.409704] to Kirill Korolev) and by the startup fund from Boston University to Kirill Korolev. Simulations were carried out on Shared Computing Cluster at Boston University. Rajita Menon was partially supported by a Hariri Graduate Fellowship from Boston University. Harland Winter, MD received support from Martin Schlaff and the Diane and Dorothy Brooks Foundation.

REFERENCES

1 **Kappelman MD**, Moore KR, Allen JK, Cook SF. Recent trends in the prevalence of Crohn's disease and ulcerative colitis in a commercially insured US population. *Dig Dis Sci* 2013; **58**: 519-525 [PMID: 22926499 DOI: 10.1007/s10620-012-2371-5]

2 **Burisch J**, Pedersen N, Čuković-Čavka S, Brinar M, Kaimakliotis I, Duricova D, Shonová O, Vind I, Avnstrøm S, Thorsgaard N, Andersen V, Krabbe S, Dahlerup JF, Salupere R, Nielsen KR, Olsen J, Manninen P, Collin P, Tsianos EV, Katsanos KH, Ladefoged K, Lakatos L, Björnsson E, Ragnarsson G, Bailey Y, Odes S, Schwartz D, Martinato M, Lupinacci G, Milla M, De Padova A, D'Incà R, Beltrami M, Kupcinskas L, Kiudelis G, Turcan S, Tighineanu O, Mihu I, Magro F, Barros LF, Goldis A, Lazar D, Belousova E, Nikulina I, Hernandez V, Martinez-Ares D, Almer S, Zhulina Y, Halfvarson J, Arebi N, Sebastian S, Lakatos PL, Langholz E,

Munkholm P; EpiCom-group. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. *Gut* 2014; **63**: 588-597 [PMID: 23604131]
3 **Benchimol EI**, Fortinsky KJ, Gozdyra P, Van den Heuvel M, Van Limbergen J, Griffiths AM. Epidemiology of pediatric inflammatory bowel disease: a systematic review of international trends. *Inflamm Bowel Dis* 2011; **17**: 423-439 [PMID: 20564651 DOI: 10.1002/ibd.21349]
4 **El Mouzan MI**, Saadah O, Al-Saleem K, Al Edreesi M, Hasosah M, Alanazi A, Al Mofarreh M, Asery A, Al Qourain A, Nouli K, Al Hussaini A, Telmesani A, AlReheili K, Alghamdi S, Alrobiaa N, Alzaben A, Mehmadi A, Al Hebbi H, Al Sarkhy A, Al Mehaidib A, Al Saleem B, Assiri A, Wali S. Incidence of pediatric inflammatory bowel disease in Saudi Arabia: a multicenter national study. *Inflamm Bowel Dis* 2014; **20**: 1085-1090 [PMID: 24788219 DOI: 10.1097/MIB.0000000000000048]
5 **Leone V**, Chang EB, Devkota S. Diet, microbes, and host genetics: the perfect storm in inflammatory bowel diseases. *J Gastroenterol* 2013; **48**: 315-321 [PMID: 23475322 DOI: 10.1007/s00535-013-0777-2]
6 **Chapman-Kiddell CA**, Davies PS, Gillen L, Radford-Smith GL. Role of diet in the development of inflammatory bowel disease. *Inflamm Bowel Dis* 2010; **16**: 137-151 [PMID: 19462428 DOI: 10.1002/ibd.20968]
7 **Wu GD**, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105-108 [PMID: 21885731 DOI: 10.1126/science.1208344]
8 **Sokol H**, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, Cohen D, Liguori G, Bourrier A, Nion-Larmurier I, Cosnes J, Seksik P, Langella P, Skurnik D, Richard ML, Beaugerie L. Fungal microbiota dysbiosis in IBD. *Gut* 2017; **66**: 1039-1048 [PMID: 26843508 DOI: 10.1136/gutjnl-2015-310746]
9 **Liguori G**, Lamas B, Richard ML, Brandi G, da Costa G, Hoffmann TW, Di Simone MP, Calabrese C, Poggioli G, Langella P, Campieri M, Sokol H. Fungal Dysbiosis in Mucosa-associated Microbiota of Crohn's Disease Patients. *J Crohns Colitis* 2016; **10**: 296-305 [PMID: 26574491 DOI: 10.1093/ecco-jcc/jjv209]
10 **El Mouzan M**, Wang F, Al Mofarreh M, Menon R, Al Barrag A, Korolev KS, Al Sarkhy A, Al Asmi M, Hamed Y, Saeed A, Dowd SE, Assiri A, Winter H. Fungal Microbiota Profile in Newly Diagnosed Treatment-naïve Children with Crohn's Disease. *J Crohns Colitis* 2017; **11**: 586-592 [PMID: 27811291 DOI: 10.1093/ecco-jcc/jjw197]
11 **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]
12 **Gevers D**, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, Schwager E, Knights D, Song SJ, Yassour M, Morgan XC, Kostic AD, Luo C, González A, McDonald D, Haberman Y, Walters T, Baker S, Rosh J, Stephens M, Heyman M, Markowitz J, Baldassano R, Griffiths A, Sylvester F, Mack D, Kim S, Crandall W, Hyams J, Huttenhower C, Knight R, Xavier RJ. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* 2014; **15**: 382-392 [PMID: 24629344 DOI: 10.1016/j.chom.2014.02.005]
13 **Wang F**, Kaplan JL, Gold BD, Bhasin MK, Ward NL, Kellermayer R, Kirschner BS, Heyman MB, Dowd SE, Cox SB, Dogan H, Steven B, Ferry GD, Cohen SA, Baldassano RN, Moran CJ, Garnett EA, Drake L, Otu HH, Mirny LA, Libermann TA, Winter HS, Korolev KS. Detecting Microbial Dysbiosis Associated with Pediatric Crohn Disease Despite the High Variability of the Gut Microbiota. *Cell Rep* 2016; **14**: 945-955 [PMID: 26804920 DOI: 10.1016/j.celrep.2015.12.088]
14 **Levine A**, Koletzko S, Turner D, Escher JC, Cucchiara S, de Ridder L, Kolho KL, Veres G, Russell RK, Paerregaard A, Buderus S, Greer ML, Dias JA, Veereman-Wauters G, Lionetti P, Sladek M, Martin de Carpi J, Staiano A, Ruemmele FM, Wilson DC; European Society of Pediatric Gastroenterology, Hepatology, and Nutrition. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014; **58**: 795-806 [PMID: 24231644 DOI: 10.1097/MPG.0000000000000239]
15 **Dowd SE**, Callaway TR, Wolcott RD, Sun Y, McKeehan T, Hagevoort RG, Edrington TS. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol* 2008; **8**: 125 [PMID: 18652685 DOI: 10.1186/1471-2180-8-125]
16 **Pedregosa F**, Varoquaux G, Gramfort A, Michel V, Thirion B. Scikit-learn: Machine Learning in Python. *J Mach Learn Res* 2011; **12**: 2825-2830
17 **Benjamini Y**, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B* 1995; **57**: 289-300
18 **Petersen C**, Round JL. Defining dysbiosis and its influence on host immunity and disease. *Cell Microbiol* 2014; **16**: 1024-1033 [PMID: 24798552 DOI: 10.1111/cmi.12308]
19 **Mukhopadhya I**, Hansen R, Meharg C, Thomson JM, Russell RK, Berry SH, El-Omar EM, Hold GL. The fungal microbiota of de-novo paediatric inflammatory bowel disease. *Microbes Infect* 2015; **17**: 304-310 [PMID: 25522934 DOI: 10.1016/j.micinf.2014.12.001]
20 **Chehoud C**, Albenberg LG, Judge C, Hoffmann C, Grunberg S, Bittinger K, Baldassano RN, Lewis JD, Bushman FD, Wu GD. Fungal Signature in the Gut Microbiota of Pediatric Patients With Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2015; **21**: 1948-1956 [PMID: 26083617 DOI: 10.1097/MIB.0000000000000454]
21 **Suhr MJ**, Hallen-Adams HE. The human gut mycobiome: pitfalls and potentials--a mycologist's perspective. *Mycologia* 2015; **107**: 1057-1073 [PMID: 26354806 DOI: 10.3852/15-147]
22 **Hallen-Adams HE**, Kachman SD, Kim J, Legg RM, Martinez I. Fungi inhabiting the healthy human gastrointestinal tract: A diverse and dynamic community. *Fungal Ecol* 2015; **15**: 9-17 [DOI: 10.1016/j.funeco.2015.01.006]
23 **Nash AK**, Auchtung TA, Wong MC, Smith DP, Gesell JR, Ross MC, Stewart CJ, Metcalf GA, Muzny DM, Gibbs RA, Ajami NJ, Petrosino JF. The gut mycobiome of the Human Microbiome Project healthy cohort. *Microbiome* 2017; **5**: 153 [PMID: 29178920 DOI: 10.1186/s40168-017-0373-4]
24 **Wang ZK**, Yang YS, Stefka AT, Sun G, Peng LH. Review article: fungal microbiota and digestive diseases. *Aliment Pharmacol Ther* 2014; **39**: 751-766 [PMID: 24612332 DOI: 10.1111/apt.12665]
25 **Iliev ID**, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, Brown J, Becker CA, Fleshner PR, Dubinsky M, Rotter JI, Wang HL, McGovern DP, Brown GD, Underhill DM. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* 2012; **336**: 1314-1317 [PMID: 22674328 DOI: 10.1126/science.1221789]
26 **Zwolinska-Wcislo M**, Brzozowski T, Budak A, Kwiecien S, Sliwowski Z, Drozdowicz D, Trojanowska D, Rudnicka-Sosin L, Mach T, Konturek SJ, Pawlik WW. Effect of Candida colonization on human ulcerative colitis and the healing of inflammatory changes of the colon in the experimental model of colitis ulcerosa. *J Physiol Pharmacol* 2009; **60**: 107-118 [PMID: 19439813]

P- Reviewer: Day AS, Gazouli M **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 21; 24(39): 4517-4518

DOI: 10.3748/wjg.v24.i39.4517

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

CORRECTION

Correction to "Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model [*World J Gastroenterol* 2018 March 14; 24(10): 1107-1119]"

Yi-Fan Tong, Xiu-Jun Cai

Yi-Fan Tong, Xiu-Jun Cai, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310000, Zhejiang Province, China

ORCID number: Yi-Fan Tong (0000-0001-9028-5756); Xiu-Jun Cai (0000-0002-3615-4680).

Author contributions: Yi-Fan Tong and Xiu-Jun Cai contributed to this correction.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Manuscript source: Unsolicited manuscript

Correspondence to: Xiu-Jun Cai, FRSC, MD, Professor, Surgeon, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Qingchun East Road No. 3, Hangzhou 310016, Zhejiang Province, China. srrsh_cxj@zju.edu.cn
Telephone: +86-571-86006605
Fax: +86-571-86006605

Received: June 20, 2018
Peer-review started: July 4, 2018
First decision: July 31, 2018
Revised: August 1, 2018
Accepted: August 24, 2018
Article in press: August 24, 2018
Published online: October 21, 2018

Tong YF, Cai XJ. Correction to "Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model [*World J Gastroenterol* 2018 March 14; 24(10): 1107-1119]". *World J Gastroenterol* 2018; 24(39): 4517-4518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i39/4517.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i39.4517

CORRECTION

Correction to: Tong YF, Meng N, Chen MQ, Ying HN, Xu M, Lu B, Hong JJ, Wang YF, Cai XJ. Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model (*World J Gastroenterol* 2017; 24(10): 1107-1119)[1].

Erratum: In the "Conclusion of Abstract", "Core tip", "Discussion" and "Research perspectives", the description regarding the relationship between the volumetric and functional proliferation during ALPPS-derived liver regeneration should be revised. Specifically, the sentence that reads "as the ALPPS-derived proliferation in volume lags behind the functional regeneration" should be revised to "as the ALPPS-derived functional regeneration lags behind the proliferation in volume".

Conclusion of Abstract: The sentence "This could be convincing evidence that the stage Ⅱ of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived proliferation in volume lags

behind the functional regeneration" should be revised to "This could be convincing evidence that stage Ⅱ of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived functional regeneration lags behind the proliferation in volume".

Core tip: The last sentence "as the ALPPS-derived proliferation in volume lags behind the functional regeneration" should be revised to "as the ALPPS-derived functional regeneration lags behind the proliferation in volume".

Discussion: In line 8, the sentence "rapid increase in volume derived from ALPPS lags behind the functional proliferation" should be revised to "that ALPPS-derived functional regeneration lags behind the proliferation in volume". In addition, the last sentence "as the ALPPS-derived proliferation in volume lags behind the functional regeneration" should be revised to "as the ALPPS-derived functional regeneration lags behind the proliferation in volume".

Research perspectives: In line 2, the sentence "as the ALPPS-derived proliferation in volume lags behind the functional regeneration" should be revised to "as the ALPPS-derived functional regeneration lags behind the proliferation in volume".

REFERENCES

1 **Tong YF**, Meng N, Chen MQ, Ying HN, Xu M, Lu B, Hong JJ, Wang YF, Cai XJ. Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model. *World J Gastroenterol* 2018; **24**: 1107-1119 [PMID: 29563755 DOI: 10.3748/wjg.v24.i10.1107]

P- Reviewer: Campos RR, Sandri GB **S- Editor**: Wang XJ
L- Editor: Filipodia **E- Editor**: Yin SY

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327

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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 October 28; 24(40): 4519-4616

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 40 October 28, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Mortada HF El-Shabrawi, MD, Professor, Department of Pediatrics, Cairo University, Cairo 11559, Egypt

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 642 experts in gastroenterology and hepatology from 59 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 (*WJG*) 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 and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

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
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
October 28, 2018

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4519-4526

DOI: 10.3748/wjg.v24.i40.4519

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

EDITORIAL

Extracellular vesicles in liver disease and beyond

Laura Morán, Francisco Javier Cubero

Laura Morán, Francisco Javier Cubero, Department of Immunology, Ophthalmology and ORL, Complutense University School of Medicine, Madrid 28040, Spain

Francisco Javier Cubero, 12 de Octubre Health Research Institute (imas12), Madrid 28041, Spain

ORCID number: Laura Morán (0000-0002-2305-631X); Francisco Javier Cubero (0000-0003-1499-650X).

Author contributions: Morán L and Cubero FJ outlined the editorial, wrote the manuscript and designed the figures.

Supported by the MINECO Retos, No. SAF2016-78711; the EXOHEP-CM, No. S2017/BMD-3727; the AMMF Cholangiocarcinoma Charity, No. 2018/117; the COST Action, No. CA17112; Ramón y Cajal Researcher Grant, No. RYC-2014-15242; and Gilead Liver Research Scholar, 2018.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: Francisco Javier Cubero, BSc, MSc, PhD, Assistant Professor, Department of Immunology, Ophthalmology and ORL, Complutense University School of Medicine, c/Doctor Severo Ochoa, 9, Madrid 28040, Spain. fcubero@ucm.es
Telephone: +34-91-3941385
Fax: +34-91-3941641

Received: July 26, 2018
Peer-review started: July 26, 2018
First decision: August 27, 2018
Revised: September 2, 2018
Accepted: October 5, 2018

Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

Extracellular vesicles (EVs) are membrane-derived vesicles which can be released by different cell types, including hepatocytes, hepatic stellate cells and immune cells in normal and pathological conditions. EVs carry lipids, proteins, coding and non-coding RNAs and mitochondrial DNA causing modifications on the recipient cells. These vesicles are considered potential biomarkers and therapeutic agents for human diagnostic and prognostic due to their function as intercellular mediators of cell-cell communication within the liver and between other organs. However, the development and optimization of methods for EVs isolation is required to characterize their biological functions as well as their potential as a treatment option in the clinic. Nevertheless, many questions remain unanswered related to the function of EVs under physiological and pathological conditions. In the current editorial, the results obtained in different studies that investigated the role of intrahepatic EVs during liver diseases, including drug-induced liver injury, non-alcoholic fatty liver, non-alcoholic steatohepatitis, alcoholic liver disease and hepatocellular carcinoma and extrahepatic EVs in remote organs during pathological events such as pulmonary disease, cardiovascular diseases, neurodegenerative disorders *e.g.*, Alzheimer's disease, Parkinson's disease and multiple sclerosis as well as in immunopathological processes, are discussed. Although much light needs to be shed on the mechanisms of EVs, these membrane-derived vesicles represent both a novel promising diagnostic, and a therapeutic tool for clinical use that we emphasize in the current editorial.

Key words: Extracellular vesicles; MicroRNA; Hepatocytes; Drug-induced liver injury; Alcoholic liver disease; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Hepatocellular carcinoma

Core tip: It has become increasingly clear that extracellular vesicles (EVs) are particularly important intercellular messenger vesicles during pathophysiological processes. EVs can provide more information about the processes that occur in remote organs during the development of diseases contributing to improving our tools for diagnosis, prognosis and therapy.

Morán L, Cubero FJ. Extracellular vesicles in liver disease and beyond. *World J Gastroenterol* 2018; 24(40): 4519-4526 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4519.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4519

INTRODUCTION

The emergence of extracellular vesicles (EVs) as critical mediators of cell-cell communication has gained great interest from the scientific community due to its implication for human diagnostic and therapeutic applications[1,2]. The role of EVs in intercellular transport was reported for the first time in 1980[3]. However, in the past decades, EVs have exponentially attracted the interest of researchers.

There are different mechanisms of formation of these vesicles, creating a complex repertoire of EVs which are secreted and differ in size and origin, such as exosomes, ectosomes, apoptotic bodies, oncosomes and large oncosomes[1]. Exosomes are the smallest EVs (30-100 nm). The process of formation the exosomes is originated during endosome maturation[2]. First, the early endosome is formed by invagination of the plasma membrane and the consequent fusion of endocytic vesicles. The endocytic vesicles can follow two pathways: (1) The endocytic material is recycled and returns to the plasmatic membrane; and (2) exosomes become multivesicular bodies (MVBs) which are a type of late endosomes containing membrane-bound vesicles (intraluminal vesicles)[4].

MVBs are formed by the invagination of the limiting membrane, a process during which a small portion of cytosol is trapped into the vesicle. Finally, there are MVBs which are degraded in the lysosome or release their membrane-bound vesicles known as exosomes to extracellular media by the fusion of MVBs to the plasma membrane (Figure 1).

The process of generation of vesicles is mediated by the endosomal sorting complex responsible for transport and other components, such as ceramide lipids and tetraspanins. Rab GTPases are involved in exosome secretion but the requirements for specific Rabs may differ depending on the cell type[5,6].

Ectosomes (also known as microvesicles) are a population of extracellular vesicles whose size is 50-1000 nm[7]. They are formed by outward budding of the cell plasma membrane[8]. These vesicles are shed by different cell types and express a subset of cell surface proteins that depend on the component of the cells plasma membranes of origin[9].

Apoptotic bodies are presented in a wide range of sizes (50-2000 nm). Programmed cell death or apoptosis triggers the formation and release of apoptotic bodies[10].

Oncosomes and large oncosomes are presented in a range of size between 100-500 nm and they are generated by budding of the plasma membrane. These types of vesicles are only released by cancer cells[11] carrying oncogenic cargo which modulate tumor environment promoting the proliferation, differentiation and metabolism of tumors[12].

Composition of EVs

Independently of their biogenesis, the composition of EVs includes proteins, lipids, and nucleic acids (coding and non-coding RNA and mitochondrial DNA)[13]. Lipidomic analysis shows that the membrane of EVs contains abundant cholesterol, sphingomyelin, ceramide, saturated fatty acids and phosphatidylserine. Furthermore, proteomic analysis shows that EVs share common marker proteins, such as heat shock proteins (Hsp70 and Hsp90), tetraspanins (CD9, CD63, CD81, CD82), endosomal sorting complex required for transport (Alix and Tsg101) and membrane trafficking and merging proteins (GTPases, Flotillin and Annexins) (Figure 2)[14].

Location of EVs

EVs are released to the extracellular media circulating in the adjacent extracellular space and appear in biological fluids, such as blood, saliva, breast milk, bronchial lavage fluid, cerebral spinal fluid, amniotic fluid and urine[15]. However, due to their heterogeneous size, there is a current lack of purification methods. Moreover, these molecules are included in a big group known as EVs since they are also very difficult to isolate and fully discriminate[16].

Circulating EVs can be captured by other cells *via* three ways: Direct membrane fusion, receptor mediated fusion or endocytosis. The recipient cells accept their cargo and, consequently, may suffer modifications in their normal cellular processes[17,18]. EVs-mediated pathological processes can be interrupted by inhibiting EVs release. Emerging studies have recently shown that the inhibition of neutral sphingomyelinase 2 (nSmase2) with GW4869 block exosome release or exosome mediated signalling in different cell types[19].

EVs in liver

The liver has great interest in the scientific research due to this implication in many processes, such as detoxification of blood, filtering all harmful elements and in production, processing and transport of lipids. Furthermore, the liver is a multicellular organ formed by parenchymal cells (hepatocytes) and non-parenchymal cells including Kupffer

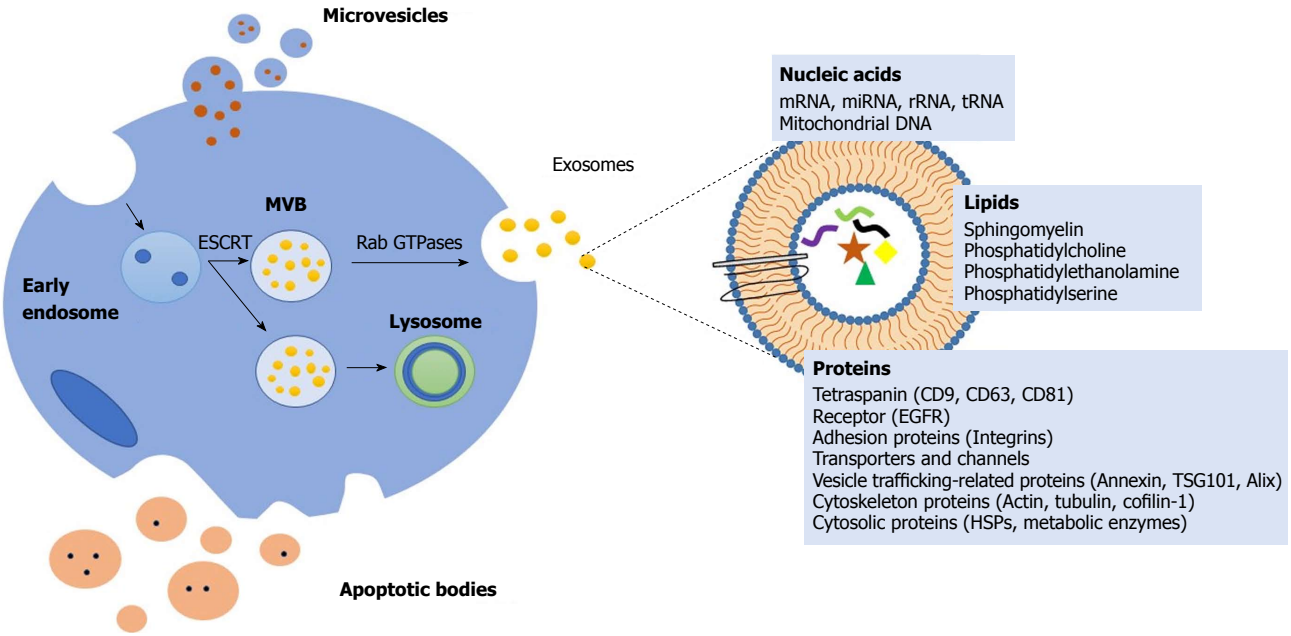


Figure 1 Mechanisms of formation extracellular vesicles and composition. The early endosome is generated by invagination of the plasma membrane. The consequent fusion of endocytic vesicles mediated by the endosomal sorting complex responsible for transport (ESCRTs), formed multivesicular bodies (MVBs). MVBs can be degraded in the lysosome or released the intraluminal vesicles known as exosomes by the fusion of MVBs to the plasma membrane mediated by Rab GTPases. Microvesicles are generated by outward budding from the plasmatic membrane. Apoptotic bodies are generated during programmed cell death or apoptosis. The composition of extracellular vesicles (EVs) includes proteins (tetraspanins, receptors including epidermal growth factor receptor (EGFR), adhesion proteins, transporters and channels, vesicle trafficking-related proteins, cytoskeleton proteins and cytosolic proteins), lipids (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine) and nucleic acids (messenger RNA (mRNA), microRNA (miRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), mitochondrial DNA (mtDNA)). ESCRTs: Endosomal sorting complex responsible for transport; MVBs: Multivesicular bodies; EVs: Extracellular vesicles; EGFR: Epidermal growth factor receptor; mRNA: Messenger RNA; miRNA: MicroRNA; rRNA: Ribosomal RNA; tRNA: Transfer RNA; mtDNA: Mitochondrial DNA.

cells (KCs), sinusoidal endothelial cells (SECs), hepatic stellate cells (HSCs)[20]. The coexistence of different cell types creates a need for intercellular communication network in order to maintain liver homeostasis[21]. Many pathophysiological events are regulated by EVs which can be transferred from donor cells to recipient cells and can activate or regulate cell functions including protein expression, cell proliferation and differentiation and/or antiviral responses. This intercellular communication might be done through EVs, and for this reason, it is necessary to shed light into the physiology and pathology of hepatic EVs[21].

Primary hepatocytes secrete EVs proteins that include exosomal marker proteins (*e.g.*, Tsg101, CD63 and CD81), hepatic-specific proteins, like the asialoglycoprotein receptor, and different proteins associated with metabolic disorder which need further investigation and identification[22].

EVs in drug-induced liver injury

Nowadays, traditional standard biomarkers for liver injury are based on the measurement of hepatic enzymes in plasma or serum including AST, ALT, alkaline phosphatase (AP) and gamma-glutamil-transpeptidase[23]. However, serum or plasma levels of these enzymes do not always reflect the stage of liver disease, therefore causing significant limitations in the diagnosis and staging of different chronic and acute liver disorders. For this reason, miRNAs have emerged as new potential biomarkers of liver injury.

Liver-derived miRNAs may originate from resident parenchymal and non-parenchymal cells and can be significantly altered in certain liver diseases. It can be found as non-vesicle associated miRNA (free circulating miRNA) or associated with vesicles (EVs miRNA) being the last one, the more stable biomarkers[24].

The use of miRNAs as potential biomarker of liver injury was demonstrated in a mouse model of APAP-induced acute liver injury. It was found a significant increase in miR-122 levels in EVs released from hepatocytes[25]. The same results were observed in a rat model of APAP-induced liver injury with increased levels of circulating EVs. These results correlated with a study in primary human hepatocytes (PHH)[26]. These results strongly support that miRNAs might be used as potential biomarkers of liver diseases, being miR-122 associated with EVs proposed as biomarker in drug-induced liver injury (DILI).

EVs in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is characterized by over-accumulation of fat in the liver producing hepatic steatosis triggering an inflammatory reaction which results in the development of non-alcoholic steatohepatitis (NASH). Both diseases are characterized by an increase of circulating EVs. In order to characterize EVs cargo, it was demonstrated that hepatocyte-derived EVs released

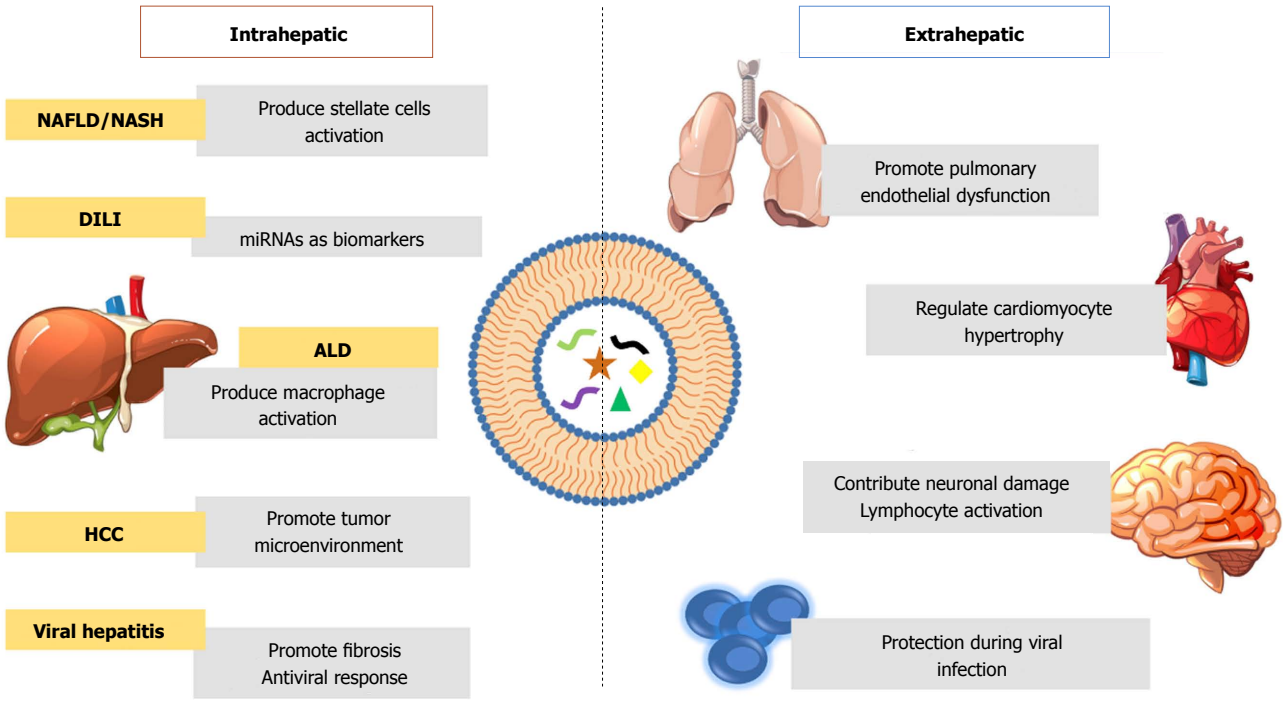


Figure 2 Role of extracellular vesicles during pathologic processes inside and outside the liver. Intrahepatic extracellular vesicles in non-alcoholic fatty liver (NAFLD), non-alcoholic steatohepatitis (NASH), drug-induced liver injury (DILI), alcoholic liver disease (ALD) and hepatocellular carcinoma (HCC). Extrahepatic EVs play a fundamental role in pulmonary disease, cardiovascular diseases (CVDs), neurodegenerative disorders and immunopathological disorders. EVs: Extracellular vesicles; NAFLD: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis; DILI: Drug-induced liver injury; ALD: Alcoholic liver disease; HCC: Hepatocellular carcinoma; CVDs: Cardiovascular diseases.

during lipotoxic fatty acids are enriched in Vanin-1 (VNN1) and miR128-3p. VNN1 is responsible of the internalization of EVs into HSCs and miR128-3p inhibits the expression of PPAR-gamma provoking an activation of stellate cells inducing fibrosis in the liver[27]. Altogether these results indicate that VNN1 and miR128-3p released by hepatocytes associated with EVs during lipotoxicity might be important during HSCs activation in NAFLD/NASH.

EVs in alcoholic liver disease

In an attempt to further characterize the critical role of EVs *in vivo* during alcoholic liver disease (ALD), Saha and colleagues[28], used an experimental model of ALD. The authors first found a significant increase in the total number of EVs in the serum of mice with an alcohol diet and the effect of serum EVs derived from ALD mice in naïve recipient mice. To characterize the different components in EVs release to ALD mice they found an increase in miR-192 and miR-30a levels compared to control EVs. Moreover, hepatocyte released EVs causing an increase in the percentage of F4/80^{hi} CD11b^{low} (KCs) and TNF-α, suggesting the link between innate immune cell activation and hepatocyte intoxication during the process of alcoholic liver injury.

Hepatic resident macrophages (KCs) and infiltrating macrophages play a pivotal role in ALD pathogenesis whose production of proinflammatory cytokines exhibited the inflammatory process characteristic of alcoholic hepatitis (AH). For this reason, it is necessary to characterize specific proteins implicated in macrophage activation.

In order to evaluate the *in vivo* role of macrophages, Verma and collaborators[29] described that cultured hepatocytes released CD40L in EVs in response to alcohol exposure which leads to macrophage activation. In contrast, Saha *et al*[28] showed that Hsp90 as the cause of macrophage activation, demonstrating that there was a significant increase in levels of Hsp90 EVs secreted from hepatocytes in ALD. These studies reveal that Hsp90 and CD40L carried by EVs released from hepatocytes in response to alcohol intake, have an important role in macrophage activation during ALD.

EVs in hepatocellular carcinoma

Several studies suggest that EVs contribute to proliferation and propagation of hepatocellular carcinoma (HCC) cells during HCC[30]. It was demonstrated that EVs released by CD90^{+} cells provoked an increase in vascular endothelial growth factor 1 in endothelial cells which lead with metastasis. Moreover, it has suggested that EVs collaborate with the microenvironment that promote tumor survival and growth[31]. It was found that EVs released by metastatic HCC cells induce hepatocytes to secrete metalloproteinase-2 and -9 which facilitate the invasion of HCC cells[32].

Kogure *et al*[33], characterized the cargo of EVs release by HCC cells *in vitro* identifying several miRNA, such as miR-584, miR-517c, miR-378, miR-520f, miR142-5p, miR-451, miR-518d, miR-215, miR-376a, miR-133b, and miR-367. These studies indicate that oncogenic cargo

released by HCC cells modulate tumor environment facilitating the invasion of HCC cells promoting the proliferation and differentiation of tumors.

Viral hepatitis

The role of CCL5 released by HCV-infected macrophages/KCs thereby inducing the activation of HSCs through the phosphorylation of ERK was demonstrated. In fact, the neutralization of CCL5 in HSCs in culture using supernatant from HCV-infected macrophages caused a significant down-regulation of inflammatory and profibrotic genes[34]. Another study demonstrated that liver cells treated with IFN-α induced resistance to HBV replication in infected liver cells by cell-cell communication through EVs[35]. These results provide evidence that EVs have an important role during viral infection and antiviral response.

Extrahepatic EVs

So far, the role of EVs in different pathophysiological events in the liver was discussed. However, several articles revealed the role of EVs in remote organs taking part of different events under pathological conditions, such as pulmonary disease, neurodegenerative disorders, cardiovascular diseases and during immunopathological processes.

EVs in pulmonary disease

The liver takes an important role in maintaining systemic homeostasis[36]. The injured liver can induce different pathogenic processes in remote organs. Indeed, EVs are linked with different pathological conditions inside and outside the liver[37]. For this reason, hepatocyte-derived-EVs are suggested to have an important role in the pathogenesis of pulmonary disease.

To characterize the critical role of hepatic pathogenic processes, and their implications in the lung, Royo *et al*[37] investigated the role of Arg1 carried by EVs as one of the factors responsible for the lung damage. The study confirms that hepatic EVs and the effect of Arg1 might propagate the injury in the lung inducing pulmonary endothelial dysfunction. It concludes that EVs take an important part in communication between the liver and lung, could be Arg1 the responsible for pulmonary endothelial dysfunction.

EVs in neurodegenerative disorders

On the other hand, we discuss the role of EVs in different neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS) as a potential source of information in neurodegenerative disorders[38].

It has been suggested that lipids cargo in EVs released from neurons promoting the formation of β-amyloid (βA) peptides contributing to neuronal damage in AD[39]. Furthermore, it was found that AD patients have lower levels of miR-193b in blood which are correlated with levels in cerebral spinal fluid (CSF)[40]. In addition, miR-132 and miR-212 are downregulated in neurodegenerative disorders including AD[41].

PD is characterized by an accumulation of α-synuclein protein. Therefore the cargo inside EVs was analysed and showed that this protein is present outside and inside of EVs, and their secretion contribute to the development of the disease[42].

In order to understand the role of EVs in MS, researchers showed that EVs are released from brain endothelium and have increased levels of β2-microglobulin, MHC Ⅱ, CD40 and ICOSL. Moreover, they are involved in the activation of $CD4^+$ and $CD8^+$ lymphocytes[43]. Furthermore, serum EVs were able to decrease the levels of miR-122-5p, miR-196b-5p, miR-301a-3p, miR-532-5p[44].

Considering these results, EVs might contribute to the progression of neurodegenerative diseases and thus be used in the clinical setting as biomarkers or drug delivery tools.

EVs in cardiovascular diseases

Emerging studies reveals that EVs have regulatory effects in cardiovascular diseases being released by endothelial cells, cardiomyocytes, fibroblasts and stem cells and participating in pathophysiological processes contributing to the development of disease[45].

EVs have been involved in the regulation of cardiomyocyte hypertrophy and cardiac fibrosis. It was demonstrated that EVs released from myocytes carry Hsp90 together with IL-6. Both molecules are involved in the activation of cardiac fibroblasts causing increased collagen production and deposition during cardiac hypertrophy[46]. Furthermore, it was found a significantly increase in the levels of miR-21-3p in pericardial fluid in a mice model of transverse aortic constriction-induced hypertrophy. This miR-21-3p associated with EVs was released by fibroblasts and was uptake by cardiomyocytes leading to an activation of intercellular signalling pathways which provoke cellular hypertrophy[47]. Interestingly, EVs play a critical role in intercellular communication between fibroblasts and cardiomyocytes during the hypertrophic process contributing to cardiac fibrosis.

EVs in immunopathology

Another important issue is the role of EVs in antiviral immune response. Torralba and colleagues[48], investigated that EVs released from T cells contained mitochondrial DNA and this genetic material can be transferred unidirectionally from T cells to dendritic cells (DCs) during the formation of antigen-dependent contacts. The possible signalling pathways which are activating in DCs were analysed, finding a significantly increase in the expression of different genes. Most of them were involved in the antiviral response mediated by IFN-I resulting into immune protection effect against virus infection leading a decrease viral infection. Altogether these results indicate that EVs from T cells conferred protection to DCs against virus infection

Table 1 Summary of extracellular vesicles biomarkers in hepatic and extracellular vesicles

	Type of disease	Sample	Species	Biomarker	Ref.
Intrahepatic	Drug-induced liver injury (DILI)	Plasma/serum/cell culture	Mouse/rat/human	miR-122 [↑]	[25,26]
	Non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)	Cell culture	Mouse	miR-128-3p [↑]; VNN1	[27]
	Alcoholic liver disease (ALD)	Serum/cell culture	Mouse/human	miR-192, miR-30a [↑]; CD40L, Hsp90	[28,29]
	Hepatocellular carcinoma (HCC)	Cell culture	Human	Vascular endotelial growth factor 1, MMP2, MMP9; miR-584, miR-517c, miR-378, miR-520f, miR142-5p, miR-451, miR-518d, miR-215, miR376a, miR-133b, and miR-367	[31-33]
	Viral hepatitis (HBV/HCV)	Cell culture	Human	Viral RNA; CCL5	[34]
Extrahepatic	Pulmonary disease	Serum/cell culture	Rat	Arg 1	[37]
	Alzheimer's disease (AD)	CSF/blood/tissue	Human	β-amiloyd; miR-193b, miR-132 [↓]	[39,41]
	Parkinson's disease (PD)	Cell culture	Mouse	α-sinucleyn	[42]
	Multiple Sclerosis (MS)	Serum/cell culture	Mouse/human	Beta-2-microglobulin, MHC-Ⅱ, CD40,ICOSL; miR-122-5p, miR-196b-5p, miR-301a-3p, miR-5p [↓]	[43,44]
	Cardiovascular disease (CVDs)	Cell culture/pericardial fluid	Rat/mouse	Hsp90, IL-6; miR-21-3p [↑]	[46,47]
	Immunopathology	Cell culture	Human	mtDNA	[48]

VNN1: Vanin-1; MMP: Matrix metalloproteinase.

through antigen-driven contacts.

CONCLUSION

In summary, the data show that EVs can be used not only as diagnostic but theranostic tool for the treatment of acute and chronic liver disease (Table 1). EVs can be released by hepatocytes carrying miRNA as potential biomarkers in DILI or triggering macrophage activation in ALD and an activation of HSCs in NAFLD/NASH. Emerging evidences suggests that EVs promotes the proliferation and migrations of tumor cells. Additionally, circulating EVs have an effect outside the liver as seen in the lung taking particularly interest the link between EVs released by hepatocytes and the effect in pulmonary disease. The effect of EVs in the brain as seen in different neurodegenerative disorders contributing to the progress and development of the diseases; in the heart, having regulatory effects in cardiovascular diseases and finally during viral infection for their immune protection effect.

In conclusion, EVs are important intercellular communication mediators during pathology and physiology events. It would be interesting in future studies to investigate the particularly role of EVs in the development of diseases. However, little data support the function of EVs in physiopathological processes suggests the need for further research.

REFERENCES

1 **Cho YE**, Song BJ, Akbar M, Baek MC. Extracellular vesicles as potential biomarkers for alcohol- and drug-induced liver injury and their therapeutic applications. *Pharmacol Ther* 2018; **187**: 180-194 [PMID: 29621595 DOI: 10.1016/j.pharmthera.2018.03.009]

2 **Devhare PB**, Ray RB. Extracellular vesicles: Novel mediator for cell to cell communications in liver pathogenesis. *Mol Aspects Med* 2018; **60**: 115-122 [PMID: 29122679 DOI: 10.1016/j.mam.2017.11.001]

3 **Trams EG**, Lauter CJ, Salem N Jr, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta* 1981; **645**: 63-70 [PMID: 6266476 DOI: 10.1016/0005-2736(81)90512-5]

4 **Simpson RJ**, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. *Expert Rev Proteomics* 2009; **6**: 267-283 [PMID: 19489699 DOI: 10.1586/epr.09.17]

5 **Henne WM**, Buchkovich NJ, Emr SD. The ESCRT pathway. *Dev Cell* 2011; **21**: 77-91 [PMID: 21763610 DOI: 10.1016/j.devcel.2011.05.015]

6 **Hirsova P**, Ibrahim SH, Verma VK, Morton LA, Shah VH, LaRusso NF, Gores GJ, Malhi H. Extracellular vesicles in liver pathobiology: Small particles with big impact. *Hepatology* 2016; **64**: 2219-2233 [PMID: 27628960 DOI: 10.1002/hep.28814]

7 **Lemoinne S**, Thabut D, Housset C, Moreau R, Valla D, Boulanger CM, Rautou PE. The emerging roles of microvesicles in liver diseases. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 350-361 [PMID: 24492276 DOI: 10.1038/nrgastro.2014.7]

8 **Cocucci E**, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009; **19**: 43-51 [PMID: 19144520 DOI: 10.1016/j.tcb.2008.11.003]

9 **Mathivanan S**, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* 2010; **73**: 1907-1920 [PMID: 20601276 DOI: 10.1016/j.jprot.2010.06.006]

10 **Hristov M**, Erl W, Linder S, Weber PC. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. *Blood* 2004; **104**: 2761-2766 [PMID: 15242875 DOI: 10.1182/blood-2003-10-3614]

11 **Minciacchi VR**, Freeman MR, Di Vizio D. Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. *Semin Cell Dev Biol* 2015; **40**: 41-51 [PMID: 25721812 DOI: 10.1016/j.semcdb.2015.02.010]

12 **Wendler F**, Stamp GW, Giamas G. Tumor-Stromal Cell Communication: Small Vesicles Signal Big Changes. *Trends Cancer* 2016; **2**: 326-329 [PMID: 28741534 DOI: 10.1016/j.trecan.2016.05.007]

13 **Sun D**, Zhuang X, Zhang S, Deng ZB, Grizzle W, Miller D, Zhang HG. Exosomes are endogenous nanoparticles that can deliver biological information between cells. *Adv Drug Deliv Rev* 2013; **65**: 342-347 [PMID: 22776312 DOI: 10.1016/j.addr.2012.07.002]

14 **Escola JM**, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie

O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* 1998; **273**: 20121-20127 [PMID: 9685355 DOI: 10.1074/jbc.273.32.20121]

15 **Lässer C**, Alikhani VS, Ekström K, Eldh M, Paredes PT, Bossios A, Sjöstrand M, Gabrielsson S, Lötvall J, Valadi H. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med* 2011; **9**: 9 [PMID: 21235781 DOI: 10.1186/1479-5876-9-9]

16 **Szabo G**, Momen-Heravi F. Extracellular vesicles in liver disease and potential as biomarkers and therapeutic targets. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 455-466 [PMID: 28634412 DOI: 10.1038/nrgastro.2017.71]

17 **Bátiz LF**, Castro MA, Burgos PV, Velásquez ZD, Muñoz RI, Lafourcade CA, Troncoso-Escudero P, Wyneken U. Exosomes as Novel Regulators of Adult Neurogenic Niches. *Front Cell Neurosci* 2016; **9**: 501 [PMID: 26834560 DOI: 10.3389/fncel.2015.00501]

18 **Sato K**, Meng F, Glaser S, Alpini G. Exosomes in liver pathology. *J Hepatol* 2016; **65**: 213-221 [PMID: 26988731 DOI: 10.1016/j.jhep.2016.03.004]

19 **Lang JK**, Young RF, Ashraf H, Canty JM Jr. Inhibiting Extracellular Vesicle Release from Human Cardiosphere Derived Cells with Lentiviral Knockdown of nSMase2 Differentially Effects Proliferation and Apoptosis in Cardiomyocytes, Fibroblasts and Endothelial Cells In Vitro. *PLoS One* 2016; **11**: e0165926 [PMID: 27806113 DOI: 10.1371/journal.pone.0165926]

20 **Crispe IN**. The liver as a lymphoid organ. *Annu Rev Immunol* 2009; **27**: 147-163 [PMID: 19302037 DOI: 10.1146/annurev.immunol.021908.132629]

21 **Cai S**, Cheng X, Pan X, Li J. Emerging role of exosomes in liver physiology and pathology. *Hepatol Res* 2017; **47**: 194-203 [PMID: 27539153 DOI: 10.1111/hepr.12794]

22 **Chen L**, Chen R, Kemper S, Brigstock DR. Pathways of production and delivery of hepatocyte exosomes. *J Cell Commun Signal* 2018; **12**: 343-357 [PMID: 29063370 DOI: 10.1007/s12079-017-0421-7]

23 **Kim WR**, Flamm SL, Di Bisceglie AM, Bodenheimer HC; Public Policy Committee of the American Association for the Study of Liver Disease. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology* 2008; **47**: 1363-1370 [PMID: 18366115 DOI: 10.1002/hep.22109]

24 **Köberle V**, Pleli T, Schmithals C, Augusto Alonso E, Haupenthal J, Bönig H, Peveling-Oberhag J, Biondi RM, Zeuzem S, Kronenberger B, Waidmann O, Piiper A. Differential stability of cell-free circulating microRNAs: implications for their utilization as biomarkers. *PLoS One* 2013; **8**: e75184 [PMID: 24073250 DOI: 10.1371/journal.pone.0075184]

25 **Bala S**, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, Alao H, Kodys K, Szabo G. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology* 2012; **56**: 1946-1957 [PMID: 22684891 DOI: 10.1002/hep.25873]

26 **Holman NS**, Mosedale M, Wolf KK, LeCluyse EL, Watkins PB. Subtoxic Alterations in Hepatocyte-Derived Exosomes: An Early Step in Drug-Induced Liver Injury? *Toxicol Sci* 2016; **151**: 365-375 [PMID: 26962055 DOI: 10.1093/toxsci/kfw047]

27 **Povero D**, Panera N, Eguchi A, Johnson CD, Papouchado BG, de Araujo Horcel L, Pinatel EM, Alisi A, Nobili V, Feldstein AE. Lipid-induced hepatocyte-derived extracellular vesicles regulate hepatic stellate cell *via* microRNAs targeting PPAR-γ. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 646-663.e4 [PMID: 26783552 DOI: 10.1016/j.jcmgh.2015.07.007]

28 **Saha B**, Momen-Heravi F, Furi I, Kodys K, Catalano D, Gangopadhyay A, Haraszti R, Satishchandran A, Iracheta-Vellve A, Adejumo A, Shaffer SA, Szabo G. Extracellular vesicles from mice with alcoholic liver disease carry a distinct protein cargo and induce macrophage activation through heat shock protein 90. *Hepatology* 2018; **67**: 1986-2000 [PMID: 29251792 DOI: 10.1002/hep.29732]

29 **Verma VK**, Li H, Wang R, Hirsova P, Mushref M, Liu Y, Cao S, Contreras PC, Malhi H, Kamath PS, Gores GJ, Shah VH. Alcohol stimulates macrophage activation through caspase-dependent hepatocyte derived release of CD40L containing extracellular vesicles. *J Hepatol* 2016; **64**: 651-660 [PMID: 26632633 DOI: 10.1016/j.jhep.2015.11.020]

30 **Santangelo L**, Battistelli C, Montaldo C, Citarella F, Strippoli R, Cicchini C. Functional Roles and Therapeutic Applications of Exosomes in Hepatocellular Carcinoma. *Biomed Res Int* 2017; **2017**: 2931813 [PMID: 28265569 DOI: 10.1155/2017/2931813]

31 **Conigliaro A**, Costa V, Lo Dico A, Saieva L, Buccheri S, Dieli F, Manno M, Raccosta S, Mancone C, Tripodi M, De Leo G, Alessandro R. CD90+ liver cancer cells modulate endothelial cell phenotype through the release of exosomes containing H19 lncRNA. *Mol Cancer* 2015; **14**: 155 [PMID: 26272696 DOI: 10.1186/s12943-015-0426-x]

32 **He M**, Qin H, Poon TC, Sze SC, Ding X, Co NN, Ngai SM, Chan TF, Wong N. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncogenic proteins and RNAs. *Carcinogenesis* 2015; **36**: 1008-1018 [PMID: 26054723 DOI: 10.1093/carcin/bgv081]

33 **Kogure T**, Lin WL, Yan IK, Braconi C, Patel T. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. *Hepatology* 2011; **54**: 1237-1248 [PMID: 21721029 DOI: 10.1002/hep.24504]

34 **Sasaki R**, Devhare PB, Steele R, Ray R, Ray RB. Hepatitis C virus-induced CCL5 secretion from macrophages activates hepatic stellate cells. *Hepatology* 2017; **66**: 746-757 [PMID: 28318046 DOI: 10.1002/hep.29170]

35 **Li J**, Liu K, Liu Y, Xu Y, Zhang F, Yang H, Liu J, Pan T, Chen J, Wu M, Zhou X, Yuan Z. Exosomes mediate the cell-to-cell transmission of IFN-α-induced antiviral activity. *Nat Immunol* 2013; **14**: 793-803 [PMID: 23832071 DOI: 10.1038/ni.2647]

36 **Robinson MW**, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol* 2016; **13**: 267-276 [PMID: 27063467 DOI: 10.1038/cmi.2016.3]

37 **Royo F**, Moreno L, Mleczko J, Palomo L, Gonzalez E, Cabrera D, Cogolludo A, Vizcaino FP, van-Liempd S, Falcon-Perez JM. Hepatocyte-secreted extracellular vesicles modify blood metabolome and endothelial function by an arginase-dependent mechanism. *Sci Rep* 2017; **7**: 42798 [PMID: 28211494 DOI: 10.1038/srep42798]

38 **Croese T**, Furlan R. Extracellular vesicles in neurodegenerative diseases. *Mol Aspects Med* 2018; **60**: 52-61 [PMID: 29137922 DOI: 10.1016/j.mam.2017.11.006]

39 **Perez-Gonzalez R**, Gauthier SA, Kumar A, Levy E. The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space. *J Biol Chem* 2012; **287**: 43108-43115 [PMID: 23129776 DOI: 10.1074/jbc.M112.404467]

40 **Liu CG**, Song J, Zhang YQ, Wang PC. MicroRNA-193b is a regulator of amyloid precursor protein in the blood and cerebrospinal fluid derived exosomal microRNA-193b is a biomarker of Alzheimer's disease. *Mol Med Rep* 2014; **10**: 2395-2400 [PMID: 25119742 DOI: 10.3892/mmr.2014.2484]

41 **Lau P**, Frigerio CS, De Strooper B. Variance in the identification of microRNAs deregulated in Alzheimer's disease and possible role of lincRNAs in the pathology: the need of larger datasets. *Ageing Res Rev* 2014; **17**: 43-53 [PMID: 24607832 DOI: 10.1016/j.arr.2014.02.006]

42 **Chang C**, Lang H, Geng N, Wang J, Li N, Wang X. Exosomes of BV-2 cells induced by alpha-synuclein: important mediator of neurodegeneration in PD. *Neurosci Lett* 2013; **548**: 190-195 [PMID: 23792198 DOI: 10.1016/j.neulet.2013.06.009]

43 **Wheway J**, Latham SL, Combes V, Grau GE. Endothelial microparticles interact with and support the proliferation of T cells. *J Immunol* 2014; **193**: 3378-3387 [PMID: 25187656 DOI: 10.4049/jimmunol.1303431]

44 **Selmaj I**, Cichalewska M, Namiecinska M, Galazka G, Horzelski W, Selmaj KW, Mycko MP. Global exosome transcriptome profiling reveals biomarkers for multiple sclerosis. *Ann Neurol* 2017; **81**: 703-717 [PMID: 28411393 DOI: 10.1002/ana.24931]

45 **Bei Y**, Das S, Rodosthenous RS, Holvoet P, Vanhaverbeke M,

Monteiro MC, Monteiro VVS, Radosinska J, Bartekova M, Jansen F, Li Q, Rajasingh J, Xiao J. Extracellular Vesicles in Cardiovascular Theranostics. *Theranostics* 2017; **7**: 4168-4182 [PMID: 29158817 DOI: 10.7150/thno.21274]

46 **Datta R**, Bansal T, Rana S, Datta K, Datta Chaudhuri R, Chawla-Sarkar M, Sarkar S. Myocyte-Derived Hsp90 Modulates Collagen Upregulation *via* Biphasic Activation of STAT-3 in Fibroblasts during Cardiac Hypertrophy. *Mol Cell Biol* 2017; **37**: [PMID: 28031326 DOI: 10.1128/MCB.00611-16]

47 **Bang C**, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J, Zimmer K, Zeug A, Ponimaskin E, Schmiedl A, Yin X, Mayr M, Halder R, Fischer A, Engelhardt S, Wei Y, Schober A, Fiedler J, Thum T. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. *J Clin Invest* 2014; **124**: 2136-2146 [PMID: 24743145 DOI: 10.1172/JCI70577]

48 **Torralba D**, Baixauli F, Villarroya-Beltri C, Fernández-Delgado I, Latorre-Pellicer A, Acín-Pérez R, Martín-Cófreces NB, Jaso-Tamame ÁL, Iborra S, Jorge I, González-Aseguinolaza G, Garaude J, Vicente-Manzanares M, Enríquez JA, Mittelbrunn M, Sánchez-Madrid F. Priming of dendritic cells by DNA-containing extracellular vesicles from activated T cells through antigen-driven contacts. *Nat Commun* 2018; **9**: 2658 [PMID: 29985392 DOI: 10.1038/s41467-018-05077-9]

P- Reviewer: Koizume S, Kanda T, Link A, Marcos R
S- Editor: Wang XJ **L- Editor**: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4527-4535

DOI: 10.3748/wjg.v24.i40.4527

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

EDITORIAL

Better surgical treatment method for hepatocellular carcinoma with portal vein tumor thrombus

Shu-You Peng, Xu-An Wang, Cong-Yun Huang, Jiang-Tao Li, De-Fei Hong, Yi-Fang Wang, Bin Xu

Shu-You Peng, Jiang-Tao Li, Department of Hepatobiliary and Pancreatic Surgery, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310006, Zhejiang Province, China

Xu-An Wang, Department of General Surgery, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Cong-Yun Huang, Department of General Surgery, Yuebei People's Hospital Affiliated to Shantou University School of Medicine, Shaoguan 512025, Guangdong Province, China

De-Fei Hong, Yi-Fang Wang, Bin Xu, Department of Hepatobiliary and Pancreatic Surgery, Sir Run Run Shaw Hospital Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310016, Zhejiang Province, China

ORCID number: Shu-You Peng (0000-0003-3989-3056); Xu-An Wang (0000-0001-9437-457X); Cong-Yun Huang (0000-0003-3886-4130); Jiang-Tao Li (0000-0001-7538-2910); De-Fei Hong (0000-0001-8665-1119); Yi-Fan Wang (0000-0002-8828-4268); Bin Xu (0000-0003-0889-5737).

Author contributions: Peng SY and Wang XA contributed equally to this work; Peng SY and Wang XA wrote and revised the manuscript; Peng SY designed the "thrombectomy first" approach. All the authors participated in the operations.

Supported by Shanghai Pujiang Program, No.17PJD025; and Shanghai Natural Science Foundation, No.17ZR1418500.

Conflict-of-interest statement: All the authors deny any form of conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: Xu-An Wang, MD, PhD, Department of General Surgery, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, No. 1665, Kongjiang Road, Shanghai 200092, China. wangxuan@xinhuamed.com.cn
Telephone: +86-21-25078813

Received: August 21, 2018
Peer-review started: August 22, 2018
First decision: August 30, 2018
Revised: September 11, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

Hepatocellular carcinoma (HCC) with portal vein tumor thrombus (PVTT) is a disease that is not uncommon, but the treatments vary drastically between Eastern and Western countries. In Europe and America, the first line of treatment is systemic therapy such as sorafenib and the surgical treatment is not a recommend option. While an increasing number of studies from China and Japan have suggested that surgical treatment results in better outcomes when compared to transcatheter arterial chemoembolization (TACE), sorafenib, or other nonsurgical treatments, and two classification systems, Japanese Vp classification and Chinese Cheng's classification, were very useful to guide the surgical treatment. We have also found that surgical treatment may be more effective, as we have performed surgical treatment for HCC-PVTT patients over a period of approximately 15 years and achieved good results with the longest surviving time being 13 years and onward. In this study, we review the efficacy and principles of current surgical treatments and introduce our new, more effective surgical technique named "thrombectomy first", which means the tumor thrombus in the main portal vein, the bifurcation or the contralateral portal vein should be removed prior to liver resection. Thus, compression and crushing of PVTT during the operation could be avoided and new intrahepatic

metastases caused by tumor thrombus to the remnant liver minimized. The new technique is even beneficial to the prognosis of Cheng's classification Types Ⅲ and Ⅳ PVTT. The vital tips and tricks for the surgical approach are described.

Key words: Portal vein tumor thrombus; Thrombectomy first; Surgery; Hepatocellular carcinoma

Core tip: The treatments for hepatocellular carcinoma with portal vein tumor thrombus between Eastern and Western countries vary drastically. In Western countries, the first-line treatment is systemic therapy such as sorafenib, while studies from China and Japan suggest that surgical treatment results in better outcomes. We review the efficacy and principles of current surgical treatments and introduce our new, more effective surgical technique named "thrombectomy first", which means the tumor thrombus would be removed prior to liver resection. We have performed this technique over approximately 15 years and achieved good results with the longest surviving time being 13 years and onward.

Peng SY, Wang XA, Huang CY, Li JT, Hong DF, Wang YF, Xu B. Better surgical treatment method for hepatocellular carcinoma with portal vein tumor thrombus. *World J Gastroenterol* 2018; 24(40): 4527-4535 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4527.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4527

INTRODUCTION

Portal vein tumor thrombus (PVTT) is not rare, but it is correlated with an extremely poor prognosis, with approximately 40% of patients with hepatocellular carcinoma (HCC) having PVTT when diagnosed[1]. HCC with PVTT is recognized as an advanced stage disease (BCLC-C) according to the Barcelona Clinic for Liver Cancer (BCLC) staging system[2]. Surgical resection is not a recommended treatment option; the only proposed treatment option is systemic therapy. The tyrosine kinase inhibitors sorafenib and regorafenib have been reported to prolong survival and have been selected as treatment options[3]. Both the American Association for the Study of the Liver Disease (AASLD) and the European Association for the Study of the Liver (EASL) accepted the BCLC treatment algorithm[4,5].

Eastern countries have totally opposite views. Experts from mainland China[6] and Japan[7] as well as other eastern experts[8] have recognized that HCC with PVTT is not a contradiction of surgical approach. In selected HCC-PVTT patients, surgical treatment could not only improve the life quality but also prolong the survival time, even though a R0 resection for this malignant disease could not be achieved. The criteria for surgical resection are defined as follows: (1) PVTT type Ⅰ/Ⅱ or type Ⅲ/Ⅳ (but these classifications remain controversial); (2) Good general medical conditions with Eastern Cooperative Oncology Group (ECOG) scores of 0-2; (3) Liver function was Child-Pugh class A or selected class B; (4) Resectable primary tumor, without metastasis; and (5) Enough future liver remnant.

We have performed surgical treatment for HCC-PVTT patients over a period of approximately 15 years and achieved good results, with the longest surviving time being 13 years and onward. In this study, we review the efficacy and principles of current surgical treatments and introduce our new, more effective surgical technique named "thrombectomy first".

EFFICACY OF SURGICAL TREATMENT

Surgical vs nonsurgical treatments

A Japanese nationwide survey[9] of 6474 patients from 645 registered institutions showed that the median survival time (MST) of the operation group was prolonged by 1.77 years compared to the nonsurgical group (2.87 years *vs* 1.10 years; $P < 0.001$) for the overall cohort and by 0.88 years (2.45 years *vs* 1.57 years; $P < 0.001$) for the propensity score-matched cohort. Furthermore, the survival rates at 1, 3, and 5 years after diagnosis for the operation group were significantly higher compared to the conventional treatment group, with the survival rates being 70.9%, 43.5%, and 32.9% compared to 62.9%, 31.6%, and 20.1%, respectively. Moreover, subgroup analysis after propensity score matching revealed that surgical treatment provided a survival benefit regardless of age, etiology, serum α-fetoprotein elevation, tumor number, or hepatitis B or C infection; however, a previous study[7] showed that the survival benefits of surgical treatment in Vp4 patient groups were not statistically significant, and most of them could not achieve R0 or R1 resection. Thus, the authors only recommended liver resection as a first line of treatment when PVTT was limited to the first-order branch of the portal vein.

Another large cohort study from China[10] analyzed 1580 patients diagnosed with HCC with PVTT from four of the largest tertiary hospitals. The MSTs of the surgical treatment group for types Ⅰ and Ⅱ patients were 15.9 and 12.5 mo, respectively, and these MSTs were much better compared to their nonsurgical counterparts. The authors concluded that surgical approaches are the best treatment for type Ⅰ/Ⅱ PVTT patients with good liver function. Likewise, several other studies from Eastern countries also suggested the potential survival benefits of surgical treatment in HCC-PVTT patients[11-14]; however, most of these studies suggested that operation is not useful for type Ⅳ PVTT patients who had a poor prognosis.

Surgical vs TACE treatment

Transcatheter arterial chemoembolization (TACE) is

commonly used to help patients with advanced HCC including PVTT. Recently, many studies reported that surgical treatment had a better prognosis compared to those undergoing TACE, especially for Cheng's types Ⅰ and Ⅱ PVTT. Peng *et al*[15] compared 201 HCC patients with PVTT who received surgical treatment and 402 matched cases treated with TACE. The median survival of the surgical treatment and TACE groups was 20.0 mo ± 1.8 mo and 13.1 mo ± 0.6 mo, respectively. The 1-, 3-, and 5-year overall survival rates for the surgical and TACE groups were 42.0%, 14.1%, and 11.1% and 37.8%, 7.3%, and 0.5%, respectively ($P < 0.001$). The survival curve for the operation group was better than that for the TACE group. Subgroup analyses showed that the overall survival for the operation group was better than that of the TACE group for type Ⅰ/Ⅱ PVTT but not for type Ⅲ/Ⅳ PVTT. This result was concordant with a stratified meta-analysis[16]. Other typical studies[10,17] also favor the surgical treatment, which showed that the survival rates were better for the surgical resection group than the TACE group for Cheng's type Ⅰ/Ⅱ PVTT.

Combining preoperative TACE with surgical resection alone was also compared by Yamamoto[18] and Ban[19]; neither were able to find a survival benefit with preoperative TACE for HCC-PVTT patients, especially for Cheng's types Ⅲ and Ⅳ PVTT.

Surgical vs sorafenib treatment

In Western countries, the first-line treatment option for HCC-PVTT is sorafenib, but the MST was short at 6.5 mo in the Eastern countries[20] and 10.7 mo in Western areas[3]. In a study by Jeong *et al*[21], 30 patients with HCC-PVTT (Vp3 or Vp4) treated with sorafenib monotherapy had a median survival period of only 3.1 mo. Another typical study by Nakazawa *et al*[22] also revealed that the MST(4.3 mo) was similar for PVTT in the main trunk or in the first branch after treated with sorafenib alone. These data demonstrated that the MST was quite shorter for HCC-PVTT treated with surgical method.

SURGICAL CLASSIFICATION

The tumor extent and the range and position of PVTT affect surgical planning and correlate with overall survival after surgical resection. Thus, the surgical method should aim to achieve a complete resection of the primary tumor and removal of PVTT with a sufficient future remnant liver. Therefore, classification of PVTT is necessary to determine the surgical process.

Currently, there are two classifications that are well accepted and widely used in the Asia-Pacific: the Japanese Vp classification[23,24] and the classification suggested by Cheng, who is a Chinese professor[12,25].

Japanese classification

The Liver Cancer Study Group of Japan proposed a helpful macroscopic classification divided into five grades (Vp0-Vp4)[23,25] for HCC with PVTT. Each one is defined as follows: (1) no tumor thrombus in the portal vein, Vp0; (2) existence of a tumor thrombus distal to, but not in, the 2nd-order branches of the portal vein, Vp1; (3) presence of a tumor thrombus in the 2nd-order branches of the portal vein, Vp2; (4) existence of a tumor thrombus in the 1st-order branches of the portal vein, Vp3; and (5) presence of a tumor thrombus in the main trunk of the portal vein or a portal vein branch contralateral to the mainly involved lobe (or both), Vp4.

Cheng's classification

Cheng's new classification system comprises five grades: (1) Type Ⅰ0: tumor thrombus formation found under a microscope; (2) Type Ⅰ: tumor thrombus involving segmental branches of the portal vein or above; (3) Type Ⅱ: tumor thrombus involving the right/left portal vein; (4) Type Ⅲ: tumor thrombus involving the main portal vein trunk; and (5) Type Ⅳ: tumor thrombus involving the superior mesenteric vein or the inferior vein cava. Furthermore, two subgroups were classified for each type: (1) For Type Ⅰ: Ⅰa, tumor thrombus involving segmental branches of the portal vein or above, and Ⅰb, tumor thrombus involving segmental branches of the portal vein extending to sectoral branch; (2) For Type Ⅱ: Ⅱa, tumor thrombus involving the right/left portal vein, and Ⅱb, tumor thrombus involving both the left and right portal veins; (3) For Type Ⅲ: Ⅲa, tumor thrombus involving the main portal vein trunk for no more than 2 cm below the confluence of the left and right portal veins, and Ⅲb, tumor thrombus involving the main portal vein trunk for more than 2 cm below the confluence of the left and right portal veins; and (4) For Type Ⅳ: Ⅳa, tumor thrombus involving the superior mesenteric vein (SMV), and IVb, tumor thrombus involving the inferior vein cava (IVC).

Compared to the Cheng's classification, the Vp classification does not mention the extension of PVTT involving the SMV or IVC. Vp0 is equal to Type 0, Vp1 is equal to Type Ⅰa, Vp2 is equal to Type Ⅰb, Vp3 is equal to Type Ⅱa, and Vp4 is equal to Type Ⅲ and Type Ⅱb. Most Japanese studies concluded that surgical treatment did not achieve a better survival outcome compared to nonsurgical treatments in Vp4 PVTT. There are, however, disadvantages of the two classifications: (1) both of them do not consider liver function or tumor-correlated factors, such as tumor number and size; and (2) no pathological information of PVTT is described, such as necrotic degree, proliferative type, necrotic type and organized type. Several studies recognized that the Cheng's classification was more applicable than the Vp classification for disease assessment and treatment.

SURGICAL METHODS AND EFFICACY

Based on the two classifications, several surgical resection methods and techniques were introduced.

Tumor thrombus lies within the liver resection line

En bloc resection of PVTT with the tumor could be achieved: (1) Segmental hepatectomy is suitable for

Japanese classification Vp1-2 or Cheng's classification Type Ⅰ; (2) Hemihepatectomy is suitable for Japanese classification Vp3 or Cheng's classification Type Ⅱa, and the primary tumor should be located in the same liver without extending beyond the hemiliver; and (3) Extended hemihepatectomy is suitable for Cheng's classification Type Ⅱb and Japanese classification Vp4 (tumor thrombus in the bifurcation of the portal vein).

Tumor thrombus lies beyond the liver resection line

Hepatectomy plus thrombectomy or *en bloc* resection (with or without reconstruction of the main portal vein) can be selected in this kind of HCC-PVTT, which includes Japanese classification Vp4 and Cheng's classification Types Ⅲ and Ⅳ. The PVTT is typically extracted through an incision in the portal vein after resection to the liver.

Tumor thrombus invades the main portal vein wall

Combined with the main portal resection, a direct end-to-end reconstruction is usually needed after the hepatectomy when the wall of the main portal vein is invaded by the tumor thrombus or if the removal is difficult. However, this concept is not recommended by the study based on the findings of the Japanese registry, and the result showed that when the tumor thrombus lies beyond the liver resection line or invades the main portal vein wall (type Ⅲ/Ⅳ PVTT), there was no significant improvement in survival after surgical treatment.

Efficacy of different surgical methods

Several studies compared the surgical approaches, complications, survival benefit, and recurrence between hepatectomy with thrombectomy and hepatectomy with *en bloc* resection. Most of reports showed that hepatectomy plus thrombectomy or *en bloc* resection was not significantly different no matter for patients with Vp4 or Vp3 PVTT when compared for overall survival and disease-free survival at 1, 3, and 5 years, but hepatectomy had a lower mortality and morbidity[26,27]. While some other studies suggested that thrombectomy had a risk of residual tumor and hepatectomy plus *en bloc* resection with or without reconstruction of the main portal vein would theoretically improve the oncological outcomes. Another typical study[28] also revealed that the 5-year overall survival and disease-free survival rates were not significantly decreased in HCC patients with PVTT extended to the bifurcation of the portal vein if *en bloc* resection was selected. However, the decision of such surgical manipulations could be based on various factors including the surgeon's expertise in portal vein resection and reconstruction as well as the nature of the thrombus and no randomized controlled trial had been done to compare these techniques.

"THROMBECTOMY FIRST" INSTEAD OF "LIVER RESECTION FIRST"

Most surgical procedures are "liver resection first"

Most of the studies report that the prognosis is poor for Vp4 or Types Ⅲ and Ⅳ patients with HCC and PVTT. Shi *et al*[25] compared the surgical effects of Type Ⅰ/Ⅱ and Type Ⅲ/Ⅳ PVTT and found that the overall survival and disease-free survival rates of patients with type Ⅲ or Ⅳ PVTT were significantly worse than those of type Ⅰ or Ⅱ. The 1- and 3-year overall survival and disease-free survival rates of 78 Type Ⅲ patients were 24.7% and 3.6% and 3.0% and 0%, respectively. The 1- and 3-year overall survival and disease-free survival rates of 20 Type Ⅳ patients were 18.0% and 0% and 0% and 0%, respectively. In Li's report[27], the 1-, 3-, and 5-year OS and DFS rates for the patients whose PVTT extended beyond the resection line were 42.6%, 11.4%, and 5.7% and 15.4%, 5.1%, and 5.1%, respectively.

We analyzed these reports and found that almost all of them performed the thrombectomy after the liver resection. Only Daisuke[19] performed the removal of PVTT prior to the mobilization and transection of the liver, and the MST and OS were much higher than those of the other studies. The MST of 19 Vp4 patients was 28 mo and the 3- and 5-year overall survival rates were 41.8% and 20.9%, respectively. The MST of 26 Vp3 patients was 18 mo and the 3- and 5-year overall survival rates were 25.2% and 21.2%, respectively.

Efficacy of "thrombectomy first" instead of "liver resection first"

We have performed surgical treatments for Types Ⅲ and Ⅳ PVTT since 2003, and we proposed a "thrombectomy first" surgical concept to provide superior surgical therapeutic effects and simplify the management approach. The tumor thrombus in the main portal vein, the bifurcation or the contralateral portal vein would be removed prior to liver resection. The main advantages of this concept are as follows: (1) Compression and crushing of PVTT during the operative process could be avoided; thus, new intrahepatic metastases caused by tumor scattering from the PVTT to the remnant liver were minimized; (2) The contralateral portal venous flow could be recovered at an early stage of the operation, which is a beneficial measure for the preservation of remnant liver function; and (3) The portal venous pressure decreases as a result of complete removal of tumor thrombus.

The following considers a right hemihepatectomy as an example to explain the detailed surgical process. The main portal vein (MPV) and the right portal vein (RPV) were clearly revealed by dissecting the fascia and Glisson sheath along the right edge of the hepatoduodenal ligament until the point where it entered the right liver. The tissues in the front were then pushed forward to fully expose the anterior wall of the RPV. Both the right hepatic artery (HA) and the right hepatic duct (HD) were then cut and ligated separately with a 1 cm distance from the entry point of the right liver (Figure 1A and B). The right HA and right HD along with the anterior tissues of the Glisson sheath was called the hepatic artery-duct flap (HA-HD flap) (Figures 2 and 3). The left hepatic portal vein was revealed by pulling up and dissecting the flap, and a small branch of the portal vein that entered the caudate lobe was cut and ligated in this process. The

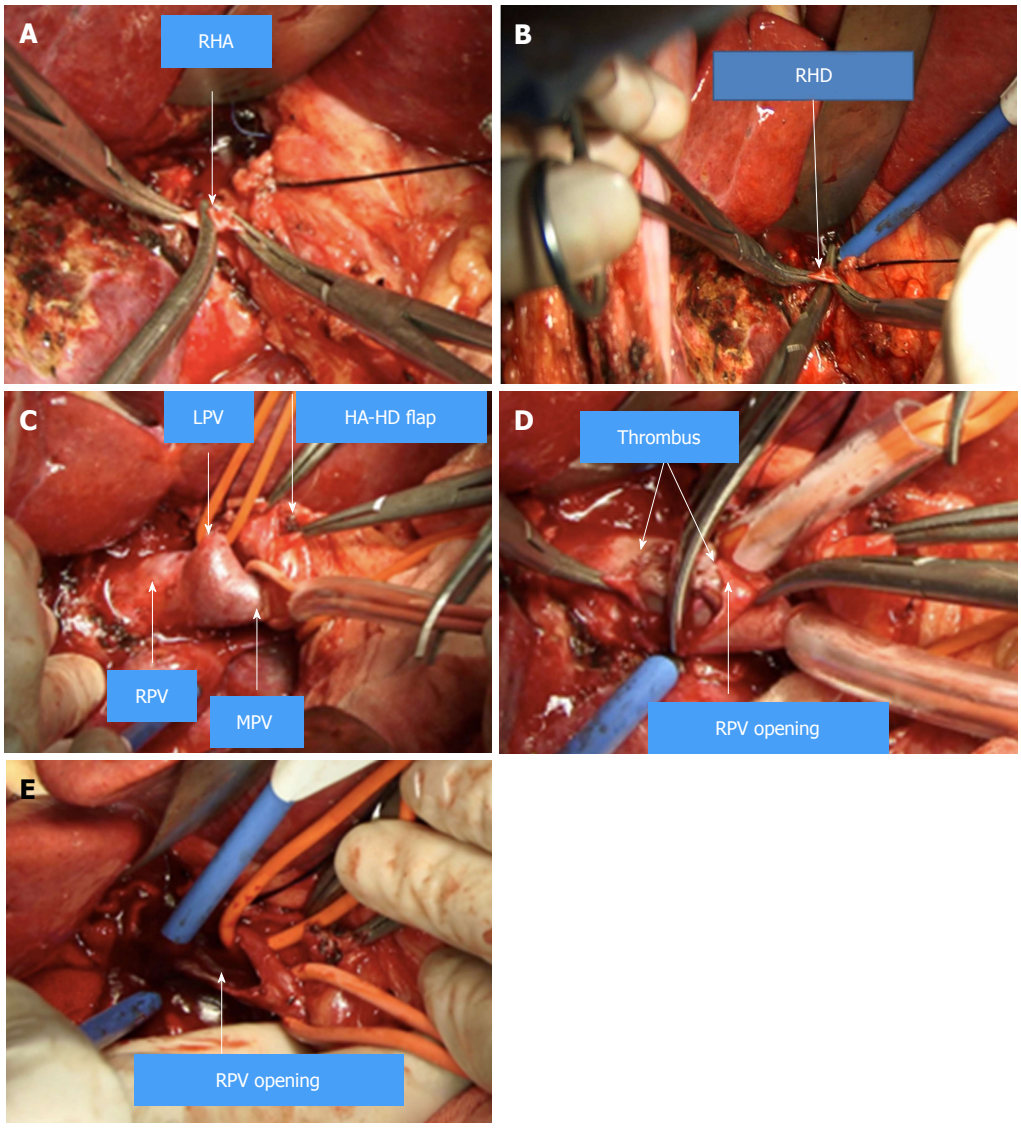


Figure 1 The "thrombectomy first" approach. A: The right hepatic artery (RHA) was isolated and dissected. B: The right hepatic duct (RHD) was isolated and dissected. C: The left portal vein (LPV), right portal vein (RPV) and the main portal vein (PV) were clearly revealed by dissecting the hepatic artery-hepatic duct (HA-HD) flap. D: The tumor thrombus in the RPV and main portal vein (MPV) was extracted. E: The tape of LPV and PV were both released to flush the remnant tumor thrombus. RHA: Right hepatic artery; RHD: Right hepatic duct; LPV: Left portal vein; RPV: Right portal vein; PV: Portal vein; MPV: Main portal vein; HA-HD: Hepatic artery-hepatic duct.

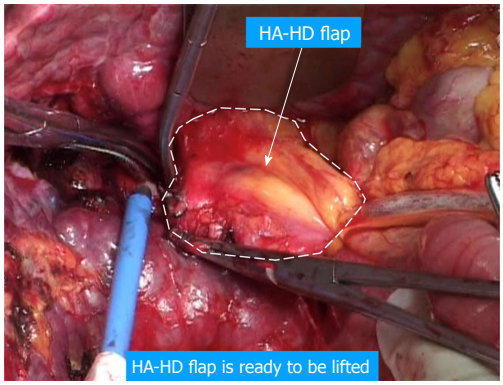


Figure 2 The hepatic artery-hepatic duct flap was ready to be lifted. HA-HD: Hepatic artery-hepatic duct.

dissection and taping of the RPV, the left portal vein (LPV) and MPV by vascular slings continued prior to ligation of the RPV distant to the bifurcation, followed by clamping of the LPV and MPV distal to the PVTT (Figure 1C). The anterior wall of the RPV was opened to a 1 cm distance to the confluence (the incision could be extended to the portal trunk longitudinally if necessary). The PVTT in the RPV and MPV was then extracted from the opening (Figure 1D), followed by flushing the potential residual tumor thrombus by the portal vein blood flow by releasing the vascular occlusion band of the MPV. The MPV was occluded again, and the PVTT in the LPV was extracted. The left remnant tumor thrombus was flushed by the reverse portal vein blood through releasing the vascular occlusion band of LPV (the left hepatic artery was not occluded), and a catheter was used to help flushing when necessary. After flushing and confirming that no PVTT remained (Figure 1E), the stump was closed by a

Table 1 Clinical characteristics of the three cases

Gender	Age	HBV	Tumor location	Tumor size	PVTT classification	Operation	Pre-op treatment	Post-op treatment	Tumor recurrence	Survival time (mo)
M	40	+	Right liver	8 cm × 6 cm	Type Ⅳ or Vp4 (right branch extending to the main PV)	Thrombectomy and right hemihepatectomy	TACE (once)	TACE (4 times)	No	162 (still alive)
M	52	+	Right liver	6 cm × 6 cm	Type Ⅲ or Vp4 (left and right branch)	Thrombectomy and right hemihepatectomy	TACE (once)	TACE (twice)	No	108 (still alive)
M	50	+	Left liver	20 cm × 16 cm	Type Ⅳ or Vp4 (left branch and main PV)	Thrombectomy and left hemihepatectomy	No	TACE (once)	No	55 (still alive)

M: Male; HBV: Hepatitis B virus; TACE: Transcatheter arterial chemoembolization; PV: Portal vein; PVTT: Portal vein tumor thrombus.

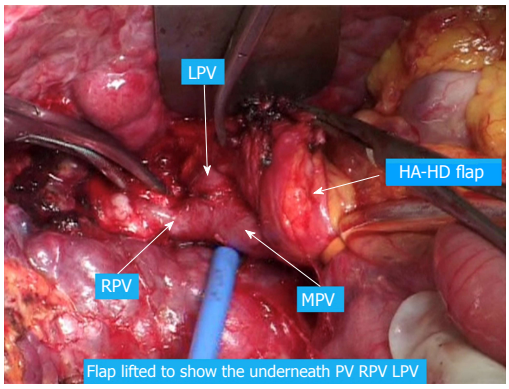


Figure 3 The hepatic artery-hepatic duct flap was lifted to show the portal vein, right portal vein, and left portal vein. RPV: Right portal vein; LPV: Left portal vein; MPV: Main portal vein; HA-HD: Hepatic artery-hepatic duct.

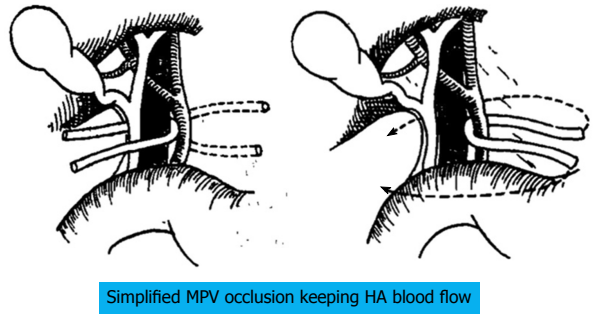


Figure 4 A simplified method to occlude the main portal vein while keeping hepatic artery blood flow. MPV: Main portal vein; HA: Hepatic artery.

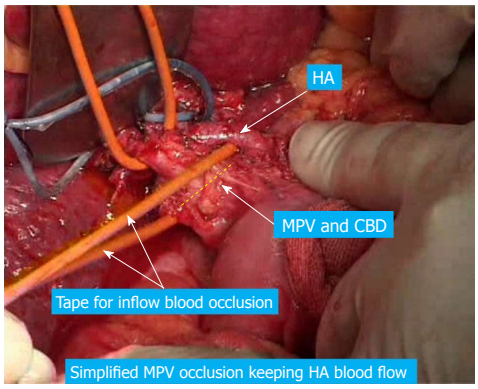


Figure 5 A simplified method to occlude the main portal vein while keeping hepatic artery blood flow. HA: Hepatic artery; MPV: Main portal vein; CBD: Common bile duct.

continuous Prolene suture, and the vascular occlusion was released. If the tumor thrombus strongly adhered to the portal vein wall, resection of the portal vein would be required. Direct end-to-end anastomosis was conducted to reconstruct the portal vein with the use of a 6/0 Prolene continuous suture. After suturing, the right liver was mobilized and transected *via* standard procedure, or the anterior approach was performed using PMOD (Peng's Multiple Operative Dissector). To achieve long-term survival, the thrombectomy must be completely cleared. A key element to successfully performing the operation is doing so with no time pressure to prevent sloppiness. When blocking the MPV, the HA blood flow should be not occluded to protect remnant liver function. If the MPV was difficult to dissect, the selected portal vein occlusion method was used by isolating the common hepatic artery and occluding the portal trunk and common hepatic duct together (Figures 4 and 5).

We have performed the "thrombectomy first" approach at two campuses of the Second Hospital affiliated with Zhejiang University School of Medicine, two campuses of the Sir Run Run Shaw Hospital affiliated with Zhejiang University School of Medicine and Yuebei People's Hospital affiliated with Shantou University School of Medicine for roughly 15 years. Detailed data is still being collected and analyzed (unpublished), but we would like to share three typical Cheng's Type Ⅲ/Ⅳ (Vp4) cases that had long-term survival. The disease-free survival time was 13, 9 and 4.6 years, respectively (Table 1).

Case 1 (Figure 6, the pictures were taken by camera 13 years ago and had poor quality): A 40-year-old man had HBV infection for more than 10 years. The CT scan showed an 8 cm × 6 cm mass with satellite lesions in the right liver, and tumor thrombus was found in the right branch of the portal vein and extended to the MPV, and the liver function was Class A according to Child-Pugh classification. The patient was diagnosed with HCC-PVTT and received TACE once before surgery.

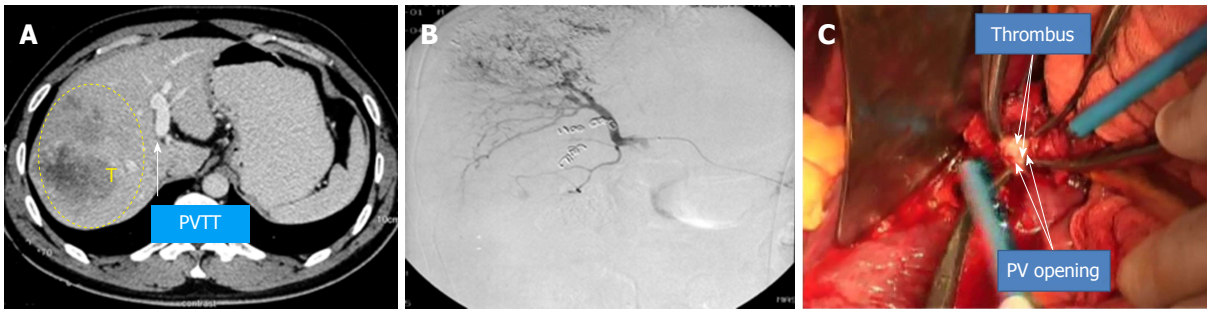


Figure 6 Case 1. A: The computed tomography scan showed tumor thrombus in the right portal vein. B: Transcatheter arterial chemoembolization was performed before the operation. C: The thrombus was extracted from the right portal vein opening. PV: Portal vein; PVTT: Portal vein tumor thrombus.

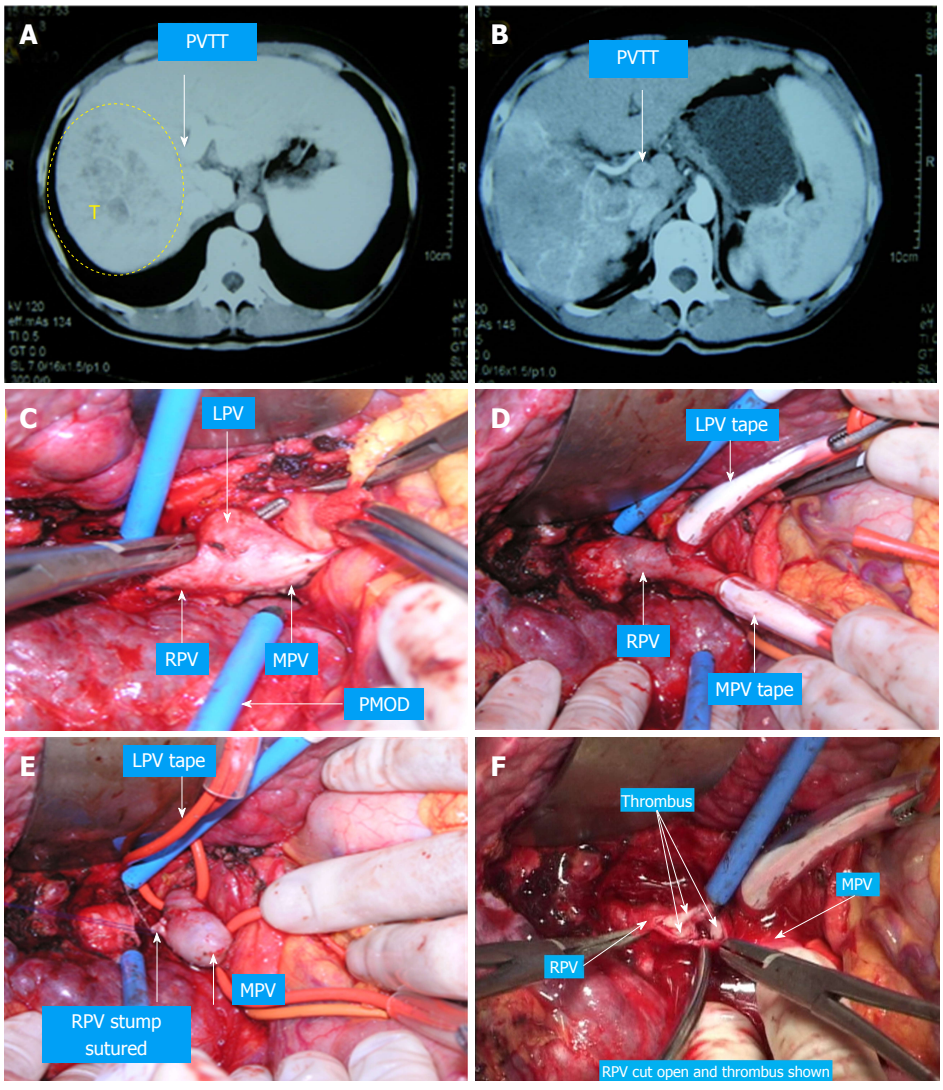


Figure 7 Case 2. A: The computed tomography scan showed tumor thrombus in the right portal vein (RPV) and portal vein (PV). B: Transcatheter arterial chemoembolization was performed before the operation. C and D: The left portal vein (LPV), RPV and PV were clearly revealed by dissecting the hepatic artery-hepatic duct flap. E: The LPV and PV were taped. F: Tumor thrombus in the RPV and PV was extracted. G: The tumor thrombus in the LPV was extracted, and the RPV stump was closed. H: Live transection was performed by the anterior approach. PV: Portal vein; PVTT: Portal vein tumor thrombus; RPV: Right portal vein; LPV: Left portal vein; MPV: Main portal vein; HA-HD: Hepatic artery-hepatic duct.

The "thrombectomy first" approach plus right hemi-hepatectomy was performed in February 2005. The patient recovered smoothly after surgery and continued with TACE treatment four times after the operation. The patient is still alive and working normally without recurrence.

Case 2 (Figure 7): A 52-year-old man had HBV infection for more than 15 years. The CT scan showed a 6

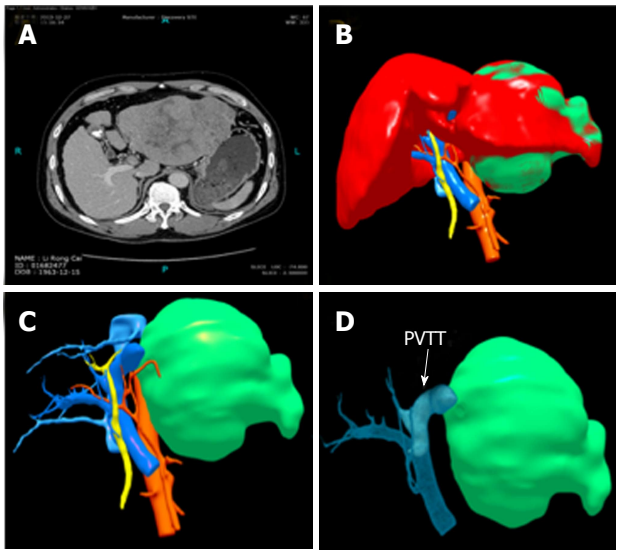


Figure 8 Case 3. A: The computed tomography scan showed the left hepatocellular carcinoma (HCC) and tumor thrombus in the left portal vein (LPV) and portal vein (PV). B and C: 3D reconstruction of the left HCC. D: 3D reconstruction of tumor thrombus in the LPV and PV. PVTT: Portal vein tumor thrombus.

cm × 6 cm mass in the right liver, a 3 cm × 3 cm tumor thrombus in the right branch of the portal vein, and two tumors approximately 0.9 cm × 1.0 cm and thrombus in the left branch. The liver function was Class A according to Child-Pugh classification. The "thrombectomy first" approach plus right hemihepatectomy was performed in August 2009. The patient recovered without any complications and was treated with TACE twice postoperatively. The patient is still alive and working normally without recurrence.

Case 3 (Figure 8): A 50-year-old man had HBV infection for more than 10 years. The CT scan showed a huge mass in the left liver larger than 20 cm × 16 cm, and tumor thrombus was found in the left branch of the portal vein and the main portal vein. The liver function was Child-Pugh Class A. The "thrombectomy first" approach plus left hemihepatectomy was performed in December 2013. The patient received anti-HBV treatment and TACE once after the operation. He is still alive without recurrence.

We usually classify PVTT as follows: (1) Segmental PVTT (thrombus confined in a segment); (2) Lobar PVTT (thrombus confined in the right/left lobe), which is then subdivided into R lobar and L lobar; (3) Main PVTT (thrombus extending to main trunk portal vein), which is then subdivided into R Main PVTT and L Main PVTT; (4) R + M PVTT (Right PVTT extending to both main trunk and left portal vein); and (5) L + M PVTT (Left PVTT extending to both main trunk and right portal vein). Accordingly, case one belongs to R Main PVTT; case two belongs to R + Main PVTT; case three belongs to L Main PVTT. Our classification seems more helpful for determining the method of surgical intervention when compared with other classifications. It is simpler and easier to remember, and thus, more practical for clinical use.

CONCLUSION

HCC with PVTT is not a surgical contraindication, and the accumulation of experience and development of technology favor surgical treatments. Treatment guidelines led by China and Japan have confirmed the therapeutic effects of surgical treatment in Type Ⅰ/Ⅱ, but the benefit of surgical treatment for Type Ⅲ/Ⅳ is still controversial. Through our experience, we have found that the appropriate surgical approach, especially the new "thrombectomy first" method, would remarkably improve the survival outcomes in patients with Cheng's Ⅲ/Ⅳ PVTT.

ACKNOWLEDGMENTS

Many thanks to Yuan-Quan Yu, Yun Jin, Guo-Jun Chen and Xiao-Long Liu for their help collecting the data for the three cases.

REFERENCES

1 **Llovet JM**, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M, Brú C, Rodés J, Bruix J. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. *Hepatology* 1999; **29**: 62-67 [PMID: 9862851 DOI: 10.1002/hep.510290145]

2 **Forner A**, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; **379**: 1245-1255 [PMID: 22353262 DOI: 10.1016/S0140-6736(11)61347-0]

3 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

4 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

5 **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 DOI: 10.1016/j.jhep.2011.12.001]

6 **Cheng S**, Chen M, Cai J; National Research Cooperative Group for Diagnosis and Treatment of Hepatocellular Carcinoma with Tumor Thrombus. Chinese expert consensus on multidisciplinary diagnosis and treatment of hepatocellular carcinoma with portal vein tumor thrombus: 2016 edition. *Oncotarget* 2017; **8**: 8867-8876 [PMID: 27780939 DOI: 10.18632/oncotarget.12817]

7 **Kudo M**, Matsui O, Izumi N, Iijima H, Kadoya M, Imai Y, Okusaka T, Miyayama S, Tsuchiya K, Ueshima K, Hiraoka A, Ikeda M, Ogasawara S, Yamashita T, Minami T, Yamakado K; Liver Cancer Study Group of Japan. JSH Consensus-Based Clinical Practice Guidelines for the Management of Hepatocellular Carcinoma: 2014 Update by the Liver Cancer Study Group of Japan. *Liver Cancer* 2014; **3**: 458-468 [PMID: 26280007 DOI: 10.1159/000343875]

8 **Ho MC**, Hasegawa K, Chen XP, Nagano H, Lee YJ, Chau GY, Zhou J, Wang CC, Choi YR, Poon RT, Kokudo N. Surgery for Intermediate and Advanced Hepatocellular Carcinoma: A

Consensus Report from the 5th Asia-Pacific Primary Liver Cancer Expert Meeting (APPLE 2014). *Liver Cancer* 2016; **5**: 245-256 [PMID: 27781197 DOI: 10.1159/000449336]

9 **Kokudo T**, Hasegawa K, Matsuyama Y, Takayama T, Izumi N, Kadoya M, Kudo M, Ku Y, Sakamoto M, Nakashima O, Kaneko S, Kokudo N; Liver Cancer Study Group of Japan. Survival benefit of liver resection for hepatocellular carcinoma associated with portal vein invasion. *J Hepatol* 2016; **65**: 938-943 [PMID: 27266618 DOI: 10.1016/j.jhep.2016.05.044]

10 **Wang K**, Guo WX, Chen MS, Mao YL, Sun BC, Shi J, Zhang YJ, Meng Y, Yang YF, Cong WM, Wu MC, Lau WY, Cheng SQ. Multimodality Treatment for Hepatocellular Carcinoma With Portal Vein Tumor Thrombus: A Large-Scale, Multicenter, Propensity Mathching Score Analysis. *Medicine (Baltimore)* 2016; **95**: e3015 [PMID: 26986115 DOI: 10.1097/MD.0000000000003015]

11 **Kojima H**, Hatano E, Taura K, Seo S, Yasuchika K, Uemoto S. Hepatic Resection for Hepatocellular Carcinoma with Tumor Thrombus in the Major Portal Vein. *Dig Surg* 2015; **32**: 413-420 [PMID: 26316188 DOI: 10.1159/000437375]

12 **Shi J**, Lai EC, Li N, Guo WX, Xue J, Lau WY, Wu MC, Cheng SQ. Surgical treatment of hepatocellular carcinoma with portal vein tumor thrombus. *Ann Surg Oncol* 2010; **17**: 2073-2080 [PMID: 20131013 DOI: 10.1245/s10434-010-0940-4]

13 **Chen XP**, Qiu FZ, Wu ZD, Zhang ZW, Huang ZY, Chen YF, Zhang BX, He SQ, Zhang WG. Effects of location and extension of portal vein tumor thrombus on long-term outcomes of surgical treatment for hepatocellular carcinoma. *Ann Surg Oncol* 2006; **13**: 940-946 [PMID: 16788755 DOI: 10.1245/ASO.2006.08.007]

14 **Minagawa M**, Makuuchi M. Treatment of hepatocellular carcinoma accompanied by portal vein tumor thrombus. *World J Gastroenterol* 2006; **12**: 7561-7567 [PMID: 17171782 DOI: 10.3748/wjg.v12.i47.7561]

15 **Peng ZW**, Guo RP, Zhang YJ, Lin XJ, Chen MS, Lau WY. Hepatic resection versus transcatheter arterial chemoembolization for the treatment of hepatocellular carcinoma with portal vein tumor thrombus. *Cancer* 2012; **118**: 4725-4736 [PMID: 22359112 DOI: 10.1002/cncr.26561]

16 **Xue TC**, Xie XY, Zhang L, Yin X, Zhang BH, Ren ZG. Transarterial chemoembolization for hepatocellular carcinoma with portal vein tumor thrombus: a meta-analysis. *BMC Gastroenterol* 2013; **13**: 60 [PMID: 23566041 DOI: 10.1186/1471-230X-13-60]

17 **Liu PH**, Lee YH, Hsia CY, Hsu CY, Huang YH, Chiou YY, Lin HC, Huo TI. Surgical resection versus transarterial chemoembolization for hepatocellular carcinoma with portal vein tumor thrombosis: a propensity score analysis. *Ann Surg Oncol* 2014; **21**: 1825-1833 [PMID: 24499831 DOI: 10.1245/s10434-014-3510-3]

18 **Yamamoto Y**, Ikoma H, Morimura R, Shoda K, Konishi H, Murayama Y, Komatsu S, Shiozaki A, Kuriu Y, Kubota T, Nakanishi M, Ichikawa D, Fujiwara H, Okamoto K, Sakakura C, Ochiai T, Otsuji E. Post-hepatectomy survival in advanced hepatocellular carcinoma with portal vein tumor thrombosis. *World J Gastroenterol* 2015; **21**: 246-253 [PMID: 25574098 DOI: 10.3748/wjg.v21.i1.246]

19 **Ban D**, Shimada K, Yamamoto Y, Nara S, Esaki M, Sakamoto Y, Kosuge T. Efficacy of a hepatectomy and a tumor thrombectomy for hepatocellular carcinoma with tumor thrombus extending to the main portal vein. *J Gastrointest Surg* 2009; **13**: 1921-1928 [PMID: 19727969 DOI: 10.1007/s11605-009-0998-0]

20 **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase Ⅲ randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

21 **Jeong SW**, Jang JY, Shim KY, Lee SH, Kim SG, Cha SW, Kim YS, Cho YD, Kim HS, Kim BS, Kim KH, Kim JH. Practical effect of sorafenib monotherapy on advanced hepatocellular carcinoma and portal vein tumor thrombosis. *Gut Liver* 2013; **7**: 696-703 [PMID: 24312711 DOI: 10.5009/gnl.2013.7.6.696]

22 **Nakazawa T**, Hidaka H, Shibuya A, Okuwaki Y, Tanaka Y, Takada J, Minamino T, Watanabe M, Kokubu S, Koizumi W. Overall survival in response to sorafenib versus radiotherapy in unresectable hepatocellular carcinoma with major portal vein tumor thrombosis: propensity score analysis. *BMC Gastroenterol* 2014; **14**: 84 [PMID: 24886354 DOI: 10.1186/1471-230X-14-84]

23 **Ikai I**, Yamamoto Y, Yamamoto N, Terajima H, Hatano E, Shimahara Y, Yamaoka Y. Results of hepatic resection for hepatocellular carcinoma invading major portal and/or hepatic veins. *Surg Oncol Clin N Am* 2003; **12**: 65-75, ix [PMID: 12735130 DOI: 10.1016/S1055-3207(02)00082-0]

24 **Kudo M**, Izumi N, Kokudo N, Matsui O, Sakamoto M, Nakashima O, Kojiro M, Makuuchi M; HCC Expert Panel of Japan Society of Hepatology. Management of hepatocellular carcinoma in Japan: Consensus-Based Clinical Practice Guidelines proposed by the Japan Society of Hepatology (JSH) 2010 updated version. *Dig Dis* 2011; **29**: 339-364 [PMID: 21829027 DOI: 10.1159/000327577]

25 **Shi J**, Lai EC, Li N, Guo WX, Xue J, Lau WY, Wu MC, Cheng SQ. A new classification for hepatocellular carcinoma with portal vein tumor thrombus. *J Hepatobiliary Pancreat Sci* 2011; **18**: 74-80 [PMID: 20686792 DOI: 10.1007/s00534-010-0314-0]

26 **Chok KS**, Cheung TT, Chan SC, Poon RT, Fan ST, Lo CM. Surgical outcomes in hepatocellular carcinoma patients with portal vein tumor thrombosis. *World J Surg* 2014; **38**: 490-496 [PMID: 24132826 DOI: 10.1007/s00268-013-2290-4]

27 **Shaohua L**, Qiaoxuan W, Peng S, Qing L, Zhongyuan Y, Ming S, Wei W, Rongping G. Surgical Strategy for Hepatocellular Carcinoma Patients with Portal/Hepatic Vein Tumor Thrombosis. *PLoS One* 2015; **10**: e0130021 [PMID: 26076461 DOI: 10.1371/journal.pone.0130021]

28 **Wu CC**, Hsieh SR, Chen JT, Ho WL, Lin MC, Yeh DC, Liu TJ, P'eng FK. An appraisal of liver and portal vein resection for hepatocellular carcinoma with tumor thrombi extending to portal bifurcation. *Arch Surg* 2000; **135**: 1273-1279 [PMID: 11074879 DOI: 10.1001/archsurg.135.11.1273]

P- Reviewer: Tomizawa M **S- Editor**: Ma RY **L- Editor**: Wang TQ
E- Editor: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4536-4547

DOI: 10.3748/wjg.v24.i40.4536

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

MINIREVIEWS

Dimensions of hepatocellular carcinoma phenotypic diversity

Romain Désert, Natalia Nieto, Orlando Musso

Romain Désert, Orlando Musso, Institut NuMeCan, Université de Rennes 1, Institut national de la recherche agronomique (INRA), Institut national de la santé et de la recherche médicale (INSERM), Rennes F-35000, France

Romain Désert, Natalia Nieto, Department of Pathology, Department of Medicine (Gastroenterology and Hepatology), University of Illinois at Chicago, IL 60612, United States

ORCID number: Romain Désert (0000-0003-3787-3801); Natalia Nieto (0000-0002-2706-0295); Orlando Musso (0000-0001-8881-6925).

Author contributions: Désert R and Musso O designed research; Désert R, Nieto N and Musso O wrote and edited the manuscript; Désert R and Musso O designed figures and tables.

Supported by INSERM (to Musso O); United States Department of Defense Office of the Congressionally Directed Medical Research Programs Grant, No. CA170172 (to Nieto N and Désert R).

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Correspondence to: Orlando Musso, MD, PhD, Senior Scientist, U-1241 Institut NuMeCan, Université de Rennes 1, INRA, INSERM, Nutrition, Métabolismes et Cancer, Hôpital Pontchaillou, rue Henri Le Guilloux, Rennes F-35000, France. orlando.musso@inserm.fr
Telephone: +33-2-23234565
Fax: +33-2-99540137

Received: July 3, 2018
Peer-review started: July 4, 2018
First decision: August 1, 2018
Revised: August 31, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

Hepatocellular carcinoma (HCC) is the 3rd leading cause of cancer-related death worldwide. More than 80% of HCCs arise within chronic liver disease resulting from viral hepatitis, alcohol, hemochromatosis, obesity and metabolic syndrome or genotoxins. Projections based on Western lifestyle and its metabolic consequences anticipate a further increase in incidence, despite recent breakthroughs in the management of viral hepatitis. HCCs display high heterogeneity of molecular phenotypes, which challenges clinical management. However, emerging molecular classifications of HCCs have not yet formed a unified corpus translatable to the clinical practice. Thus, patient management is currently based upon tumor number, size, vascular invasion, performance status and functional liver reserve. Nonetheless, an impressive body of molecular evidence emerged within the last 20 years and is becoming increasingly available to medical practitioners and researchers in the form of repositories. Therefore, the aim this work is to review molecular data underlying HCC classifications and to organize this corpus into the major dimensions explaining HCC phenotypic diversity. Major efforts have been recently made worldwide toward a unifying "clinically-friendly" molecular landscape. As a result, a consensus emerges on three major dimensions explaining the HCC heterogeneity. In the first dimension, tumor cell proliferation and differentiation enabled allocation of HCCs to two major classes presenting profoundly different clinical aggressiveness. In the second dimension, HCC microenvironment and tumor immunity underlie recent therapeutic breakthroughs prolonging patients' survival. In the third dimension,

metabolic reprogramming, with the recent emergence of subclass-specific metabolic profiles, may lead to adaptive and combined therapeutic approaches. Therefore, here we review recent molecular evidence, their impact on tumor histopathological features and clinical behavior and highlight the remaining challenges to translate our cognitive corpus into patient diagnosis and allocation to therapeutic options.

Key words: Liver metabolism; Liver zonation; Hepatocellular carcinoma classification; Wnt/β-catenin; *TP53*; Tumor microenvironment; Inflammation; Tumor immunity; Hepatocyte proliferation; Hepatocyte differentiation

Core tip: Recent work revealed substantial steps toward a unifying molecular classification of human hepatocellular carcinomas. The expected translation of high-throughput assays to the clinical practice will further refine evidence-based patient management in terms of prognosis and response to treatment.

Désert R, Nieto N, Musso O. Dimensions of hepatocellular carcinoma phenotypic diversity. *World J Gastroenterol* 2018; 24(40): 4536-4547 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4536.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4536

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide[1] and its incidence has doubled over the past 20 years in Western countries. More than 80% of HCCs emerge within chronic inflammatory liver diseases resulting from chronic viral hepatitis, alcohol abuse, hemochromatosis, obesity and metabolic syndrome or DNA-damaging food or environmental toxins. Based on the current epidemics of obesity and metabolic syndrome in the Western world, projections anticipate an increase in HCC incidence, despite recent breakthroughs in the management of viral hepatitis[2]. HCCs display a high heterogeneity of molecular phenotypes, which raises major challenges in clinical management[3]. Currently, patient management is based on tumor number, size, vascular invasion, performance status and functional reserve of the liver[2]. A further obstacle complicating the understanding of basic human HCC biology (and of patient management) is that liver biopsy cannot routinely be used for HCC diagnosis because of the risk of complications[4,5]. Thus, surveillance for HCC emergence in patients with chronic liver diseases relies on biannual ultrasound or magnetic resonance imaging (MRI). As a surrogate for tumor liver biopsy, functional MRI-based metabolic imaging will become increasingly relevant for diagnosis and allocation to treatments in early-stage HCCs[6-8]. Surveillance programs currently detect small early-stage tumors that are candidates for potentially curative therapies (local ablation, resection or transplantation) with 5-year survival rates of approximately 50%-70%. However, high 5-year recurrence rates (up to 70%) after HCC resection or even higher after percutaneous ablation make liver transplantation the best possible treatment, with a recurrence rate of 10%[2]. As a result, the prediction of early recurrence and of the potential for cancer aggressiveness are key factors leading patient eligibility for liver transplantation[4]. Therefore, gaining insight into the diversity of HCC phenotypes and deepening our understanding of the mechanisms of progression from low-grade HCCs to aggressive tumors, that will ultimately kill the patient, will shed basic knowledge to improve patient management[4,7,9]. Systemic therapies for intermediate and advanced HCC patients not eligible for surgical approaches have made encouraging progress in the past 10 years. Sorafenib was the first systemic therapy to be approved in HCC[10-12]. Then, regorafenib and nivolumab were approved in the second-line setting after sorafenib, significantly improving the original survival benefit[13]. Importantly, HCC patients presenting beyond transplant or resection eligibility may benefit from locoregional therapies [tumor ablation, transarterial chemoembolization (TACE) and radioembolization with yttrium-90 microspheres (Y90)], according to recent Clinical Practice Guidelines[14]. Whereas tumor ablation is recommended as a first-line therapy for early-stage HCC[14]; TACE has been recommended for intermediate-stage HCC[14]. Y90 has been investigated as an alternative to TACE, with a good safety profile in delaying tumor progression[15] and has been proposed as a treatment of choice for down-staging HCC patients as a bridging strategy toward liver transplantation[16]. However, Y90 has not shown overall survival benefit over sorafenib in intermediate and locally advanced HCC patients after unsuccessful TACE[14,17].

The well-recognized phenotypic and genetic heterogeneity of HCCs occurs after a marathon of cellular changes driven by chronic inflammation, which progressively leads to severe fibrosis and profound remodeling of the tissue architecture. The two major consequences of this process are impaired liver function and the emergence of HCC[18]. In fact, chronic liver inflammation and progressive liver fibrosis generate a pro-tumorigenic microenvironment known as "the field effect"[19,20], whereby the diseased liver turns into a "minefield" riddled with pre-neoplastic and neoplastic foci. This longtime process can explain the relatively important number of mutations within each tumor, approximately 40[21-23], and the high heterogeneity between patients, as well as the frequent intra-tumor heterogeneity[3,24,25].

HCC heterogeneity is also amplified by the potential multiplicity of cell origin. One possibility for hepatocellular carcinogenesis is a well-established multistep process

Table 1 Molecular classifications of human hepatocellular carcinomas

First author	Year	Number of HCCs	Number of groups	Class/subclass names	Ref.
Lee	2004	91	2	Cluster A-Cluster B	[36,37]
Boyault	2006	56	6	G1-G6	[42]
Chiang	2008	91	5	*CTNNB1*-proliferation	[58]
Hoshida	2009	232	3	S1-S3	[59]
Désert	2017	1133	4	PP-PV-ECM-STEM	[43]
TCGA network	2017	559	3	iCluster 1-iCluster 3	[92]

HCCs: Hepatocellular carcinomas; TCGA : The Cancer Genome Atlas; *CTNNB1*: Gene encoding β-catenin; PP: Periportal; PV: Perivenous; ECM: Extracellular matrix; STEM: Stem/progenitor cells.

defined by a precise sequence of lesions, from cirrhosis to low-grade, then high-grade dysplastic nodules followed by early and advanced HCC[9]. This process is strongly enhanced by the *TERT* promoter mutation[26] and by *MYC* activation[27] and may involve retro-differentiation of hepatocytes to liver progenitor cells in an inflammatory environment[28-31]. In a different pathological context, HCCs can result from the malignant transformation of a hepatocellular adenoma carrying exon 3 mutations of *CTNNB1,* the gene encoding β-catenin[32]. Regardless of the case, a parallel can be stablished between the diversity of histopathological patterns of HCCs observed in the routine Anatomic Pathology laboratory and specific molecular programs[33-35].

HCC classifications (Table 1) have revealed multi-dimensional mechanisms leading to HCC heterogeneity. Here, we provide an overview of the major dimensions explaining the HCC phenotypic diversity with the aim of providing a unifying perspective of HCC classifications.

TUMOR CELL PROLIFERATION AND DIFFERENTIATION

Pioneering transcriptomics studies classified 91 HCCs into two groups: Cluster A and Cluster B[36]. Cluster A showed high alpha fetoprotein (AFP), high Edmondson-Steiner's scores indicating low cyto-architectural differentiation and unfavorable survival. These clinical features correlated with high expression of genes associated with cell proliferation and low hepatocyte differentiation. By contrast, genes typical of cluster B were liver-specific, indicating that these tumors were composed of well-differentiated, hepatocyte-like cells. Subsequent studies integrated the gene expression program of the 91-HCC dataset with the orthologous genes from several mouse models[37], whereby poorly differentiated human HCCs matched *Myc-Tgfα* transgenic mice. These mice typically showed early and high incidence rates of HCC development with ensuing high mortality. Their HCCs showed high rates of genomic instability and of expression of transcripts indicating poor prognosis[38]. Poorly differentiated human HCCs also matched a *MET* activation signature in genetically modified mice[39]. Moreover, integration of gene expression data from fetal hepatoblasts and adult hepatocytes with 61 cases of human HCCs revealed a group sharing gene expression patterns with fetal hepatoblasts[40]. These tumors fell into the category of proliferative poorly differentiated HCCs (cluster A). These pioneering studies shed light on a first layer of HCC heterogeneity and set the basis for the well-established classification of HCCs into two classes: non-proliferative and proliferative. Proliferative tumors are in general poorly differentiated, highly aggressive and associated with unfavorable patient outcome. On the other hand, non-proliferative HCCs tend to preserve a certain degree of hepatocyte differentiation and they are associated with more favorable patient outcome[2,9].

Further work searched for underlying dimensions that could explain phenotypic diversity within proliferative and non-proliferative HCCs by combining analysis of tumor transcriptomics' programs and genetic mutations. Fifty-seven HCCs were classified into six groups (G1 to G6) and the expression of genes of interest was confirmed by real time qPCR in an independent collection of 63 HCCs. The aim of this approach was two-fold: first, to gain insight into the mechanisms leading to HCC heterogeneity; second, to find molecular markers enabling researchers, surgeons and oncologists to identify patients who may benefit from adaptive therapeutic approaches. However, one of the major pitfalls of biomarker identification is that the number of features (genes) entering the analysis is exceedingly higher than the number of observations (patients/tumors), which involves the risk of generating overfitting models. Overfitting may lead the classifiers to better describe a particular set of observations, but may not be equally performant to describe and classify sets of observations collected in different contexts. To circumvent this pitfall, statistical methods such as partial least squares are used to reduce the number of discriminant genes[41-43]. Importantly, the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria are applied to warrant that a set of markers performs consistently[44,45]. These include, among other recommendations, the use of training and validation sets to ensure consistency of results, followed by reproducibility studies in external validation cohorts.

Applying these procedures, studies revealed group G1, expressing high AFP levels in young women; group G2 associated with hemochromatosis and both groups G1 and G2 showing *AKT* activation as well as frequent *AXIN1* mutation. G2 and G3 tumors were enriched in *TP53* mutations, G3 containing the most poorly differentiated HCCs. Together, G1, G2 and G3 HCCs shared activation of biological pathways leading to cell proliferation and chromosome instability. G4 was a heterogeneous group of HCCs. Finally, groups G5 and G6 were associated with *CTNNB1* mutation, which occurs in 30%-40% of HCCs, and showed strong expression of β-catenin target genes[9,42]. These tumors

fit within the non-proliferative HCC class[9] because they proliferate at lower rates than G1-G3 tumors and tend to show higher cyto-architectural differentiation. However, two studies analyzing three different cohorts totaling 819 patients demonstrated that HCCs with mutated *CTNNB1* do not differ in clinical outcome from HCCs with wild-type *CTNNB1*[43,46]. In fact, the survival curves and clinical features of patients undergoing resection of HCCs with mutated *CTNNB1* suggest that the intrinsic aggressiveness of these tumors is intermediate between well-differentiated, non-proliferative HCCs with wild-type *CTNNB1* and poorly-differentiated, proliferative HCCs[43,47].

As AXIN1 is part of the molecular scaffold involved in β-catenin inactivation, it was first suggested that *AXIN1* mutations, which occur in 10% of HCCs, would lead to activation of the β-catenin pathway in HCCs[48,49]. However, later studies showed no association between *AXIN1* mutations and activation of the β-catenin pathway in HCCs[50]. Indeed, AXIN1 deficiency induces HCCs in mice in the absence of activation of the β-catenin pathway[51]. HCCs with mutated *AXIN1* differ in histology, genomic signature and outcome from those with *CTNNB1* mutations[51]. Indeed, *AXIN1*-mutated HCCs impact the Notch and YAP pathways[51]. By contrast, although *APC* mutations are infrequent in HCCs (1%-2%), they are associated with activation of the β-catenin pathway[52,53].

The analysis of the clinical features associated with G1 to G6 subgroups in a cohort of 82 HCC patients from Singapore[54] confirmed the significant correlation between high AFP and groups G1 and 2, and showed an association between microvascular invasion and group G3. In a large cohort of 343 HCCs, associations were confirmed between the groups G1-G6 and clinical and pathological features as well as genetic aberrations[34]. G1 tumors were associated will female gender and high AFP, showed strong levels of KRT19, EPCAM and phospho-ERK protein expression. Also, groups G1 and G2 were enriched in *AXIN1* mutations. Group G3 confirmed a high rate of macrovascular invasion and were associated with macrotrabecular massive subtype and poor outcome[33,34]. Groups G1, G2 and G3 had increased AFP serum levels and high *TP53* mutation rates. On the other hand, group G4 was negatively associated with tumor size, vascular invasion, *CTNNB1* and *TP53* mutations and positively associated with the steatohepatitic subtype, inflammatory infiltrates and CRP protein expression. Groups G5 and G6 showed a high *CTNNB1* mutation rate (80%), were positively associated with tumor differentiation and, by immunohistochemistry, were positive for β-catenin in the nuclei and cytoplasm and for GLUL (glutamine synthetase), a β-catenin target gene expressed in well-differentiated hepatocytes[33,34,50]. Finally, patients with G3 HCCs showed early tumor recurrence after resection and poor overall survival by univariate and multivariate analysis in a large cohort of 244 HCCs[55].

Taken together, a vast body of evidence confirms that HCCs can be grouped in two large classes: non-proliferative and proliferative (Figure 1), which are, respectively, well-differentiated and poorly differentiated tumors[2,9,31,47,56-58]. Proliferative and non-proliferative HCCs are respectively characterized by almost mutually exclusive mutational patterns. Indeed, *CTNNB1* and *TP53* mutations rarely coexist; they are respectively associated with the non-proliferative and proliferative HCC classes[31,52,56,57]. Non-proliferative, well-to-moderately differentiated HCCs, with low AFP serum levels match groups G5-G6[42]; S3[59,60] and B[36,40]. Our recent work showed that the class of non-proliferative HCCs can be split into two subclasses: Periportal-type (wild-type *CTNNB1)* and Perivenous-type (mutated *CTNNB1)*, according to their respective metabolic liver zonation programs (Figure 1). Of note, patients undergoing resection of Periportal-type HCCs showed the lowest early (< 2 years) recurrence and the highest survival rates among all HCC patients treated by tumor resection in two cohorts of 247 and 210 subjects[43,47]. At the opposite, proliferative HCCs are poorly differentiated and highly aggressive. They match the G1-G2-G3[42], S1-S2[59,60], A[36,40] and ECM-STEM[43] subclasses and form a consensual class of tumors sharing a common background of large tumor size, high *TP53* mutation rates, with loss of the hepatocyte-like phenotype, high AFP serum levels, extracellular matrix remodeling and angiogenesis[61-63]. Of note, the relationships between tumor size, differentiation, proliferation and development of a rich vascular network enabling tumor growth had been illustrated before the advent of molecular HCC classifications. In fact, modeling of HCC growth revealed two curves which were anti-parallel, but had similar slopes[64]. The first represented the loss of the hepatocyte-like phenotype through the expression of a liver-specific gene marker. The second represented the increase in tumor size and in the density of the vascular network[64]. Further studies revealed that this pattern was concomitant with an increase in extracellular matrix remodeling[65,66], suggesting plasticity of the tumor microenvironment across HCC progression.

TUMOR MICROENVIRONMENT: INFLAMMATION, FIBROSIS AND IMMUNITY

HCC classification based on three publicly available transcriptome datasets (90, 82 and 60 HCCs, respectively) applying three unsupervised clustering methods (hierarchical clustering, non-negative matrix factorization and k-means clustering) defined HCC subclasses independently in the training sets before integration by a subclass mapping algorithm[59]. Three robust subclasses (S1, S2 and S3) were validated in six datasets (totaling 371 HCCs). Further work using whole genome sequencing and transcriptomics in 88 human HCCs confirmed that the outcome of the S1 and S2 subclasses was less favorable than that of the S3 subclass[67]. The S1 subclass was associated with signatures of Wnt/β-catenin,

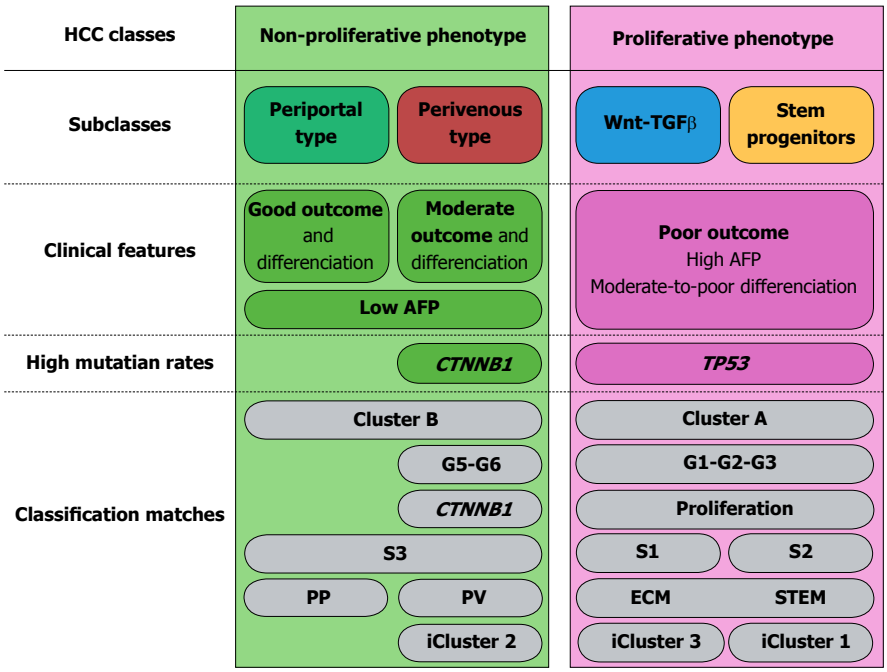


Figure 1 Toward a unifying molecular classification of human hepatocellular carcinomas. Two major hepatocellular carcinoma (HCC) classes, non-proliferative and proliferative can be subdivided into four subclasses. Non-proliferative, well-differentiated HCCs comprise two subclasses with mutually exclusive metabolic features and regulatory signaling pathways: Periportal-type (HNF4α-driven) and Perivenous-type (β-catenin-driven). Proliferative, moderately-to-poorly differentiated HCCs comprise two subclasses: Wnt/TGF-β (regulated by interplays between Wnt and TGF-β ligands, leading to expression of extracellular matrix glycoproteins) and Stem/Progenitors (showing features of liver progenitor cells). Major clinical features, gene mutations and matches between the different HCC classifications are indicated. HCC: Hepatocellular carcinoma; TGF-β: Transforming growth factor beta; AFP: Alpha-fetoprotein; *CTNNB1*: Gene encoding β-catenin; *TP53*: Gene encoding p53; PP: Periportal; PV: Perivenous; ECM: Extracellular matrix; STEM: Stem/progenitor cells.

transforming growth factor beta (TGF-β) activation, epithelial-to-mesenchymal transition, vascular invasion and early recurrence by univariate and multivariate analyses.

It is interesting to note that both the S1 and S3 subclasses showed β-catenin pathway activation[59]. However, the mechanisms of pathway activation and the target genes responding to such stimuli were shown to be quite different. In the S3 subclass of non-proliferating, well-differentiated HCCs, grossly 50% of HCCs carried *CTNNB1* mutations and expressed the so-called "liver-specific" β-catenin target genes, involved in hepatocyte metabolism, such as GLUL[59]. By contrast, the S1 subclass was not enriched in *CTNNB1* mutations and expressed high levels of "classical Wnt genes", *i.e.*, Wnt targets involved in tumor cell proliferation, angiogenesis, epithelial-to-mesenchymal transition and extracellular matrix remodeling, such as *CCND1, VEGF* and *MMP7*[60]. In HCCs of the S3 subclass, activation of the β-catenin pathway results from *CTNNB1* mutations in exon 3. By contrast, in HCCs of the S1 subclass activation of the β-catenin pathway was associated with upregulation in the expression of Wnt ligands, *FZD* receptors and *TGFB1* target genes, that suggested Wnt/TGFB1 pathway cross-talks[60]. *In vitro*, well-differentiated hepatocyte-like HepaRG human HCC cells (wild-type *CTNNB1*), when plated at low density, spontaneously retro-differentiate to liver progenitors through a transient phase of epithelial-to-mesenchymal transition. This process involves translocation of β-catenin to the cell nuclei, indicating activation of the Wnt/β-catenin pathway[68]. These cells express a transcriptomic program that matches the S1 subclass of HCCs[28,29]. *In vitro* modeling of the microenvironment of the S1 subclass of HCC by incubating HepaRG liver progenitor cells with soluble Wnt3a ligand enhances and perpetuates the S1-like HCC phenotype, increasing HCC cell proliferation, invasive activity and driving the expression of extracellular matrix remodeling genes, as well as progenitor/stem cell markers associated with signatures of unfavorable HCC outcome[69]. This molecular phenotype can be reverted *in vitro* with small interfering RNAs targeting β-catenin, as well as with the soluble Wnt inhibitors FZD7_CRD and FZD8_CRD, that block the interaction of Wnt ligands with their cognate FZD receptors[69].

Although HCC are soft, cellular tumors with very scant extracellular matrix, some HCCs contain intratumor foci enriched in extracellular matrix glycoproteins, myofibroblasts and stem/progenitor cell markers that we called "fibrous nests"[69]. HCCs containing fibrous nests express high levels of the Wnt2 ligand, the FZD1 and FZD7 receptors, the Wnt inhibitors SFRP1, 2, 5; DKK1, the β-catenin target genes *CD44*, *LEF1*, *LGR5*, *SOX9*, *GPC3* and, in particular, a minimal signature composed of *COL4A1*, *LAMC1*, *DKK1* and *SFRP1*, associated with poor HCC outcome[69]. Of note, although the Wnt3 ligand is up-regulated in HCCs[70], HCCs containing fibrous nests are enriched in Wnt2 (and not Wnt3)[35].

In fact, Wnt2 is expressed by liver endothelial cells and stimulates liver regeneration from progenitor cells upon tissue damage[71]. In addition, Wnt2 secreted by tumor fibroblasts promotes progression of esophageal cancer[72]. These mechanisms are in line with the evidence that progenitor cell markers predicted outcome in 132 HCC patients undergoing liver transplantation[73].

The above body of evidence points to the clinical relevance of inflammation and the immune response of the host to the emergence and progression of HCCs. Transcriptomics analysis of a training set of 228 and a validation set of 728 HCCs revealed that approximately 25% of the tumors exhibit an immune profile compatible with a favorable response to immunotherapy, *i.e.*, expression of the immune checkpoint modulators Programmed Death-Ligand 1 (CD274, a.k.a., PDL1) and Programmed Cell Death 1 (PDCD1, a.k.a., PD1)[74]. Thus, 25% of HCCs can be considered as the Immune HCC class, which contains two subtypes, exhibiting markers of adaptive T-cell response (active immune response) or of T-cell exhaustion (exhausted immune response). The latter may result from TGFB1 signaling mediating immunosuppression and is associated with S1 *(i.e.*, Wnt/TGFB1-type) HCCs. The potential clinical relevance of these findings resides in the identification of an HCC class that may predict responses to checkpoint inhibitors in terms of survival benefits[75,76]. At least 80% of HCCs worldwide arise in a background of chronic inflammation and immune reactivity; thus, inflammatory mediators in the underlying non-tumor liver impact cancer HCC aggressiveness[19,77]. Cross-talk between the HCC cells and their microenvironment affect numerous biological functions. Therefore, it is not surprising that a wealth of transcriptome-based studies have identified signatures reflecting vascular invasion[78], metastasis[79] or angiogenesis[80] in patients with HCC with poor outcome. HCCs expressing cholangiocarcinoma traits[81], as well as those expressing the stem/biliary epithelial marker *EPCAM*[82,83] had also poor outcome, as well as a subset of HCCs expressing a signature reflecting late *Tgfb1* activation in mice[84]. Most of these signatures were compared in a large cohort of 287 HCCs[55]. The study showed that most of the signatures fell within the same group of tumors, matching G3[42], proliferation class[58], and S1-S2[59] HCC subclasses. Therefore, inflammation, fibrosis and immunity are important components highly related to HCC phenotypic diversity.

LIVER METABOLISM

Cancer cells proliferate and modify their microenvironment, which leads to extracellular matrix degradation, tissue invasion and metastases. These cancer cell functions call for increased energy production and macromolecule synthesis, which explains why malignant cells display a profound reprogramming of their metabolic pathways[85,86]. Non-targeted metabolic profiling on liquid chromatography-mass spectrometry of 50 HCCs revealed increased glycolysis, β-oxidation and gluconeogenesis; with reduced tricarboxylic acid (TCA) cycle activity. These changes were accompanied by increased levels of antioxidant molecules such as glutathione, as well as by lower levels of inflammatory-related polyunsaturated fatty acids. In particular, betaine and propionyl carnitine were proposed as markers to distinguish HCC from chronic hepatitis and cirrhosis[87]. A large-scale multicenter serum metabolite biomarker study identified a metabolite panel of interest in the detection of early HCC in patients at risk[88]. Combined transcriptomics and metabolomics in two tumor collections of 31 and 59 HCCs by gas chromatography-mass spectrometry-based metabolomics similarly showed increased glycolysis over mitochondrial oxidative phosphorylation and, in particular, increased lipid catabolism in the subgroup G1 of HCCs[89]. A transcriptomics study of 2761 metabolic genes in eight microarray datasets gathering 521 human HCCs confirmed down-downregulation of genes involved in physiologic hepatocyte metabolic functions, such as xenobiotic detoxification, fatty acid and amino acid metabolism[90]. The same study identified up-regulation of genes involved in glycolysis, pentose phosphate pathway, nucleotide biosynthesis, TCA cycle, oxidative phosphorylation and glycan metabolism; with several metabolic genes being associated with patient outcome and tumor progression markers[90]. These data fit the Warburg model of energy metabolism in cancer cells, whereby they bypass the TCA cycle and utilize glycolysis as the primary source of energy, that enables a less efficient but faster production of ATP[85,86]. However, up-regulation of genes involved in the TCA cycle[90] suggests that the metabolic landscape in HCCs is much more complex than a simple shift from oxidative phosphorylation to glycolysis, because cancer cells may rely on the TCA cycle for macromolecule synthesis[85]. Also, β-catenin-activated HCCs are not glycolytic, but oxidize fatty acids at high rates as an energy source, under the control of the transcription factor PPARα[91].

The Cancer Genome Atlas (TCGA) research network integrated data from multiple platforms, comprising whole exome sequencing, copy number analysis, RNA sequencing, microRNA sequencing, methylomics and proteomics[57,92]. This network analyzed an international cohort of 363 HCC cases by whole-exome sequencing and DNA copy number and 196 cases by DNA methylation, RNA, miRNA and proteomics analysis (Table 1). This comprehensive work confirmed previous HCC classifications (Figure 1) into two large non-proliferative and proliferative classes and subclasses corresponding to the S1, S2 and S3 subclasses[57,59,60] and identified gene expression changes resulting from mutation or down-regulation by hypermethylation in genes likely to participate in metabolic reprogramming, such as *ALB, APOB* and *CSP1.* In particular, isocitrate dehydrogenase (*IDH*) mutations are associated with poor patient outcome in the S2 HCC subclass[92], in line with the previous demonstration that mutant *IDH* inhibits HNF4α, thus blocking hepatocyte differentiation[93].

Well-differentiated HCCs, *i.e.*, groups B[36,40],

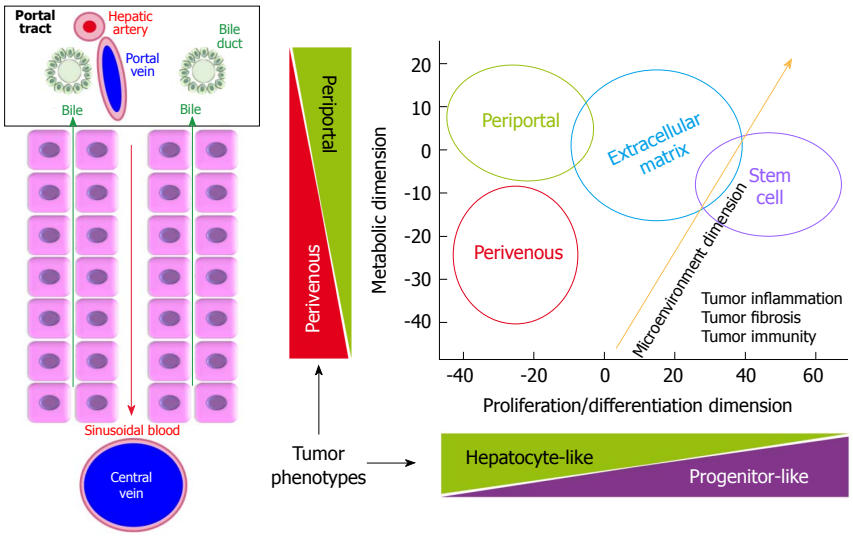


Figure 2 The dimensions of hepatocellular carcinoma phenotypic diversity. In normal liver, the interplay between HNF4A and β-catenin governs the differential distribution of metabolic functions along the portal-to-central vein axis, which is known as liver zonation. The phenotypic spectrum of hepatocellular carcinomas across the increasing proliferation/differentiation ratios is orthogonal to the metabolic dimension, with opposing periportal *vs* perivenous tumor metabolic phenotypes. The third dimension, the tumor microenvironment, comprises specific features of tumor inflammation, fibrosis and immunity characterizing each hepatocellular carcinoma (HCC) subclass. Adapted from Désert *et al*[43].

G5-G6[2,42,54], S3[59,60], iCluster2[57,92] (Figure 1) and morphologically low-grade tumors, as defined by the Edmondson-Steiner's score and other histological classification systems[94-96] are composed of hepatocyte-like cells, which are easily recognizable by routine microscopic analysis of standard hematoxylin-eosin stained tumor tissue slides. Well-differentiated, hepatocyte-like HCCs in these groups were classically described as enriched in *CTNNB1* mutations, because approximately 50% of them presented mutations in the third exon of this gene in humans, thus expressing a perivenous-type metabolic program comprising β-catenin-regulated, liver-specific genes.

In normal liver, the interplay between HNF4α and β-catenin governs the differential distribution of metabolic functions along the periportal to perivenous axis, which is known as liver zonation (Figure 2). Periportal HNF4α-regulated networks include such functions as amino-acid catabolism and gluconeogenesis. At the opposite, perivenous β-catenin-regulated networks include, for example, glycolysis and glutamine synthesis[97-100]. Thus, parenchymal cells near the portal triads are called periportal (PP) hepatocytes; whereas those close to the centrilobular vein are known as perivenous (PV) hepatocytes. We recently showed that well-differentiated HCCs display mutually exclusive liver zonation programs, *i.e.*, they express either a periportal or a perivenous metabolic program[43] and they respectively display periportal (wild-type β-catenin) or perivenous (mutant β-catenin) metabolic phenotypes. Periportal-type HCCs show the highest 2-year recurrence-free survival rates by multivariate analysis, suggesting that these tumors have the lowest potential for early recurrence among all HCCs. They can be identified because they express high levels of an 8-gene signature composed of genes involved in periportal metabolic functions[43]. Periportal, extracellular matrix (ECM) and stem cell (STEM) HCC subclasses seem to be distributed in a continuum across the spectrum of proliferation/differentiation ratios (Figure 2). In addition, orthogonally to the hepatocyte proliferation/differentiation dimension, well-differentiated HCCs distribute across the metabolic zonation dimension[43,47]. This body of evidence indicates that HCC subclasses may show specific metabolic reprogramming profiles.

CONCLUSION

Over the past decade, transcriptome-based classifications increased our knowledge on the molecular heterogeneity of HCCs. They demonstrated that HCCs could be divided into two subtypes of less aggressive tumors (PP and PV) and two subtypes of more aggressive tumors (S1 and S2 or ECM and STEM). They also showed that *TP53* mutation was associated with the most aggressive HCC subtypes and that *CTNNB1* mutation defined a homogenous subtype displaying intermediate outcomes (Figure 1).

We can imagine a future where HCC patients would benefit from high-throughput technologies. However, a major obstacle complicating further understanding of basic human HCC biology and patient management is that liver tumor biopsy cannot routinely be used for HCC diagnosis and follow-up because of the risk of complications. As a surrogate for tumor liver biopsy, MRI-based metabolic imaging may become increasingly relevant for diagnosis and allocation to treatments in early-stage HCCs[6-8]. Future challenges will call for validation of circulating protein markers and molecular studies on liquid biopsies. Data management will call for further bio-statistical refinements, such as defining

standard methods enabling the classification of a single sample by itself, without requiring an entire cohort. But the effort could pay off.

It is however important to bear in mind three major features in the natural history of HCCs. First, these tumors arise in more than 80% of the cases in severely fibrotic livers, with impaired liver function. Second, HCCs show high intra-tumor heterogeneity[24,25] despite a limited number of trunk mutational events[56]. Third, despite their metastatic capacity, HCC may develop locally advanced disease given the vascular anatomy of the liver. The natural history of HCC explains why tumor diagnosis, staging and treatment allocation is based upon tumor size and number, vascular invasion, location with respect to main vascular structures, underlying functional liver reserve and patient's performance status. As a consequence, liver and HCC imaging are thriving fields of research and development. They will benefit from statistical refinements in HCC texture analyses by MRI[7,101,102], in the light of molecular tumor profiles. This body of cognitive data will spur translational efforts toward evidence-based patient management.

ACKNOWLEDGMENTS

The authors thank the following members of the Institut NuMeCan, at INSERM in Rennes, France: Anne Corlu, for helpful discussion and inspiring remarks; Bruno Clément, for critical reading of the manuscript and helpful suggestions; Michèle Le Guennec, Patricia Jouas and Thomas Poussou for efficient secretarial assistance. We thank Christelle Reynès and Robert Sabatier, Biostatistics and Informatics laboratory, Pharmacy School, University of Montpellier, France and Pierre-Antoine Eliat, Small Animal Imaging Core Facility, Rennes 1 University, INSERM, CNRS, for enlightening discussions.

REFERENCES

1 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

2 **Llovet JM**, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016; **2**: 16018 [PMID: 27158749 DOI: 10.1038/nrdp.2016.18]

3 **Nault JC**, Villanueva A. Intratumor molecular and phenotypic diversity in hepatocellular carcinoma. *Clin Cancer Res* 2015; **21**: 1786-1788 [PMID: 25628398 DOI: 10.1158/1078-0432.CCR-14-2602]

4 **Sapisochin G**, Bruix J. Liver transplantation for hepatocellular carcinoma: outcomes and novel surgical approaches. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 203-217 [PMID: 28053342 DOI: 10.1038/nrgastro.2016.193]

5 **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

6 **Yamashita T**, Kitao A, Matsui O, Hayashi T, Nio K, Kondo M, Ohno N, Miyati T, Okada H, Yamashita T, Mizukoshi E, Honda M, Nakanuma Y, Takamura H, Ohta T, Nakamoto Y, Yamamoto M, Takayama T, Arii S, Wang X, Kaneko S. Gd-EOB-DTPA-enhanced magnetic resonance imaging and alpha-fetoprotein predict prognosis of early-stage hepatocellular carcinoma. *Hepatology*2014; **60**: 1674-1685 [PMID: 24700365 DOI: 10.1002/hep.27093]

7 **Hectors SJ**, Wagner M, Bane O, Besa C, Lewis S, Remark R, Chen N, Fiel MI, Zhu H, Gnjatic S, Merad M, Hoshida Y, Taouli B. Quantification of hepatocellular carcinoma heterogeneity with multiparametric magnetic resonance imaging. *Sci Rep* 2017; **7**: 2452 [PMID: 28550313 DOI: 10.1038/s41598-017-02706-z]

8 **Ueno A**, Masugi Y, Yamazaki K, Komuta M, Effendi K, Tanami Y, Tsujikawa H, Tanimoto A, Okuda S, Itano O, Kitagawa Y, Kuribayashi S, Sakamoto M. OATP1B3 expression is strongly associated with Wnt/β-catenin signalling and represents the transporter of gadoxetic acid in hepatocellular carcinoma. *J Hepatol* 2014; **61**: 1080-1087 [PMID: 24946283 DOI: 10.1016/j.jhep.2014.06.008]

9 **Zucman-Rossi J**, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015; **149**: 1226-1239.e4 [PMID: 26099527 DOI: 10.1053/j.gastro.2015.05.061]

10 **Llovet JM**, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015; **12**: 408-424 [PMID: 26054909 DOI: 10.1038/nrclinonc.2015.103]

11 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

12 **Roberts LR**. Sorafenib in liver cancer--just the beginning. *N Engl J Med* 2008; **359**: 420-422 [PMID: 18650519 DOI: 10.1056/NEJMe0802241]

13 **Raoul JL**, Kudo M, Finn RS, Edeline J, Reig M, Galle PR. Systemic therapy for intermediate and advanced hepatocellular carcinoma: Sorafenib and beyond. *Cancer Treat Rev* 2018; **68**: 16-24 [PMID: 29783126 DOI: 10.1016/j.ctrv.2018.05.006]

14 **European Association for the Study of the Liver**. Electronic address: easloffice@easloffice.eu.; European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

15 **Salem R**, Gordon AC, Mouli S, Hickey R, Kallini J, Gabr A, Mulcahy MF, Baker T, Abecassis M, Miller FH, Yaghmai V, Sato K, Desai K, Thornburg B, Benson AB, Rademaker A, Ganger D, Kulik L, Lewandowski RJ. Y90 Radioembolization Significantly Prolongs Time to Progression Compared With Chemoembolization in Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **151**: 1155-1163.e2 [PMID: 27575820 DOI: 10.1053/j.gastro.2016.08.029]

16 **Garin E**, Pallard X, Edeline J. Does Y90 Radioembolization Prolong Overall Survival Compared With Chemoembolization in Patients With Hepatocellular Carcinoma? *Gastroenterology* 2017; **152**: 1624-1625 [PMID: 28371622 DOI: 10.1053/j.gastro.2017.01.061]

17 **Vilgrain V**, Pereira H, Assenat E, Guiu B, Ilonca AD, Pageaux GP, Sibert A, Bouattour M, Lebtahi R, Allaham W, Barraud H, Laurent V, Mathias E, Bronowicki JP, Tasu JP, Perdrisot R, Silvain C, Gerolami R, Mundler O, Seitz JF, Vidal V, Aubé C, Oberti F, Couturier O, Brenot-Rossi I, Raoul JL, Sarran A, Costentin C, Itti E, Luciani A, Adam R, Lewin M, Samuel D, Ronot M, Dinut A, Castera L, Chatellier G; SARAH Trial Group. Efficacy and safety of selective internal radiotherapy with yttrium-90 resin microspheres compared with sorafenib in locally advanced and inoperable hepatocellular carcinoma (SARAH): an open-label randomised controlled phase 3 trial. *Lancet Oncol* 2017; **18**: 1624-1636 [PMID: 29107679 DOI: 10.1016/S1470-2045(17)30683-6]

18 **Wallace MC**, Friedman SL. Hepatic fibrosis and the microenvironment: fertile soil for hepatocellular carcinoma development.

Gene Expr 2014; **16**: 77-84 [PMID: 24801168 DOI: 10.3727/105221614X13919976902057]

19 **Hoshida Y**, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, Reich M, Chan JA, Glickman JN, Ikeda K, Hashimoto M, Watanabe G, Daidone MG, Roayaie S, Schwartz M, Thung S, Salvesen HB, Gabriel S, Mazzaferro V, Bruix J, Friedman SL, Kumada H, Llovet JM, Golub TR. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 1995-2004 [PMID: 18923165 DOI: 10.1056/NEJMoa0804525]

20 **Hernandez-Gea V**, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 2013; **144**: 512-527 [PMID: 23313965 DOI: 10.1053/j.gastro.2013.01.002]

21 **Vogelstein B**, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW. Cancer genome landscapes. *Science* 2013; **339**: 1546-1558 [PMID: 23539594 DOI: 10.1126/science.1235122]

22 **Schulze K**, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, Calatayud AL, Pinyol R, Pelletier L, Balabaud C, Laurent A, Blanc JF, Mazzaferro V, Calvo F, Villanueva A, Nault JC, Bioulac-Sage P, Stratton MR, Llovet JM, Zucman-Rossi J. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* 2015; **47**: 505-511 [PMID: 25822088 DOI: 10.1038/ng.3252]

23 **Fujimoto A**, Furuta M, Totoki Y, Tsunoda T, Kato M, Shiraishi Y, Tanaka H, Taniguchi H, Kawakami Y, Ueno M, Gotoh K, Ariizumi S, Wardell CP, Hayami S, Nakamura T, Aikata H, Arihiro K, Boroevich KA, Abe T, Nakano K, Maejima K, Sasaki-Oku A, Ohsawa A, Shibuya T, Nakamura H, Hama N, Hosoda F, Arai Y, Ohashi S, Urushidate T, Nagae G, Yamamoto S, Ueda H, Tatsuno K, Ojima H, Hiraoka N, Okusaka T, Kubo M, Marubashi S, Yamada T, Hirano S, Yamamoto M, Ohdan H, Shimada K, Ishikawa O, Yamaue H, Chayama K, Miyano S, Aburatani H, Shibata T, Nakagawa H. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat Genet* 2016; **48**: 500-509 [PMID: 27064257 DOI: 10.1038/ng.3547]

24 **Friemel J**, Rechsteiner M, Frick L, Böhm F, Struckmann K, Egger M, Moch H, Heikenwalder M, Weber A. Intratumor heterogeneity in hepatocellular carcinoma. *Clin Cancer Res* 2015; **21**: 1951-1961 [PMID: 25248380 DOI: 10.1158/1078-0432.CCR-14-0122]

25 **Xue R**, Li R, Guo H, Guo L, Su Z, Ni X, Qi L, Zhang T, Li Q, Zhang Z, Xie XS, Bai F, Zhang N. Variable Intra-Tumor Genomic Heterogeneity of Multiple Lesions in Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 998-1008 [PMID: 26752112 DOI: 10.1053/j.gastro.2015.12.033]

26 **Nault JC**, Mallet M, Pilati C, Calderaro J, Bioulac-Sage P, Laurent C, Laurent A, Cherqui D, Balabaud C, Zucman-Rossi J. High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions. *Nat Commun* 2013; **4**: 2218 [PMID: 23887712 DOI: 10.1038/ncomms3218]

27 **Nault JC**, Calderaro J, Di Tommaso L, Balabaud C, Zafrani ES, Bioulac-Sage P, Roncalli M, Zucman-Rossi J. Telomerase reverse transcriptase promoter mutation is an early somatic genetic alteration in the transformation of premalignant nodules in hepatocellular carcinoma on cirrhosis. *Hepatology* 2014; **60**: 1983-1992 [PMID: 25123086 DOI: 10.1002/hep.27372]

28 **Dubois-Pot-Schneider H**, Fekir K, Coulouarn C, Glaise D, Aninat C, Jarnouen K, Le Guével R, Kubo T, Ishida S, Morel F, Corlu A. Inflammatory cytokines promote the retrodifferentiation of tumor-derived hepatocyte-like cells to progenitor cells. *Hepatology* 2014; **60**: 2077-2090 [PMID: 25098666 DOI: 10.1002/hep.27353]

29 **Cabillic F**, Corlu A. Regulation of Transdifferentiation and Retrodifferentiation by Inflammatory Cytokines in Hepatocellular Carcinoma. *Gastroenterology* 2016; **151**: 607-615 [PMID: 27443822 DOI: 10.1053/j.gastro.2016.06.052]

30 **He G**, Dhar D, Nakagawa H, Font-Burgada J, Ogata H, Jiang Y, Shalapour S, Seki E, Yost SE, Jepsen K, Frazer KA, Harismendy O, Hatziapostolou M, Iliopoulos D, Suetsugu A, Hoffman RM, Tateishi R, Koike K, Karin M. Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. *Cell* 2013; **155**: 384-396 [PMID: 24120137 DOI: 10.1016/j.cell.2013.09.031]

31 **Sia D**, Villanueva A, Friedman SL, Llovet JM. Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis. *Gastroenterology* 2017; **152**: 745-761 [PMID: 28043904 DOI: 10.1053/j.gastro.2016.11.048]

32 **Pilati C**, Letouzé E, Nault JC, Imbeaud S, Boulai A, Calderaro J, Poussin K, Franconi A, Couchy G, Morcrette G, Mallet M, Taouji S, Balabaud C, Terris B, Canal F, Paradis V, Scoazec JY, de Muret A, Guettier C, Bioulac-Sage P, Chevet E, Calvo F, Zucman-Rossi J. Genomic profiling of hepatocellular adenomas reveals recurrent FRK-activating mutations and the mechanisms of malignant transformation. *Cancer Cell* 2014; **25**: 428-441 [PMID: 24735922 DOI: 10.1016/j.ccr.2014.03.005]

33 **Ziol M**, Poté N, Amaddeo G, Laurent A, Nault JC, Oberti F, Costentin C, Michalak S, Bouattour M, Francoz C, Pageaux GP, Ramos J, Decaens T, Luciani A, Guiu B, Vilgrain V, Aubé C, Derman J, Charpy C, Zucman-Rossi J, Barget N, Seror O, Ganne-Carrié N, Paradis V, Calderaro J. Macrotrabecular-massive hepatocellular carcinoma: A distinctive histological subtype with clinical relevance. *Hepatology* 2018; **68**: 103-112 [PMID: 29281854 DOI: 10.1002/hep.29762]

34 **Calderaro J**, Couchy G, Imbeaud S, Amaddeo G, Letouzé E, Blanc JF, Laurent C, Hajji Y, Azoulay D, Bioulac-Sage P, Nault JC, Zucman-Rossi J. Histological subtypes of hepatocellular carcinoma are related to gene mutations and molecular tumour classification. *J Hepatol* 2017; **67**: 727-738 [PMID: 28532995 DOI: 10.1016/j.jhep.2017.05.014]

35 **Désert R**, Mebarki S, Desille M, Sicard M, Lavergne E, Renaud S, Bergeat D, Sulpice L, Perret C, Turlin B, Clément B, Musso O. "Fibrous nests" in human hepatocellular carcinoma express a Wnt-induced gene signature associated with poor clinical outcome. *Int J Biochem Cell Biol* 2016; **81**: 195-207 [PMID: 27545991 DOI: 10.1016/j.biocel.2016.08.017]

36 **Lee JS**, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, Durnez A, Demetris AJ, Thorgeirsson SS. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology* 2004; **40**: 667-676 [PMID: 15349906 DOI: 10.1002/hep.20375]

37 **Lee JS**, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, Thorgeirsson SS. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. *Nat Genet* 2004; **36**: 1306-1311 [PMID: 15565109 DOI: 10.1038/ng1481]

38 **Sargent LM**, Zhou X, Keck CL, Sanderson ND, Zimonjic DB, Popescu NC, Thorgeirsson SS. Nonrandom cytogenetic alterations in hepatocellular carcinoma from transgenic mice overexpressing c-Myc and transforming growth factor-alpha in the liver. *Am J Pathol* 1999; **154**: 1047-1055 [PMID: 10233843]

39 **Kaposi-Novak P**, Lee JS, Gòmez-Quiroz L, Coulouarn C, Factor VM, Thorgeirsson SS. Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype. *J Clin Invest* 2006; **116**: 1582-1595 [PMID: 16710476 DOI: 10.1172/JCI27236]

40 **Lee JS**, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006; **12**: 410-416 [PMID: 16532004 DOI: 10.1038/nm1377]

41 **Wold H.** Estimation of principal components and related models by iterative least squares. In: Krishnaiah PR. Multivariate analysis. New York: Academic Press, 1966: 391-420

42 **Boyault S**, Rickman DS, de Reyniès A, Balabaud C, Rebouissou S, Jeannot E, Hérault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007; **45**: 42-52 [PMID: 17187432 DOI: 10.1002/hep.21467]

43 **Désert R**, Rohart F, Canal F, Sicard M, Desille M, Renaud S, Turlin B, Bellaud P, Perret C, Clément B, Lê Cao KA, Musso O. Human hepatocellular carcinomas with a periportal phenotype have the lowest potential for early recurrence after curative resection. *Hepatology* 2017; **66**: 1502-1518 [PMID: 28498607 DOI: 10.1002/hep.29254]

44 **McShane LM**, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM; Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst* 2005; **97**: 1180-1184 [PMID: 16106022 DOI: 10.1093/jnci/dji237]

45 **Altman DG**, McShane LM, Sauerbrei W, Taube SE. Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. *PLoS Med* 2012; **9**: e1001216 [PMID: 22675273 DOI: 10.1371/journal.pmed.1001216]

46 **Rebouissou S**, Franconi A, Calderaro J, Letouzé E, Imbeaud S, Pilati C, Nault JC, Couchy G, Laurent A, Balabaud C, Bioulac-Sage P, Zucman-Rossi J. Genotype-phenotype correlation of CTNNB1 mutations reveals different β-catenin activity associated with liver tumor progression. *Hepatology* 2016; **64**: 2047-2061 [PMID: 27177928 DOI: 10.1002/hep.28638]

47 **Ng CKY**, Piscuoglio S, Terracciano LM. Molecular classification of hepatocellular carcinoma: The view from metabolic zonation. *Hepatology* 2017; **66**: 1377-1380 [PMID: 28599064 DOI: 10.1002/hep.29311]

48 **Clevers H**. Axin and hepatocellular carcinomas. *Nat Genet* 2000; **24**: 206-208 [PMID: 10700164 DOI: 10.1038/73396]

49 **Satoh S**, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y, Nakamura Y. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat Genet* 2000; **24**: 245-250 [PMID: 10700176 DOI: 10.1038/73448]

50 **Zucman-Rossi J**, Benhamouche S, Godard C, Boyault S, Grimber G, Balabaud C, Cunha AS, Bioulac-Sage P, Perret C. Differential effects of inactivated Axin1 and activated beta-catenin mutations in human hepatocellular carcinomas. *Oncogene* 2007; **26**: 774-780 [PMID: 16964294 DOI: 10.1038/sj.onc.1209824]

51 **Abitbol S**, Dahmani R, Coulouarn C, Ragazzon B, Mlecnik B, Senni N, Savall M, Bossard P, Sohier P, Drouet V, Tournier E, Dumont F, Sanson R, Calderaro J, Zucman-Rossi J, Vasseur-Cognet M, Just PA, Terris B, Perret C, Gilgenkrantz H. AXIN deficiency in human and mouse hepatocytes induces hepatocellular carcinoma in the absence of β-catenin activation. *J Hepatol* 2018; **68**: 1203-1213 [PMID: 29525529 DOI: 10.1016/j.jhep.2017.12.018]

52 **Guichard C**, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, Calderaro J, Bioulac-Sage P, Letexier M, Degos F, Clément B, Balabaud C, Chevet E, Laurent A, Couchy G, Letouzé E, Calvo F, Zucman-Rossi J. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat Genet* 2012; **44**: 694-698 [PMID: 22561517 DOI: 10.1038/ng.2256]

53 **Colnot S**, Decaens T, Niwa-Kawakita M, Godard C, Hamard G, Kahn A, Giovannini M, Perret C. Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. *Proc Natl Acad Sci USA* 2004; **101**: 17216-17221 [PMID: 15563600 DOI: 10.1073/pnas.0404761101]

54 **Allen JC Jr**, Nault JC, Zhu G, Khor AY, Liu J, Lim TK, Zucman-Rossi J, Chow PK. The transcriptomic G1-G6 signature of hepatocellular carcinoma in an Asian population: Association of G3 with microvascular invasion. *Medicine* (Baltimore) 2016; **95**: e5263 [PMID: 27893662 DOI: 10.1097/MD.0000000000005263]

55 **Villanueva A**, Hoshida Y, Battiston C, Tovar V, Sia D, Alsinet C, Cornella H, Liberzon A, Kobayashi M, Kumada H, Thung SN, Bruix J, Newell P, April C, Fan JB, Roayaie S, Mazzaferro V, Schwartz ME, Llovet JM. Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. *Gastroenterology* 2011; **140**: 1501-1512.e2 [PMID: 21320499 DOI: 10.1053/j.gastro.2011.02.006]

56 **Torrecilla S**, Sia D, Harrington AN, Zhang Z, Cabellos L, Cornella H, Moeini A, Camprecios G, Leow WQ, Fiel MI, Hao K, Bassaganyas L, Mahajan M, Thung SN, Villanueva A, Florman S, Schwartz ME, Llovet JM. Trunk mutational events present minimal intra- and inter-tumoral heterogeneity in hepatocellular carcinoma. *J Hepatol* 2017; **67**: 1222-1231 [PMID: 28843658 DOI: 10.1016/j.jhep.2017.08.013]

57 **Sia D**, Llovet JM. Liver cancer: Translating '-omics' results into precision medicine for hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 571-572 [PMID: 28765583 DOI: 10.1038/nrgastro.2017.103]

58 **Chiang DY**, Villanueva A, Hoshida Y, Peix J, Newell P, Minguez B, LeBlanc AC, Donovan DJ, Thung SN, Solé M, Tovar V, Alsinet C, Ramos AH, Barretina J, Roayaie S, Schwartz M, Waxman S, Bruix J, Mazzaferro V, Ligon AH, Najfeld V, Friedman SL, Sellers WR, Meyerson M, Llovet JM. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. *Cancer Res*2008; **68**: 6779-6788 [PMID: 18701503 DOI: 10.1158/0008-5472.CAN-08-0742]

59 **Hoshida Y**, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, Watanabe G, Gabriel S, Friedman SL, Kumada H, Llovet JM, Golub TR. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009; **69**: 7385-7392 [PMID: 19723656 DOI: 10.1158/0008-5472.CAN-09-1089]

60 **Lachenmayer A**, Alsinet C, Savic R, Cabellos L, Toffanin S, Hoshida Y, Villanueva A, Minguez B, Newell P, Tsai HW, Barretina J, Thung S, Ward SC, Bruix J, Mazzaferro V, Schwartz M, Friedman SL, Llovet JM. Wnt-pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. *Clin Cancer Res* 2012; **18**: 4997-5007 [PMID: 22811581 DOI: 10.1158/1078-0432.CCR-11-2322]

61 **Yuan RH**, Jeng YM, Hu RH, Lai PL, Lee PH, Cheng CC, Hsu HC. Role of p53 and β-catenin mutations in conjunction with CK19 expression on early tumor recurrence and prognosis of hepatocellular carcinoma. *J Gastrointest Surg* 2011; **15**: 321-329 [PMID: 21061181 DOI: 10.1007/s11605-010-1373-x]

62 **Mise K**, Tashiro S, Yogita S, Wada D, Harada M, Fukuda Y, Miyake H, Isikawa M, Izumi K, Sano N. Assessment of the biological malignancy of hepatocellular carcinoma: relationship to clinicopathological factors and prognosis. *Clin Cancer Res* 1998; **4**: 1475-1482 [PMID: 9626465]

63 **Hayashi H**, Sugio K, Matsumata T, Adachi E, Takenaka K, Sugimachi K. The clinical significance of p53 gene mutation in hepatocellular carcinomas from Japan. *Hepatology* 1995; **22**: 1702-1707 [PMID: 7489977 DOI: 10.1016/0270-9139(95)90194-9]

64 **Musso O**, Rehn M, Théret N, Turlin B, Bioulac-Sage P, Lotrian D, Campion JP, Pihlajaniemi T, Clément B. Tumor progression is associated with a significant decrease in the expression of the endostatin precursor collagen XVIII in human hepatocellular carcinomas. *Cancer Res* 2001; **61**: 45-49 [PMID: 11196195]

65 **Théret N**, Musso O, Turlin B, Lotrian D, Bioulac-Sage P, Campion JP, Boudjéma K, Clément B. Increased extracellular matrix remodeling is associated with tumor progression in human hepatocellular carcinomas. *Hepatology* 2001; **34**: 82-88 [PMID: 11431737 DOI: 10.1053/jhep.2001.25758]

66 **Le Pabic H**, Bonnier D, Wewer UM, Coutand A, Musso O, Baffet G, Clément B, Théret N. ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling. *Hepatology* 2003; **37**: 1056-1066 [PMID: 12717386 DOI: 10.1053/jhep.2003.50205]

67 **Kan Z**, Zheng H, Liu X, Li S, Barber TD, Gong Z, Gao H, Hao K, Willard MD, Xu J, Hauptschein R, Rejto PA, Fernandez J, Wang G, Zhang Q, Wang B, Chen R, Wang J, Lee NP, Zhou W, Lin Z, Peng Z, Yi K, Chen S, Li L, Fan X, Yang J, Ye R, Ju J, Wang K, Estrella H, Deng S, Wei P, Qiu M, Wulur IH, Liu J, Ehsani ME, Zhang C, Loboda A, Sung WK, Aggarwal A, Poon RT, Fan ST, Wang J, Hardwick J, Reinhard C, Dai H, Li Y, Luk JM, Mao M. Whole-genome sequencing identifies recurrent mutations in hepatocellular carcinoma. *Genome Res* 2013; **23**: 1422-1433 [PMID: 23788652

DOI: 10.1101/gr.154492.113]
68 **Cerec V**, Glaise D, Garnier D, Morosan S, Turlin B, Drenou B, Gripon P, Kremsdorf D, Guguen-Guillouzo C, Corlu A. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology* 2007; **45**: 957-967 [PMID: 17393521 DOI: 10.1002/hep.21536]
69 **Mebarki S**, Désert R, Sulpice L, Sicard M, Desille M, Canal F, Dubois-Pot Schneider H, Bergeat D, Turlin B, Bellaud P, Lavergne E, Le Guével R, Corlu A, Perret C, Coulouarn C, Clément B, Musso O. De novo HAPLN1 expression hallmarks Wnt-induced stem cell and fibrogenic networks leading to aggressive human hepatocellular carcinomas. *Oncotarget* 2016; **7**: 39026-39043 [PMID: 27191501 DOI: 10.18632/oncotarget.9346]
70 **Bengochea A**, de Souza MM, Lefrançois L, Le Roux E, Galy O, Chemin I, Kim M, Wands JR, Trepo C, Hainaut P, Scoazec JY, Vitvitski L, Merle P. Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma. *Br J Cancer* 2008; **99**: 143-150 [PMID: 18577996 DOI: 10.1038/sj.bjc.6604422]
71 **Wang B**, Zhao L, Fish M, Logan CY, Nusse R. Self-renewing diploid Axin2(+) cells fuel homeostatic renewal of the liver. *Nature* 2015; **524**: 180-185 [PMID: 26245375 DOI: 10.1038/nature14863]
72 **Fu L**, Zhang C, Zhang LY, Dong SS, Lu LH, Chen J, Dai Y, Li Y, Kong KL, Kwong DL, Guan XY. Wnt2 secreted by tumour fibroblasts promotes tumour progression in oesophageal cancer by activation of the Wnt/β-catenin signalling pathway. *Gut* 2011; **60**: 1635-1643 [PMID: 21672941 DOI: 10.1136/gut.2011.241638]
73 **Miltiadous O**, Sia D, Hoshida Y, Fiel MI, Harrington AN, Thung SN, Tan PS, Dong H, Revill K, Chang CY, Roayaie S, Byrne TJ, Mazzaferro V, Rakela J, Florman S, Schwartz M, Llovet JM. Progenitor cell markers predict outcome of patients with hepatocellular carcinoma beyond Milan criteria undergoing liver transplantation. *J Hepatol* 2015; **63**: 1368-1377 [PMID: 26220754 DOI: 10.1016/j.jhep.2015.07.025]
74 **Sia D**, Jiao Y, Martinez-Quetglas I, Kuchuk O, Villacorta-Martin C, Castro de Moura M, Putra J, Camprecios G, Bassaganyas L, Akers N, Losic B, Waxman S, Thung SN, Mazzaferro V, Esteller M, Friedman SL, Schwartz M, Villanueva A, Llovet JM. Identification of an Immune-specific Class of Hepatocellular Carcinoma, Based on Molecular Features. *Gastroenterology* 2017; **153**: 812-826 [PMID: 28624577 DOI: 10.1053/j.gastro.2017.06.007]
75 **Sim HW**, Knox J. Hepatocellular carcinoma in the era of immunotherapy. *Curr Probl Cancer* 2018; **42**: 40-48 [PMID: 29150141 DOI: 10.1016/j.currproblcancer.2017.10.007]
76 **Iñarrairaegui M**, Melero I, Sangro B. Immunotherapy of Hepatocellular Carcinoma: Facts and Hopes. *Clin Cancer Res* 2018; **24**: 1518-1524 [PMID: 29138342 DOI: 10.1158/1078-0432.CCR-17-0289]
77 **Ringelhan M**, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. *Nat Immunol* 2018; **19**: 222-232 [PMID: 29379119 DOI: 10.1038/s41590-018-0044-z]
78 **Mínguez B**, Hoshida Y, Villanueva A, Toffanin S, Cabellos L, Thung S, Mandeli J, Sia D, April C, Fan JB, Lachenmayer A, Savic R, Roayaie S, Mazzaferro V, Bruix J, Schwartz M, Friedman SL, Llovet JM. Gene-expression signature of vascular invasion in hepatocellular carcinoma. *J Hepatol* 2011; **55**: 1325-1331 [PMID: 21703203 DOI: 10.1016/j.jhep.2011.02.034]
79 **Roessler S**, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, Thorgeirsson SS, Sun Z, Tang ZY, Qin LX, Wang XW. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. *Cancer Res* 2010; **70**: 10202-10212 [PMID: 21159642 DOI: 10.1158/0008-5472.CAN-10-2607]
80 **Villa E**, Critelli R, Lei B, Marzocchi G, Cammà C, Giannelli G, Pontisso P, Cabibbo G, Enea M, Colopi S, Caporali C, Pollicino T, Milosa F, Karampatou A, Todesca P, Bertolini E, Maccio L, Martinez-Chantar ML, Turola E, Del Buono M, De Maria N, Ballestri S, Schepis F, Loria P, Enrico Gerunda G, Losi L, Cillo U. Neoangiogenesis-related genes are hallmarks of fast-growing hepatocellular carcinomas and worst survival. Results from a prospective study. *Gut* 2016; **65**: 861-869 [PMID: 25666192 DOI: 10.1136/gutjnl-2014-308483]
81 **Woo HG**, Lee JH, Yoon JH, Kim CY, Lee HS, Jang JJ, Yi NJ, Suh KS, Lee KU, Park ES, Thorgeirsson SS, Kim YJ. Identification of a cholangiocarcinoma-like gene expression trait in hepatocellular carcinoma. *Cancer Res* 2010; **70**: 3034-3041 [PMID: 20395200 DOI: 10.1158/0008-5472.CAN-09-2823]
82 **Yamashita T**, Forgues M, Wang W, Kim JW, Ye Q, Jia H, Budhu A, Zanetti KA, Chen Y, Qin LX, Tang ZY, Wang XW. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. *Cancer Res* 2008; **68**: 1451-1461 [PMID: 18316609 DOI: 10.1158/0008-5472.CAN-07-6013]
83 **Yamashita T**, Ji J, Budhu A, Forgues M, Yang W, Wang HY, Jia H, Ye Q, Qin LX, Wauthier E, Reid LM, Minato H, Honda M, Kaneko S, Tang ZY, Wang XW. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* 2009; **136**: 1012-1024 [PMID: 19150350 DOI: 10.1053/j.gastro.2008.12.004]
84 **Coulouarn C**, Factor VM, Thorgeirsson SS. Transforming growth factor-beta gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. *Hepatology* 2008; **47**: 2059-2067 [PMID: 18506891 DOI: 10.1002/hep.22283]
85 **Anderson NM**, Mucka P, Kern JG, Feng H. The emerging role and targetability of the TCA cycle in cancer metabolism. *Protein Cell* 2018; **9**: 216-237 [PMID: 28748451 DOI: 10.1007/s13238-017-0451-1]
86 **Shang RZ**, Qu SB, Wang DS. Reprogramming of glucose metabolism in hepatocellular carcinoma: Progress and prospects. *World J Gastroenterol* 2016; **22**: 9933-9943 [PMID: 28018100 DOI: 10.3748/wjg.v22.i45.9933]
87 **Huang Q**, Tan Y, Yin P, Ye G, Gao P, Lu X, Wang H, Xu G. Metabolic characterization of hepatocellular carcinoma using nontargeted tissue metabolomics. *Cancer Res* 2013; **73**: 4992-5002 [PMID: 23824744 DOI: 10.1158/0008-5472.CAN-13-0308]
88 **Luo P,** Yin P, Hua R, Tan Y, Li Z, Qiu G, Yin Z, Xie X, Wang X, Chen W, Zhou L, Wang X, Li Y, Chen H, Gao L, Lu X, Wu T, Wang H, Niu J, Xu G. A Large-scale, multicenter serum metabolite biomarker identification study for the early detection of hepatocellular carcinoma. *Hepatology* 2017; Epub ahead of print [PMID: 28960374 DOI: 10.1002/hep.29561]
89 **Beyoğlu D**, Imbeaud S, Maurhofer O, Bioulac-Sage P, Zucman-Rossi J, Dufour JF, Idle JR. Tissue metabolomics of hepatocellular carcinoma: tumor energy metabolism and the role of transcriptomic classification. *Hepatology* 2013; **58**: 229-238 [PMID: 23463346 DOI: 10.1002/hep.26350]
90 **Nwosu ZC**, Megger DA, Hammad S, Sitek B, Roessler S, Ebert MP, Meyer C, Dooley S. Identification of the Consistently Altered Metabolic Targets in Human Hepatocellular Carcinoma. *Cell Mol Gastroenterol Hepatol* 2017; **4**: 303-323.e1 [PMID: 28840186 DOI: 10.1016/j.jcmgh.2017.05.004]
91 **Senni N**, Savall M, Cabrerizo Granados D, Alves-Guerra MC, Sartor C, Lagoutte I, Gougelet A, Terris B, Gilgenkrantz H, Perret C, Colnot S, Bossard P. β-catenin-activated hepatocellular carcinomas are addicted to fatty acids. *Gut* 2018; Epub ahead of print [PMID: 29650531 DOI: 10.1136/gutjnl-2017-315448]
92 **Cancer Genome Atlas Research Network**. Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]
93 **Saha SK**, Parachoniak CA, Ghanta KS, Fitamant J, Ross KN, Najem MS, Gurumurthy S, Akbay EA, Sia D, Cornella H, Miltiadous O, Walesky C, Deshpande V, Zhu AX, Hezel AF, Yen KE, Straley KS, Travins J, Popovici-Muller J, Gliser C, Ferrone CR, Apte U, Llovet JM, Wong KK, Ramaswamy S, Bardeesy N. Mutant IDH inhibits HNF-4α to block hepatocyte differentiation and promote biliary cancer. *Nature* 2014; **513**: 110-114 [PMID: 25043045 DOI: 10.1038/nature13441]
94 **Edmondson HA**, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954; **7**:

462-503 [PMID: 13160935 DOI: 10.1002/1097-0142(195405)7:3<462::AID-CNCR2820070308>3.0.CO;2-E]
95 **Bosman FT**, World Health Organization., International Agency for Research on Cancer. Who classification of tumours of the digestive system. 4th ed. Lyon: International Agency for Research on Cancer, 2010
96 **Goodman ZD**. Neoplasms of the liver. *Mod Pathol* 2007; **20** Suppl 1: S49-S60 [PMID: 17486052 DOI: 10.1038/modpathol.3800682]
97 **Berasain C**, Avila MA. Deciphering liver zonation: new insights into the β-catenin, Tcf4, and HNF4α triad. *Hepatology* 2014; **59**: 2080-2082 [PMID: 24407883 DOI: 10.1002/hep.27000]
98 **Braeuning A**, Ittrich C, Köhle C, Hailfinger S, Bonin M, Buchmann A, Schwarz M. Differential gene expression in periportal and perivenous mouse hepatocytes. *FEBS J* 2006; **273**: 5051-5061 [PMID: 17054714 DOI: 10.1111/j.1742-4658.2006.05503.x]
99 **Holloway MG**, Miles GD, Dombkowski AA, Waxman DJ. Liver-specific hepatocyte nuclear factor-4alpha deficiency: greater impact on gene expression in male than in female mouse liver. *Mol Endocrinol* 2008; **22**: 1274-1286 [PMID: 18276827 DOI: 10.1210/me.2007-0564]
100 **Gougelet A**, Torre C, Veber P, Sartor C, Bachelot L, Denechaud PD, Godard C, Moldes M, Burnol AF, Dubuquoy C, Terris B, Guillonneau F, Ye T, Schwarz M, Braeuning A, Perret C, Colnot S. T-cell factor 4 and β-catenin chromatin occupancies pattern zonal liver metabolism in mice. *Hepatology* 2014; **59**: 2344-2357 [PMID: 24214913 DOI: 10.1002/hep.26924]
101 **De Certaines JD**, Larcher T, Duda D, Azzabou N, Eliat P-A, Escudero LM, Pinheiro AM, Yang G, Coatrieux J-L, Snezkho E, Shukelovich A, Pereira M, Lerski RA. Application of texture analysis to muscle mri: 1-what kind of information should be expected from texture analysis? *EPJ Nonlinear Biomed Phys* 2015; **3**: 3 [DOI: 10.1140/epjnbp/s40366-015-0017-1]
102 **Lerski RA**, de Certaines JD, Duda D, Klonowski W, Yang G, Coatrieux JL, Azzabou N, Eliat P-A. Application of texture analysis to muscle mri: 2 - technical recommendations. *EPJ Nonlinear Biomed Phys* 2015; **3**: 2 [DOI: 10.1140/epjnbp/s40366-015-0018-0]

P- Reviewer: Liedtke C, Tomizawa M **S- Editor**: Ma RY
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4548-4553

DOI: 10.3748/wjg.v24.i40.4548

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

MINIREVIEWS

Second-line rescue treatment of *Helicobacter pylori* infection: Where are we now?

Te-Fu Lin, Ping-I Hsu

Te-Fu Lin, Ping-I Hsu, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan

ORCID number: Te-Fu Lin (0000-0003-1996-5008); Ping-I Hsu (0000-0003-3905-4674).

Author contributions: Hsu PI designed the study, reviewed the articles and drafted the manuscript; Lin TF reviewed the articles and drafted the manuscript.

Conflict-of-interest statement: Both authors have no conflicts of interest.

Manuscript source: Invited manuscript

Correspondence to: Ping-I Hsu, MD, Attending Doctor, Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, No. 386, Ta-Chung 1st Rd, Kaohsiung 813, Taiwan. williamhsup@yahoo.com.tw
Telephone: +886-7-3468233
Fax: +886-7-3468237

Received: August 7, 2018
Peer-review started: August 7, 2018
First decision: August 27, 2018
Revised: September 20, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

At present, the best rescue therapy for *Helicobacter pylori* (*H. pylori*) infection following failure of first-line eradication remains unclear. The Maastricht V /Florence Consensus Report recommends bismuth quadruple therapy, or fluoroquinolone-amoxicillin triple/quadruple therapy as the second-line therapy for *H. pylori* infection. Meta-analyses have shown that bismuth quadruple therapy and levofloxacin-amoxicillin triple therapy have comparable eradication rates, while the former has more adverse effects than the latter. There are no significant differences between the eradication rates of levofloxacin-amoxicillin triple and quadruple therapies. However, the eradication rates of both levofloxacin-containing treatments are suboptimal. An important caveat of levofloxacin-amoxicillin triple or quadruple therapy is poor eradication efficacy in the presence of fluoroquinolone resistance. High-dose dual therapy is an emerging second-line therapy and has an eradication efficacy comparable with levofloxacin-amoxicillin triple therapy. Recently, a 10-d tetracycline-levofloxacin (TL) quadruple therapy comprised of a proton pump inhibitor, bismuth, tetracycline and levofloxacin has been developed, which achieves a markedly higher eradication rate compared with levofloxacin-amoxicillin triple therapy (98% *vs* 69%) in patients with failure of standard triple, bismuth quadruple or non-bismuth quadruple therapy. The present article reviews current second-line anti-*H. pylori* regimens and treatment algorisms. In conclusion, bismuth quadruple therapy, levofloxacin-amoxicillin triple/quadruple therapy, high-dose dual therapy and TL quadruple therapy can be used as second-line treatment for *H. pylori* infection. Current evidence suggests that 10-d TL quadruple therapy is a simple and effective regimen, and has the potential to become a universal rescue treatment following eradication failure by all first-line eradication regimens for *H. pylori* infection.

Key words: *Helicobacter pylori*; Rescue treatment; Levofloxacin-amoxicillin triple therapy; Bismuth quadruple therapy; Tetracycline-levofloxacin quadruple therapy; High-dose dual therapy

Core tip: The present article reviews current second-line anti-*Helicobacter pylori* (*H. pylori*) regimens. Bismuth quadruple therapy and levofloxacin-amoxicillin triple therapy have comparable eradication rates in the rescue treatment of *H. pylori* infection, while the former has more adverse effects than the latter. High-dose dual therapy has an eradication rate comparable with levofloxacin-amoxicillin triple therapy. Ten-day tetracycline-levofloxacin quadruple therapy achieves a markedly higher eradication rate compared with levofloxacin-amoxicillin triple therapy (98% *vs* 69%) in patients with failure of standard triple, bismuth quadruple or non-bismuth quadruple therapy. In conclusion, tetracycline-levofloxacin quadruple therapy has the potential to become a universal second-line treatment for *H. pylori* infection.

Lin TF, Hsu PI. Second-line rescue treatment of *Helicobacter pylori* infection: Where are we now? *World J Gastroenterol* 2018; 24(40): 4548-4553 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4548.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4548

INTRODUCTION

Helicobacter pylori (*H. pylori*) infects > 50% of humans globally. It is a major cause of chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma[1,2]. With the rising prevalence of global antibiotic resistance, the eradication rate of *H. pylori* with standard triple therapy has decreased to < 80% worldwide[3]. Although there are other emerging 1st-line therapies, including bismuth quadruple therapy and non-bismuth quadruple (sequential, concomitant or hybrid) therapy, which can increase the eradication rate, *H. pylori* eradication still fails in 3%-24% of infected patients[4-7]. At present, the optimal choice for second-line anti-*H. pylori* therapy has not been well established. The present article aims to review and update the current options for second-line therapy against *H. pylori* infections.

ANTIBIOTIC RESISTANCE IN ANTI-*H. PYLORI* THERAPY

Causes of treatment failure of anti-*H. pylori* therapies include antibiotic resistant bacteria, poor patient compliance, low gastric pH and a high bacterial load. Among these reasons, antibiotic resistance is the main factor which determines the efficacy of an eradication therapy[8]. Primary resistance to amoxicillin is either null or < 1% in most countries[9]. In contrast, the rate of primary clarithromycin-resistance ranges from 49% (Spain) to 1% (the Netherlands) worldwide[10]. High primary resistance to clarithromycin and low resistance to metronidazole have been observed in Japan; moderate resistance to clarithromycin and high resistance to metronidazole were reported in South Korea; and high primary resistance to both clarithromycin and metronidazole was observed in China[11]. High primary resistance to both clarithromycin and metronidazole has also been reported in some other countries, such as Italy, Spain, Mexico and Vietnam. Low clarithromycin resistance is generally observed in northern Europe, including the Netherlands, Sweden and Ireland[10,11].

In patients who experience eradication failure following standard triple therapy, the rates of drug resistance to clarithromycin, metronidazole, levofloxacin, amoxicillin and tetracycline are 65%-75%, 30%-56%, 26%-37%, 0%-6.1% and 0%-10%, respectively[12-16]. Whereas for patients who experience failure of non-bismuth quadruple therapy, the rates of drug resistance to clarithromycin, metronidazole, levofloxacin, amoxicillin and tetracycline are 75%, 75%, 25%, 0%, and 0%, respectively[17,18]. This data implies that amoxicillin, tetracycline and levofloxacin are good choices of antibiotics for rescue treatment of *H. pylori* infection.

Point mutations play a primary role in the antimicrobial resistance of *H. pylori, and* different mutations involving the *rdxA* gene have been identified in metronidazole resistant strains[19]. Resistance to clarithromycin in *H. pylori* is commonly caused by point mutations in the *rrl* gene encoding two 23S rRNA nucleotides, namely 2142 and 2143[20]. Another mechanism associated with the development of clarithromycin resistance is the efflux pump system[21,22]. Fluoroquinolone acts on the site of the type A DNA gyrase enzyme, which is encoded by the *gyrA* gene, to inhibit DNA cleavage and rejoining[23]. Gene mutations in *gyrA* are associated with fluoroquinolone resistance. In particular double mutations at both N87 and D91 in *gyrA* have been reported to increase fluoroquinolone resistance[24].

UPDATED SECOND-LINE THERAPIES

Current updated second-line therapies include bismuth quadruple therapy, fluoroquinolone-amoxicillin triple therapy, fluoroquinolone-amoxicillin quadruple therapy, tetracycline-levofloxacin (TL) quadruple therapy and high-dose dual therapy.

Bismuth quadruple therapy

Bismuth quadruple therapy consists of a proton pump inhibitor (PPI), bismuth, metronidazole and tetracycline (Table 1). The standard regimen comprises PPI twice daily, colloidal bismuth subcitrate 120 mg four times daily, tetracycline 500 mg four times daily and metronidazole 500 mg three times daily for 10 to 14 d. A pool analysis demonstrated that bismuth quadruple therapy fails in 5%-63% of patients as a second-line therapy and achieves a mean 76% eradication rate[25-27]. Its efficacy is related to metronidazole resistance in

Table 1 Regimens for second-line anti-*Helicobacter pylori* therapy

Regimen	Drug						Duration of therapy
	PPI	Bismuth	Levo	Amox	Tetra	Metro	
Bismuth-containing quadruple therapy	SD, *b.i.d.*	120 mg, *q.i.d.*			500 mg, *q.i.d.*	500 mg, *t.i.d.*	10-14 d
Levofloxacin-containing triple therapy	SD, *b.i.d.*		500 mg, *q.d.*	1 g, *b.i.d.*			10-14 d
Levofloxacin-amoxicillin quadruple therapy	SD, *b.i.d.*	120 mg, *q.i.d.*	500 mg, *q.d.*	1 g, *b.i.d.*			10-14 d
Tetracycline-levofloxacin quadruple therapy	SD, *b.i.d.*	120 mg, *q.i.d.*	500 mg, *q.d.*		500 mg, *q.i.d.*		10 d
High-dose dual therapy	SD, *q.i.d.*			750 mg, *b.i.d.*			14 d

PPI: Proton pump inhibitor; Levo: Levofloxacin; Amox: Amoxicillin; Tetra: Tetracycline; Metro: Metronidazole.

H. pylori strains and the duration of the regimens[28]. Meta-analysis of randomized controlled trials of bismuth quadruple therapy as a rescue treatment after failure of clarithromycin triple therapy revealed a significantly higher eradication rate for the 14-d regimen compared with the 7-d regimen[29]. Therefore, it is reasonable to encourage a 14-d regimen duration for bismuth quadruple therapy when used as a second-line treatment for *H. pylori* infection.

Fluoroquinolone-based triple/quadruple therapy

The most commonly used fluoroquinolone-based triple therapy is composed of levofloxacin 500 mg daily, amoxicillin 1 g twice daily and a PPI (standard dose) twice daily for 10 to 14 d (Table 1). Meta-analyses revealed that levofloxacin-amoxicillin triple therapy and bismuth quadruple therapy had comparable eradication rates, whereas the former had fewer adverse effects than the latter[30]. A systemic review and meta-analysis revealed that levofloxacin-amoxicillin triple therapy achieved an overall eradication rate of 78% after failure of a non-bismuth quadruple therapy[31]. It was similarly effective after failure of sequential and concomitant therapies (81% *vs* 78%, respectively), and the cure rate of levofloxacin-amoxicillin triple therapy following hybrid therapy was 50%.

An important drawback of levofloxacin-amoxicillin triple therapy is poor eradication efficacy in the presence of fluoroquinolone resistance. Bismuth salts have a synergistic effect on antibiotics and have been used to increase eradication rates[32]. The Maastricht Ⅴ/Florence Consensus Report also recommended the application of fluoroquinolone-amoxicillin quadruple therapy as a second-line therapy for *H. pylori* infection[31]. Levofloxacin-amoxicillin quadruple therapy is composed of levofloxacin 500 mg daily, amoxicillin 1 g twice daily, PPI (standard dose) twice daily and bismuth 240 mg twice daily for 10 to 14 d (Table 1). A randomized controlled trial showed there were no significant differences between the eradication rates of second-line 14-d levofloxacin-amoxicillin quadruple therapy and 14-d levofloxacin-amoxicillin triple therapy (87% *vs* 83%, respectively)[33]. However, the former had a higher eradication rate for levofloxacin-resistant strains than the latter (71% *vs* 37%)[33].

TL quadruple therapy

Recently, Hsu *et al*[17] developed a novel TL quadruple therapy as a rescue treatment for *H. pylori* infection. It consists of esomeprazole 40 mg twice daily, tripotassium dicitrato bismuthate 120 mg four times daily, tetracycline 500 mg four times daily, and levofloxacin 500 mg once daily for 10 d (Table 1). The simple regimen maintains a high eradication rate for *H. pylori* strains with levofloxacin resistance[17]. A randomized control study showed that as a second-line anti-*H. pylori* treatment, 10-d of TL quadruple therapy achieved a much higher eradication rate compared with 10-d levofloxacin triple therapy containing esomeprazole, amoxicillin and levofloxacin (98% *vs* 68%, respectively)[34]. Subgroup analysis revealed that the former was superior to the latter in patients with failure of either standard triple therapy (100% *vs* 75%) or non-bismuth quadruple therapy (95% *vs* 53%). There were only 7 patients recruited into the study with eradication failure by bismuth quadruple therapy as a first-line treatment, and both TL quadruple and levofloxacin-amoxicillin triple therapies had a 100% eradication rate in this subgroup of patients. The data suggests that 10-d TL quadruple therapy is a good option for second-line treatment after failure of standard triple, concomitant and bismuth quadruple therapies.

High-dose dual therapy

High-dose dual therapy is another emerging second-line treatment for *H. pylori* infection[35]. The new therapy consists of high-dose PPI and amoxicillin (Table 1), which keep the intragastric pH higher than 6.5 regardless of *CYP2C19* genotype[36], and maintain a steady plasma concentration of amoxicillin above the minimal inhibitory concentration for *H. pylori*[37]. A randomized control trial from Taiwan revealed that 14-d high-dose dual therapy achieved a higher eradication rate than 10-d sequential therapy as a second-line treatment for *H. pylori* infection (89% *vs* 52%), and had an eradication rate comparable with 7-d levofloxacin-amoxicillin triple therapy (79%)[35]. Another randomized controlled trial from Germany demonstrated that 14-d high-dose dual therapy and 14-d bismuth quadruple therapy had comparable efficacies as a rescue treatment for *H. pylori* infection (76% *vs* 81%, respectively)[38].

TREATMENT ALGORISM

After a first failure of *H. pylori* treatment, if an endoscopy is arranged, the Maastricht Ⅴ/Florence Consensus Report recommends antimicrobial susceptibility testing

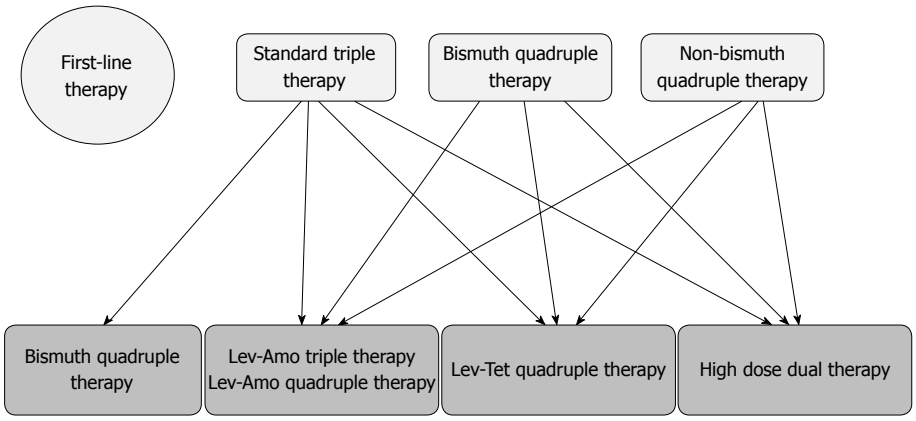


Figure 1 Algorism for second-line therapy of *Helicobacter pylori* infection. Lev: Levofloxacin; Amo: Amoxicillin; Tet: Tetracycline.

(AST)[31] to enable tailoring of the rescue eradication therapy. However, AST is not routinely performed in clinical practice due to the invasiveness of the endoscopy procedure, the availablity of laboratory culture facilities and cost considerations. If AST data are not available, 10-d TL quadruple therapy can be used as a rescue treatment since it achieves an eradication rate of > 90% following failure of standard triple, concomitant and bismuth quadruple therapies. In addition, the novel 10-d TL quadruple regimen can maintain a high eradication rate (> 90%) for *H. pylori* strains with levofloxacin resistance[34]. However, the choice of second line rescue regimen also depends on regional factors. In Japan, PPI-containing triple therapy with metronidazole and amoxicillin is the standard second line regimen and is covered under Japan's national health insurance. This second-line therapy can also achieve an eradication rate of around 90% because metronidazole resistance rate is relatively low in Japan.

After failure of a standard triple therapy

According to the Maastricht Ⅴ/Florence Consensus Report[31], bismuth-containing quadruple therapy, fluoroquinolone-containing triple therapy or fluoroquinolone-amoxicillin quadruple therapy are recommended following failure of standard triple therapy. As TL quadruple therapy achieves a higher eradication rate than levofloxacin triple therapy, and high-dose dual therapy has a comparable eradication rate with levofloxacin-amoxicillin triple therapy in patients with failure of standard triple therapy[34,35], both TL quadruple and high-dose dual therapies can be recommended as a rescue regimen following failure of standard triple therapy (Figure 1).

After failure of a non-bismuth quadruple therapy

The Maastricht Ⅴ/Florence Consensus Report recommends bismuth quadruple therapy, levofloxacin-amoxicillin triple therapy and levofloxacin-amoxicillin quadruple therapy as rescue treatments after failure of a non-bismuth quadruple therapy[31]. As 10-d TL quadruple therapy is superior to 10-d levofloxacin-amoxicillin triple therapy, it is reasonable to recommend TL-quadruple therapy as the rescue treatment for patients with eradication failure by non-bismuth quadruple therapy (Figure 1).

After failure of a bismuth quadruple therapy

According to the Maastricht Ⅴ/Florence Consensus Report[31], fluoroquinolone-containing triple or fluoroquinolone-amoxicillin quadruple therapy can be recommended for patients with eradication failure by bismuth quadruple therapy for *H. pylori* infection. As both TL quadruple and levofloxacin-amoxicillin triple therapies achieved a 100% cure rate in this setting. TL quadruple therapy may also be considered as an option for the rescue treatment of bismuth quadruple therapy (Figure 1).

CONCLUSION

The current updated second-line therapies include bismuth quadruple therapy, fluoroquinolone-amoxicillin triple therapy, fluoroquinolone-amoxicillin quadruple therapy, TL quadruple therapy and high-dose dual therapy. Ten-day TL quadruple therapy has great potential to become a universal rescue treatment following eradication failure by all first-line eradication regimens for *H. pylori* infection, and warrants further investigation.

REFERENCES

1 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186 [PMID: 12374879 DOI: 10.1056/NEJMra020542]
2 **Zucca E**, Dreyling M; ESMO Guidelines Working Group. Gastric marginal zone lymphoma of MALT type: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* 2009; **20** Suppl 4: 113-114 [PMID: 19454427 DOI: 10.1093/annonc/mdp146]
3 **Camargo MC**, García A, Riquelme A, Otero W, Camargo CA, Hernandez-García T, Candia R, Bruce MG, Rabkin CS. The problem of Helicobacter pylori resistance to antibiotics: a systematic review in Latin America. *Am J Gastroenterol* 2014; **109**: 485-495 [PMID: 24589670 DOI: 10.1038/ajg.2014.24]
4 **Gatta L**, Vakil N, Leandro G, Di Mario F, Vaira D. Sequential therapy or triple therapy for Helicobacter pylori infection: systematic review and meta-analysis of randomized controlled trials in adults and children. *Am J Gastroenterol* 2009; **104**: 3069-79; quiz

1080 [PMID: 19844205 DOI: 10.1038/ajg.2009.555]
5 **Hsu PI**, Wu DC, Wu JY, Graham DY. Is there a benefit to extending the duration of Helicobacter pylori sequential therapy to 14 days? *Helicobacter* 2011; **16**: 146-152 [PMID: 21435093 DOI: 10.1111/j.1523-5378.2011.00829.x]
6 **Hsu PI**, Wu DC, Wu JY, Graham DY. Modified sequential Helicobacter pylori therapy: proton pump inhibitor and amoxicillin for 14 days with clarithromycin and metronidazole added as a quadruple (hybrid) therapy for the final 7 days. *Helicobacter* 2011; **16**: 139-145 [PMID: 21435092 DOI: 10.1111/j.1523-5378.2011.00828.x]
7 **Kao SS**, Chen WC, Hsu PI, Lai KH, Yu HC, Cheng HH, Peng NJ, Lin CK, Chan HH, Tsai WL, Wang HM, Tsai TJ, Lin KH, Tsay FW. 7-Day Nonbismuth-Containing Concomitant Therapy Achieves a High Eradication Rate for Helicobacter pylori in Taiwan. *Gastroenterol Res Pract* 2012; **2012**: 463985 [PMID: 22888337 DOI: 10.1155/2012/463985]
8 **Graham DY**, Fischbach L. Helicobacter pylori treatment in the era of increasing antibiotic resistance. *Gut* 2010; **59**: 1143-1153 [PMID: 20525969 DOI: 10.1136/gut.2009.192757]
9 **Kobayashi I**, Murakami K, Kato M, Kato S, Azuma T, Takahashi S, Uemura N, Katsuyama T, Fukuda Y, Haruma K, Nasu M, Fujioka T. Changing antimicrobial susceptibility epidemiology of Helicobacter pylori strains in Japan between 2002 and 2005. *J Clin Microbiol* 2007; **45**: 4006-4010 [PMID: 17942652 DOI: 10.1128/JCM.00740-07]
10 **Chuah SK**, Tsay FW, Hsu PI, Wu DC. A new look at anti-Helicobacter pylori therapy. *World J Gastroenterol* 2011; **17**: 3971-3975 [PMID: 22046084 DOI: 10.3748/wjg.v17.i35.3971]
11 **Suzuki H**, Mori H. World trends for H. pylori eradication therapy and gastric cancer prevention strategy by H. pylori test-and-treat. *J Gastroenterol* 2018; **53**: 354-361 [PMID: 29138921 DOI: 10.1007/s00535-017-1407-1]
12 **Wu IT**, Chuah SK, Lee CH, Liang CM, Lu LS, Kuo YH, Yen YH, Hu ML, Chou YP, Yang SC, Kuo CM, Kuo CH, Chien CC, Chiang YS, Chiou SS, Hu TH, Tai WC. Five-year sequential changes in secondary antibiotic resistance of Helicobacter pylori in Taiwan. *World J Gastroenterol* 2015; **21**: 10669-10674 [PMID: 26457027 DOI: 10.3748/wjg.v21.i37.10669]
13 **Kuo CH**, Hu HM, Kuo FC, Hsu PI, Chen A, Yu FJ, Tsai PY, Wu IC, Wang SW, Li CJ, Weng BC, Chang LL, Jan CM, Wang WM, Wu DC. Efficacy of levofloxacin-based rescue therapy for Helicobacter pylori infection after standard triple therapy: a randomized controlled trial. *J Antimicrob Chemother* 2009; **63**: 1017-1024 [PMID: 19246508 DOI: 10.1093/jac/dkp034]
14 **Rendell-Baker L**. Nineteenth-century resuscitation apparatus. *Anaesthesia* 1981; **36**: 1058-1059 [PMID: 7032348 DOI: 10.1111/j.1365-2362.2006.01725.x]
15 **Liou JM**, Bair MJ, Chen CC, Lee YC, Chen MJ, Chen CC, Tseng CH, Fang YJ, Lee JY, Yang TH, Luo JC, Wu JY, Chang WH, Chang CC, Chen CY, Chen PY, Shun CT, Hsu WF, Hung HW, Lin JT, Chang CY, Wu MS; Taiwan Gastrointestinal Disease and Helicobacter Consortium. Levofloxacin Sequential Therapy vs Levofloxacin Triple Therapy in the Second-Line Treatment of Helicobacter pylori: A Randomized Trial. *Am J Gastroenterol* 2016; **111**: 381-387 [PMID: 26832653 DOI: 10.1038/ajg.2015.439]
16 **Cao Z**, Chen Q, Zhang W, Liang X, Liao J, Liu W, Xiao S, Lu H. Fourteen-day optimized levofloxacin-based therapy versus classical quadruple therapy for Helicobacter pylori treatment failures: a randomized clinical trial. *Scand J Gastroenterol* 2015; **50**: 1185-1190 [PMID: 25881966 DOI: 10.3109/00365521.2015.1037345]
17 **Hsu PI**, Chen WC, Tsay FW, Shih CA, Kao SS, Wang HM, Yu HC, Lai KH, Tseng HH, Peng NJ, Chen A, Kuo CH, Wu DC; Taiwan Acid-Related Disease (TARD) Study Group. Ten-day Quadruple therapy comprising proton-pump inhibitor, bismuth, tetracycline, and levofloxacin achieves a high eradication rate for Helicobacter pylori infection after failure of sequential therapy. *Helicobacter* 2014; **19**: 74-79 [PMID: 24033865 DOI: 10.1111/hel.12085]
18 **Gisbert JP**, Molina-Infante J, Marin AC, Vinagre G, Barrio J, McNicholl AG. Second-line rescue triple therapy with levofloxacin after failure of non-bismuth quadruple "sequential" or "concomitant" treatment to eradicate H. pylori infection. *Scand J Gastroenterol* 2013; **48**: 652-656 [PMID: 23556551 DOI: 10.3109/00365521.2013.786132]
19 **Masaoka T**, Suzuki H, Kurabayashi K, Nomoto Y, Nishizawa T, Mori M, Hibi T. Could frameshift mutations in the frxA and rdxA genes of Helicobacter pylori be a marker for metronidazole resistance? *Aliment Pharmacol Ther* 2006; **24** Suppl 4: 81-87 [DOI: 10.1111/j.1746-6342.2006.00029.x]
20 **Arslan N**, Yılmaz Ö, Demiray-Gürbüz E. Importance of antimicrobial susceptibility testing for the management of eradication in *Helicobacter pylori* infection. *World J Gastroenterol* 2017; **23**: 2854-2869 [PMID: 28522904 DOI: 10.3748/wjg.v23.i16.2854]
21 **Bina JE**, Alm RA, Uria-Nickelsen M, Thomas SR, Trust TJ, Hancock RE. Helicobacter pylori uptake and efflux: basis for intrinsic susceptibility to antibiotics in vitro. *Antimicrob Agents Chemother* 2000; **44**: 248-254 [PMID: 10639345 DOI: 10.1128/AAC.44.2.248-254.2000]
22 **Hirata K**, Suzuki H, Nishizawa T, Tsugawa H, Muraoka H, Saito Y, Matsuzaki J, Hibi T. Contribution of efflux pumps to clarithromycin resistance in Helicobacter pylori. *J Gastroenterol Hepatol* 2010; **25** Suppl 1: S75-S79 [PMID: 20586871 DOI: 10.1111/j.1440-1746.2009.06220.x]
23 **Nishizawa T**, Suzuki H. Mechanisms of Helicobacter pylori antibiotic resistance and molecular testing. *Front Mol Biosci* 2014; **1**: 19 [PMID: 25988160 DOI: 10.3389/fmolb.2014.00019]
24 **Mori H**, Suzuki H, Matsuzaki J, Masaoka T, Kanai T. Acquisition of double mutation in *gyrA* caused high resistance to sitafloxacin in *Helicobacter pylori* after unsuccessful eradication with sitafloxacin-containing regimens. *United European Gastroenterol J* 2018; **6**: 391-397 [PMID: 29774152 DOI: 10.1177/2050640617737215]
25 **Wu DC**, Hsu PI, Tseng HH, Tsay FW, Lai KH, Kuo CH, Wang SW, Chen A. Helicobacter pylori infection: a randomized, controlled study comparing 2 rescue therapies after failure of standard triple therapies. *Medicine (Baltimore)*2011; **90**: 180-185 [PMID: 21512411 DOI: 10.1097/MD.0b013e31821c9d1c]
26 **Gisbert JP**. "Rescue" regimens after Helicobacter pylori treatment failure. *World J Gastroenterol* 2008; **14**: 5385-5402 [PMID: 18803350 DOI: 10.3748/wjg.14.5385]
27 **Hojo M**, Miwa H, Nagahara A, Sato N. Pooled analysis on the efficacy of the second-line treatment regimens for Helicobacter pylori infection. *Scand J Gastroenterol* 2001; **36**: 690-700 [PMID: 11444467 DOI: 10.1080/00365520116825]
28 **Lee BH**, Kim N, Hwang TJ, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Jung HC, Song IS. Bismuth-containing quadruple therapy as second-line treatment for Helicobacter pylori infection: effect of treatment duration and antibiotic resistance on the eradication rate in Korea. *Helicobacter* 2010; **15**: 38-45 [PMID: 20302588 DOI: 10.1111/j.1523-5378.2009.00735.x]
29 **Chey WD**, Leontiadis GI, Howden CW, Moss SF. ACG Clinical Guideline: Treatment of Helicobacter pylori Infection. *Am J Gastroenterol* 2017; **112**: 212-239 [PMID: 28071659 DOI: 10.1038/ajg.2016.563]
30 **Di Caro S**, Fini L, Daoud Y, Grizzi F, Gasbarrini A, De Lorenzo A, Di Renzo L, McCartney S, Bloom S. Levofloxacin/amoxicillin-based schemes vs quadruple therapy for Helicobacter pylori eradication in second-line. *World J Gastroenterol* 2012; **18**: 5669-5678 [PMID: 23155306 DOI: 10.3748/wjg.v18.i40.5669]
31 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
32 **Goodwin CS**, Marshall BJ, Blincow ED, Wilson DH, Blackbourn S, Phillips M. Prevention of nitroimidazole resistance in Campylobacter pylori by coadministration of colloidal bismuth subcitrate: clinical and in vitro studies. *J Clin Pathol* 1988; **41**: 207-210 [PMID: 3280609 DOI: 10.1136/jcp.41.2.207]

33 **Liao J**, Zheng Q, Liang X, Zhang W, Sun Q, Liu W, Xiao S, Graham DY, Lu H. Effect of fluoroquinolone resistance on 14-day levofloxacin triple and triple plus bismuth quadruple therapy. *Helicobacter* 2013; **18**: 373-377 [PMID: 23581720 DOI: 10.1111/hel.12052]

34 **Hsu PI**, Tsai FW, Kao SS, Hsu WH, Cheng JS, Peng NJ, Tsai KW, Hu HM, Wang YK, Chuah SK, Chen A, Wu DC. Ten-Day Quadruple Therapy Comprising Proton Pump Inhibitor, Bismuth, Tetracycline, and Levofloxacin is More Effective than Standard Levofloxacin Triple Therapy in the Second-Line Treatment of Helicobacter pylori Infection: A Randomized Controlled Trial. *Am J Gastroenterol* 2017; **112**: 1374-1381 [PMID: 28719592 DOI: 10.1038/ajg.2017.195]

35 **Yang JC**, Lin CJ, Wang HL, Chen JD, Kao JY, Shun CT, Lu CW, Lin BR, Shieh MJ, Chang MC, Chang YT, Wei SC, Lin LC, Yeh WC, Kuo JS, Tung CC, Leong YL, Wang TH, Wong JM. High-dose dual therapy is superior to standard first-line or rescue therapy for Helicobacter pylori infection. *Clin Gastroenterol Hepatol* 2015; **13**: 895-905.e5 [PMID: 25460556 DOI: 10.1016/j.cgh.2014.10.036]

36 **Sugimoto M**, Furuta T, Shirai N, Kajimura M, Hishida A, Sakurai M, Ohashi K, Ishizaki T. Different dosage regimens of rabeprazole for nocturnal gastric acid inhibition in relation to cytochrome P450 2C19 genotype status. *Clin Pharmacol Ther* 2004; **76**: 290-301 [PMID: 15470328 DOI: 10.1016/j.clpt.2004.06.008]

37 **Craig WA**. Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clin Infect Dis* 1998; **26**: 1-10; quiz 11-12 [PMID: 9455502 DOI: 10.1086/516284]

38 **Miehlke S**, Kirsch C, Schneider-Brachert W, Haferland C, Neumeyer M, Bästlein E, Papke J, Jacobs E, Vieth M, Stolte M, Lehn N, Bayerdörffer E. A prospective, randomized study of quadruple therapy and high-dose dual therapy for treatment of Helicobacter pylori resistant to both metronidazole and clarithromycin. *Helicobacter* 2003; **8**: 310-319 [PMID: 12950604 DOI: 10.1046/j.1523-5378.2003.00158.x]

P- Reviewer: Nishida T, Suzuki H **S- Editor**: Ma RY
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4554-4564

DOI: 10.3748/wjg.v24.i40.4554

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

ORIGINAL ARTICLE

Basic Study

Recovery of natural killer cells is mainly in post-treatment period in chronic hepatitis C patients treated with sofosbuvir plus ledipasvir

Xiao-Xiao Wang, Bi-Fen Luo, Han-Ji Jiang, Xu Cong, Qian Jin, Dan-Li Ma, Lai Wei, Bo Feng

Xiao-Xiao Wang, Bi-Fen Luo, Han-Ji Jiang, Xu Cong, Qian Jin, Dan-Li Ma, Lai Wei, Bo Feng, Peking University Hepatology Institute, Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Diseases, Peking University People's Hospital, Beijing 100044, China

ORCID number: Xiao-Xiao Wang (0000-0003-1654-7302); Bi-Fen Luo (0000-0001-7166-278X); Han-Ji Jiang (0000-0003-2118-0584); Xu Cong (0000-0001-8922-7321); Qian Jin (0000-0002-5213-8680); Dan-Li Ma (0000-0002-0936-6792); Lai Wei (0000-0003-2326-1257); Bo Feng (0000-0001-5084-6715).

Author contributions: Wang XX, Luo BF, Wei L and Feng B designed the study, performed data analysis, and wrote and edited the manuscript; Luo BF, Jiang HJ, Jin Q and Ma DL enrolled the patients; Wang XX, Jiang HJ, and Cong X performed sample testing; all authors reviewed and approved the manuscript.

Supported by the National S&T Major Projects for Infectious Diseases Control, No. 2017ZX10302201-004-001.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Peking University People's Hospital.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Bo Feng, PhD, Chief Doctor, Professor, Research Fellow, Peking University Hepatology Institute, Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Diseases, Peking University People's Hospital, No. 11, Xizhimen South Street, Beijing 100044, China. fengbo@pkuph.edu.cn
Telephone: +86-10-88325980
Fax: +86-10-66515490

Received: July 27, 2018
Peer-review started: July 27, 2018
First decision: August 27, 2018
Revised: September 2, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

AIM

To investigate how natural killer (NK) cells are affected in the elimination of hepatitis C virus (HCV) by sofosbuvir/ledipasvir, two highly effective direct-acting antivirals (DAAs).

METHODS

Thirteen treatment-naïve and treatment-experienced chronic hepatitis C (CHC) patients were treated with sofosbuvir/ledipasvir, and NK cells were detected at baseline, weeks 2, 4, 8 and 12 during therapy, and week post of treatment (Pt)-12 and 24 after the end of therapy by multicolor flow cytometry and compared with those from 13 healthy controls.

RESULTS

All patients achieved sustained virological response. There was a significant decline in $CD56^{bright}$ NK cell frequencies at week 8 (P = 0.002) and week 12 (P = 0.003), which were altered to the level comparable to healthy controls at week Pt-12, but no difference

was observed in the frequency of CD56^{dim} NK cells. Compared with healthy controls, the expression levels of NKG2A, NKp30 and CD94 on NK cells from CHC patients at baseline were higher. NKG2A, NKp30 and CD94 started to recover at week 12 and reached the levels similar to those of healthy controls at week Pt-12 or Pt-24. Before treatment, patients have higher interferon (IFN)-γ and perforin levels than healthy controls, and IFN-γ started to recover at week 8 and reached the normalized level at week Pt-12.

CONCLUSION

NK cells of CHC patients can be affected by DAAs, and phenotypes and function of NK cells recover not at early stage but mainly after the end of sofosbuvir/ledipasvir treatment.

Key words: Direct-acting antivirals therapy; Hepatitis C virus; Natural killer cells; Natural killer subsets

Core tip: In our study, we observed the dynamic changes of natural killer (NK) cell subsets, phenotypes and functional parameters during and after direct-acting antivirals (DAAs) treatment and investigated the effect of sofosbuvir/ledipasvir therapy on innate immunity in genotype 1b hepatitis C virus (HCV)-infected patients. We illustrated that NK cells of chronic hepatitis C patients can be affected by DAAs and phenotypes and function of NK cells recovered not at early stage but mainly after the end of sofosbuvir/ledipasvir treatment. These findings may provide an explanation for HCV reinfection or liver carcinogenesis after HCV elimination.

Wang XX, Luo BF, Jiang HJ, Cong X, Jin Q, Ma DL, Wei L, Feng B. Recovery of natural killer cells is mainly in post-treatment period in chronic hepatitis C patients treated with sofosbuvir plus ledipasvir. *World J Gastroenterol* 2018; 24(40): 4554-4564 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4554.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4554

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a disease that affects about 71 million people worldwide[1]. It can lead to mortality from hepatic as well as extra-hepatic causes[2]. In the era of HCV treatment with pegylated-interferon (PEG-IFN)/ribavirin (RBV), the sustained virological response (SVR) rate is different according to HCV genotypes: Approximately 40% to 50% in patients with genotype 1 and 80% in patients with genotype 2 or 3[3-5]. However, because of their higher SVR rate (> 95%) and less toxicity[6], IFN-free direct antiviral agents (DAAs) have replaced PEG-IFN/RBV as a first-line treatment option recommended by international guidelines[7,8].

IFN-α can induce immunomodulatory effects by acting the innate and adaptive immune systems of various cells[9,10]. Since IFN-free DAAs take the virus life cycle as a target, they can inhibit NS3 protease, NS5A replication complex or NS5B polymerase activity specifically[11]. These regimens can help us clarify the interaction between HCV clearance and the innate immune response, regardless of the IFNα induced immune modulation[12]. This provides a unique opportunity to analyze whether DAAs can change natural killer (NK) cell activation when HCV replication is inhibited. Previous studies have explored the effect of IFN therapy on the NK cells. It has been shown that chronic hepatitis C (CHC) patients with SVR to IFN therapy exhibited greater levels of NK cell degranulation and enhanced NK cytotoxicity[13,14]. Combination therapy of PEG-IFN-α/RBV reversed NK subtype distribution and function in HCV-eliminated patients[15]. Until now, only few studies have reported the effect of DAAs on NK cells[16-18], in which the results and conclusions were controversial.

NK cells are enriched among lymphocytes in the blood (5%-20%), and their percentage increases further in viral hepatitis[19]. NK cells play an important role in the antiviral immune defense and undergo great changes in subsets, phenotypes and function during persistent viral infection[20]. NK cells can be divided into three subgroups according to the expression levels of CD56 and CD16, including CD56^{bright} NK cells, CD56^{dim} NK cells and CD56^{neg} (CD16^{positive}) NK cells[21]. CD56^{bright} NK cells, which can produce IFN-γ mainly and inhibit viral replication, are the less-mature subset that can differentiate into CD56^{dim} NK cells[22]. CD56^{dim} NK cells are cytotoxic NK cell subset expressing higher levels of killer immunoglobulin-like receptors (KIR), CD16 and perforin[21]. CD56^{neg} NK cells express lower perforin and exhibit lower cytotoxicity[23].

The function of NK cells is regulated by interaction of NK cell receptors (NKRs), which can be divided into activating and inhibitory NKRs, and their respective ligands[24]. Activating NKRs include NKG2C, NKG2D and the "natural cytotoxicity receptors", for example, NKp30, NKp44 and NKp46. Inhibitory receptors comprise NKG2A and the KIR family members[25]. During viral infection, the balance shifts from inhibition to activation because the threshold value of activation receptors exceeds that of inhibition[26]. For example, the integration of all signals results in activation of blood and liver NK cells in HCV infection[27] and altered functional phenotypes with increased cytotoxicity and decreased antiviral cytokine production[27,28].

Up to now, more and more DAAs have been approved for clinical practice. In China, sofosbuvir/ledipasvir have been increasingly used among CHC patients, especially those with genotype 1 HCV infection. Sofosbuvir is an NS5B polymerase inhibitor and ledipasvir is an NS5A replication complex inhibitor. In the current study, we aimed to observe the dynamic changes of NK cell subsets, phenotypes and functional parameters during and after DAAs treatment, and to investigate the effect of DAAs (sofosbuvir/ledipasvir) treatment on innate

immunity in genotype 1b HCV-infected patients.

MATERIALS AND METHODS

Study cohort

NK cells were studied in 13 genotype 1b HCV infected patients at baseline, weeks 2, 4, 8 and 12 in a 12-wk treatment course with DAAs (90 mg ledipasvir once daily and 400 mg sofosbuvir once daily), and then at week post of treatment (Pt)-12 and 24 after the end of therapy. There are six treatment-experienced patients who relapsed after treatment with PEG-IFN/RBV and seven treatment-naïve patients in our study. Thirteen age- and sex-matched uninfected subjects were enrolled for comparison. Informed consent was obtained from all participants. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Peking University People's Hospital. Patients had no signs or evidence of coinfection with hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus and human immunodeficiency virus. Patients with evidence of hepatocellular carcinoma or cirrhosis were excluded. Besides, pregnant patients or patients with psychiatric disorders were also excluded.

Serologic analysis

Serum HCV RNA level was quantitated using the Cobas TaqMan automated real-time PCR platform reaction (Roche Molecular System, Pleasanton, CA, United States) with a lowest limit of detection of 15 IU/mL and a lower limit of quantification of 43 IU/mL.

Lymphocyte isolation

Peripheral blood mononuclear cells (PBMCs) were separated from EDTA- anticoagulated blood on Ficoll Histopaque (GE Healthcare Bio-Science AB, Germany) density gradients, washed three times with phosphate-buffered saline (BD, Bioscience, Franklin Lakes, NJ, United States)[17], cryopreserved at -80 ℃ and transferred to the liquid nitrogen after 24 h.

NK cell frequency and phenotypes

For each patient, cryopreserved PBMCs from week 0 to week Pt-24 were thawed and tested. PBMCs of healthy donors were included in this experiment. Thawed PBMCs were stained with anti-CD45-APC-H7, anti-CD3-PerCP-Cy5.5, anti-CD56-APC, anti-CD16-BV510, anti-CD94-PE, anti-CD335 (NKp46) -PE-Cy7, anti-CD336 (NKp44) -BB515, anti-CD337 (NKp30) - BV421 (BD Bioscience), anti-CD314 (NKG2D)-PE-Cy7 (Biolegend), anti-CD159a (NKG2A)-PE (R and D Systems) and anti-CD159C (NKG2C)-VioBright™FITC (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min and with 7-AAD (BD Bioscience) for 10 min before being detected by flow cytometry (BD FACSAria Ⅱ, BD Bioscience). Data were analyzed with BD FACSDiva Software v7.0.

NK cell cytokine production

Thawed PBMCs were incubated with Leukocyte Activation Cocktail with BD GolgiPlug (BD Bioscience) 2 μL/1 × 10^6 cells for 4 h. Cells were washed, fixed, and permeabilized with the BD IntraSure™ Kit and stained with anti-IFN-γ-FITC, anti-Perforin-BV421 and anti-Granzyme B-PE-CF594 (all from BD Biosciences) for 30 min before being detected by flow cytometry (BD FACSAria Ⅱ, BD Bioscience). Data were analyzed with BD FACSDiva Software v7.0.

Statistical analysis

Statistical analyses were performed using Graphpad Prism Version 5.0a (Graphpad Software Inc., San Diego, CA, United States) and SPSS 16.0 (SPSS, Chicago, IL, United States). Normal distribution was tested by the Kolmogorov-Smirnov test. Values in our study were not normally distributed, and comparisons of expression levels among different time points in CHC patients were performed by the Wilcoxon matched pairs test. Comparisons of expression levels between the CHC patients and healthy controls were performed by the Mann-Whitney test. Two-sided *P*-values less than 0.05 were considered significant.

RESULTS

Baseline characteristics of the study population and effect of sofosbuvir/ledipasvir therapy on HCV viremia and liver inflammation

Table 1 describes the main demographical and clinical characteristics of the CHC patients at baseline. The median log HCV RNA level was 6.39 (range, 4.60-6.98). The median alanine aminotransferase (ALT) level was 34 U/L (range, 11-55 U/L). All 13 patients were treated with sofosbuvir/ledipasvir within 12 wk and reached SVR24. Treatment with DAAs induced rapid and early clearance of serum HCV RNA within the first 2 wk, accompanied by a significant reduction in liver inflammation as demonstrated by a decrease in ALT ($P = 0.007$) and AST levels ($P = 0.015$) (Figure 1).

Effect of sofosbuvir/ledipasvir therapy on NK cell subsets

NK cells were identified as $CD3^-CD56^+$ cells in the PBMC population, and $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were determined by sequential gating on $CD3^-CD56^+$ NK cells (Figure 2A).

Our study showed that during the 12 wk of IFN-free DAAs therapy, there was a significant decline in $CD56^{bright}$ NK cell frequencies at week 8 ($P = 0.002$) and week 12 ($P = 0.003$), which were lower than that of healthy controls at week 12. The frequency of $CD56^{bright}$ NK cells was altered to the level comparable to that of healthy controls at week Pt-12 (Figure 3B). There was no difference in the frequency of $CD56^+$ NK cells or $CD56^{dim}$ NK cells between chronically HCV-infected patients and healthy controls at baseline. No difference

Table 1 Demographical and clinical characteristics of chronic hepatitis C patients

Sex (F/M)	Age (yr)	BMI (kg/m^2)	Fibroscan index	Treatment-naïve /experienced	ALT/AST (U/L)-baseline	ALT/AST (U/L)-week 2	Log HCV RNA	Response to sofosbuvir/ledipasvir
F	27	18.71	3.8	N	25/25	10/19	6.16	SVR24
M	62	15.85	6.5	N	55/47	24/23	6.09	SVR24
F	30	20.07	5.9	N	18/20	10/19	6.38	SVR24
F	53	20.10	3.9	N	11/25	6/19	6.98	SVR24
M	25	24.39	6.1	N	43/22	23/18	4.60	SVR24
F	27	19.38	7.9	N	20/20	11/22	6.87	SVR24
F	29	26.67	6.4	N	32/24	16/14	5.90	SVR24
M	24	29.33	7.6	E	85/75	10/16	6.45	SVR24
M	63	25.10	4.8	E	50/32	31/23	6.51	SVR24
M	55	23.62	4.5	E	34/30	24/20	6.56	SVR24
F	57	22.31	4.7	E	20/26	15/20	6.76	SVR24
F	63	26.75	4.3	E	27/29	11/19	6.76	SVR24
M	25	26.53	6.0	E	30/23	11/22	6.60	SVR24

F: Female; M: Male; N: Treatment-naïve; E: Treatment-experienced; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HCV: Hepatitis C virus; SVR: Sustained virological response.

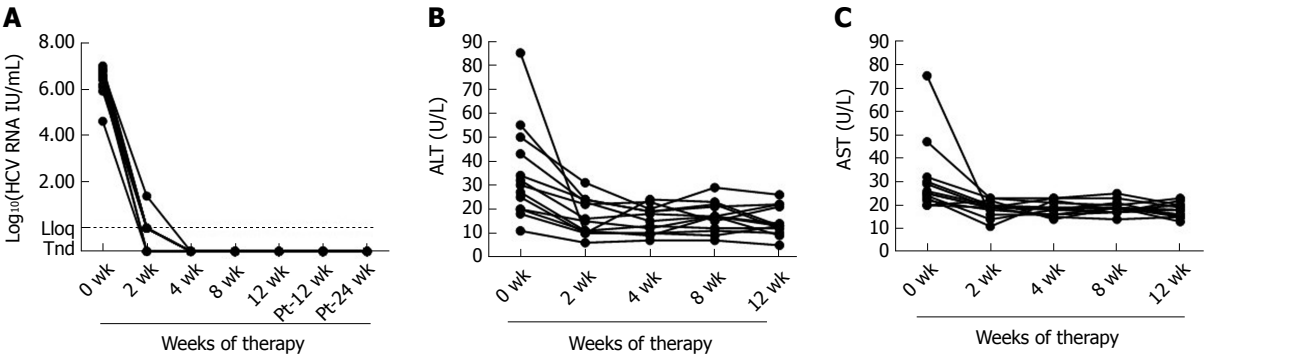


Figure 1 Serum hepatitis C virus RNA levels and liver inflammation decrease rapidly with sofosbuvir and ledipasvir therapy. A: Serum hepatitis C virus RNA levels of patients who all responded to therapy (n = 13). A response to sofosbuvir and ledipasvir therapy was defined as undetectable viremia at end of treatment (week 24); (B) Serum alanine aminotransferase. (C) Serum aspartate aminotransferase. HCV: Hepatitis C virus; Lloq: Lower limit of quantitation; Tnd: Target not detected; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

was found in the frequency of CD56$^+$ NK cells or CD56^{dim} NK cells among different time points during and after DAAs therapy (Figure 3A and C).

Effect of sofosbuvir/ledipasvir therapy on NK cell phenotypes

To illustrate the effect of the rapid DAA-mediated decrease in HCV RNA levels on NK cell phenotypes, we detected activating and inhibitory receptors on the surface of NK cells by multicolor flow cytometry (Figure 2B).

Compared with uninfected healthy controls, the frequencies of the inhibitory NKG2A and activating NKp30 on NK cells from CHC patients were higher at baseline (P < 0.001) (Figures 4A and 5A). The frequency and mean fluorescence intensity (MFI) of NKG2A and the frequency of NKp30 started to decline at week 12 of treatment and reached the levels similar to those of NK cells from healthy controls at week Pt-12 (Figure 4A and B, Figure 5A). However, MFI of NKp30 did not differ on NK cells from CHC patients and healthy controls and did not change during and after the end of sofosbuvir/ledipasvir therapy (Figure 5B).

There was no difference in the frequency of CD94$^+$ NK cells between CHC patients at baseline and healthy controls, and the frequency of CD94$^+$NK cells from CHC patients did not change significantly during and after sofosbuvir/ledipasvir therapy (Figure 4C). The MFI of CD94 on NK cells started to decline in CHC patients at week 12 of treatment and normalized at week Pt-24 (Figure 4D).

There was no difference in the frequency or MFI of NKp46 on NK cells between healthy controls and CHC patients at baseline. During DAAs treatment, there was a significant decline in the frequency of NKp46 on NK cells at week 8 and week 12, but NKp46 expression levels increased to those of uninfected controls at week Pt-12 and week Pt-24 (Figure 5C and D).

Frequencies and MFI of NKp44, NKG2C and NKG2D on NK cells from CHC patients at baseline did not differ from those from healthy controls and did not change during and after sofosbuvir/ledipasvir therapy.

Effect of sofosbuvir/ledipasvir therapy on NK cell function

The frequency and MFI of IFN-γ^+ NK cells from CHC

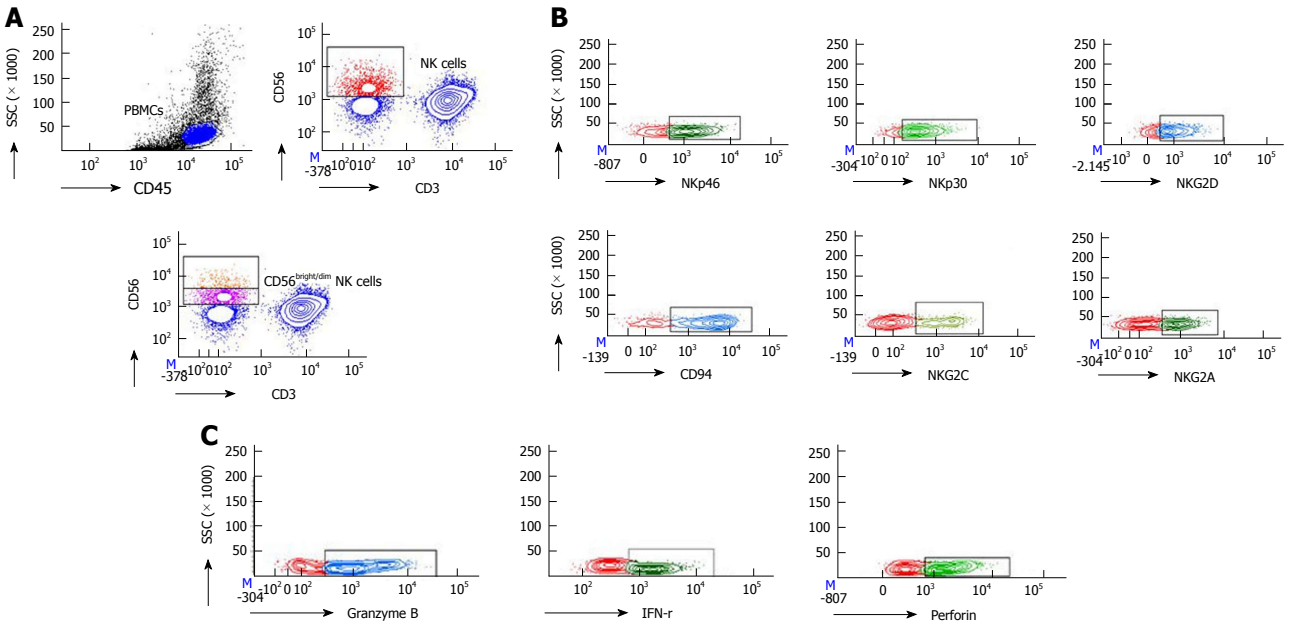


Figure 2 Flow cytometry results. A: Isolation of human peripheral blood natural killer (NK) cells and subsets; B: The expression of NKp46, NKp30, NKG2D, CD94, NKG2C and NKG2A during and after the end of direct-acting antivirals (DAAs) treatment; C: The expression of Granzyme B, IFN-γ and perforin during and after the end of DAAs treatment. NK: Natural killer; IFN: Interferon; DAAs: Direct-acting antivirals.

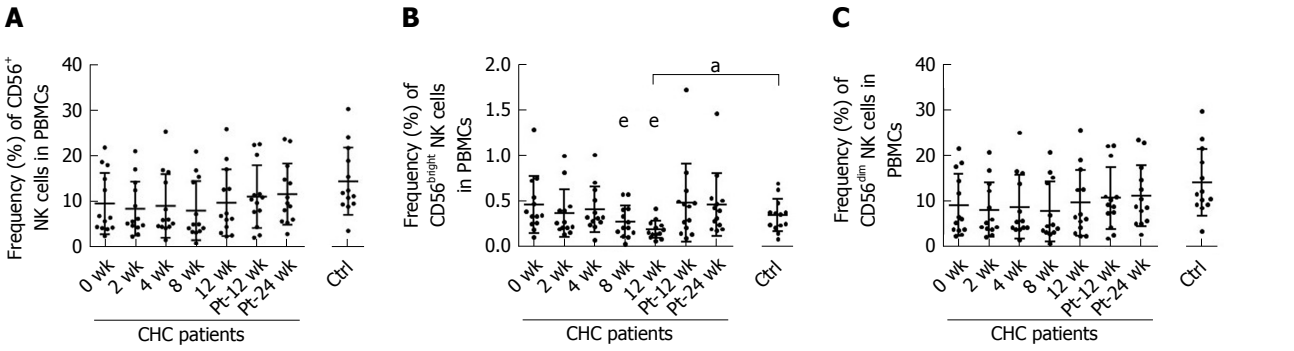


Figure 3 Effect of sofosbuvir and ledipasvir therapy on the frequencies of natural killer cell subsets from chronic hepatitis C patients. A: Frequencies of CD56^{+} natural killer (NK) cells in peripheral blood mononuclear cells (PBMCs); B: Frequencies of CD56^{bright} NK cells in PBMCs; C: Frequencies of CD56^{dim} NK cells in PBMCs. $^{a}P < 0.05$ and $^{b}P \leqslant 0.01$ and $^{c}P \leqslant 0.001$, different time points of CHC patients *vs* healthy controls; $^{d}P < 0.05$ and $^{e}P \leqslant 0.01$ and $^{f}P \leqslant 0.001$, different time points of CHC patients (2 wk, 4 wk, 8 wk, 12 wk, Pt-12 wk, Pt-24 wk *vs* 0 wk). NK: Natural killer; CHC: Chronic hepatitis C; Ctrl: Healthy controls; Pt: Post of treatment; PBMCs: Peripheral blood mononuclear cells.

patients at baseline were significantly higher than those from uninfected subjects ($P < 0.001$). Frequency of IFN-r^{+} NK cells started to decline in CHC patients at week 8 of treatment and reached the level similar to that of healthy controls at week Pt-12 (Figure 6A). However, no difference was shown in MFI of IFN-γ^{+} NK cells from CHC patients during and after DAAs therapy (Figure 6B). There was no difference in the frequency of perforin^{+} NK cells between healthy controls and CHC patients at each time point (Figure 6C). MFI of perforin^{+} NK cells from CHC patients was significantly higher than that from uninfected subjects from week 0 to week Pt-24W persistently (Figure 6D). However, there was no difference in the frequency (Figure 6E) or MFI (Figure 6F) of Granzyme B of NK cell groups between CHC patients at baseline and healthy controls, and the expression of NKp46 did not change during and after sofosbuvir/ledipasvir therapy.

Effect of sofosbuvir/ledipasvir therapy on NK cells between treatment-naïve and treatment-experienced CHC patients

There was no difference in the frequency of CD56^{+} NK cells or the expression of NKG2A, NKG2D, NKG2C, CD94, NKp30, NKp46, IFN-γ, perforin and Granzyme B between treatment-naïve and treatment-experienced CHC patients during and after DAAs treatment. The changing trends of these phenotypes and cytokines of NK cells between these two groups were similar. (Figure 7A-J)

DISCUSSION

In our study, we evaluated the innate immune effects in 13 CHC patients successfully treated with sofosbuvir/

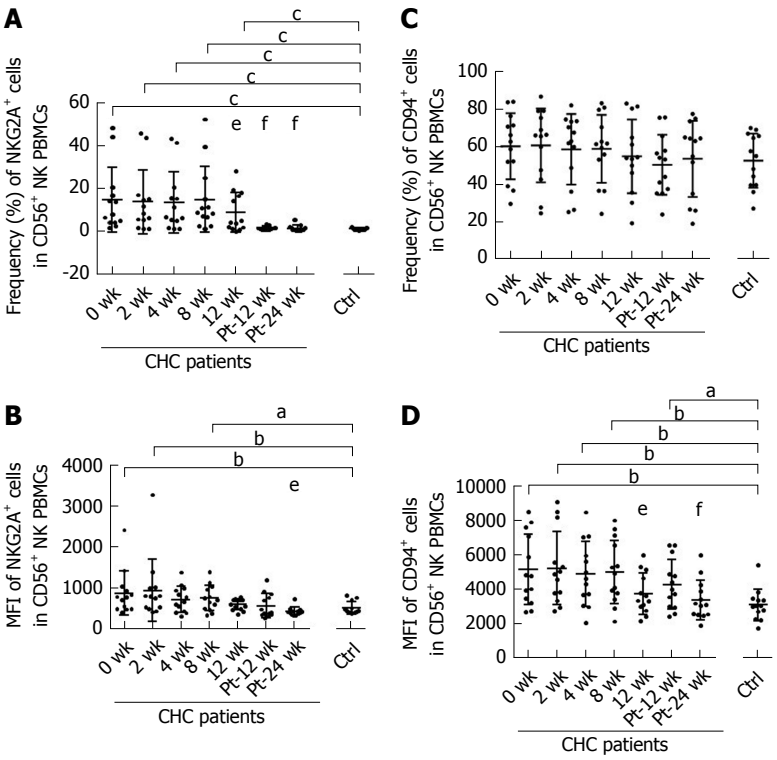


Figure 4 Sofosbuvir and ledipasvir therapy modulates the expression of NKG2A and CD94 on natural killer cells. Flow cytometric analyses of inhibitory receptor NKG2A and CD94 on natural killer (NK) cells. The graphs display the frequencies and mean fluorescence intensity (MFI) of NKG2A (A-B) and CD94 (C-D) on NK cells from chronic hepatitis C (CHC) patients during and after direct-acting antivirals therapy and healthy controls (n = 13). $^{a}P < 0.05$ and $^{b}P \leqslant 0.01$ and $^{c}P \leqslant 0.001$, different time points of CHC patients *vs* healthy controls; $^{d}P < 0.05$ and $^{e}P \leqslant 0.01$ and $^{f}P \leqslant 0.001$, different time points of CHC patients (2 wk, 4 wk, 8 wk, 12 wk, Pt-12 wk, Pt-24 wk *vs* 0 wk). NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; CHC: Chronic hepatitis C; Ctrl: Healthy controls; Pt: Post of treatment; MFI: Mean fluorescence intensity.

ledipasvir. All patients achieved SVR12 and SVR24, and no patients had virologic breakthrough.

The development of DAAs has opened a new era of HCV treatment[29]. IFN-free regimens for HCV infection provide a unique opportunity to study the interaction between HCV and the immune system because DAAs rapidly decrease viremia to undetectable levels regardless of the IFNα induced immune modulation[12]. Due to the direct effect of IFN-α on NK cells, the consequence of viral load decline on NK cells could not be examined precisely. Our study examined the innate immune effects of sofosbuvir/ledipasvir therapy induced HCV RNA level decline. There was no difference in the frequency of total NK cells from CHC patients at baseline and healthy controls, which is inconsistent with other two studies assuming that NK cell frequency decreases in the blood in chronic HCV infection[28,30]. We found that only $CD56^{bright}$ NK cells showed a slightly decline at week 8 and week 12 during DAAs treatment and normalized at week Pt-12. There was no change in total NK cells or $CD56^{dim}$ NK cells. However, another study reported that DAA therapy enhances the frequency of $CD56^{dim}$ and decreases $CD56^{bright}$ cells in chronically HCV-infected patients[30].

As for NK cell phenotypes and function of CHC patients, we showed that reduced HCV RNA load altered NK cell phenotype and function, including NKG2A, CD94, NKp30, NKp46, IFN-γ and perforin. Most of these phenotypes and cytokines became to change at week 12 approximately and normalized to the levels of healthy controls at week Pt-12 or Pt-24, which are different from previous studies[17,30]. Serti *et al*[17] demonstrated that the expression levels of NKp46 and NKG2A normalized in patients with undetectable viremia by week 8 in daclatasvir (DCV) and asunaprevir (ASV) therapy, and they assumed that the percentage of IFN-γ producing NK cells and the IFN-γ expression level were significantly lower in chronically HCV-infected patients compared with healthy controls and increased within the first 8 wk. Spaan *et al*[30] illustrated that NK cell phenotype is already normalized at week 12 during DCV/ASV therapy, and they assumed that DAAs treatment did not alter the frequency of NK cells producing perforin. However, we found that the MFI of $perforin^{+}$ NK cells was higher than that of healthy controls before, during and after DAAs therapy persistently, which has not been found in previous studies. Importantly, researchers in these two studies have not demonstrated the changes of NK cells after the end of DAAs therapy.

Patients in other two studies were treated with DCV/ASV and all patients were HCV-infected non-responders to previous PEG-IFN/RBV therapy[17,30]. However, patients in our study were treatment-naïve CHC patients and treatment-experienced CHC patients who had a relapse after PEG-IFN/RBV therapy. NK cells might be affected by PEG-IFN/RBV therapy[13,14]. Combination therapy of

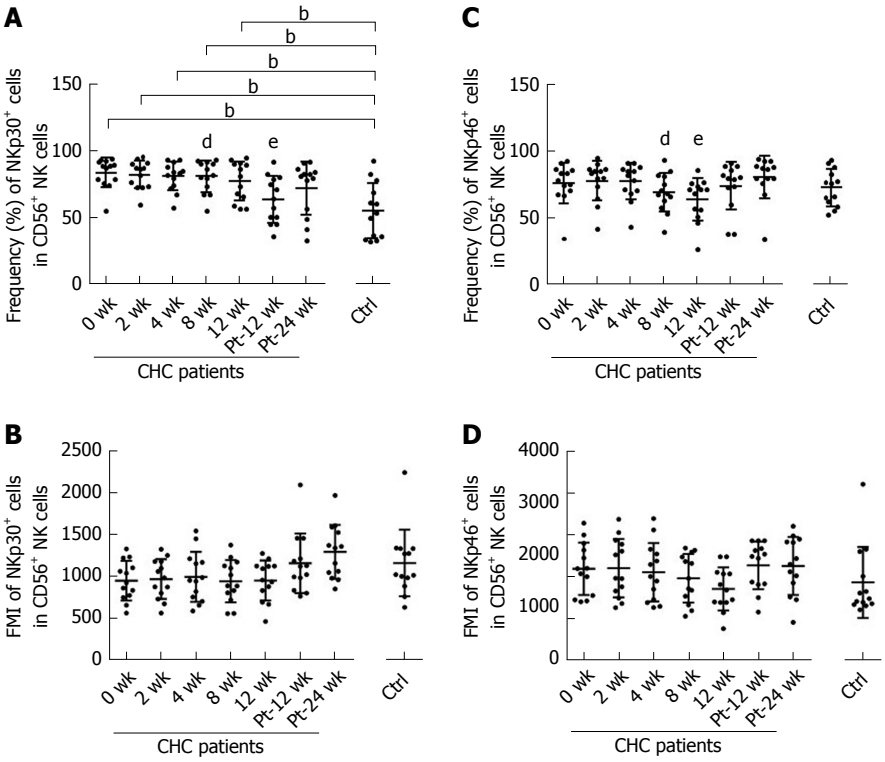


Figure 5 Sofosbuvir and ledipasvir therapy modulates the expression of NKp30 and NKp46 on natural killer cells. Flow cytometric analyses of activating receptors NKp30 and NKp46 on natural killer (NK) cells. The graphs display the frequencies and mean fluorescence intensity (MFI) of NKp30 (A-B) and NKp46 (C-D) on NK cells from chronic hepatitis C (CHC) patients during and after Direct-acting antivirals therapy and healthy controls (n = 13). $^{a}P < 0.05$ and $^{b}P \leqslant 0.01$ and $^{c}P \leqslant 0.001$, different time points of CHC patients *vs* healthy controls; $^{d}P < 0.05$ and $^{e}P \leqslant 0.01$ and $^{f}P \leqslant 0.001$, different time points of CHC patients (2 wk, 4 wk, 8 wk, 12 wk, Pt-12 wk, Pt-24 wk *vs* 0 wk). NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; CHC: Chronic hepatitis C; Ctrl: Healthy controls; Pt: Post of treatment; MFI: Mean fluorescence intensity.

PEG-IFN-α/RBV reversed NK subtype distribution and functions in HCV-eliminated patients[15]. However, we found that there was no difference in the changes of the NK cells during and after DAAs treatment between patients who were treatment-naïve and those who had a relapse after PEG-IFN/RBV therapy. And the effect of non-response to PEG-IFN/RBV treatment on the changes of NK cells during and after DAA treatment should not be excluded. Because it is unclear whether the response pattern to PEG-IFN/RBV treatment can affect the subsequent changes of NK cells induced by DAAs in treatment-experienced patients. Second, HCV genotype may affect dynamic changes of NK cells during DAAs therapy. In a study of Ning *et al*[31] in which patients with different genotypes were included, they assumed that different HCV genotypes may have an impact on their results. After treatment with sofosbuvir/ledipasvir or sofosbuvir/daclatasvir, the frequency of $CD16^{+}CD56^{+}$ NK cells gradually increased to normal levels of healthy controls at week 12. Third, different DAAs may have different impact on NK cells. Sofosbuvir is an NS5B polymerase inhibitor, ledipasvir and DCV are NS5A replication complex inhibitor and ASV is an NS3 protease inhibitor. As Ning *et al*[31] described, we cannot exclude the possibility that different DAA regimens induce different dynamic changes of NK cells. Fourth, the race of CHC patients may be an important factor. Whether in era of PEG-IFN/RBV or DAAs treatment, race is one of the most important factors which can affect SVR[32,33]. Therefore, we cannot rule out the distinction induced by different races between our studies and others. Additionally, we assumed that it may take a long term for NK cell recovery and NK cells cannot recover immediately after the influence from DAAs was relieved.

We confirm earlier studies on increased inhibitory NKG2A expression on NK cells in HCV infection[18,27]. NKG2A is a major and prominent inhibitory NK cell receptor and is known as lectin superfamily group A[34]. DAA-induced NKG2A level reduction might be the consequence of compensatory mechanisms exerted upon declining activating signals. Besides, CD94 is mainly expressed as a heterodimer with NKG2A, NKG2B or NKG2C protein[35]. The inhibitory signals of NK cells are mainly mediated by HLA class I-binding receptors, including KIRs and CD94/NKG2A[36]. Accordingly, there are something in common of the changing trends between NKG2A and CD94 MFI in our study.

Frequency of $NKp30^{+}$ NK cells at baseline in our cohort was higher than those of healthy controls. During DAAs treatment, the percentage of $NKp30^{+}$ NK cells was reduced to the level of healthy controls, which is consistent with the studies of Serti *et al*[17] and Spaan *et al*[30], but there was no difference in the MFI of NKp30.

In all changed phenotypes, NKp46 is a special one. The changing trend of NKp46 is completely different from that of other receptors during sofosbuvir/ledipasvir

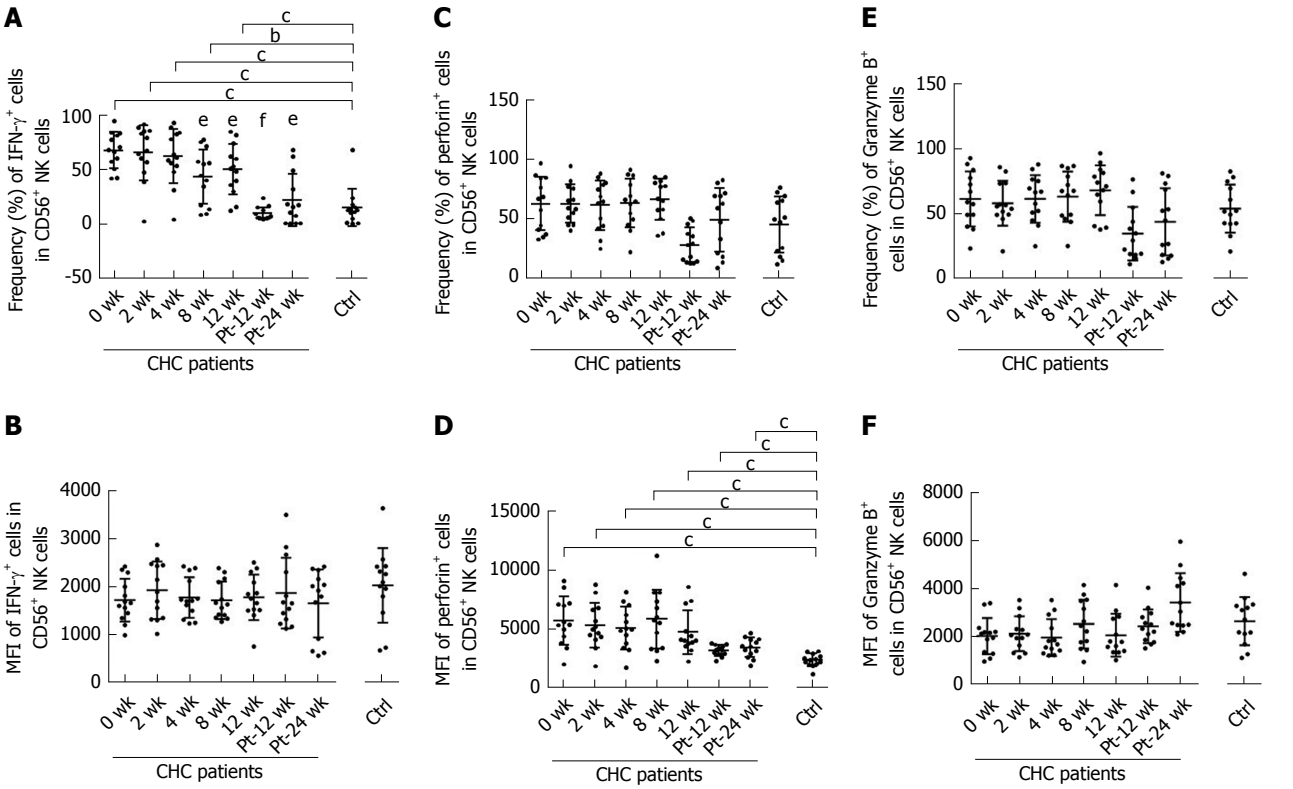


Figure 6 Sofosbuvir and ledipasvir therapy modulates the expression of natural killer cell-related cytokine interferon-γ, perforin and Granzyme B. Flow cytometric analyses of interferon (IFN)-γ, perforin and Granzyme B expression in natural killer (NK) cells. The graphs display the frequencies and mean fluorescence intensity (MFI) of IFN-γ (A-B), perforin (C-D) and Granzyme B (E-F) in NK cells from CHC patients during and after direct-acting antivirals therapy and healthy controls (n = 13). $^{a}P < 0.05$ and $^{b}P \leqslant 0.01$ and $^{c}P \leqslant 0.001$, different time points of CHC patients *vs* healthy controls; $^{d}P < 0.05$ and $^{e}P \leqslant 0.01$ and $^{f}P \leqslant 0.001$, different time points of CHC patients (2 wk, 4 wk, 8 wk, 12 wk, Pt-12 wk, Pt-24 wk *vs* 0 wk). IFN: Interferon; NK: Natural killer; CHC: Chronic hepatitis C; Ctrl: Healthy controls; Pt: Post of treatment; MFI: Mean fluorescence intensity.

treatment. There was a significant decline only at week 8 and week 12. As we all know, NKp46, a member of the natural cytotoxicity receptor family, is a main activating NK-cell receptor[37]. NK cells with high expression of NKp46 were characterized by a high functional capacity (*e.g.*, high cytotoxicity and IFN-γ production) and a high antiviral activity *in vitro*[38,39]. However, the percentage of IFN-γ^+ NK cells was similar to the level of healthy controls at week Pt-12, and this level was maintained until week Pt-24.

The effect of HCV infection on NKG2D was controversial. Oliviero *et al*[28] reported that CHC patients have increased NKG2D expression, while Dessouki *et al*[15] demonstrated that the frequency of NKG2D$^+$ NK cells decreased in HCV infection. In our study, the expression of NKp44, NKG2C, NKG2D and Granzyme B did not differ between NK cells from CHC patients at baseline and healthy donors in our study, and there was no change in the expression during and after the end of treatment (EOT) of DAAs, which is consistent with the published articles[19,40].

Our results showed that NK cells phenotypes and function started to change in the later period of sofosbuvir/ledipasvir treatment and reversed to the normalized level of healthy individuals mainly after EOT. What we found in our research is different from previous studies which assumed that HCV clearance induced by DAAs can mediate NK recovery rapidly. Whether dynamic changes of NK cells in DAA-treated patients are related to HCV reinfection or liver carcinogenesis after HCV elimination is a great topic in the future.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis C virus (HCV) infection can lead to mortality from hepatic as well as extra-hepatic causes. Until now, direct-acting antivirals (DAAs) have replaced pegylated-interferon (PEG-IFN)/ribavirin as a first-line treatment option. IFN-free DAAs take the virus life cycle as a target and can help us clarify the interaction between HCV clearance and the innate immune response, regardless of the IFN-α induced immune modulation. Previous studies showed that PEG-IFN-α can change natural killer (NK) cell subtype distribution and function in HCV-eliminated patients. However, it is controversial whether DAAs can change the phenotypes and function of NK cells.

Research motivation

More and more DAAs have been approved for clinical practice. In China, sofosbuvir/ledipasvir have been increasingly used among chronic hepatitis C (CHC) patients, especially those with genotype 1 HCV infection. Previous studies illustrated that NK cells play an important role in the antiviral immune defense and undergo great changes in subsets, phenotype and function during persistent viral infections. Therefore, it is meaningful to investigate how NK cells are affected in the elimination of HCV by sofosbuvir/ledipasvir.

Research objectives

The objectives of this study are to observe the dynamic changes of NK cell subsets, phenotypes and functional parameters during and after DAAs

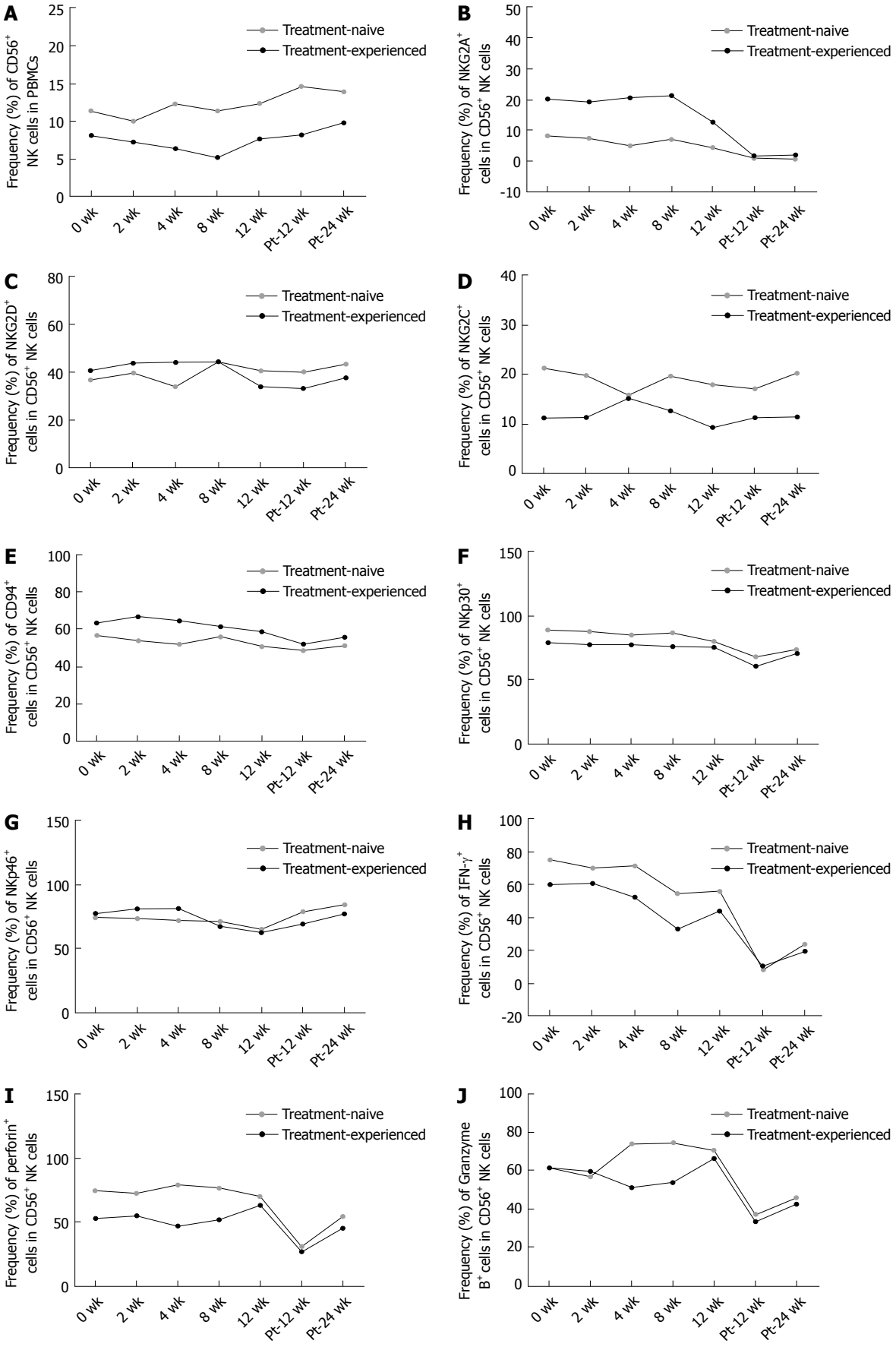


Figure 7 Frequency of CD56$^+$ natural killer cells, phenotypes and cytokine expression of natural killer cells between treatment-naïve and treatment-experienced chronic hepatitis C patients. Flow cytometric analyses of the frequency of CD56$^+$ natural killer (NK) cells (A), the expression of NKG2A (B), NKG2D (C), NKG2C (D), CD94 (E), NKp30 (F), NKp46 (G), IFN-γ (H), perforin (I) and Granzyme B (J) expression of NK cells and the changing trend between treatment-naïve (n = 7) and treatment-experienced (n = 6) chronic hepatitis C patients during and after direct-acting antivirals therapy. Pt: Post of treatment; IFN: Interferon; NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; CHC: Chronic hepatitis C.

treatment, and investigate the effect of DAAs (sofosbuvir/ledipasvir) treatment on innate immunity in genotype 1b HCV-infected patients.

Research methods

Thirteen treatment-naïve and treatment-experienced CHC patients were treated with sofosbuvir/ledipasvir, and NK cells were detected at baseline, week 2 to 12 during therapy, and week post of treatment (Pt)-12 and 24 after the end of therapy by multicolor flow cytometry and compared with 13 healthy controls.

Research results

There was a significant decline in $CD56^{bright}$ NK cell frequencies at week 8 (P = 0.002) and week 12 (P = 0.003), which were altered to the level comparable to that of healthy controls at week Pt-12, but there was no difference in the frequency of $CD56^{dim}$ NK cells. Compared with healthy controls, the expression levels of NKG2A, NKp30 and CD94 on NK cells from CHC patients at baseline were higher. NKG2A, NKp30 and CD94 started to recover at week 12 and reached the levels similar to those of healthy controls at week Pt-12 or Pt-24. Before treatment, patients had higher IFN-γ and perforin levels than healthy controls, and IFN-γ started to recover at week 8 and reached the normalized level at week Pt-12.

Research conclusions

NK cells of CHC patients can be affected by DAAs, and NK cell phenotypes and function started to change at the later period of sofosbuvir/ledipasvir treatment and reversed to the normalized level of healthy individuals mainly after end of treatment. What we found in our research is different from previous studies which assumed that HCV clearance induced by DAAs can mediate NK recovery rapidly.

Research perspectives

In hepatitis B virus (HBV)/HCV coinfected patients, HBV reactivation often occurred at the later period or even after the end of DAAs treatment. Our study may provide an explanation for this observation. Whether dynamic changes of NK cells in DAA-treated patients are related to HCV reinfection or liver carcinogenesis after HCV elimination is a great topic in the future.

REFERENCES

1 **World Health Organization**. Hepatitis C. Available on October 13, 2017. Updated July 2017. Available from: URL: http://www.who.int/mediacentre/factsheets/fs164/en/

2 **Lee MH**, Yang HI, Lu SN, Jen CL, You SL, Wang LY, Wang CH, Chen WJ, Chen CJ; R.E.V.E.A.L.-HCV Study Group. Chronic hepatitis C virus infection increases mortality from hepatic and extrahepatic diseases: a community-based long-term prospective study. *J Infect Dis* 2012; **206**: 469-477 [PMID: 22811301 DOI: 10.1093/infdis/jis385]

3 **Hadziyannis SJ**, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM; PEGASYS International Study Group. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355 [PMID: 14996676 DOI: 10.7326/0003-4819-140-5-200403020-00010]

4 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982 [PMID: 12324553 DOI: 10.1056/NEJMoa020047]

5 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965 [PMID: 11583749 DOI: 10.1016/S0140-6736(01)06102-5]

6 **Chu PS**, Nakamoto N, Taniki N, Ojiro K, Amiya T, Makita Y, Murata H, Yamaguchi A, Shiba S, Miyake R, Katayama T, Ugamura A, Ikura A, Takeda K, Ebinuma H, Saito H, Kanai T. On-treatment decrease of NKG2D correlates to early emergence of clinically evident hepatocellular carcinoma after interferon-free therapy for chronic hepatitis C. *PLoS One* 2017; **12**: e0179096 [PMID: 28617830 DOI: 10.1371/journal.pone.0179096]

7 **European Association for Study of Liver**. EASL Recommendations on Treatment of Hepatitis C 2015. *J Hepatol* 2015; **63**: 199-236 [PMID: 25911336 DOI: 10.1016/j.jhep.2015.03.025]

8 **Omata M**, Kanda T, Wei L, Yu ML, Chuang WL, Ibrahim A, Lesmana CR, Sollano J, Kumar M, Jindal A, Sharma BC, Hamid SS, Dokmeci AK, Mamun-Al-Mahtab, McCaughan GW, Wasim J, Crawford DH, Kao JH, Yokosuka O, Lau GK, Sarin SK. APASL consensus statements and recommendation on treatment of hepatitis C. *Hepatol Int* 2016; **10**: 702-726 [PMID: 27130427 DOI: 10.1007/s12072-016-9717-6]

9 **García-Sastre A**, Biron CA. Type 1 interferons and the virus-host relationship: a lesson in détente . *Science* 2006; **312**: 879-882 [PMID: 16690858 DOI: 10.1126/science.1125676]

10 **Markova AA**, Mihm U, Schlaphoff V, Lunemann S, Filmann N, Bremer B, Berg T, Sarrazin C, Zeuzem S, Manns MP, Cornberg M, Herrmann E, Wedemeyer H. PEG-IFN alpha but not ribavirin alters NK cell phenotype and function in patients with chronic hepatitis C. *PLoS One* 2014; **9**: e94512 [PMID: 24751903 DOI: 10.1371/journal.pone.0094512]

11 **Pawlotsky JM**. New hepatitis C therapies: the toolbox, strategies, and challenges. *Gastroenterology* 2014; **146**: 1176-1192 [PMID: 24631495 DOI: 10.1053/j.gastro.2014.03.003]

12 **Lin JC**, Habersetzer F, Rodriguez-Torres M, Afdhal N, Lawitz EJ, Paulson MS, Zhu Y, Subramanian GM, McHutchison JG, Sulkowski M, Wyles DL, Schooley RT. Interferon γ-induced protein 10 kinetics in treatment-naive versus treatment-experienced patients receiving interferon-free therapy for hepatitis C virus infection: implications for the innate immune response. *J Infect Dis* 2014; **210**: 1881-1885 [PMID: 24907384 DOI: 10.1093/infdis/jiu325]

13 **Edlich B**, Ahlenstiel G, Zabaleta Azpiroz A, Stoltzfus J, Noureddin M, Serti E, Feld JJ, Liang TJ, Rotman Y, Rehermann B. Early changes in interferon signaling define natural killer cell response and refractoriness to interferon-based therapy of hepatitis C patients. *Hepatology* 2012; **55**: 39-48 [PMID: 21898483 DOI: 10.1002/hep.24628]

14 **Ahlenstiel G**, Edlich B, Hogdal LJ, Rotman Y, Noureddin M, Feld JJ, Holz LE, Titerence RH, Liang TJ, Rehermann B. Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. *Gastroenterology* 2011; **141**: 1231-1239, 1239.e1-1239.e2 [PMID: 21741920 DOI: 10.1053/j.gastro.2011.06.069]

15 **Dessouki O**, Kamiya Y, Nagahama H, Tanaka M, Suzu S, Sasaki Y, Okada S. Chronic hepatitis C viral infection reduces NK cell frequency and suppresses cytokine secretion: Reversion by anti-viral treatment. *Biochem Biophys Res Commun* 2010; **393**: 331-337 [PMID: 20138830 DOI: 10.1016/j.bbrc.2010.02.008]

16 **Meissner EG**, Kohli A, Virtaneva K, Sturdevant D, Martens C, Porcella SF, McHutchison JG, Masur H, Kottilil S. Achieving sustained virologic response after interferon-free hepatitis C virus treatment correlates with hepatic interferon gene expression changes independent of cirrhosis. *J Viral Hepat* 2016; **23**: 496-505 [PMID: 26840694 DOI: 10.1111/jvh.12510]

17 **Serti E**, Chepa-Lotrea X, Kim YJ, Keane M, Fryzek N, Liang TJ, Ghany M, Rehermann B. Successful Interferon-Free Therapy of Chronic Hepatitis C Virus Infection Normalizes Natural Killer Cell Function. *Gastroenterology* 2015; **149**: 190-200.e2 [PMID: 25754160 DOI: 10.1053/j.gastro.2015.03.004]

18 **Serti E**, Park H, Keane M, O'Keefe AC, Rivera E, Liang TJ, Ghany M, Rehermann B. Rapid decrease in hepatitis C viremia by direct acting antivirals improves the natural killer cell response to IFNα. *Gut* 2017; **66**: 724-735 [PMID: 26733671 DOI: 10.1136/gutjnl-2015-310033]

19 **Doherty DG**, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. *Immunol Rev* 2000; **174**: 5-20 [PMID: 10807503

DOI: 10.1034/j.1600-0528.2002.017416.x]
20 **Pollmann J**, Rölle A, Hofmann M, Cerwenka A. Hepatitis C Virus and Human Cytomegalovirus-Natural Killer Cell Subsets in Persistent Viral Infections. *Front Immunol* 2017; **8**: 566 [PMID: 28567042 DOI: 10.3389/fimmu.2017.00566]
21 **Lunemann S**, Schlaphoff V, Cornberg M, Wedemeyer H. NK cells in hepatitis C: role in disease susceptibility and therapy. *Dig Dis* 2012; **30** Suppl 1: 48-54 [PMID: 23075868 DOI: 10.1159/000341680]
22 **Saha B**, Szabo G. Innate immune cell networking in hepatitis C virus infection. *J Leukoc Biol* 2014; **96**: 757-766 [PMID: 25001860 DOI: 10.1189/jlb.4MR0314-141R]
23 **Tatsumi T**, Takehara T. Impact of natural killer cells on chronic hepatitis C and hepatocellular carcinoma. *Hepatol Res* 2016; **46**: 416-422 [PMID: 26574168 DOI: 10.1111/hepr.12619]
24 **Cooper MA**, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001; **22**: 633-640 [PMID: 11698225 DOI: 10.1016/S1471-4906(01)02060-9]
25 **Kokordelis P**, Krämer B, Körner C, Boesecke C, Voigt E, Ingiliz P, Glässner A, Eisenhardt M, Wolter F, Kaczmarek D, Nischalke HD, Rockstroh JK, Spengler U, Nattermann J. An effective interferon-gamma-mediated inhibition of hepatitis C virus replication by natural killer cells is associated with spontaneous clearance of acute hepatitis C in human immunodeficiency virus-positive patients. *Hepatology* 2014; **59**: 814-827 [PMID: 24382664 DOI: 10.1002/hep.26782]
26 **Mondelli MU**, Varchetta S, Oliviero B. Natural killer cells in viral hepatitis: facts and controversies. *Eur J Clin Invest* 2010; **40**: 851-863 [PMID: 20597961 DOI: 10.1111/j.1365-2362.2010.02332.x]
27 **Ahlenstiel G**, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, Ghany MG, Hoofnagle JH, Liang TJ, Heller T, Rehermann B. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. *Gastroenterology* 2010; **138**: 325-335.e1-2 [PMID: 19747917 DOI: 10.1053/j.gastro.2009.08.066]
28 **Oliviero B**, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, De Filippi F, Bruno S, Mondelli MU. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* 2009; **137**: 1151-1160, 1160.e1-1160.e7 [PMID: 19470388 DOI: 10.1053/j.gastro.2009.05.047]
29 **Liang TJ**, Ghany MG. Current and future therapies for hepatitis C virus infection. *N Engl J Med* 2013; **368**: 1907-1917 [PMID: 23675659 DOI: 10.1056/NEJMra1213651]
30 **Spaan M**, van Oord G, Kreefft K, Hou J, Hansen BE, Janssen HL, de Knegt RJ, Boonstra A. Immunological Analysis During Interferon-Free Therapy for Chronic Hepatitis C Virus Infection Reveals Modulation of the Natural Killer Cell Compartment. *J Infect Dis* 2016; **213**: 216-223 [PMID: 26223768 DOI: 10.1093/infdis/jiv391]
31 **Ning G**, Li YT, Chen YM, Zhang Y, Zeng YF, Lin CS. Dynamic Changes of the Frequency of Classic and Inflammatory Monocytes Subsets and Natural Killer Cells in Chronic Hepatitis C Patients Treated by Direct-Acting Antiviral Agents. *Can J Gastroenterol Hepatol* 2017; **2017**: 3612403 [PMID: 28567369 DOI: 10.1155/2017/3612403]
32 **Yan KK**, Guirgis M, Dinh T, George J, Dev A, Lee A, Zekry A. Treatment responses in Asians and Caucasians with chronic hepatitis C infection. *World J Gastroenterol* 2008; **14**: 3416-3420 [PMID: 18528940 DOI: 10.3748/wjg.14.3416]
33 **Su F**, Green PK, Berry K, Ioannou GN. The association between race/ethnicity and the effectiveness of direct antiviral agents for hepatitis C virus infection. *Hepatology* 2017; **65**: 426-438 [PMID: 27775854 DOI: 10.1002/hep.28901]
34 **Borrego F**, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med* 1998; **187**: 813-818 [PMID: 9480992 DOI: 10.1084/jem.187.5.813]
35 **Borrego F**, Masilamani M, Marusina AI, Tang X, Coligan JE. The CD94/NKG2 family of receptors: from molecules and cells to clinical relevance. *Immunol Res* 2006; **35**: 263-278 [PMID: 17172651 DOI: 10.1385/IR:35:3:263]
36 **Enqvist M**, Nilsonne G, Hammarfjord O, Wallin RP, Björkström NK, Björnstedt M, Hjerpe A, Ljunggren HG, Dobra K, Malmberg KJ, Carlsten M. Selenite induces posttranscriptional blockade of HLA-E expression and sensitizes tumor cells to CD94/NKG2A-positive NK cells. *J Immunol* 2011; **187**: 3546-3554 [PMID: 21890659 DOI: 10.4049/jimmunol.1100610]
37 **Moretta A**, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Immunol Today* 2000; **21**: 228-234 [PMID: 10782054 DOI: 10.1016/S0167-5699(00)01596-6]
38 **Krämer B**, Körner C, Kebschull M, Glässner A, Eisenhardt M, Nischalke HD, Alexander M, Sauerbruch T, Spengler U, Nattermann J. Natural killer p46High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. *Hepatology* 2012; **56**: 1201-1213 [PMID: 22532190 DOI: 10.1002/hep.25804]
39 **Golden-Mason L**, Stone AE, Bambha KM, Cheng L, Rosen HR. Race- and gender-related variation in natural killer p46 expression associated with differential anti-hepatitis C virus immunity. *Hepatology* 2012; **56**: 1214-1222 [PMID: 22505144 DOI: 10.1002/hep.25771]
40 **Nattermann J**, Feldmann G, Ahlenstiel G, Langhans B, Sauerbruch T, Spengler U. Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis C. *Gut* 2006; **55**: 869-877 [PMID: 16322112 DOI: 10.1136/gut.2005.076463]

P- Reviewer: Pellicano R, Rezaee-Zavareh MS, Said ZN
S- Editor: Wang XJ **L- Editor**: Wang TQ **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4565-4577

DOI: 10.3748/wjg.v24.i40.4565

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

ORIGINAL ARTICLE

Basic Study

Helicobacter pylori promotes invasion and metastasis of gastric cancer by enhancing heparanase expression

Li-Ping Liu, Xi-Ping Sheng, Tian-Kui Shuai, Yong-Xun Zhao, Bin Li, Yu-Min Li

Li-Ping Liu, The Second Clinical Medical School of Lanzhou University, Lanzhou 730000, Gansu Province, China

Li-Ping Liu, Bin Li, Department of Critical Care Medicine, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Li-Ping Liu, Tian-Kui Shuai, Department of Critical Care Medicine, The Donggang District of First Hospital of Lanzhou University, Lanzhou 730030, Gansu Province, China

Xi-Ping Sheng, Institute of Epidemiology and Health Statistics, School of Public Health, Lanzhou University, Lanzhou 730000, Gansu Province, China

Yong-Xun Zhao, Department of Surgical Oncology, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Yu-Min Li, Key Laboratory of Digestive System Tumors of Gansu Province, The Second Clinical Medical School of Lanzhou University, Lanzhou 730000, Gansu Province, China

ORCID number: Li-Ping Liu (0000-0002-8339-0256); Xi-Ping Sheng (0000-0001-9641-7633); Tian-Kui Shuai (0000-0001-6733-3377); Yong-Xun Zhao (0000-0001-9448-1623); Bin Li (0000-0002-7366-4997); Yu-Min Li (0000-0002-9267-1412).

Author contributions: Liu LP and Li YM designed the experiments; Sheng XP and Li B contributed to the statistical analyses; Zhao YX performed the experiments; Liu LP prepared the manuscript; Shuai TK and Li B conducted data collection.

Supported by the Natural Science Foundation of Gansu Province, No. 1506RJZA255; the National Natural Science Foundation of China, No. 81572437; the Open Topics of the Key Laboratory of Biological Treatment and Regenerative Medicine in Gansu Province, No. zdsyskfkt-201702; and the Fund of Donggang Branch, The First Hospital of Lanzhou University, No. ldyydgyn-201705.

Institutional review board statement: The study was approved by the Ethics Review Board of the First Hospital of Lanzhou University.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: No additional unpublished data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Yu-Min Li, MD, PhD, Chief Doctor, Professor, Key Laboratory of Digestive System Tumors of Gansu Province, The Second Clinical Medical School of Lanzhou University, No. 82, Cuiyingmen, Chengguan District, Lanzhou 730000, Gansu Province, China. liym@lzu.edu.cn
Telephone: +86-931-8912127
Fax: +86-931-8912127

Received: August 11, 2018
Peer-review started: August 13, 2018
First decision: August 27, 2018
Revised: September 2, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

AIM

To detect the mechanisms of *Helicobacter pylori* (*H. pylori*) infection in the invasion and metastasis of gastric cancer (GC).

METHODS

Specimens from 99 patients with GC were collected. The

correlation among *H. pylori* infection, heparanase (HPA) and mitogen-activated protein kinase (MAPK) expression, which was determined by immunohistochemistry, and the clinical features of GC was analysed using SPSS 22.0. Overall survival (OS) and relapse-free survival (RFS) of GC patients were estimated by the Kaplan-Meier method. Independent and multiple factors of HPA and MAPK with prognosis were determined with COX proportional hazards models. HPA and MAPK expression in MKN-45 cells infected with *H. pylori* was analysed using Western blot.

RESULTS

H. pylori infection was observed in 70 of 99 patients with GC (70.7%), which was significantly higher than that in healthy controls. *H. pylori* infection was related to lymph metastasis and expression of HPA and MAPK ($P < 0.05$); HPA expression was relevant to MAPK expression ($P = 0.024$). HPA and MAPK expression in MKN-45 cells was significantly upregulated following *H. pylori* infection and peaked at 24 h and 60 min, before decreasing ($P < 0.05$). SB203580, an inhibitor of MAPK, significantly decreased HPA expression. HPA was related to lymph metastasis and invasive depth. HPA positive GC cases and *H. pylori* positive GC cases showed poorer prognosis than HPA negative cases ($P < 0.05$). COX models showed that the prognosis of GC was connected with HPA expression, lymph metastasis, tissue differentiation, and invasive depth.

CONCLUSION

H. pylori may promote the invasion and metastasis of GC by increasing HPA expression that may associate with MAPK activation, thus causing a poorer prognosis of GC.

Key words: Gastric cancer; *Helicobacter pylori*; Heparanase; Mitogen-activated protein kinase; Overall survival; Relapse-free survival

Core tip: The mechanism of *Helicobacter pylori* (*H. pylori*) infection in the invasion and metastasis of gastric cancer (GC) is still unknown. This paper studied heparanase (HPA) and mitogen-activated protein kinase (MAPK) expression in GC tissues and GC cells and their relationship with *H. pylori* infection. *H. pylori* infection may promote the invasion and metastasis of GC by increasing the expression of HPA that may be increased by activation of MAPK signal and HPA expression in GC tissue. *H. pylori* positive GC had a poorer prognosis.

Liu LP, Sheng XP, Shuai TK, Zhao YX, Li B, Li YM. *Helicobacter pylori* promotes invasion and metastasis of gastric cancer by enhancing heparanase expression. *World J Gastroenterol* 2018; 24(40): 4565-4577 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4565.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4565

INTRODUCTION

Helicobacter pylori (*H. pylori*) has been classified as a class Ⅰ carcinogen by the International Agency for Research on Cancer[1]. *H. pylori* can live in the acidic environment of the stomach for a long time, and its prolonged infection can destroy the gastric mucosa and result in changes in the release of gastric mucosal hormones, thus affecting the physiological state of the stomach. Therefore, *H. pylori* infection represents the most significant risk factor for malignant gastric tumours[2,3]. Approximately 50% of the world's population are infected with *H. pylori*[4,5]. The infection rate in China may be as high as 73.3%[6], especially in the Beijing region, where the infection rate is as high as 83.4%[7]. Although there have been increasing numbers of studies indicating that *H. pylori* infection can result in gastric cancer (GC), the underlying mechanism is still unknown.

Heparanase (HPA) is an endoglycosidase that is capable of degrading heparan sulfate (HS) in the extracellular matrix (ECM) and basement membrane (BM)[8,9], the process of which releases many types of biological mediators, such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF), in response to local or systemic signals[10,11]. Thus, HPA is involved in tissue remodelling and cell migration, which lead to inflammation, angiogenesis, and tumour metastasis[12-15]. The degradation of the ECM is one of the key steps involved in the invasion and metastasis of malignant tumours, and matrix degradation primarily depends on proteolytic enzymes. An increasing number of studies have demonstrated that the invasion and metastasis of tumour cells are closely associated with HPA production[16-18], including those derived from the stomach, pancreas, colon, and bladder. In addition to its enzymatic activity, recent studies have shown that the non-enzyme activity of HPA promotes the aggregation of heparan sulfate proteoglycans (HSPGs), causing a cascade of intracellular signal amplification that results in the activation of protein kinase C (PKC), Src, and Rac. HSPGs act on HPA receptors located on the cell surface, such as 6-phosphate mannose receptor (MPR), cationic non-6-phosphate-dependent mannose receptor (CD222), and low-density lipoprotein receptor-related protein (LRP), to cause signalling cascades. In addition, HPA plays an important role in inflammation and autoimmune diseases (*e.g.*, colitis, arthritis, psoriasis, and sepsis)[19,20].

Some studies have shown that *H. pylori* infection leads the development of gastric adenocarcinoma by activating mitogen-activated protein kinase (MAPK)[21,22]. Once activated, MAPK is translocated to the nucleus and leads to the activation of transcription factors, such as NF-κB[23,24]. A recent study[25] has also shown that the activation of the MAPK pathway is closely related to the expression of HPA. However, it is not clear whether MAPK is involved in the regulation of HPA expression following *H. pylori* infections that lead to GC.

The present research aimed to explore the role of *H.*

pylori infection in GC and analyse the connections among *H. pylori* infection, HPA and MAPK expression, and the pathological state of GC. By detecting the expression of HPA and MAPK and *H. pylori* in GC tissue and the expression of HPA and MAPK in MKN-45 cells infected by *H. pylori*, we analysed their associations with the clinical and pathological features of GC, and the survival and prognosis of *H. pylori* or HPA/MAPK positive GC.

MATERIALS AND METHODS

Cells and H. pylori strain

MKN-45 (human GC cell line) cells were obtained from the Chinese Academy of Sciences (Shanghai, China), preserved in the Key Laboratory of Digestive System Tumours of the Second Clinical Medical School of Lanzhou University, and cultivated in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, United States) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc.), 100 U/mL penicillin, and 100 mg/mL streptomycin (North China Pharmaceutical Co., Inc., Shijiazhuang, China) in humidified air containing 5% carbon dioxide at 37 ℃.

H. pylori NCTC11637 was provided by the Key Institute of Digestion and Oncology of Gansu Province and was cultured on Columbia agar plates (Solarbio, Shanghai, China) containing 7% filtered goat blood in an anaerobic tank.

Infection of MKN-45 cells with H. pylori

After digestion, MKN-45 cells were inoculated in three culture dishes with an equal cell volume and cultured under normal growth conditions until they reached the logarithmic growth phase, at which point the old medium was discarded and replaced with culture medium containing serum and antibiotics. *H. pylori* was cultured at 37 ℃ for 72 h under microaerophilic conditions using an anaerobic box. *H. pylori* was then added to the above cell medium at a bacteria:cell ratio of 100:1. The bacteria and cells were co-cultured for 6, 12, 24, and 48 h at 37 ℃ in 5% CO_2 and saturated humidity to detect HPA and MAPK expression.

Western blot assay

Total protein of the treated cells was extracted using RIPA lysis buffer (Beyotime Biotechnology, Haimen, China), phenylmethanesulfonyl fluoride (PMSF) (Beyotime Biotechnology), and a protein phosphatase inhibitor (Solarbio Biotechnology Co., Shanghai, China) after being washed with ice-cold phosphate-buffered saline (PBS). The protein concentration was measured by the BCA protein assay (Beyotime Biotechnology) after centrifugation at 14000 rpm for 30 min. The protein was separated on a 10% SDS polyacrylamide gel and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Solarbio Biotechnology), which was then blocked in the blocking solution for 2 h at a constant temperature. Then, the above membrane was incubated with the following primary antibodies: anti-heparanase1 (Abcam Biotechnology, Cambridge, United Kingdom), anti-phospho-p38, anti-p38 (Abcam Biotechnology), and anti-β-actin (Zhongshan Golden Bridge Biotech, Beijing, China). After overnight incubation, the membrane was washed three times with Tris Buffered Saline-Tween (TBST) (Solarbio Biotechnology) and then incubated with a horseradish peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge Biotech; dilution 1:10000) at room temperature for 1 h. The SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Inc., Rockford, IL, United States) was used to detect signals, which were displayed with a VersaDoc Imaging System (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States). Data were analysed using Bio-Rad Quantity One Software v4.62 (Bio-Rad Laboratories Co., Ltd.). To further illustrate whether the *H. pylori*-induced upregulation of HPA was mediated through the MAPK pathway, 20 μmol/L of the MAPK inhibitor SB203580 (Selleck, United States) was added to MKN-45 cells for 2 h before they were cultured with *H. pylori*.

Transwell assay

A Transwell assay was used to detect the invasion capability of the tumour cells. A 1:8 mixture of Matrigel gel:RPMI-1640 medium (100 μL) was added to the lower chamber, which was placed in 24-well plates at 37 ℃ for 24 h. A serum-free cell suspension (200 μL) at a concentration of 2.5×10^5 cells/mL was added to the upper chamber, while serum-containing medium was added to the lower chamber. Then, the cells were cultured at 37 ℃ in an atmosphere with 5% CO_2 and saturated humidity for 24 h. The remaining cells in the upper chamber were carefully wiped off, and the lower chamber was rinsed twice with PBS and fixed in methanol for 15 min. The cells were stained with crystal violet solution in methanol for 30 min, and then excess crystal violet was washed off. The cells were observed and images were obtained using a microscope. The number of migrated cells was counted in several fields of view.

Scratch test

The scratch test was used to detect the migration ability of tumour cells. Horizontal lines were drawn every 0.5-1 cm on the back of a 6-well plate and 5×10^5 cells were added to each well. The cells were incubated overnight and when cells were 100% confluent, a cut was made across the dish with a 200-mL pipette. The cells were rinsed twice with PBS, serum-free medium was added, and the cells were incubated at 37 ℃ in an atmosphere with 5% CO_2. Following the incubation, images of the cells were obtained and the migration distance was measured.

Patients and clinicopathological characteristics

Ninety-nine cases of pathologically diagnosed and surgically resected primary gastric carcinoma tissues

Table 1 Heparanase and mitogen-activated protein kinase expression in specimens of gastric cancer *n* (%)

	Cases (*n*)	Immunohistochemical result				HR (*P* value)
		-	+	+ +	+ + +	
HPA						
Gastric cancer	99	30 (30.3)	16 (16.2)	25 (25.3)	28 (28.3)	35.547 (0.000)
Para-cancer	99	57 (57.6)	11 (11.1)	17 (17.2)	16 (16.2)[b]	
Normal tissue	25	24 (96.0)	1 (4.0)	0 (0.0)	0 (0.0)[bd]	
MAPK						
Gastric cancer	99	49 (49.5)	12 (12.1)	19 (19.2)	19 (19.2)	33.303 (0.000)
Para-cancer	99	82 (82.8)	5 (5.1)	6 (6.1)	6 (6.1)[b]	
Normal tissue	25	23 (92.0)	2 (8.0)	0 (0.0)	0 (0.0)[b]	

P values < 0.05 were determined using one way analysis of variance (ANOVA): [b]*P*: Comparison with gastric cancer; [d]*P*: Comparison with para-cancer tissue. HPA: Heparanase; MAPK: Mitogen-activated protein kinase; HR: Hazard ratio.

and tumour-adjacent tissues (> 5 cm from the edge of the neoplastic foci and non-tumour tissue confirmed by pathology) without preoperative chemotherapy or radiotherapy from the Department of Gastroenterological and Oncological Surgery of the First Hospital of Lanzhou University were collected between June 2013 and June 2014. Patients with GC were aged between 31 and 77 years, with a mean age of 59.5 ± 9.8 years, and included 61 males and 38 females. Of the 99 cases of GC, there were 48 cases of highly and moderately differentiated carcinoma, 51 cases of poorly differentiated carcinoma, 55 cases of lymph node metastasis, and 44 cases without lymph node metastasis. With respect to the extent of infiltration, there were 42 cases of the T1/2 stage and 57 cases of the T3/4 stage. The clinicopathological characteristics are shown in Table 1. All cases of GC had received D2 radical surgery, and paraffin sections of GC tissue were taken from the Department of Pathology, First Hospital of Lanzhou University. The patient's clinical data, pathological results, and follow-up data were all recorded in detail. Except for stage Ia patients with GC, patients were given postoperative chemotherapy regimens of 6 cycles of XELOX. A total of 25 healthy controls were selected from the Department of Gastroenterology, who required gastroscopy to exclude digestive system tumours and diseases, including 16 males and 9 females, with an average age of 40 years. The follow-up of patients with GC was performed by a specialist. Overall survival time was measured from the date of surgery to the date of death due to any cause. All postoperative cases were followed for 3-60 mo. All research complied with the "Methods for Ethical Review of Biomedical Research Involving Human Beings (Trial)" and the Declaration of Helsinki. Prior written informed consent was obtained from every subject, and the study was approved by the Ethics Review Board of the First Hospital of Lanzhou University.

Immunohistochemistry

Protein expression of HPA and MAPK was detected by immunohistochemical staining (the SP method) in GC and para-carcinoma tissues. Formaldehyde fixed and paraffin embedded sections of samples were dewaxed, rehydrated with different concentrations of alcohol, and prepared for antigen retrieval with citrate by the high-temperature and high-pressure method. Primary antibodies were added to the sections and incubated at 37 ℃ in the dark for 1 h. Then, secondary antibodies were added for 30 min. Next, DAB chromogenic reagent was added to develop and hematoxylin was added to stain. The working concentrations of the primary antibodies against HPA and MAPK were both 1:200, and the negative control group used PBS instead of the primary antibody. Positive staining for HPA and MAPK was both primarily located in the cytoplasm. The semi-quantitative scoring criteria were as follows: according to the percentage of positive cells and staining intensity of each slice, the positive staining cell ratio was recorded as 0 points, 1 point, 2 points and 3 points for < 5%, 5% to 25%, 26% to 50%, and > 50%, respectively; the staining intensity was defined as 0 points, 1 point, 2 points and 3 points for no staining, pale yellow, brownish yellow and tan, respectively.

H. pylori infection status

To identify the infection status of *H. pylori* in GC and adjacent tissues, immunohistochemistry was used to test for *H. pylori* infection with a rabbit polyclonal anti-*H. pylori* antibody (DAKO). All diagnosed GC patients underwent a C13 breath test before surgery to check for *H. pylori* infection. If the clinical C13 exhalation test and immunohistochemical staining of the surgical pathology section were both confirmed to be positive for *H. pylori*, the patient was defined as *H. pylori* positive.

Statistical analysis

Data were analysed using SPSS 22.0 statistical software (IBM, Amonk, NY, United States). The classified data are described as the number of cases and rate or constituent ratio (%). The Chi-square test was used to compare classified disordered data groups. The Kruskal-Wallis H test was used to compare the classified ordered data. Association analysis between *H. pylori* infection and the expression of HPA and MAPK was analysed by the chi-square test, and the contingency coefficient was calculated. The survival rates of the different groups were analysed by the Kaplan-Meier method and log-rank (mantel-COX) test. Univariate and multivariate COX

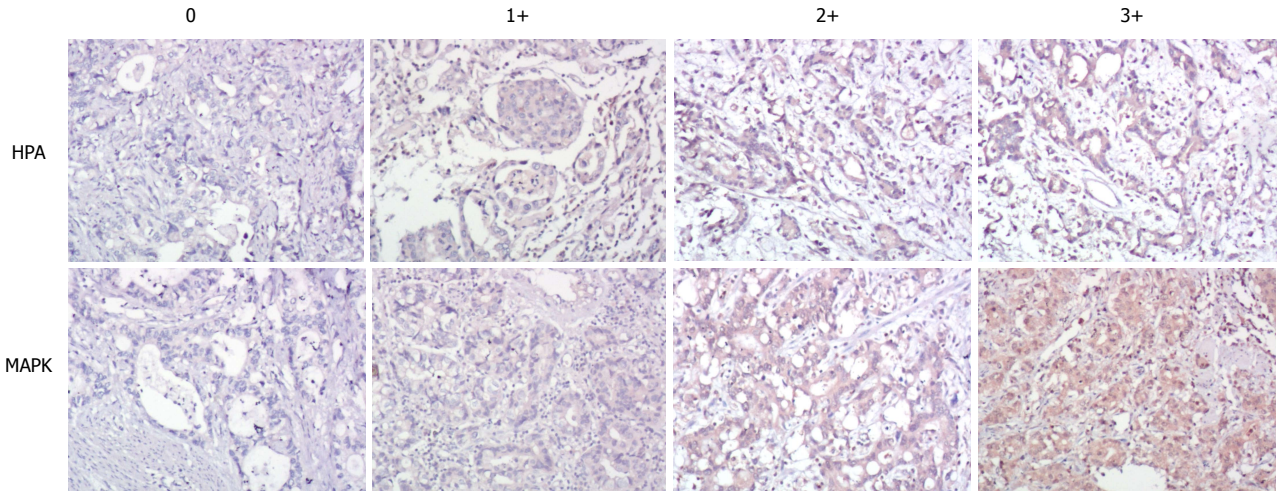


Figure 1 Immunohistochemical analysis of heparanase and mitogen-activated protein kinase protein expression in gastric cancer. Expression of heparanase (HPA) and mitogen-activated protein kinase (MAPK) was detected by immunohistochemical staining in normal gastric tissue. Representative immunohistochemical staining images are shown (magnification, 200 ×). HPA: Heparanase; MAPK: Mitogen-activated protein kinase.

regression analyses were used to explore the influencing factors of the survival time. $P < 0.05$ was considered statistically significant.

RESULTS

HPA and MAPK protein expression in GC tissue is higher than that in para-carcinoma tissue and normal gastric tissue

To detect HPA and MAPK protein expression in GC, immunohistochemical staining was carried out. Representative results from the immunohistochemical staining are shown in Figure 1 and detailed data are shown in Table 1. As shown in Figure 1, HPA and MAPK were not basally expressed in normal gastric tissue and were seldom expressed in para-carcinoma tissue; however, expression of HPA and MAPK was significantly positive in GC tissue. As seen in Table 1, HPA and MAPK were not basally expressed in normal gastric tissue, but expression of HPA and MAPK was positive in GC and para-carcinoma tissues, and expression of HPA and MAPK in GC tissue was significantly higher than that in para-carcinoma and normal tissues.

H. pylori infection in GC is relevant to the expression of HPA and MAPK and leads to higher expression than that in normal gastric tissue

To detect the *H. pylori* infection levels, the clinical C13 exhalation test and immunohistochemical staining of surgical sections were used. There were 70 cases of *H. pylori* infection out of 99 GC cases, with a positive rate of 70.71%, and 10 cases of *H. pylori* infection out of 25 healthy control cases, with a positive rate of 40.0%. There was a significant difference between the levels of *H. pylori* infection in GC and those of normal gastric tissue ($P < 0.05$), as revealed in Table 2. This result suggests that there is a significant correlation between *H. pylori* infection and GC.

Correlation between H. pylori infection, expression of HPA and MAPK proteins, and pathological characteristics of GC

To ascertain the effects of *H. pylori* infection and HPA and MAPK protein expression in GC, the associations among the clinical pathological characteristics of gastric cancer and the above factors were analysed. Positive *H. pylori* infection and positive expression of HPA were both associated with lymph node metastasis ($P < 0.05$), but not with age, gender, diameter of tumour, or differentiation degree ($P > 0.05$). Similarly, positive HPA expression and positive MAPK expression were both associated with the depth of invasion ($P < 0.05$), as shown in Table 3.

There were 54 cases of HPA positive expression in 70 *H. pylori* positive GC cases, with a positive rate of 54.5%, and 15 cases of HPA positive expression in 29 *H. pylori* negative GC cases, with a positive rate of 15.2%. Obviously, significantly higher levels of HPA positive expression in *H. pylori* positive GC cases were observed than those in *H. pylori* negative GC cases ($P < 0.05$), as shown in Table 4. This result suggests that there is significant HPA expression in GC with *H. pylori* infection.

There were 40 cases with MAPK positive expression in 70 *H. pylori* positive GC cases, with a positive rate of 40.4%, and 10 cases of MAPK positive expression in 29 *H. pylori* negative GC cases, with a positive rate of 10.1%. Significantly higher levels of MAPK positive expression in *H. pylori* positive GC cases were observed than those in *H. pylori* negative GC cases ($P < 0.05$), as shown in Table 5.

There were 40 cases of MAPK positive expression in 69 HPA positive GC cases, with a positive rate of 39.4%, and 10 cases of MAPK positive expression in 30 HPA negative GC cases, with a positive rate of 11.1%. Significantly higher MAPK positive expression in HPA positive GC cases were observed than those in HPA negative GC cases ($P < 0.05$), as shown in Table 6. This result suggests that there is a significant correlation between HPA expression and MAPK expression in GC.

Table 2 Correlation between *Helicobacter pylori* infection and gastric cancer *n* (%)

H. pylori infection	Gastric cancer (n = 99)	Normal tissue (n = 25)	χ^2	*P* value	C
Negative	29 (29.29)	15 (60.00)	8.221	0.004	0.229
Positive	70 (70.71)	10 (40.00)			

H. pylori: *Helicobacter pylori*.

Table 3 Correlation analyses of *Helicobacter pylori* infection, heparanase and mitogen-activated protein kinase expression, and gastric cancer pathological characteristics *n* (%)

Parameter	Cases (n)	*H. pylori* infection		*P* value	HPA expression		*P* value	MAPK expression		*P* value
		Positive	Negative		Positive	Negative		Positive	Negative	
Age (yr)										
< 60	46	32 (32.3)	14 (14.1)	0.816	31 (31.3)	14 (14.1)	0.8731	22 (22.2)	24 (24.2)	0.619
⩾ 60	53	38 (38.4)	15 (15.2)		38 (38.4)	16 (16.2)		28 (28.3)	25 (25.3)	
Gender										
Male	61	46 (46.5)	15 (15.2)	0.193	42 (42.4)	18 (18.2)	0.935	27 (27.3)	34 (34.3)	0.115
Female	38	24 (24.2)	14 (14.1)		27 (27.3)	12 (12.1)		23 (23.2)	15 (15.2)	
Lymph node metastasis										
Without	42	24 (24.2)	18 (18.2)	0.011	23 (23.2)	19 (19.2)	0.006	24 (24.2)	18 (18.2)	0.257
With	57	46 (46.5)	11 (11.1)		46 (46.5)	11 (11.1)		26 (26.3)	31 (31.3)	
Tumour diameter										
< 40 mm	57	40 (40.4)	17 (17.2)	0.892	39 (39.4)	17 (17.2)	0.989	25 (25.3)	32 (32.3)	0.123
⩾ 40 mm	42	30 (30.3)	12 (12.1)		30 (30.3)	13 (13.1)		25 (25.3)	17 (17.2)	
Differentiation degree										
Poorly	40	28 (28.3)	12 (12.1)	0.899	26 (26.3)	14 (14.1)	0.402	23 (23.2)	17 (17.2)	0.252
Highly and moderately	59	42 (42.4)	17 (17.2)		43 (43.3)	16 (16.2)		27 (27.3)	32 (32.3)	
Invasive depth										
T1/2	39	31 (31.3)	8 (8.1)	0.122	33 (33.3)	6 (6.1)	0.009	24 (24.2)	15 (15.2)	0.077
T3/4	60	39 (39.4)	21 (21.2)		36 (36.4)	24 (24.2)		26 (26.3)	34 (34.3)	

HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*.

Table 4 Correlation between heparanase expression and *Helicobacter pylori* infection in gastric cancer

H. pylori infection	HPA expression		χ^2	*P* value	C
	Negative	Positive			
Negative	14 (14.1)	15 (15.2)	6.273	0.012	0.244
Positive	16 (16.2)	54 (54.5)			

HPA: Heparanase; *H. pylori*: *Helicobacter pylori*.

H. pylori mediates the increase of HPA expression in MKN-45 cells via the MAPK signalling pathway

To detect the effect of *H. pylori* infection on HPA expression in GC cells, *H. pylori* and MKN-45 were co-cultured at a ratio of 100 bacteria: 1 cell for 0 h, 6 h, 12 h, 24 h, and 48 h. Western blot assay confirmed the enhancement of HPA expression at the protein level, and this level peaked at 24 h in *H. pylori*-infected GC cells (Figure 2A and B). To illustrate whether MAPK signalling is involved in *H. pylori*-induced expression of HPA, the expression of phosphorylated p38 MAPK (p-p38MAPK) was detected by Western blot analysis when *H. pylori* and MKN-45 were co-cultured for 0 min, 30 min, 60 min, 120 min, and 480 min. The expression of p-p38MAPK was significantly higher after 30 min and peaked at 60 min, whereas the total amount of p38MAPK remained unchanged (Figure 2C and D). To further illustrate whether *H. pylori*-induced upregulation of HPA is mediated through the MAPK pathway, 20 μmol/L of the MAPK inhibitor SB203580 was added to MKN-45 cells for 2 h before they were cultured with *H. pylori*. The expression of HPA protein was significantly higher when *H. pylori* infected MKN-45 cells, but that upregulation was significantly inhibited by SB203580 (Figure 2E and F). The Transwell invasion (Figure 2G and H) and scratch test migration (Figure 2I and J) assays confirmed that the addition of SB203580 to *H. pylori*-infected MKN-45 cells markedly decreased the invasion and migration abilities of MKN-45 cells.

H. pylori infection, HPA, and prognosis

HPA positive expression in GC significantly predicted poor overall survival (P = 0.000) and poor relapse-free survival (P = 0.006). Especially in *H. pylori*-infected GC, HPA positive expression was a more significant factor for predicting poor prognosis (overall survival, P = 0.000; relapse-free survival, P = 0.007) (Figure 3).

H. pylori infection, MAPK, and prognosis

MAPK positive expression in GC cannot predict overall survival (P = 0.063) or relapse-free survival (P = 0.163). However, in *H. pylori*-infected GCs, MAPK positive expression was a relatively significant factor for predicting poor prognosis (overall survival, P = 0.007), but did not

Table 5 Correlation between mitogen-activated protein kinase expression and *Helicobacter pylori* infection in gastric cancer

H. pylori infection	MAPK expression		χ^2	*P* value	C
	Negative	Positive			
Negative	19 (19.2)	10 (10.1)	4.212	0.04	0.202
Positive	30 (30.3)	40 (40.4)			

MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*.

Table 6 Correlation between heparanase expression and mitogen-activated protein kinase expression in gastric cancer

HPA expression	MAPK expression		χ^2	*P* value	C
	Negative	Positive			
Negative	20 (19.2)	10 (11.1)	5.077	0.024	0.221
Positive	29 (30.3)	40 (39.4)			

HPA: Heparanase; MAPK: Mitogen-activated protein kinase.

predict relapse-free survival (P = 0.557) (Figure 4).

Prognostic analysis using the COX regression model

Univariate COX regression analysis: By applying the COX regression model to single factor prognostic analysis, a stepwise regression approach was used. The results showed that there was no correlation between gender, age, and tumour diameter. However, lymph node metastasis in GC patients had a significant influence on prognosis (P = 0.003). The degree of histological differentiation had a positive impact on the prognosis of patients with GC (P = 0.003); the tumour invasion depth was also significantly associated with the prognosis of GC patients (P = 0.015). The level of HPA expression also had a very important role in the prognosis of GC patients (P = 0.000), and MAPK expression also had an influence on the prognosis of GC patients (P = 0.031). Whether *H. pylori* infection was present in patients with GC had no correlation with prognosis (P = 0.849), but *H. pylori* infection may play an important role in the initiation of GC. Therefore, the univariate COX regression analysis showed that lymph node metastasis, tissue differentiation, depth of invasion, and HPA expression in GC tissues were significantly correlated with the prognosis of patients (Table 7).

Multivariate COX regression analysis: The COX regression multivariate model was used for analysis. The results showed that age, sex, tumour diameter, MAPK expression, and *H. pylori* infection were all removed from the model and that lymph node metastasis, tissue differentiation, depth of invasion, and HPA expression were independent prognostic factors that affected the overall survival rate of GC patients. The relative risk of lymph node metastasis in GC tissues [hazard ratio (HR) = 4.443, P = 0.000] indicated that there was a higher risk of death (increased by 6.52 times) in GC patients with lymph node metastasis compared with those without. The lower the tissue differentiation of patients with GC, the higher the risk of death (HR = 3.452, P = 0.013). The greater the invasion of GC, the higher the risk of death [risk ratio (RR) = 2.542, P = 0.015]. The higher the expression of HPA protein in GC tissues, the shorter the survival time of patients (RR = 2.463, P = 0.015) (Table 8)

DISCUSSION

HPA has been documented in many primary human tumours[26-28], including GC[29], and is known to have multiple vital functions in accelerating tumour growth, angiogenesis, and tumour metastasis[30-32]. MAPK, including JNK, ERK, and p38 kinase, plays pivotal roles in proliferation, invasion, and migration of cancer cells[33-36]. There are many studies[37-39] that have shown that *H. pylori* infection leads to increased p38MAPK in GC cells, and it has also been shown[40] that p38MAPK leads to HPA elevation. This study showed that HPA and MAPK expression was significantly higher in patients with GC than that in para-carcinoma and normal gastric tissues. Our research also confirmed that HPA was highly expressed in GC cells, as has been previously reported in the literature[41,42]. To explain the correlation between HPA and MAPK expression and the clinical pathology of GC cases, we first investigated the clinicopathological characteristics. Positive expression of HPA was associated with lymph node metastasis and depth of invasion, but not with age, gender, tumour diameter, or differentiation degree. In addition, positive MAPK expression was only associated with depth of invasion, which illustrates that HPA is involved in the invasion and metastasis of GC and that MAPK is primarily involved in the proliferation of GC cells. This research also suggests that there is a significant correlation between HPA expression and MAPK expression in GC.

H. pylori, as a class Ⅰ carcinogen, causes invasion, proliferation, and metastasis of GC cells[43,44], but its specific mechanism of action remains unclear. It has been reported[45,46] that infections can cause a HPA elevation. *H. pylori*, as a bacterium, is associated with various human gastric diseases, especially gastritis and GC, but there has been no evaluation of whether *H. pylori* infection causes an increase in HPA in GC, which would contribute to the invasion and metastasis of GC. In the present study, it was revealed that *H. pylori* infection is significantly associated with HPA expression and that a positive *H. pylori* infection is connected to lymph node metastasis. To further elucidate the effect of *H. pylori* infection leading to heparanase elevation in GC, MKN-45 GC cells were infected by *H. pylori*. We showed that HPA expression was the highest at 24 h post *H. pylori* infection in these GC cells and the abilities of invasion and metastasis were increased when GC cells were infected by *H. pylori*.

There are many studies[37-39] showing that *H. pylori* infection leads to increased p38MAPK in GC cells, and it has been shown that[40] p38MAPK leads to elevation of

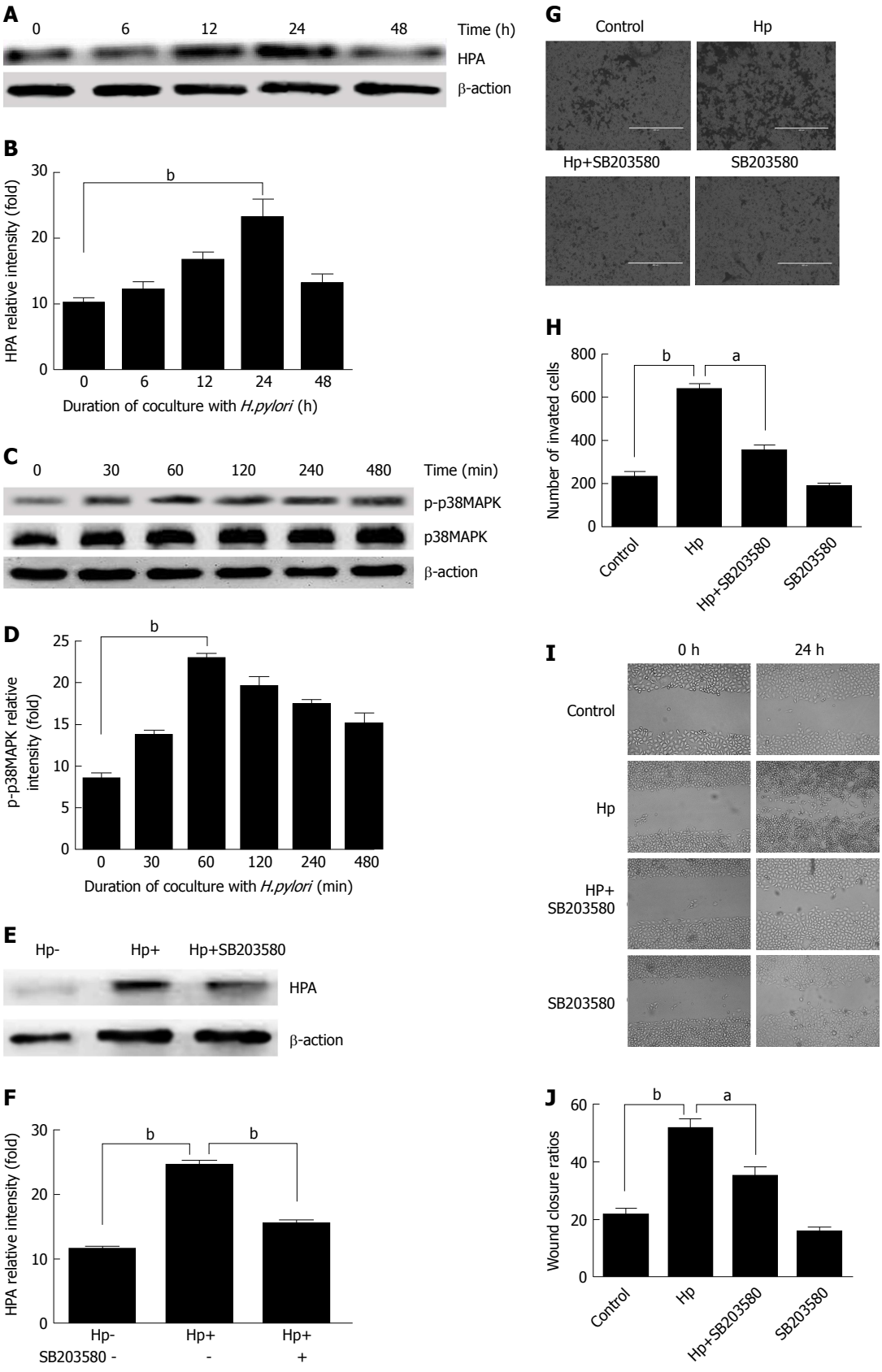


Figure 2 Heparanase protein expression following *Helicobacter pylori* infection in MKN-45 gastric cancer cells *via* the mitogen-activated protein kinase signaling pathway. A: Heparanase (HPA) expression was determined by Western blot at 0, 6, 12, 24, and 48 h after *Helicobacter pylori* (*H. pylori*) infection; B: Quantitative Western blot results of HPA; C: p-p38MAPK expression was determined by Western blot at 0, 30, 60, 120, and 480 min after *H. pylori* infection; D: Quantitative Western blot results of p-p38MAPK; E: HPA expression when the MAPK inhibitor SB203580 was given to MKN-45 cells before *H. pylori* infection; F: Quantitative Western blot results of HPA when the MAPK inhibitor SB203580 was given. $^{b}P < 0.01$ compared with the value at 0 h. G, H: Cell invasion rates in the three groups detected using a Transwell invasion assay. I, J: Migration rates in the three groups detected using a scratch migration assay. $^{a}P < 0.05$, $^{b}P < 0.01$. HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori: Helicobacter pylori*.

Table 7 Univariate COX regression analysis

Variable	Overall survival		
	HR	95%CI	*P* value
Gender (man *vs* women)	0.952	0.498-1.821	0.882
Age (≥ 60 *vs* < 60)	1.113	0.581-2.130	0.747
Tumour diameter (≥ 40 mm *vs* < 40 mm)	1.285	0.684-2.413	0.436
Lymph metastasis (yes *vs* no)	2.667	1.381-5.128	0.003
Tissue differentiation (high and medium *vs* low)	4.156	1.622-10.656	0.003
Invasive depth (T3/4 *vs* T1/2)	2.464	1.189-5.104	0.015
HPA expression (positive *vs* negative)	3.282	1.968-7.475	0.000
MARK expression (positive *vs* negative)	2.083	1.068-4.065	0.031
H. pylori (positive *vs* negative)	1.065	0.556-2.045	0.849

HPA: Heparanase; MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*; HR: Hazard ratio; CI: Confidence interval.

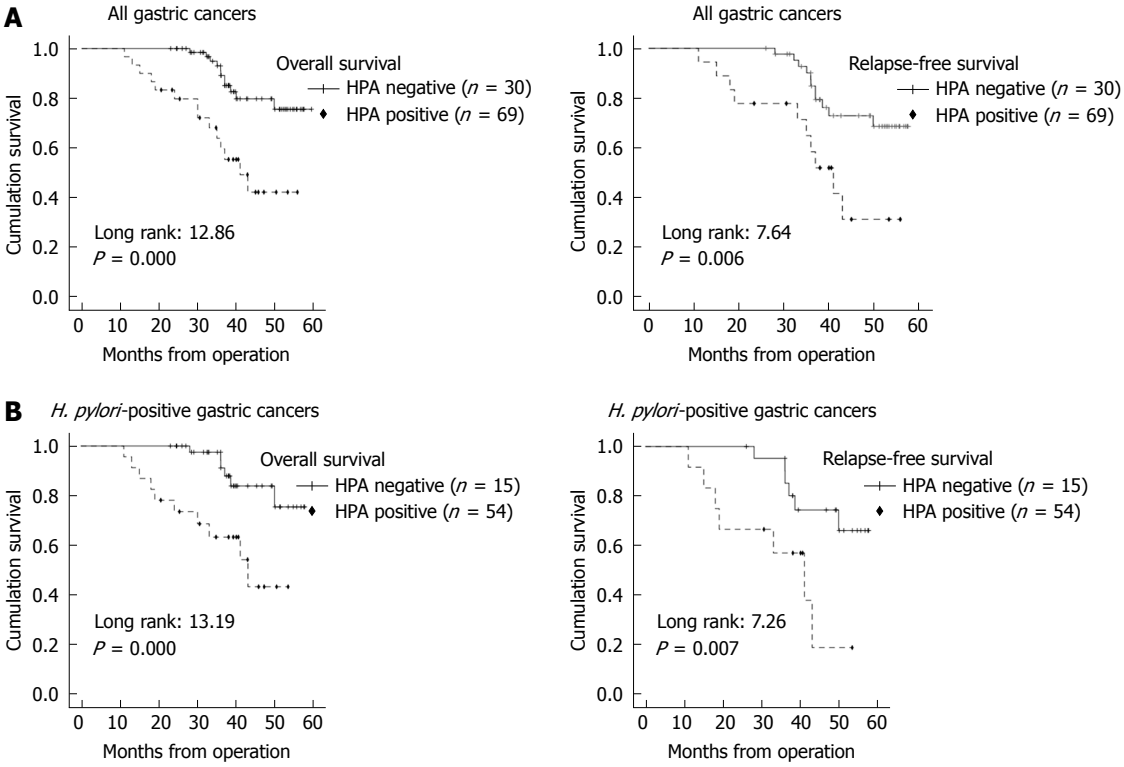


Figure 3 Kaplan-Meier survival plots for overall survival and relapse-free survival according to heparanase expression and *Helicobacter pylori* infection status. A: Heparanase (HPA) expression status (negative or positive) and the prognosis of all gastric cancer cases. HPA positive staining includes all cases of 1+, 2+, and 3+. HPA positive expression detected by immunohistochemical staining significantly predicts poor overall survival and relatively poor relapse-free survival; B: Kaplan-Meier survival according to HPA status in *Helicobacter pylori* positive gastric cancer cases. HPA positive expression significantly predicts poor overall survival as well as relapse-free survival. HPA: Heparanase; *H. pylori*: *Helicobacter pylori*.

HPA. Therefore, we hypothesized that *H. pylori* infection causes an increase in HPA in GC *via* the MAPK pathway. In this study, it was demonstrated that *H. pylori* infection was significantly associated with MAPK expression and that there was a significant correlation between HPA expression and MAPK expression in GC. In MKN-45 GC cells infected by *H. pylori*, *H. pylori* infection significantly enhanced the expression of MAPK. MAPK expression peaked at 60 min post *H. pylori* infection in MKN-45 cells. Inhibition of MAPK by SB203580 significantly decreased the expression of HPA and the invasion and metastasis of MKN-45 cells infected by *H. pylori*. Therefore, we speculate that *H. pylori* infection in GC activates MAPK signalling, leading to the activation of HPA.

HPA expression is a poor prognostic factor in some cancers[47-49], including GC[50,51]. In the present study, it was revealed that positive expression of HPA was able to predict the malignancy of GC due to its correlation with lymphatic metastasis and invasive depth. Beyond that, positive expression of HPA was a poor prognostic factor for overall survival and relapse-free survival compared with HPA negative cases, which was consistent with previously published reports[52]. Especially in GC patients with an obvious *H. pylori* infection, HPA positive expression indicated a poorer prognosis both in overall survival and in relapse-free survival, which illustrates

Table 8 Multivariate COX regression analysis

Variable	Overall survival		
	HR	95%CI	*P* value
Lymph metastasis (yes *vs* no)	4.443	2.185-9.036	0.000
Tissue differentiation (high and medium *vs* low)	3.452	1.299-9.176	0.013
Invasive depth (T3/4 *vs* T1/2)	2.542	1.198-5.392	0.015
HPA expression (positive *vs* negative)	2.463	1.195-5.076	0.015

HPA: Heparanase; HR: Hazard ratio; CI: Confidence interval.

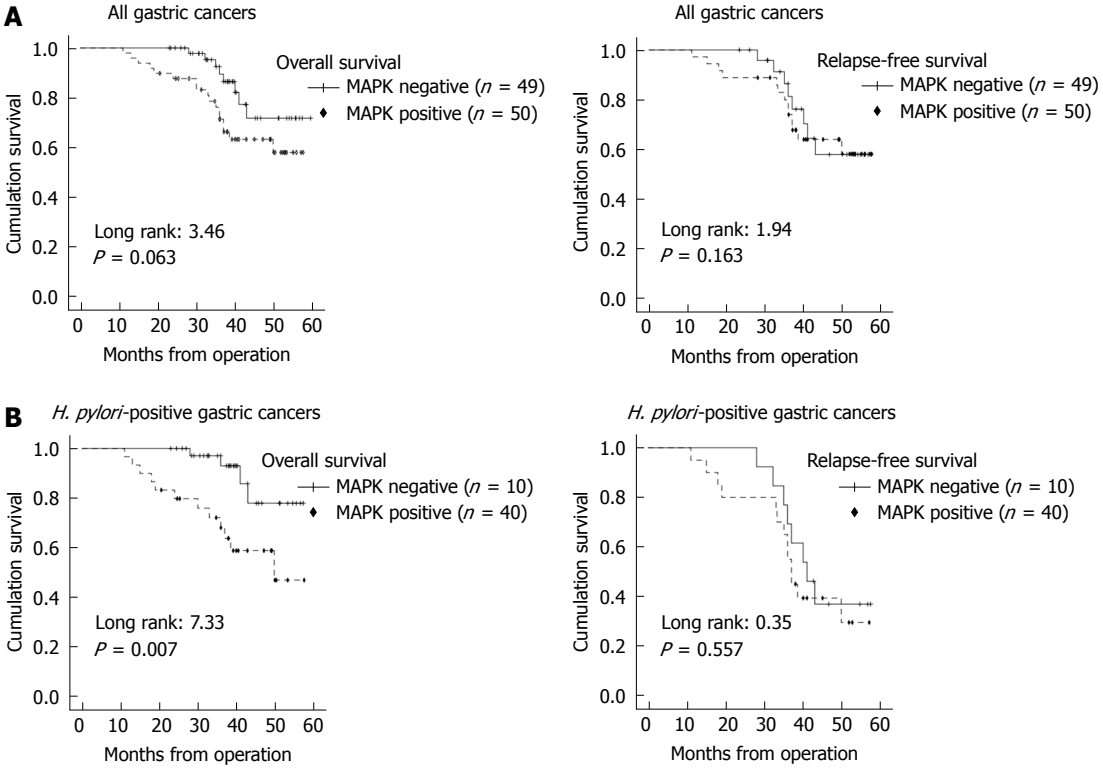


Figure 4 Kaplan-Meier survival plots for overall survival and relapse-free survival according to mitogen-activated protein kinase expression and *Helicobacter pylori* infection status. A: Mitogen-activated protein kinase (MAPK) expression status (negative or positive) and prognosis of all gastric cancer cases. MAPK positive staining includes all cases of 1+, 2+, and 3+. MAPK positive expression detected by immunohistochemical staining cannot predict overall survival or relapse-free survival; B: Kaplan-Meier survival according to MAPK status in *Helicobacter pylori* (*H. pylori*) positive gastric cancer cases. MAPK positive expression significantly predicts poor overall survival in *H. pylori* positive gastric cancer cases, but does not predict relapse free survival. MAPK: Mitogen-activated protein kinase; *H. pylori*: *Helicobacter pylori*.

that HPA is an important factor for the prediction of prognosis and relapse of GC and that *H. pylori* infection leads to an increase of HPA expression, which can worsen the prognosis of GC and make recurrence more likely. Compared to HPA, positive MAPK expression only predicted prognosis in overall survival of GC patients, which was consistent with previous reports[53], but MAPK expression could not predict a relapse in *H. pylori*-infected GC. Thus, MAPK expression cannot be used to determine the prognosis of GC patients with *H. pylori* infection, but a poor prognosis of GC patients with positive HPA expression is associated with *H. pylori* positive cases, which suggests that therapy against HPA should be taken into account when GC patients are infected with *H. pylori*.

Univariate COX regression analysis showed that lymph node metastasis, degree of histological differentiation, and invasive depth in GC patients had a significant influence on prognosis, which was identical to the results of previously published reports[54]. Moreover, in the univariate COX regression analysis, the levels of HPA and MAPK expression also had an influence on the prognosis of GC patients. Similarly, multivariate COX regression analysis showed that lymph node metastasis, tissue differentiation, depth of invasion, and HPA protein expression level were independent prognostic factors that affected the overall survival rate of GC patients. In addition to using the clinical characteristics to judge prognosis, such as lymph node metastasis, tissue differentiation, and depth of invasion, HPA is still an important factor as a biomarker to judge the prognosis of GC, which is consistent with the report by Takaoka *et al*[51].

In conclusion, the results of the current study demonstrate that *H. pylori* infection is not only the primary factor

involved in GC but is also involved in the invasion and metastasis of GC by upregulating HPA expression, which is likely mediated *via* activation of the MAPK signalling pathway. HPA is an important factor for predicting the prognosis and relapse of GC, and *H. pylori* infection increases HPA expression, which makes the prognosis of GC more aggressive and recurrence more likely, suggesting that therapy against HPA should be taken into consideration when GC patients are infected with *H. pylori.*

ARTICLE HIGHLIGHTS

Research background

The underlying mechanism that *Helicobacter pylori* (*H. pylori*) infection results in gastric cancer (GC) is still unknown. Heparanase (HPA) leads to the invasion and metastasis of GC. However, it is not clear whether *H. pylori* infection in GC increases HPA expression. Such finding suggests that HPA may become a therapeutic target for GC with *H. pylori* infection.

Research motivation

Although there have been increasing numbers of studies indicating that *H. pylori* infection results in GC, the underlying mechanism is still unknown. HPA is expressed in many tumours and leads to the invasion and metastasis of tumour, especially in GC. *H. pylori* infection can induce the development of GC by activating mitogen-activated protein kinase (MAPK) which is closely related to the expression of HPA. However, it is not clear whether MAPK is involved in the regulation of HPA expression following *H. pylori* infection that leads to GC.

Research objectives

To detect the mechanisms of *H. pylori* infection in the invasion and metastasis of GC.

Research methods

Immunohistochemistry method was used to detect *H. pylori* infection and HPA and MAPK expression in GC tissue, and their association with the clinical features of GC was analysed with SPSS 22.0. Kaplan-Meier method and COX proportional models were used to analyse prognosis. HPA and MAPK expression in MKN-45 cells infected with *H. pylori* was analysed using Western blot.

Research results

This study demonstrates that *H. pylori* infection increases HPA expression in GC, which is likely mediated *via* activation of the MAPK signaling pathway.

Research conclusions

The current study shows that *H. pylori* infection is involved in the invasion and metastasis of GC by upregulating HPA expression, which is likely mediated *via* activation of the MAPK signaling pathway. HPA is an important factor for predicting the prognosis and relapse of GC with *H. pylori* infection.

Research perspectives

HPA may become a therapeutic target for GC with *H. pylori* infection.

ACKNOWLEDGMENTS

We thank Dr. Zhong-Tian Bai for his critical reading of this manuscript. The authors appreciate Wen-Ting He for technical assistance.

REFERENCES

1 **Piazuelo MB**, Epplein M, Correa P. Gastric cancer: an infectious disease. *Infect Dis Clin North Am* 2010; **24**: 853-869, vii [PMID: 20937454 DOI: 10.1016/j.idc.2010.07.010]
2 **Wang F**, Meng W, Wang B, Qiao L. Helicobacter pylori-induced gastric inflammation and gastric cancer. *Cancer Lett* 2014; **345**: 196-202 [PMID: 23981572 DOI: 10.1016/j.canlet.2013.08.016]
3 **Wang YH**, Lv ZF, Zhong Y, Liu DS, Chen SP, Xie Y. The internalization of Helicobacter pylori plays a role in the failure of H. pylori eradication. *Helicobacter* 2017; **22**: [PMID: 27282442 DOI: 10.1111/hel.12324]
4 **Suerbaum S,** Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186 [PMID: 12374879 DOI: 10.1056/nejmra020542]
5 **Huang Y**, Wang QL, Cheng DD, Xu WT, Lu NH. Adhesion and Invasion of Gastric Mucosa Epithelial Cells by *Helicobacter pylori*. *Front Cell Infect Microbiol* 2016; **6**: 159 [PMID: 27921009 DOI: 10.3389/fcimb.2016.00159]
6 **Li Z**, Zou D, Ma X, Chen J, Shi X, Gong Y, Man X, Gao L, Zhao Y, Wang R, Yan X, Dent J, Sung JJ, Wernersson B, Johansson S, Liu W, He J. Epidemiology of peptic ulcer disease: endoscopic results of the systematic investigation of gastrointestinal disease in China. *Am J Gastroenterol* 2010; **105**: 2570-2577 [PMID: 20736940 DOI: 10.1038/ajg.2010.324]
7 **Zhang M**, Zhou YZ, Li XY, Tang Z, Zhu HM, Yang Y, Chhetri JK. Seroepidemiology of Helicobacter pylori infection in elderly people in the Beijing region, China. *World J Gastroenterol* 2014; **20**: 3635-3639 [PMID: 24707148 DOI: 10.3748/wjg.v20.i13.3635]
8 **Vlodavsky I**, Singh P, Boyango I, Gutter-Kapon L, Elkin M, Sanderson RD, Ilan N. Heparanase: From basic research to therapeutic applications in cancer and inflammation. *Drug Resist Updat* 2016; **29**: 54-75 [PMID: 27912844 DOI: 10.1016/j.drup.2016.10.001]
9 **Rivara S**, Milazzo FM, Giannini G. Heparanase: a rainbow pharmacological target associated to multiple pathologies including rare diseases. *Future Med Chem* 2016; **8**: 647-680 [PMID: 27057774 DOI: 10.4155/fmc-2016-0012]
10 **Nadir Y**, Brenner B. Heparanase multiple effects in cancer. *Thromb Res* 2014; **133** Suppl 2: S90-S94 [PMID: 24862152 DOI: 10.1016/S0049-3848(14)50015-1]
11 **Ilan N**, Elkin M, Vlodavsky I. Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int J Biochem Cell Biol* 2006; **38**: 2018-2039 [PMID: 16901744 DOI: 10.1016/j.biocel.2006.06.004]
12 **Ni M**, Elli S, Naggi A, Guerrini M, Torri G, Petitou M. Investigating Glycol-Split-Heparin-Derived Inhibitors of Heparanase: A Study of Synthetic Trisaccharides. *Molecules* 2016; **21**: [PMID: 27886097 DOI: 10.3390/molecules21111602]
13 **Vlodavsky I**, Iozzo RV, Sanderson RD. Heparanase: multiple functions in inflammation, diabetes and atherosclerosis. *Matrix Biol* 2013; **32**: 220-222 [PMID: 23499526 DOI: 10.1016/j.matbio.2013.03.001]
14 **Wilson JC**, Laloo AE, Singh S, Ferro V. 1H NMR spectroscopic studies establish that heparanase is a retaining glycosidase. *Biochem Biophys Res Commun* 2014; **443**: 185-188 [PMID: 24291708 DOI: 10.1016/j.bbrc.2013.11.079]
15 **Jin H**, Zhou S. The Functions of Heparanase in Human Diseases. *Mini Rev Med Chem* 2017; **17**: 541-548 [PMID: 27804885 DOI: 10.2174/1389557516666161101143643]
16 **Yingying X**, Yong Z, Zhenning W, Xue Z, Li J, Yang L, Huimian X. Role of heparanase-1 in gastric carcinoma invasion. *Asian Pac J Cancer Prev* 2009; **10**: 151-154 [PMID: 19469644]
17 **Meirovitz A**, Hermano E, Lerner I, Zcharia E, Pisano C, Peretz T, Elkin M. Role of heparanase in radiation-enhanced invasiveness of pancreatic carcinoma. *Cancer Res* 2011; **71**: 2772-2780 [PMID: 21447736 DOI: 10.1158/0008-5472.CAN-10-3402]
18 **Shafat I**, Pode D, Peretz T, Ilan N, Vlodavsky I, Nisman B. Clinical significance of urine heparanase in bladder cancer progression. *Neoplasia* 2008; **10**: 125-130 [PMID: 18283334 DOI: 10.1593/neo.07875]
19 **Hermano E**, Lerner I, Elkin M. Heparanase enzyme in chronic inflammatory bowel disease and colon cancer. *Cell Mol Life Sci* 2012; **69**: 2501-2513 [PMID: 22331282 DOI: 10.1007/s00018-012-0930-8]

20 **Schmidt EP**, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L, Zemans RL, Bowman JC, Koyanagi DE, Yunt ZX, Smith LP, Cheng SS, Overdier KH, Thompson KR, Geraci MW, Douglas IS, Pearse DB, Tuder RM. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med* 2012; **18**: 1217-1223 [PMID: 22820644 DOI: 10.1038/nm.2843]

21 **Ding SZ**, Smith MF Jr, Goldberg JB. Helicobacter pylori and mitogen-activated protein kinases regulate the cell cycle, proliferation and apoptosis in gastric epithelial cells. *J Gastroenterol Hepatol* 2008; **23**: e67-e78 [PMID: 18702686 DOI: 10.1111/j.1440-1746.2007.04912.x]

22 **Chen YC**, Wang Y, Li JY, Xu WR, Zhang YL. H pylori stimulates proliferation of gastric cancer cells through activating mitogen-activated protein kinase cascade. *World J Gastroenterol*2006; **12**: 5972-5977 [PMID: 17009395 DOI: 10.3748/wjg.v12.i37.5972]

23 **Xiang Y**, Ye W, Huang C, Lou B, Zhang J, Yu D, Huang X, Chen B, Zhou M. Brusatol inhibits growth and induces apoptosis in pancreatic cancer cells via JNK/p38 MAPK/NF-κb/Stat3/Bcl-2 signaling pathway. *Biochem Biophys Res Commun* 2017; **487**: 820-826 [PMID: 28455228 DOI: 10.1016/j.bbrc.2017.04.133]

24 **Chao W**, Deng JS, Li PY, Liang YC, Huang GJ. 3,4-Dihydroxybenzalactone Suppresses Human Non-Small Cell Lung Carcinoma Cells Metastasis via Suppression of Epithelial to Mesenchymal Transition, ROS-Mediated PI3K/AKT/MAPK/MMP and NFκB Signaling Pathways. *Molecules* 2017; **22**: [PMID: 28350337 DOI: 10.3390/molecules22040537]

25 **Liu X**, Fang H, Chen H, Jiang X, Fang D, Wang Y, Zhu D. An artificial miRNA against HPSE suppresses melanoma invasion properties, correlating with a down-regulation of chemokines and MAPK phosphorylation. *PLoS One* 2012; **7**: e38659 [PMID: 22719918 DOI: 10.1371/journal.pone.0038659]

26 **Zhou Y**, Song B, Qin WJ, Zhang G, Zhang R, Luan Q, Pan TJ, Yang AG, Wang H. Heparanase promotes bone destruction and invasiveness in prostate cancer. *Cancer Lett* 2008; **268**: 252-259 [PMID: 18487013 DOI: 10.1016/j.canlet.2008.04.008]

27 **Varchalama E**, Rodolakis A, Strati A, Papageorgiou T, Valavanis C, Vorgias G, Lianidou E, Antsaklis A. Quantitative analysis of heparanase gene expression in normal cervical, cervical intraepithelial neoplastic, and cervical carcinoma tissues. *Int J Gynecol Cancer* 2009; **19**: 1614-1619 [PMID: 19955948 DOI: 10.1111/IGC.0b013e3181ae3f40]

28 **Wu BW**, Li DF, Ke ZF, Ma D, Li YJ, Gang D, Zheng ZG, Zhang KJ, Zhang YH. Expression characteristics of heparanase in colon carcinoma and its close relationship with cyclooxygenase-2 and angiogenesis. *Hepatogastroenterology* 2010; **57**: 1510-1514 [PMID: 21443112]

29 **Ma XM**, Shen ZH, Liu ZY, Wang F, Hai L, Gao LT, Wang HS. Heparanase promotes human gastric cancer cells migration and invasion by increasing Src and p38 phosphorylation expression. *Int J Clin Exp Pathol* 2014; **7**: 5609-5621 [PMID: 25337202]

30 **Vlodavsky I**, Ilan N, Naggi A, Casu B. Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate. *Curr Pharm Des* 2007; **13**: 2057-2073 [PMID: 17627539 DOI: 10.2174/138161207781039742]

31 **Vlodavsky I**, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. *Nat Med* 1999; **5**: 793-802 [PMID: 10395325 DOI: 10.1038/10518]

32 **Mogler C**, Herold-Mende C, Dyckhoff G, Jenetzky E, Beckhove P, Helmke BM. Heparanase expression in head and neck squamous cell carcinomas is associated with reduced proliferation and improved survival. *Histopathology* 2011; **58**: 944-952 [PMID: 21585429 DOI: 10.1111/j.1365-2559.2011.03834.x]

33 **Chang L**, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001; **410**: 37-40 [PMID: 11242034 DOI: 10.1038/35065000]

34 **Eisenmann KM**, VanBrocklin MW, Staffend NA, Kitchen SM, Koo HM. Mitogen-activated protein kinase pathway-dependent tumor-specific survival signaling in melanoma cells through inactivation of the proapoptotic protein bad. *Cancer Res* 2003; **63**: 8330-8337 [PMID: 14678993]

35 **Johnson GL**, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002; **298**: 1911-1912 [PMID: 12471242 DOI: 10.1126/science.1072682]

36 **Achkar IW**, Abdulrahman N, Al-Sulaiti H, Joseph JM, Uddin S, Mraiche F. Cisplatin based therapy: the role of the mitogen activated protein kinase signaling pathway. *J Transl Med* 2018; **16**: 96 [PMID: 29642900 DOI: 10.1186/s12967-018-1471-1]

37 **Li Q**, Liu N, Shen B, Zhou L, Wang Y, Wang Y, Sun J, Fan Z, Liu RH. Helicobacter pylori enhances cyclooxygenase 2 expression via p38MAPK/ATF-2 signaling pathway in MKN45 cells. *Cancer Lett* 2009; **278**: 97-103 [PMID: 19201083 DOI: 10.1016/j.canlet.2008.12.032]

38 **Kim H**, Seo JH, Kim KH. The effect of p38 mitogen-activated protein kinase on mucin gene expression and apoptosis in Helicobacter pylori-infected gastric epithelial cells. *Ann N Y Acad Sci* 2003; **1010**: 90-94 [PMID: 15033700 DOI: 10.1196/annals.1299.014]

39 **Seo JH**, Lim JW, Kim H, Kim KH. Helicobacter pylori in a Korean isolate activates mitogen-activated protein kinases, AP-1, and NF-kappaB and induces chemokine expression in gastric epithelial AGS cells. *Lab Invest* 2004; **84**: 49-62 [PMID: 14631383 DOI: 10.1038/sj.labinvest.3700010]

40 **Che G,** Wang Y, Zhou B, Gao L, Wang T, Yuan F, Zhang L. Knockdown of heparanase suppresses invasion of human trophoblasts by activating p38 MAPK signaling pathway. *Dis Markers* 2018; **2018**: 1-10 [PMID: 29849826 DOI: 10.1155/2018/7413027]

41 **Zheng L,** Jiang G, Mei H, Pu J, Dong J, Hou X, Tong Q. Small RNA interference-mediated gene silencing of heparanase abolishes the invasion, metastasis and angiogenesis of gastric cancer cells. *BMC Cancer* 2010; **10**: 33 [PMID: 20137078 DOI: 10.1186/1471-2407-10-33]

42 **Tang W**, Nakamura Y, Tsujimoto M, Sato M, Wang X, Kurozumi K, Nakahara M, Nakao K, Nakamura M, Mori I, Kakudo K. Heparanase: a key enzyme in invasion and metastasis of gastric carcinoma. *Mod Pathol* 2002; **15**: 593-598 [PMID: 12065771 DOI: 10.1038/modpathol.3880571]

43 **Shichijo S**, Hirata Y. Characteristics and predictors of gastric cancer after *Helicobacter pylori* eradication. *World J Gastroenterol* 2018; **24**: 2163-2172 [PMID: 29853734 DOI: 10.3748/wjg.v24.i20.2163]

44 **Cheung KS**, Leung WK. Risk of gastric cancer development after eradication of Helicobacter pylori. *World J Gastrointest Oncol* 2018; **10**: 115-123 [PMID: 29770171 DOI: 10.4251/wjgo.v10.i5.115]

45 **Matan M**, King D, Peled E, Ackerman S, Bar-Lavi Y, Brenner B, Nadir Y. Heparanase level and procoagulant activity are reduced in severe sepsis. *Eur J Haematol* 2018; **100**: 182-188 [PMID: 29120525 DOI: 10.1111/ejh.12997]

46 **Goldberg R**, Meirovitz A, Hirshoren N, Bulvik R, Binder A, Rubinstein AM, Elkin M. Versatile role of heparanase in inflammation. *Matrix Biol* 2013; **32**: 234-240 [PMID: 23499528 DOI: 10.1016/j.matbio.2013.02.008]

47 **Vornicova O**, Naroditsky I, Boyango I, Shachar SS, Mashiach T, Ilan N, Vlodavsky I, Bar-Sela G. Prognostic significance of heparanase expression in primary and metastatic breast carcinoma. *Oncotarget* 2017; **9**: 6238-6244 [PMID: 29464068 DOI: 10.18632/oncotarget.23560]

48 **Hu B**, Wang Q, Shi Y, Lu S, Qu H, Wang L, Cui J. Significance of heparanase in metastatic lymph nodes of cervical squamous cell cancer. *Oncol Lett* 2017; **13**: 3219-3224 [PMID: 28521428 DOI: 10.3892/ol.2017.5804]

49 **Vornicova O**, Boyango I, Feld S, Naroditsky I, Kazarin O, Zohar Y, Tiram Y, Ilan N, Ben-Izhak O, Vlodavsky I, Bar-Sela G. The prognostic significance of heparanase expression in metastatic melanoma. *Oncotarget* 2016; **7**: 74678-74685 [PMID: 27732945 DOI: 10.18632/oncotarget.12492]

50 **Zhang X**, Xu S, Tan Q, Liu L. High expression of heparanase-2

is an independent prognostic parameter for favorable survival in gastric cancer patients. *Cancer Epidemiol* 2013; **37**: 1010-1013 [PMID: 24139593 DOI: 10.1016/j.canep.2013.09.012]

51 **Takaoka M**, Naomoto Y, Ohkawa T, Uetsuka H, Shirakawa Y, Uno F, Fujiwara T, Gunduz M, Nagatsuka H, Nakajima M, Tanaka N, Haisa M. Heparanase expression correlates with invasion and poor prognosis in gastric cancers. *Lab Invest* 2003; **83**: 613-622 [PMID: 12746471 DOI: 10.1097/01.lab.0000067482.84946.bd]

52 **Zhang J**, Yang J, Han X, Zhao Z, DU L, Yu T, Wang H. Over-expression of heparanase multiple antigenic peptide 2 is associated with poor prognosis in gastric cancer: Potential for therapy. *Oncol Lett* 2012; **4**: 178-182 [PMID: 22807984 DOI: 10.3892/ol.2012.703]

53 **He X**, Liu Z, Xia Y, Xu J, Lv G, Wang L, Ma T, Jiang L, Mou Y, Jiang X, Ma J, Zhao Z, Ni H, Xu W, Ru G, Huang D, Tao H. HOXB7 overexpression promotes cell proliferation and correlates with poor prognosis in gastric cancer patients by inducing expression of both AKT and MARKs. *Oncotarget* 2017; **8**: 1247-1261 [PMID: 27901487 DOI: 10.18632/oncotarget.13604]

54 **Kwon YH**. Long-Term Clinical Efficacy and Safety of Endoscopic Submucosal Dissection for Early Gastric Cancer in Korea. *Gut Liver* 2018; **12**: 371-372 [PMID: 29945421 DOI: 10.5009/gnl18216]

P- Reviewer: Cheng H, Xie X **S- Editor**: Ma RY
L- Editor: Wang TQ **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4578-4585

DOI: 10.3748/wjg.v24.i40.4578

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

ORIGINAL ARTICLE

Retrospective Study

Self-expandable metal stents in patients with postoperative delayed gastric emptying after distal gastrectomy

Seung Han Kim, Bora Keum, Hyuk Soon Choi, Eun Sun Kim, Yeon Seok Seo, Yoon Tae Jeen, Hong Sik Lee, Hoon Jai Chun, Soon Ho Um, Chang Duck Kim, Sungsoo Park

Seung Han Kim, Bora Keum, Hyuk Soon Choi, Eun Sun Kim, Yeon Seok Seo, Yoon Tae Jeen, Hong Sik Lee, Hoon Jai Chun, Soon Ho Um, Chang Duck Kim, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Institute of Gastrointestinal Medical Instrument Research, Korea University College of Medicine, Seoul 02841, South Korea

Sungsoo Park, Division of Upper GI Surgery, Department of Surgery, Korea University College of Medicine, Seoul 02841, South Korea

ORCID number: Seung Han Kim (0000-0001-9247-9175); Bora Keum (0000-0003-0391-1945); Hyuk Soon Choi (0000-0002-4343-6950); Eun Sun Kim (0000-0003-1820-459X); Yeon Seok Seo (0000-0003-4171-6331); Yoon Tae Jeen (0000-0003-0220-3816); Hong Sik Lee (0000-0001-9726-5416); Hoon Jai Chun (0000-0002-5539-361X); Soon Ho Um (0000- 0002-6390-2218); Chang Duck Kim (0000-0002-2829-6814); Sungsoo Park (0000-0001-9741-7635).

Author contributions: Kim SH, Keum B, and Chun HJ designed the research study; Kim SH and Keum B performed the research; Kim ES, Jeen YT, Lee HS , Kim CD, Park S, Choi HS, Seo YS, and Um SH analyzed the data; and Kim SH wrote the manuscript.

Supported by a Korea University Grant, No. K1809701; and the Ministry of Trade, Industry & Energy (MOTIE, Korea) under the Industrial Technology Innovation Program, No. 10060251, 'Development of diagnostic device for functional dyspepsia based on Korean-Western medicine fusion abdominal diagnosis'.

Institutional review board statement: The study was reviewed and approved by the Korea University Anam Hospital Institutional Review Board.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors have no conflicts of interest or financial ties to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Bora Keum, MD, PhD, Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Institute of Digestive Disease and Nutrition, Korea University College of Medicine, 126-1, Anam-dong 5 ga, Seongbuk-gu, Seoul 136705, South Korea. borakeum@hanmail.net
Telephone: +82-2-9206555
Fax: +82-2-9531943

Received: July 19, 2018
Peer-review started: July 19, 2018
First decision: August 25, 2018
Revised: September 11, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

AIM

To investigate the efficacy and safety of endoscopic stent insertion in patients with delayed gastric emptying after gastrectomy.

METHODS

In this study, we prospectively collected data from patients who underwent stent placement for delayed gastric emp-

tying (DGE) after distal gastrectomy between June 2010 and April 2017, at a tertiary referral academic center. Clinical improvement, complications, and consequences after stent insertion were analyzed.

RESULTS

Technical success was achieved in all patients (100%). Early symptom improvement was observed in 15 of 20 patients (75%) and clinical success was achieved in all patients. Mean follow-up period was 1178.3 ± 844.1 d and median stent maintenance period was 51 d (range 6-2114 d). During the follow-up period, inserted stents were passed spontaneously per rectum without any complications in 14 of 20 patients (70%). Symptom improvement was maintained after stent placement without the requirement of any additional intervention in 19 of 20 patients (95%).

CONCLUSION

Endoscopic stent placement provides prompt relief of obstructive symptoms. Thus, it can be considered an effective and safe salvage technique for post-operative DGE.

Key words: Self-expandable metal stent; Delayed gastric emptying; Gastrectomy; Salvage technique; Symptom improvement

Core tip: Delayed gastric emptying (DGE) after distal gastrectomy is a significant postoperative complication, and appropriate treatment measures are not yet available. This retrospective study investigated the efficacy and safety of self-expandable metallic stents in patients with DGE after gastric surgery. We found that endoscopic stent placement provided prompt relief of obstructive symptoms, with a low rate of complications, and no need for additional surgical interventions.

Kim SH, Keum B, Choi HS, Kim ES, Seo YS, Jeen YT, Lee HS, Chun HJ, Um SH, Kim CD, Park S. Self-expandable metal stents in patients with postoperative delayed gastric emptying after distal gastrectomy. *World J Gastroenterol* 2018; 24(40): 4578-4585 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4578.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4578

INTRODUCTION

Gastric cancer is one of the most common types of gastrointestinal malignancies. Every year, 950000 people are newly diagnosed with gastric cancer and 700000 gastric cancer-related deaths occur[1-4]. Although endoscopic therapy is a useful and effective therapeutic modality for early gastric cancer, to date the only curative treatment option has been surgical resection. Recently, laparoscopy-assisted gastrectomy, which is less invasive than conventional gastrectomy, with or without lymph node dissection, has become the mainstay of surgical management. With minimally invasive surgery becoming popular, the quality of life after surgical resection has improved[5-9]. Despite the use of advanced techniques, complications related to surgery are unavoidable. After gastric resection, bowel recovery can be delayed in a proportion of patients. Postgastrectomy syndromes, such as reflux esophagitis and dumping syndrome, can occur after distal gastrectomy. These complications are closely associated with the frequency of gastric emptying[10,11]. Moreover, longstanding gastric stasis following gastric resection can occur occasionally. The prevalence of postoperative delayed gastric emptying (DGE) has been shown to be between 5% and 30%[12-14]. It is characterized by an inability to consume regular meals with no evidence of anatomical narrowing or obstruction. Functional abnormality or inflammation at the anastomotic site could also lead to postoperative DGE. Sometimes, DGE may be associated with major postoperative adverse events, such as pancreatitis or pneumonia, and truncal vagotomy[12]. Moreover, it is likely that there are other unknown causes that cannot be clinically explained.

The primary therapeutic approach for postoperative DGE could be observation with nutritional support and administration of prokinetics. Reoperation to treat postoperative DGE is performed only in patients exhibiting severe and longstanding symptoms of outlet obstruction, necessitating drainage or non-oral route feeding[14,15]. However, there have been no suitable recommendations for the treatment of postoperative DGE.

Recently, self-expandable metallic stent (SEMS) placement has emerged as an effective and practical method not only for the management of gastrointestinal malignancy-associated narrowing or obstruction but also for benign stenosis or leaks of the gastrointestinal tract[16-18]. To the best of our knowledge, there has been no study mentioning the efficacy and safety of SEMS in patients with DGE after gastrectomy. We assumed that SEMS placement in the outlet area may facilitate rapid resumption of oral intake of foods and recovery of general patient conditions, resulting in a shorter hospital stay in patients with DGE after gastrectomy. We analyzed and described our experience with SEMS placement in patients with DGE exhibiting longstanding obstructive symptoms after gastrectomy.

MATERIALS AND METHODS

Patient enrolment

We prospectively collected data from June 2010 to April 2017. The total number of distal gastrectomies performed during the same period was 891. Twenty patients (2.2%) underwent stent insertion for postoperative DGE. DGE was defined as the failure to consume and/or tolerate a regular diet even after the seventh postoperative day[14,19,20]. We enrolled postoperative DGE patients who were not responsive to conservative management. Patients were kept "nil per oral" (NPO) and received conservative management with nutritional support and ad-

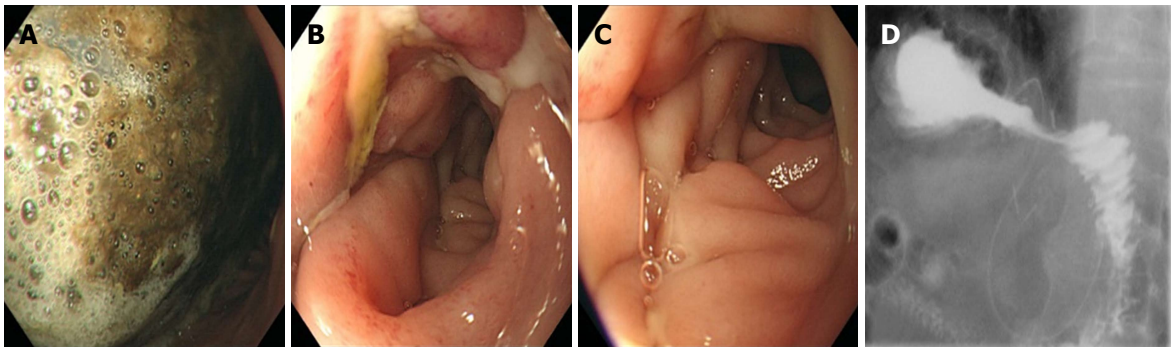


Figure 1 Delayed gastric emptying fourteen days after subtotal gastrectomy. A: Food is retained in the remnant stomach because of passage delay at the anastomotic site; B: An endoscope is passed through the anastomotic site; C: On gastroscopy, a patent lumen with edematous mucosa is observed on the efferent loop side; D: Despite the patency of the E-loop, delayed gastrojejunal passage is seen on upper gastrointestinal series.

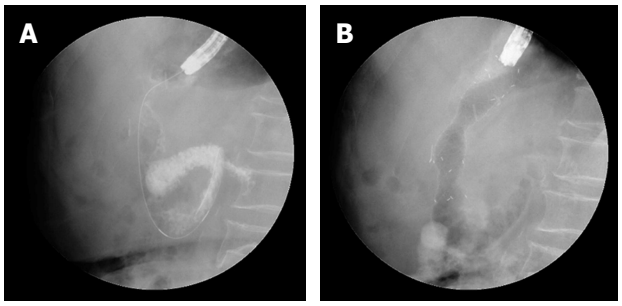


Figure 2 Placement of self-expandable metal stent. A: A catheter is placed through the lumen; B: A self-expandable metal stent (10-mm in length) is released following adjustment for the suitable position.

ministration of prokinetics before stent placement.

For each case, we recorded the diagnosis, type of gastric procedure performed, hospital course, postoperative day when oral food intake was resumed, and postoperative problems. All patients were assessed using gastroscopy and upper gastrointestinal (UGI) series with gastrografin during diagnosis and treatment (Figure 1).

Patients who were suspected of DGE, but did not undergo gastroscopy or UGI series testing, were excluded from the study. This study was reviewed by and ethical approval was obtained from the Korea University Anam Hospital Institutional Review Board (IRB No. ED13047).

Surgical procedure

Procedures were performed according to standard treatment algorithms[21]. Laparoscopy-assisted distal gastrectomy (LADG) was performed by two surgeons with considerable experience with the procedure. During surgery, resection was performed, leaving an adequate resection margin. D2 lymph node dissection was performed according to guidelines in all patients. Reconstruction after gastrectomy was performed using one of the following reconstruction methods depending on the surgeon's preference: (1) Billroth Ⅰ gastroduodenostomy and (2) Billroth Ⅱ gastrojejunostomy. During surgery, the vagal nerve was preserved because it has been shown to be helpful in improving the quality of life after gastrectomy by decreasing the occurrence of diarrhea and formation of postgastrectomy gallstones[22]. All surgical procedures were performed under general anesthesia. Postoperative oral food intake was permitted following the first bowel movement.

Stent characteristics

The stent (M. I. Tech, Seoul, South Korea) diameters ranged from 18-20 mm and were 70-, 90-, and 110-mm long. All SEMSs used were partially covered; their central body portion was covered with a silicone membrane and the flared portions of both ends were bare. The length of the stent was determined by the endoscopist based on the appearance of gastrojejunostomy anastomotic site and the efferent loop.

Stent placement

Endoscopic stent deployment was performed using a GIF-2TQ260M endoscope (Olympus Optical Co., Ltd, Tokyo, Japan). An experienced endoscopist performed stent placement following a combined fluoroscopic and endoscopic method (Figure 2). Stent placement was performed according to the procedural details described previously[23]. All procedures were performed under standard conscious sedation using propofol and/or midazolam. Patients were maintained in either the left decubitus or prone position during stent placement.

Definitions

We defined technical success of stent placement as the adequate deployment of the stent at the anastomosis site. Satisfactory relief of gastric stasis at 14 d was defined as clinical success, after which resumption of oral intake was possible. Early symptom improvement was defined as oral intake resumption within 2 d following stent placement.

Patient follow-up

Patients were followed up until they were lost to follow-up or dead. During follow-up, symptom improvement was evaluated by gastroscopy, interviews with patients, and abdominal radiographic examination. Patient symptoms

Table 1 Baseline characteristics *n* (%)

Variable	Number of patients
Sex	
Male	13 (65)
Female	7 (35)
Comorbidity	
DM	6 (30)
HTN	7 (35)
Psychological disorder	3 (15)
Histologic type	
Well differentiated	3 (15)
Moderately differentiated	5 (25)
Poorly differentiated	12 (60)
Tumor location	
Body	9 (45)
Antrum	11 (55)
Operation method	
LADG, B-Ⅰ	10 (50)
LADG, B-Ⅱ	10 (50)

DM: Diabetes mellitus; HTN: Hypertension; LADG: Laparoscopy-assisted distal gastrectomy; B-Ⅰ: Billroth-Ⅰ; B-Ⅱ: Billroth-Ⅱ.

Table 2 Baseline gastric outlet obstruction scoring system score and obstructive symptoms *n* (%)

Characteristic	Number of patients
GOOSS score	
No oral intake (0)	5 (25)
Only liquid diet (1)	10 (50)
Soft solid diet (2)	5 (25)
Low residue or normal diet (3)	0 (0)
Obstructive symptom	
Abdominal pain	
None	7 (35)
Moderate	12 (60)
Severe	1 (5)
Vomiting	
None	5 (25)
Moderate	10 (50)
Severe	5 (25)
Nausea	
None	3 (15)
Moderate	11 (55)
Severe	6 (30)
Regurgitation	
None	5 (25)
Moderate	10 (50)
Severe	5 (25)

GOOSS: Gastric outlet obstruction scoring system.

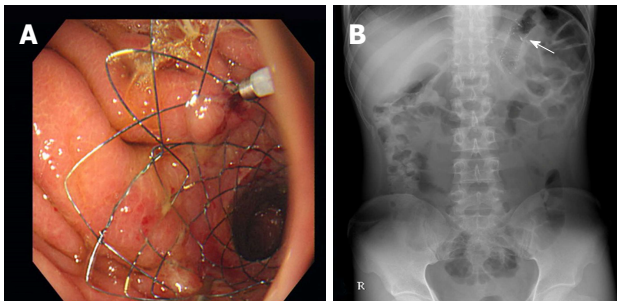


Figure 3 Inserted self-expandable metal stent. A: A stent is deployed at the anastomotic site with endoscopic clips; B: The stent is identified on an abdominal radiograph (arrow).

and obstructive signs were monitored and used to identify stent failure. When stent failure was suspected, endoscopic assessment and abdominal radiography were performed to evaluate patency. Data were acquired from endoscopic findings, radiologic reports, and clinical records.

Statistical analysis

Data are shown as mean, median, standard deviation, and percentages. The stent maintenance period determined during follow-up was assessed by Kaplan-Meier analysis. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, United States).

RESULTS

Patient population

The mean patient age was 65.9 ± 11.4 years (range 35-82 years) (Table 1). Gastroscopic examination presented no mechanical obstruction or stricture of the anastomotic site in any patient, and the gastroscope could freely pass through the gastroenteric anastomosis. The UGI series showed contrast passage delay through the anastomosis, but no obvious mechanical obstruction, stricture, or leakage of anastomosis was observed. Baseline gastric outlet obstruction scoring system (GOOSS) scores suggested that patients had adjusted their diet to compensate for DGE (Table 2).

Procedural details

A total of 20 patients underwent stent placement for DGE after distal gastrectomy (Figure 3). Technical success was achieved in all patients (100%). Procedures were performed using forward-viewing gastroscopes in all patients. The most frequently deployed stent length was 90 mm (65%), followed by 70 mm (25%) and 110 mm (10%) (Table 3). After stent placement, patients remained hospitalized for a mean of 6.8 ± 4.6 d (range 2-21 d).

Stent patency

Stent patency at 14 d was 80% (16 patients). Before the 14th d follow-up, four patients experienced stent migration, but they had no significant symptoms and did not need further intervention. Mean follow-up period was 1178.3 ± 844.1 d (Figure 4). Stent maintenance period determined during follow-up was assessed by Kaplan-Meier analysis (Figure 5). The median stent maintenance duration was 51 d (range 6-2114 d). In a patient with preserved stent maintenance, 1-year follow-up gastroscopy revealed a slightly stenotic anastomotic site with a metal stent, but the gastroscope could be passed. Granulation tissue was observed around the anastomotic site.

Clinical outcomes

During the study, over 90% of patients experienced relief in the obstructive symptoms after stent placement compared with the baseline obstructive symptoms (Table

Table 3 Comparison of details between patients

Patient No.	Tumor location	Operation type	Stent type	Duration for Symptom improvement	Stent patency	Duration from operation to stent placement	Hospital stay after stent insertion	Total hospital stay
1	Antrum	LADG (B-Ⅰ)	90-mm covered stent	2	758	17	7	31
2	Antrum	LADG (B-Ⅰ)	90-mm covered-stent	3	80	25	5	34
3	Mid body	LADG (B-Ⅰ)	70-mm covered stent	1	2114	28	7	38
4	Low body	LADG (B-Ⅱ)	90-mm covered stent	2	54	27	6	35
5	Low body	LADG (B-Ⅰ)	90-mm covered stent	2	17	31	3	36
6	Body	LADG (B-Ⅱ)	70-mm covered stent	6	6	26	10	37
7	Antrum	LADG (B-Ⅱ)	110-mm covered stent	5	194	23	13	42
8	Body	LADG (B-Ⅰ)	90-mm covered stent	3	9	28	12	42
9	Antrum	LADG (B-Ⅱ)	70-mm covered stent	2	14	14	4	25
10	Antrum	LADG (B-Ⅱ)	90-mm covered stent	1	98	12	5	21
11	Antrum	LADG (B-Ⅰ)	90-mm covered stent	5	8	24	8	35
12	Low body	LADG (B-Ⅰ)	70-mm covered stent	1	1675	7	2	21
13	Body	LADG (B-Ⅱ)	110-mm covered stent	2	24	14	6	23
14	Antrum	LADG (B-Ⅱ)	90-mm covered stent	2	40	9	8	25
15	Antrum	LADG (B-Ⅰ)	90-mm covered stent	1	51	15	3	20
16	Antrum	LADG (B-Ⅱ)	90-mm covered stent	1	42	23	2	27
17	Body	LADG (B-Ⅱ)	90-mm covered stent	1	64	11	21	35
18	Body	LADG (B-Ⅰ)	90-mm covered stent	1	23	21	3	31
19	Antrum	LADG (B-Ⅱ)	90-mm covered stent	1	4	9	9	19
20	Antrum	LADG (B-Ⅱ)	70-mm covered stent	0	52	9	2	21

LADG: Laparoscopy-assisted distal gastrectomy; B-Ⅰ: Billroth-Ⅰ; B-Ⅱ: Billroth-Ⅱ.

Table 4 Change in severity of obstructive symptoms (abdominal pain, vomiting, nausea, and regurgitation) after stent placement

Characteristics	1 yr	2 yr	3 yr	4 yr	5 yr
Number of patients available for follow-up	15	10	8	7	3
Patients with all symptoms maintained or improved compared with baseline (%)	93	90	100	100	100
Patients with any symptom worsening compared to baseline (%)	7	10	0	0	0

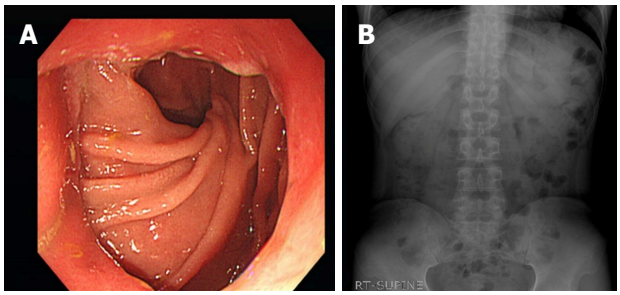


Figure 4 Patent anastomotic site on follow-up gastroscopy. A: Anastomotic lumen is patent; B: The stent is not identified on a follow-up abdominal radiograph.

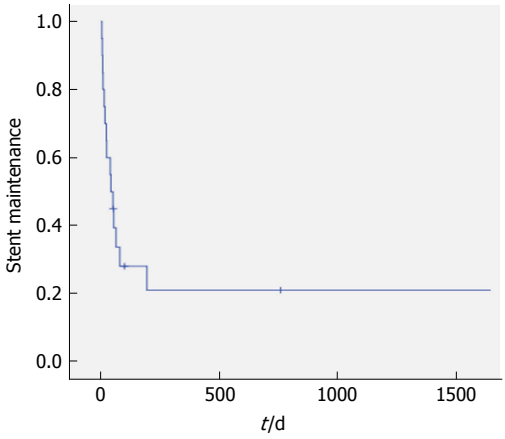


Figure 5 Kaplan–Meier estimates of the stent maintenance period. The Kaplan–Meier curve is shown. At 30 d, the estimated stent maintenance rate is 58.8%. Inserted stents were passed per rectum spontaneously in 14 of 20 patients (70%) with no significant complications.

4). After stent placement, early symptom improvement was achieved in 15 of 20 patients (75%). The rate of clinical success 14 d after stent placement was 100%. During the follow-up period, inserted stents were spontaneously passed per rectum in 14 of 20 patients (70%) and no significant complications were noted. Moreover, symptom improvement was maintained after stent placement without the requirement of any additional stent or surgical procedure in 19 of 20 patients (95%).

Adverse events

There was no procedure- or device-associated mortality in this study. After stent placement, there were no immediate adverse events, such as perforation or aspiration pneumonia (Table 5). The most common GI adverse event was stent migration. Stent migration occurred between 6 and 194 d after stent placement. Most of the migrated stents spontaneously passed per rectum. In one patient, the stent had migrated into the

Table 5 Gastrointestinal events after self-expandable metallic stent insertion

Adverse event	Total number of events	% of patients	Stent-related events	% of patients
Stent occlusion	0	0	0	0
GI bleeding	0	0	0	0
Bowel perforation	0	0	0	0
Severe abdominal pain	2	11.1	0	0
Stent migration	15	83.3	15	93.7
Stent fracture	1	5.6	1	6.3
Total	18	100	16	100

GI: Gastrointestinal.

stomach, and stent extraction was performed with rat tooth forceps. In another patient, a stent fracture was noted and an additional stent was inserted immediately. Two further patients experienced severe abdominal pain. All these events were considered to be associated with postsurgical bowel adhesion and were not stent related. There was no adverse event caused by distal stent migration.

DISCUSSION

In the present study, we evaluated the efficacy of SEMS insertion for DGE following surgical gastrectomy. Endoscopic stent placement provides prompt relief of obstructive symptoms.

During the first 1-3 wk after distal gastrectomy, postoperative DGE can occur due to different potential causes[12-14]. Of these, a mechanical problem may be the cause of the persistent increase in gastric remains. Acute angulation, kinking, long-term edema, or congestion of anastomotic lesions may also cause postoperative DGE. To date, various therapeutic approaches have been used for the treatment of postoperative DGE[19,20]. Although initial observation with conservative management including non-oral-route nutritional support is preferred, it is difficult for patients to maintain NPO status for more than 2-3 wk. Moreover, there is no appropriate way to evaluate the degree of improvement of DGE; thus, decision-making regarding treatment continuation or termination is difficult. Reoperation might be performed in situations in which nutritional support by a feeding jejunostomy is needed or when an efferent limb or stomal obstruction occurs[12]. However, reoperation increases the risk of morbidity and mortality, although it might appear to be more effective than conservative treatment. The current methods for postoperative DGE might therefore present less effective outcomes or potential morbidity associated with medications or surgical procedures.

If DGE in a patient improves after a short duration of conservative management, further therapeutic planning is not needed. If not, the subsequent therapeutic step needs to be planned. It is often preferable not to select surgery directly, but to plan a bridge or substitutive therapy that can likely fill the gap. In such a clinical situation, stent placement could be ideal, compared with medical conservative treatment or reoperation.

In this study, stent placement rapidly resolved obstructive symptoms without severe adverse events and the patient could be discharged with resumption of oral intake within a short duration after stent insertion. The stenting procedure itself is a minimally invasive therapeutic approach, which can be performed using simple fluoroscopy-guided endoscopy. Although experts are required for stent insertion, it is a relatively simple procedure. After stent insertion, patients did not require prolonged fasting or hospitalization, while the quality of life of patients improved, and adverse events did not occur. We did not need to remove the inserted stent in any patient. Deployed stents were passed per rectum spontaneously after some time. There were no associated complications.

At present, literature concerning stent placement for DGE after surgical gastrectomy is scarce. Stent deployment in patients with DGE after distal gastrectomy in this study resulted in effective and favorable outcomes. It is difficult to determine an optimal time for stent removal as early removal may decrease the effect of stenting and late removal could induce stent ingrowth. Therefore, we did not make a fixed decision regarding the appropriate timing of stent extraction. We did not extract the deployed stent because we believed that it would be better if it remained in position for as long as possible. We performed endoscopic clipping at the proximal end of the stent to prevent immediate migration after stent placement as there was no anatomical stenosis that would induce mechanical obstruction. When the stent migrated, it was passed per rectum spontaneously without any adverse events and did not need any surgical procedure.

Considering these results, physicians should consider stent placement in patients with postoperative DGE, especially, when rapid oral diet resumption could be helpful for patients. This method can relieve obstructive symptoms rapidly, shorten hospital stay, and increase patient satisfaction, and quality of life.

In conclusion, endoscopic stent placement resulted in a high technical success rate and rapid symptom improvement in patients with postoperative DGE. Further surgical intervention was not necessary in all cases. Endoscopic stenting could be considered a useful treatment option for DGE after gastrectomy.

ARTICLE HIGHLIGHTS

Research background

Delayed gastric emptying (DGE) after gastric surgery is one of the main postoperative complications. However, there have been no appropriate treatment measures for this distressing clinical situation. Recently, self-expandable metallic stent (SEMS) placement has become an effective and practical method not only for the management of gastrointestinal malignancy-associated problems but also for benign stenosis or leaks of the gastrointestinal tract.

Research motivation

The currently available methods for postoperative DGE might present less effective outcomes or potential morbidities associated with medications or surgical procedures.

Research objectives

The objective of this study was to analysis whether SEMS placement in the outlet area may facilitate rapid resumption of oral food intake and recovery of the general condition, resulting in shorter hospital stays in patients with DGE after gastrectomy.

Research methods

We prospectively collected data from 20 patients who underwent stent insertion for postoperative DGE. We recorded the diagnosis, type of gastric procedure performed, hospital course, postoperative day when oral food intake was resumed, and postoperative problems. Assessment for clinical improvement, complications, and consequences after stent insertion were performed.

Research results

Stent placement for postoperative DGE relieved obstructive symptoms rapidly, shortened hospital stay, and increased patient satisfaction and quality of life. Endoscopic stent placement presented a high technical success rate and rapid symptom improvement in patients with postoperative DGE. Moreover, no further surgical procedures were necessary in all cases. Endoscopic stenting could be considered a useful treatment option for DGE after gastrectomy.

Research conclusions

This study showed the efficacy of SEMS insertion for DGE following surgical gastrectomy. Endoscopic stent placement provides prompt relief of obstructive symptoms due to various causes after distal gastrectomy. The stenting procedure itself is a minimally invasive therapeutic alternative, which can be performed via simple fluoroscopy-guided endoscopy. After stent insertion, patients did not require prolonged fasting or hospitalization, the quality of life of the patients improved, and adverse events did not occur. Physicians could consider stent placement in patients with postoperative DGE, especially, when rapid oral diet resumption could be helpful for patients.

Research perspectives

Endoscopic stent placement, which is minimal invasive procedure, resulted in a high technical success rate and rapid symptom improvement in patients with postoperative DGE. In future research, direct comparison of clinical efficacy between stent placement and other therapeutic method could be helpful for physicians.

REFERENCES

1 **International Agency for Research on Cancer**. World Cancer Report 2014. Stewart BW, Wild CP, editor. World Health Organization, 2014

2 **Hyung WJ**, Kim SS, Choi WH, Cheong JH, Choi SH, Kim CB, Noh SH. Changes in treatment outcomes of gastric cancer surgery over 45 years at a single institution. *Yonsei Med J* 2008; **49**: 409-415 [PMID: 18581590 DOI: 10.3349/ymj.2008.49.3.409]

3 **Park JY**, von Karsa L, Herrero R. Prevention strategies for gastric cancer: a global perspective. *Clin Endosc* 2014; **47**: 478-489 [PMID: 25505712 DOI: 10.5946/ce.2014.47.6.478]

4 **Choi KS**, Suh M. Screening for gastric cancer: the usefulness of endoscopy. *Clin Endosc* 2014; **47**: 490-496 [PMID: 25505713 DOI: 10.5946/ce.2014.47.6.490]

5 **Sun YF,** Yang YG. Study for the quality of life following total gastrectomy of gastric carcinoma. *Hepatogastroenterology* 2011; **58**: 669-673 [PMID: 21661450]

6 **Ikeguchi M,** Kuroda H, Saito H, Tatebe S, Wakatsuki T. A new pouch reconstruction method after total gastrectomy (pouch-double tract method) improved the postoperative quality of life of patients with gastric cancer. *Langenbeck Arch Surg* 2011; **396**: 777-781 [PMID: 21448726 DOI: 10.1007/s00423-011-0779-6]

7 **Kim YW,** Baik YH, Yun YH, Nam BH, Kim DH, Choi IJ, Bae JM. Improved quality of life outcomes after laparoscopy-assisted distal gastrectomy for early gastric cancer results of a prospective randomized clinical trial. *Ann Surg* 2008; **248**: 721-727 [PMID: 18948798 DOI: 10.1097/SLA.0b013e318185e62e]

8 **Choi HS**, Chun HJ. Accessory Devices Frequently Used for Endoscopic Submucosal Dissection. *Clin Endosc* 2017; **50**: 224-233 [PMID: 28609818 DOI: 10.5946/ce.2017.070]

9 **Bang CS**, Park JM, Baik GH, Park JJ, Joo MK, Jang JY, Jeon SW, Choi SC, Sung JK, Cho KB. Therapeutic Outcomes of Endoscopic Resection of Early Gastric Cancer with Undifferentiated-Type Histology: A Korean ESD Registry Database Analysis. *Clin Endosc* 2017; **50**: 569-577 [PMID: 28743132 DOI: 10.5946/ce.2017.017]

10 Hulmemoir I. Role of altered gastric-emptying in the initiation of clinical dumping. *Scand J Gastroenterol* 1979; **14**: 463-467 [PMID: 482859]

11 **Fujiwara Y,** Nakagawa K, Tanaka T, Utsunomiya J. Relationship between gastroesophageal reflux and gastric emptying after distal gastrectomy. *Am J Gastroenterol* 1996; **91**: 75-79 [PMID: 8561148]

12 **Cohen AM,** Ottinger LW. Delayed gastric-emptying following gastrectomy. *Ann Surg* 1976; **184**: 689-696 [PMID: 999346 DOI: 10.1097/00000658-197612000-00005]

13 **Jordon GL Jr**, Walker LL. Severe problems with gastric emptying after gastric surgery. *Ann Surg* 1973; **177**: 660-668 [PMID: 4196670 DOI: 10.1097/00000658-197306000-00004]

14 **Bar-Natan M**, Larson GM, Stephens G, Massey T. Delayed gastric emptying after gastric surgery. *Am J Surg* 1996; **172**: 24-28 [PMID: 8686797 DOI: 10.1016/S0002-9610(96)00048-7]

15 **Behrns KE**, Sarr MG. Diagnosis and management of gastric emptying disorders. *Adv Surg* 1994; **27**: 233-255 [PMID: 8140975]

16 **Chang J**, Sharma G, Boules M, Brethauer S, Rodriguez J, Kroh MD. Endoscopic stents in the management of anastomotic complications after foregut surgery: new applications and techniques. *Surg Obes Relat Dis* 2016; **12**: 1373-1381 [PMID: 27317605 DOI: 10.1016/j.soard.2016.02.041]

17 **Bège T**, Emungania O, Vitton V, Ah-Soune P, Nocca D, Noël P, Bradjanian S, Berdah SV, Brunet C, Grimaud JC, Barthet M. An endoscopic strategy for management of anastomotic complications from bariatric surgery: a prospective study. *Gastrointest Endosc* 2011; **73**: 238-244 [PMID: 21295637 DOI: 10.1016/j.gie.2010.10.010]

18 **Kim SH**, Chun HJ, Yoo IK, Lee JM, Nam SJ, Choi HS, Kim ES, Keum B, Seo YS, Jeen YT, Lee HS, Um SH, Kim CD. Predictors of the patency of self-expandable metallic stents in malignant gastroduodenal obstruction. *World J Gastroenterol* 2015; **21**: 9134-9141 [PMID: 26290640 DOI: 10.3748/wjg.v21.i30.9134]

19 **Kurahara H**, Shinchi H, Maemura K, Mataki Y, Iino S, Sakoda M, Ueno S, Takao S, Natsugoe S. Delayed gastric emptying after pancreatoduodenectomy. *J Surg Res* 2011; **171**: e187-e192 [PMID: 22001182 DOI: 10.1016/j.jss.2011.08.002]

20 **Wente MN**, Bassi C, Dervenis C, Fingerhut A, Gouma DJ, Izbicki JR, Neoptolemos JP, Padbury RT, Sarr MG, Traverso LW, Yeo CJ, Büchler MW. Delayed gastric emptying (DGE) after pancreatic surgery: a suggested definition by the International Study Group of Pancreatic Surgery (ISGPS). *Surgery* 2007; **142**: 761-768 [PMID: 17981197 DOI: 10.1016/j.surg.2007.05.005]

21 **Japanese Gastric Cancer Association**. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011; **14**: 113-123 [PMID: 21573742 DOI: 10.1007/s10120-011-0042-4]

22 **Japanese Gastric Cancer Association**. Japanese gastric cancer

treatment guidelines 2014 (ver. 4). *Gastric Cancer* 2017; **20**: 1-19 [PMID: 27342689 DOI: 10.1007/s10120-016-0622-4]
23 **Kim CG**, Choi IJ, Lee JY, Cho SJ, Park SR, Lee JH, Ryu KW, Kim YW, Park YI. Covered versus uncovered self-expandable metallic stents for palliation of malignant pyloric obstruction in gastric cancer patients: a randomized, prospective study. *Gastrointest Endosc* 2010; **72**: 25-32 [PMID: 20381802 DOI: 10.1016/j.gie.2010.01.039]

P- Reviewer: Dumitraşcu T, Zhang XF **S- Editor**: Ma RY
L- Editor: A **E- Editor**: Yin SY

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4586-4595

DOI: 10.3748/wjg.v24.i40.4586

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

ORIGINAL ARTICLE

Retrospective Study

Second primary malignancy risk after radiotherapy in rectal cancer survivors

Ti-Hao Wang, Chia-Jen Liu, Tze-Fan Chao, Tzeng-Ji Chen, Yu-Wen Hu

Ti-Hao Wang, Department of Radiation Oncology, China Medical University Hospital, Taichung 40447, Taiwan

Chia-Jen Liu, Department of Medicine, Taipei Veterans General Hospital, Division of Hematology and Oncology, Taipei 11217, Taiwan

Tze-Fan Chao, Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Taipei 11217, Taiwan

Tzeng-Ji Chen, Department of Family Medicine, Taipei Veterans General Hospital, Taipei 11217, Taiwan

Yu-Wen Hu, Department of Oncology, Taipei Veterans General Hospital, Taipei 11217, Taiwan

Yu-Wen Hu, Institute of Public Health, National Yang-Ming University, Taipei 11217, Taiwan

ORCID number: Ti-Hao Wang (0000-0002-2105-0133); Chia-Jen Liu (0000-0001-5111-8736); Tze-Fan Chao (0000-0002-6587-3094); Tzeng-Ji Chen (0000-0002-8350-0232); Yu-Wen Hu (0000-0002-7983-415X).

Author contributions: Wang TH, Liu CJ, Chao TF, Chen TJ and Hu YW designed research; Wang TH, Liu CJ and Chao TF performed research; Wang TH, Chen TJ and Hu YW contributed new reagents or analytic tools; Wang TH and Hu YW analyzed data; Wang TH wrote the paper.

Institutional review board statement: This study was exempted from full review by the Institutional Review Board (No. 2016-05-007BC).

Conflict-of-interest statement: None.

Manuscript source: Unsolicited manuscript

Correspondence to: Yu-Wen Hu, MD, Attending Doctor, Department of Oncology, Taipei Veterans General Hospital, No.201, Sec. 2, Shipai Road, Beitou District, Taipei 11217, Taiwan. ywhu@vghtpe.gov.tw
Telephone: +886-2-28757270
Fax: +886-2-28732131

Received: June 30, 2018
Peer-review started: July 2, 2018
First decision: July 18, 2018
Revised: August 14, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

AIM

To investigate second primary malignancy (SPM) risk after radiotherapy in rectal cancer survivors

METHODS

We used Taiwan's National Health Insurance Research Database to identify rectal cancer patients between 1996 and 2011. Surgery-alone, preoperative short course, preoperative long course, and post-operative radiotherapy groups were defined. The overall and site-specific SPM incidence rates were compared among the radiotherapy groups by multivariate Cox regression, taking chemotherapy and comorbidities into account. Sensitivity tests were performed for attained-year adjustment and long-term survivors analysis.

RESULTS

A total of 28220 patients were analyzed. The 10-year cumulative SPM incidence was 7.8% [95% confidence

interval (CI): 7.2%-8.2%] using a competing risk model. The most common sites of SPM were the lung, liver, and prostate. Radiotherapy was not associated with increased SPM risk in multi-variate Cox model (hazard ratio = 1.05, 95%CI: 0.91-1.21, P = 0.494). The SPM hazard remained unchanged in 10-year-survivors. In addition, no SPM risk difference was found between the preoperative radiotherapy and postoperative radiotherapy groups.

CONCLUSION

In this large population-based cohort study, we demonstrated that radiotherapy had no increase in SPM.

Key words: Radiotherapy; Second primary malignancy; Rectal cancer; Preoperative long-course; Preoperative short-course

Core tip: Developing a second primary malignancy (SPM) after radiotherapy represents a major problem for long-term cancer survivors. In this large population-based study, no increased risk of developing SPM was found in rectal cancer patients who received pelvic radiotherapy in their initial treatment after carefully adjusted basline confounders. Also, the SPM risk remained the same among the preoperative long-course, preoperative short-course, and postoperative radiotherapy groups. However, rectal cancer survivors, similarly to other cancer survivors, are burdened with an overall higher probability of developing a second primary cancer. Life-long follow-up is recommended.

Wang TH, Liu CJ, Chao TF, Chen TJ, Hu YW. Second primary malignancy risk after radiotherapy in rectal cancer survivors. *World J Gastroenterol* 2018; 24(40): 4586-4595 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4586.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4586

INTRODUCTION

Thanks to the progress in early detection and treatment, rectal cancer survival has increased steadily over time[1]. Death rates due to colorectal cancer have declined by approximately 3% per year during the past decade[2]. Undoubtedly, radiotherapy has an established role in the multi-modal treatment of this disease[3,4]. However, radiotherapy may be related to several late adverse effects, which represents a major problem for long-term cancer survivors[5]. One of these effects, the risk of developing a second primary malignancy (SPM), has received greater attention in clinical practice. Rectal cancer survivors have a 4%-8% higher background rate of SPM compared with the normal population[6,7]. This higher rate may reflect the patients' genetic backgrounds, cancer-related treatments, lifestyles, and environmental risk factors[8]. Although several studies have investigated the relationship between radiotherapy and SPM in rectal cancer patients, the conclusions have been diverse[9-12]. Most studies have only addressed the initial treatment, which leads to results that are affected by potential confounders, such as comorbidities and other treatments during follow-up. Furthermore, whether preoperative long-course radiotherapy, preoperative short-course radiotherapy, or postoperative radiotherapy has a different contribution in increasing SPM risk is not clear. Here, we used Taiwan's National Health Insurance Research Database (NHIRD), which provides detailed diagnosis and treatment data, to assess the association between SPM and radiotherapy, taking chemotherapy and comorbidities into account.

MATERIALS AND METHODS

Data source

Taiwan's National Health Insurance, established in 1995, covers the comprehensive medical care of > 99% Taiwanese residents[13]. Taiwan's NHIRD provides encrypted nationwide data for health research, including inpatient and outpatient diagnoses, claimed procedures and drug prescriptions. The Registry of Catastrophic Illness Database (RCID), a subpart of the NHIRD, provides information on patients with a confirmed malignancy. The certification of both first primary rectal cancer and SPM requires tissue pathologic proof for peer review. This study was exempted from full review by the Institutional Review Board (No. 2016-05-007BC).

Cohort selection

The cohort was composed of patients aged 20 years or older who were diagnosed with a first primary rectal cancer (ICD-9-CM 154.0 and 154.1) from the RCID between Jan 1, 1996, and Dec 31, 2011. Because there is a lag time between radiation and SPM, we excluded patients who had SPMs within the first year of treatment or survived less than one year after treatment[14]. We also excluded patients with HIV infection. Because synchronous and metachronous colorectal cancers (CRCs) were difficult to distinguished, second primary CRCs were not analyzed. We also excluded neoplasms of the small intestine to avoid misclassification. The follow-up time for each individual began one year after the initial treatment and ended on the date of diagnosis of any SPM, death, or the end of study (Dec 31, 2011), whichever came first.

The patients were classified into four groups. The surgery-only group was composed of patients who underwent radical rectal surgery, such as abdominoperineal resection of the rectum, low anterior resection, local excision, transsacral rectosigmoidectomy, or posterior resection of the rectum, and who never received radiotherapy within the follow-up time. The postoperative radiotherapy group was composed of patients who underwent radical rectal surgery followed by radiotherapy

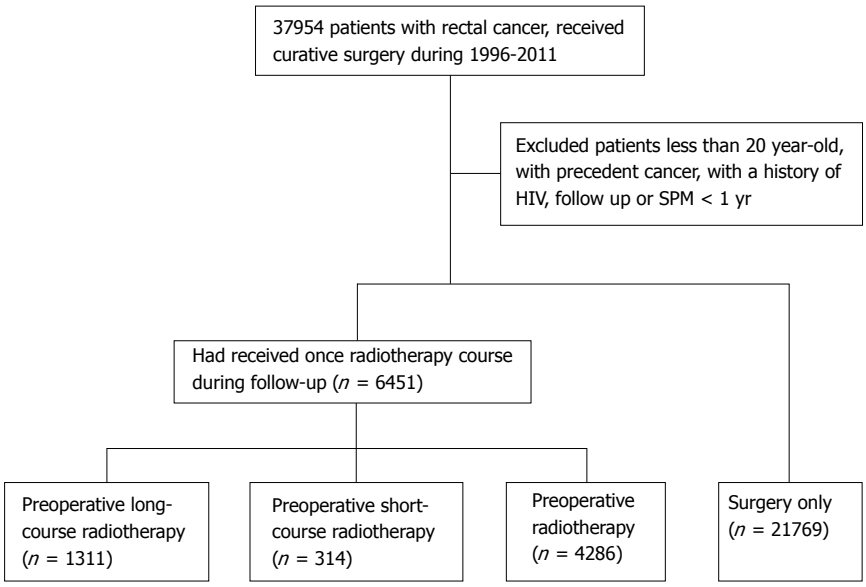


Figure 1 Study inclusion flowchart. HIV: Human immunodeficiency virus; SPM: Second primary malignancy.

within one year after surgery (considering that the radiotherapy may have been administered after 6 mo of chemotherapy). The preoperative radiotherapy group was composed of patients who received radiotherapy within 6 mo prior to radical rectal surgery. The preoperative radiotherapy group was further categorized into the short-course and the long-course radiotherapy groups according to their radiotherapy regimen, judging by claimed radiation portals. The exact dose of radiation used was not available in the NHIRD. However, the typical radiation regimen for preoperative long-course radiotherapy and postoperative radiotherapy is 45-50.4 Gy in 25-28 fractions, while 25 Gy in 5 fractions is used for preoperative short course radiotherapy. Patients who received incomplete radiotherapy regimens or re-irradiation during the follow-up period were excluded.

Treatment factors

We collected all cancer treatment information within the first 2 years after diagnosis, including surgery, radiation, and chemotherapy. The surgery procedures were coded using ICD-9-CM codes. The chemotherapy agents were classified by their Anatomical Therapeutic Chemical (ATC) code. Chemotherapy administered after and within one year of an SPM was omitted due to possible treatment of a second cancer. Demographic data such as age at rectal cancer diagnosis, year of diagnosis, attained age and year of SPM diagnosis, sex, and comorbidities, including autoimmune diseases, chronic obstructive pulmonary disease (COPD), diabetes mellitus (DM), dyslipidemia, end-stage renal disease (ESRD), liver cirrhosis, and hypertension (HTN), were collected from the NHIRD.

Statistical analysis

Because death could be considered a competing event to SPM during follow-up, a competing-risk model was used to estimate the cumulative incidence of SPM in each radiotherapy group. We used univariate and multivariate Cox proportional hazards models to identify possible risk factors for SPM development. The final Cox proportional hazard model was used to assess the significant difference between the relative risk of an SPM across the four groups after adjustment for age at and year of rectal cancer diagnosis, sex, chemotherapy, and comorbidities. A two-sided *P*-value less than 0.05 was considered statistically significant.

The data processing was performed with Microsoft SQL Server 2012 (Microsoft Corp., Redmond, WA, United States). All analyses were computed in R (version R-2.15.3; http://www.r-project.org). The cmprsk library in R was used for competing-risk analyses.

Sensitivity analysis

In addition to the final Cox model, a SPM attained-calendar-year stratified Cox proportional hazards model was tested to assess for adjusted radiotherapy effects. Subgroup analyses were also undertaken to investigate the consistency of the conclusion among different subpopulations. We generated Cox models in patients who survived more than 5 years and more than 10 years.

RESULTS

Population demographics

We identified a total of 28220 eligible rectal cancer patients based on our criteria. There were 21769, 1311, 314, and 4826 patients in the surgery-only, preoperative long-course, preoperative short-course, and postoperative radiotherapy groups, respectively. The cohort selection flow chart is shown in Figure 1.

The median follow-up for all patients was 5.2 years (range: 1 to 16.0 years) and was 5.5 years (range, 1 to 15.3 years) in the surgery-only group, 4.2 years (range,

Table 1 Patient characters and treatment factors

	All patients	Surgery-only	All radiotherapy	Postoperative	Preoperative	Long	Short
Patient number	28220	21769	6451	4826	1625	1311	314
Male (%)	16297 (58%)	12323 (57%)	3974 (62%)	2940 (61%)	1034 (64%)	831 (63%)	203 (65%)
Median follow-up (IQR), yr	5.19 (5.02)	5.47 (5.18)	4.25 (3.98)	4.29 (4.10)	4.16 (3.61)	4.18 (3.76)	4.10 (3.01)
Median rectal cancer diagnosis age (IQR)	65 (18)	66 (18)	62 (17)	62 (18)	61 (19)	60 (18)	64 (18)
Median rectal cancer diagnosis year (IQR)	2005 (7)	2004 (6)	2006 (6)	2005 (6)	2007 (5)	2007 (5)	2007 (4)
Surgery							
LAR	20416	16253	4163	2953	1210	950	260
APR	6285	4453	1832	1471	361	311	50
Other surgery	1519	1063	456	402	54	50	4
Chemotherapy							
All chemotherapy (%)	18236 (65%)	12310 (57%)	5926 (92%)	4445 (92%)	1481 (91%)	1276 (97%)	205 (65%)
Fluorouracil	12063	7399	4664	3428	1236	1105	131
Tegafur	11324	8139	3185	2547	638	517	121
Oxaliplatin	4033	2460	1573	1273	300	262	38
Irinotecan	3273	2020	1253	1069	184	151	33
Capecitabine	2620	1632	988	773	215	185	30
Comorbidities							
DM	10802	8560	2242	1696	546	427	119
Hypertension	18096	14438	3658	2742	916	720	196
Liver cirrhosis	1521	1227	294	225	69	46	23
Autoimmune disease	1763	1372	391	298	93	76	17
End stage renal disease	5456	4402	1054	825	229	168	61
COPD	10762	8709	2053	1585	468	368	100
Dyslipidemia	11695	9246	2449	1779	670	534	136

IQR: Inter-quantile range; LAR: Low anterior resection; APR: Abdominoperineal resection of rectum; DM: Diabetes mellitus; COPD: Chronic obstructive pulmonary disease.

1 to 13.2 years) in the preoperative long-course group, 4.1 years (range, 1 to 10.5 years) in the preoperative short-course group, and 4.3 years (range, 1 to 16.0 years) in the postoperative radiotherapy group. The patients in the radiotherapy group were slightly younger (mean age 61 years *vs* 66 years in those without radiotherapy), had a more recent diagnosis year (median year 2006 versus 2004 in those without radiotherapy), and had a higher chance of receiving chemotherapy (92% *vs* 56% in those without radiotherapy). The most commonly used chemotherapy agents were fluorouracil, tegafur/uracil, oxaliplatin, irinotecan, and capecitabine. Table 1 summarizes the patient and treatment characteristics.

SPM result

During the follow-up period, 1270 of the 28220 patients (4.5%) developed a SPM. In the surgery-only group, 1056 patients (8.6%) developed a second cancer, compared with 49 (3.7%) in the preoperative long-course group, 10 (3.2%) in the preoperative short-course group, and 182 (3.2%) in the postoperative radiotherapy group. The most common sites of SPM were lung (n = 284), liver (n = 183), and prostate (n = 129). The distributions of the SPMs in each group are listed in Table 2. The cumulative incidences of SPM and mortality rate are shown in Figure 2. Death is a strong competitor for SPM in both non-irradiated and irradiated patients. The cumulative incidence of mortality is higher in the irradiated patients because these patients generally had more advanced disease. The estimated cumulative incidence of SPM in the competing-risk model at the 5 year, 10 year, and 15 year marks was 3.7% (95%CI: 3.4%-3.9%), 7.8% (95%CI: 7.2%-8.2%), and 12.4% (95%CI: 10.5%-14.6%) in the surgery-only group and 3.2% (95%CI: 2.7%-3.7%), 6.7% (95%CI: 5.8%-7.6%), and 8.3% (95%CI: 7.1%-9.7%) in the irradiated groups, respectively.

Other risk factors

A univariate Cox regression model was used to test the potential risk factors for SPM. The results showed that male sex, age, liver cirrhosis, autoimmune disease, and COPD were significantly associated with a higher risk for SPMs, while dyslipidemia was significantly associated with a lower risk for SPMs. Chemotherapy and radiotherapy were not significantly associated with SPMs, although preoperative long-course radiotherapy had a trend toward increasing risk [hazard ratio (HR) = 1.25, 95%CI: 0.97-1.62; P = 0.090]. To better clarify the risk of radiotherapy for SPM, the final Cox regression model contained the covariates gender, age at and year of rectal cancer diagnosis, the use of radiotherapy, the use of chemotherapy, DM, HTN, liver cirrhosis, autoimmune disease, COPD, ESRD, and dyslipidemia. In multi-variate analysis, age (HR = 1.02 per one-year increment, 95%CI: 1.01-1.02; P < 0.001), male sex (HR = 1.47, 95%CI: 1.32-1.65; P < 0.001), DM (HR = 1.14, 95%CI: 1.02-1.28; P = 0.027), liver cirrhosis (HR = 2.40, 95%CI: 2.03-2.82; P < 0.001),

Table 2 Second primary malignancy of different treatment groups

	All patients	Surgery-only	Long	Short	Post
All SPM	1270 (100)	1056 (100)	49 (100)	10 (100)	155 (100)
Head and neck	89 (7)	69 (6.5)	7 (14.3)	2 (20)	11 (7.1)
Esophagus	31 (2.4)	28 (2.7)	0 (0)	0 (0)	3 (1.9)
Stomach	82 (6.5)	68 (6.4)	4 (8.2)	1 (10)	9 (5.8)
Liver	183 (14.4)	162 (15.3)	3 (6.1)	3 (30)	15 (9.7)
Pancreas	31 (2.4)	26 (2.5)	1 (2)	0 (0)	4 (2.6)
Lung	284 (22.4)	224 (21.2)	16 (32.7)	0 (0)	44 (28.4)
Bone	17 (1.3)	14 (1.3)	0 (0)	0 (0)	3 (1.9)
Skin	31 (2.4)	23 (2.2)	1 (2)	2 (20)	5 (3.2)
Breast	82 (6.5)	71 (6.7)	3 (6.1)	0 (0)	8 (5.2)
Cervix	18 (1.4)	17 (1.6)	0 (0)	0 (0)	1 (0.6)
Uterus	15 (1.2)	10 (0.9)	2 (4.1)	0 (0)	3 (1.9)
Ovary	10 (0.8)	10 (0.9)	0 (0)	0 (0)	0 (0)
Prostate	129 (10.2)	116 (11)	2 (4.1)	1 (10)	10 (6.5)
Bladder	83 (6.5)	63 (6)	2 (4.1)	0 (0)	18 (11.6)
Kidney	45 (3.5)	40 (3.8)	1 (2)	1 (10)	3 (1.9)
Thyroid	18 (1.4)	15 (1.4)	0 (0)	0 (0)	3 (1.9)
Hematologic	59 (4.6)	46 (4.4)	3 (6.1)	0 (0)	10 (6.5)
Others	63 (5)	54 (5.1)	4 (8.2)	0 (0)	5 (3.2)

SPM: Second primary malignancy.

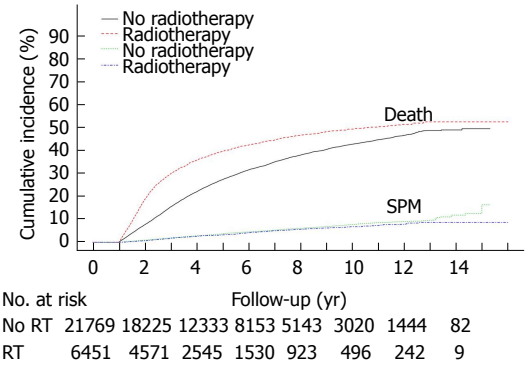


Figure 2 Competing-risk model plot for cumulative incidence of death and secondary primary malignancy, stratified by with/without radiotherapy. SPM: Secondary primary malignancy; RT: Radiotherapy.

and COPD (HR = 1.19, 95%CI: 1.06-1.33; P = 0.003) were significantly associated with a higher risk for SPMs. Hypertension (HR = 0.86, 95%CI: 0.75-0.97; P = 0.017) and dyslipidemia (HR = 0.85, 95%CI: 0.76-0.95; P = 0.006) were significantly associated with a lower risk for SPMs (Table 3). Again, no significantly elevated HR was observed among the different radiotherapy groups compared with the surgery-alone group.

Second cancer site analysis

A similar covariate-adjusted Cox model was applied to the individual SPM sites. Compared with the surgery-only group, a significantly increased HR for SPM in the radiotherapy group was only evident for lung cancer (HR = 1.42, 95%CI: 1.04-1.93; P < 0.001). The risk of bladder, uterus, skin, and hematologic cancer was elevated in irradiated patients, but the difference was not statistically significant. Irradiated patient also had less prostate and liver cancer, but again, the difference was not statistically significant (Figure 3A). We further compared the preoperative and postoperative radiotherapy groups. Due to relatively few events in each of the preoperative long/short-course groups, we combined these two groups in the second primary sites analysis. Among all SPM, the HR of the preoperative and postoperative groups compared with the surgery-only group was 1.20 (95%CI: 0.93-1.53) and 1.01 (95%CI: 0.85-1.18), respectively. Across the second cancer sites, the risk associated with radiotherapy was generally consistent between the preoperative and postoperative groups, except that patients in the preoperative radiotherapy group had a higher risk of head and neck cancers (P = 0.042) (Figure 3B).

Sensitivity analysis

A stratified Cox proportional hazards model showed the HR of radiotherapy remained unchanged after considering second primary cancer attained year (Supplementary Table 1). There were 12064 patients surviving without a SPM after 5 years of follow-up, and 3516 patients after 10 years. The HR of radiotherapy in all patients, > 5 year survivors, and > 10 year survivors was 1.05 (95%CI: 0.91-1.21), 1.17 (95%CI: 0.92-1.47), and 1.03 (95%CI: 0.56-1.89), respectively. None of these HRs was statistically significant, as listed in Supplementary Table 2.

DISCUSSION

The aim of radiotherapy in rectal cancer is to reduce the recurrence risk, and this benefit is well documented[15]. Clinical practice has shifted from postoperative chemoradiotherapy to preoperative radiotherapy as encouraging results with preoperative radiotherapy have emerged over the last decade[4]. Still, there is debate regarding short-course preoperative radiation and the

Table 3 Cox regression of second primary malignancy

	Univariate Cox regression		Multi-variate Cox regression	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Sex(M)	1.57 (1.40-1.75)[a]	< 0.001	1.47 (1.32-1.65)[a]	< 0.001
Diagnosis age (1 yr increment)	1.02 (1.01-1.02)[a]	< 0.001	1.02 (1.01-1.02)[a]	< 0.001
Diagnosis year	1.06 (1.04-1.08)[a]	< 0.001	1.06 (1.04-1.08)[a]	< 0.001
Chemotherapy	0.95 (0.86-1.06)	0.371	0.97 (0.87-1.08)	0.562
DM	1.11 (0.99-1.23)	0.062	1.14 (1.02-1.28)[a]	0.027
Hypertension	1.01 (0.90-1.13)	0.849	0.86 (0.75-0.97)[a]	0.017
Liver cirrhosis	2.47 (2.10-2.90)[a]	< 0.001	2.40 (2.03-2.82)[a]	< 0.001
Rheumatologic disease	0.78 (0.62-0.99)[a]	0.038	0.81 (0.64-1.03)	0.080
End stage renal disease	1.01 (0.89-1.16)	0.828	0.91 (0.80-1.05)	0.192
COPD	1.33 (1.20-1.48)[a]	< 0.001	1.19 (1.06-1.33)[a]	0.003
Dyslipidemia	0.87 (0.78-0.96)[a]	0.008	0.85 (0.76-0.95)[a]	0.006
Radiotherapy[1]	1.04 (0.90-1.19)	0.625	1.05 (0.91-1.21)	0.494
Long course RT[1]	1.25 (0.97-1.62)	0.090	1.28 (0.98-1.67)[2]	0.071
Short course RT[1]	1.01 (0.56-1.83)	0.976	0.91 (0.50-1.64)[2]	0.742
Post-OP RT[1]	0.98 (0.84-1.15)	0.801	1.01 (0.86-1.18)[2]	0.941

[1]Indicates surgery-only as reference; [2]Indicates calculated separately with "Radiotherapy" using same model. [a]$P < 0.05$. DM: Diabetes mellitus; COPD: Chronic obstructive pulmonary disease; Post-OP: Postoperative.

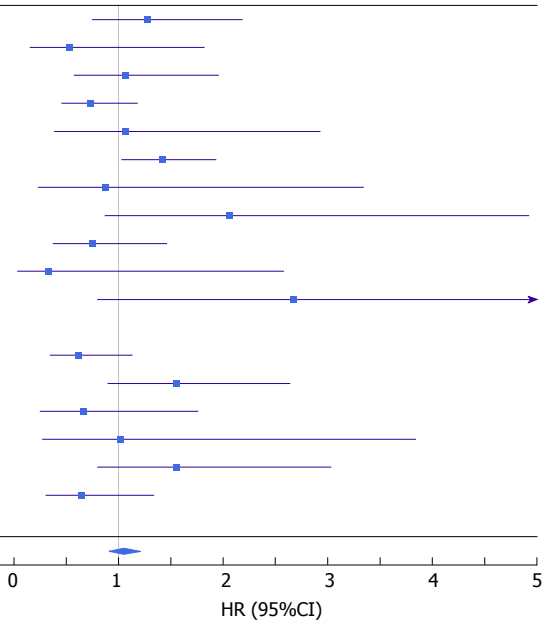
more conventional approach of long-course neoadjuvant chemoradiation. The reported efficacy of these two regimens is comparable, yet there appears to be more late gastrointestinal toxicity in short-course studies[16]. Whether different radiotherapy regimens result in different SPM risks has not been investigated. Our results showed no differences in overall SPM probability between patients in each radiotherapy regimen. To our knowledge, this is the first report to directly compare the risk of SPM among preoperative long-course radiotherapy, preoperative short-course radiotherapy, and postoperative radiotherapy.

Four previous studies have addressed the issue of SPM after rectal irradiation. Birgisson *et al*[12] analyzed pooled data from the Uppsala Trial and the Swedish Rectal Cancer Trial, and they reported an overall relative risk of 1.85 for developing a second cancer in irradiated patients. However, their results were limited by the relatively small cohort size. More recently, Martling *et al*[17]analyzed Swedish ColoRectal Cancer Registry data and reported no increased risk of second primary cancer following RT for rectal cancer within or outside of the irradiated volume up to 20 years of follow-up. Two groups have taken advantage of the large Surveillance, Epidemiology, and End Results (SEER) registry database to exam this issue, but their efforts yielded opposite results. It is noteworthy that neither of the SEER-based studies reported the radiotherapy regimen. Kendal *et al*[11] used Kaplan-Meier and Cox analyses and demonstrated no significant difference in SPM occurrence between irradiated and non-irradiated cohorts, comprising a total of 20910 patients. In a subpart of Berrington's comprehensive study, they reported that the relative risk was 1.15 in irradiated patients using a Poisson regression analysis. Although radiation-induced malignancy is a stochastic effect and risk increases in a linear-quadratic fashion with dose and exposure at younger ages, they found neither a dose response nor a correlation with patient's age at rectal cancer diagnosis. This lack may harm the validity of the casual association. In addition, the two SEER studies may have been negatively affected by occult confounding factors. For example, certain comorbidities may have a strong correlation to SPM. Liver cirrhosis is strongly associated with hepatocellular carcinoma. COPD is not only linked with smoking history but also acts as an independent risk factor for lung cancer[18]. In the present study, we demonstrated that several comorbidities were significantly associated with SPM on multivariate analysis. Any conclusion regarding radiotherapy made without adjustment for these factors is vulnerable to bias. Finally, Wiltink examined the Total Mesorectal Excision trial data[10]. They used a competing-risk model and Gray's test and found that the 10-year SPM rates were 14.8% and 15.3% in patients with and without radiotherapy, respectively. No significant difference was noted. The competing-risk model is more accurate in estimating SPM probability than the Kaplan-Meier model in that the competing circumstance is death. However, for etiological research, a proportional cause-specific hazards model may be more appropriate than the competing-risk model[19]. Here, we used competing-risk model to report the cumulative incidence of SPM and applied a Cox model to compare the HRs for different treatment groups.

Another limitation of these four studies is the lack of chemotherapy analysis. Chemotherapy is associated with SPM risk, mainly leukemias but also solid tumors[14]. However, most data on chemotherapy are derived from studies on Hodgkin lymphoma[20,21] and breast cancer[22]. The association between chemotherapy and SPM in rectal cancers has not been studied. In our study, the use of chemotherapy was not associated with increased SPM. After controlling for chemotherapy and other comorbidities, we could assess the absolute excess risk

A

Site of cancers	Event	HR (95%CI)
Head and neck	89	1.28 (0.75-2.18)
Esophagus	31	0.53 (0.16-1.82)
Stomache	82	1.07 (0.58-1.96)
Liver	183	0.73 (0.46-1.18)
Pancreas	31	1.07 (0.39-2.93)
Lung[a]	284	1.42 (1.04-1.93)
Bone	17	0.88 (0.23-3.34)
Skin	31	2.06 (0.87-4.92)
Breast	82	0.75 (0.38-1.46)
Cervix	18	0.33 (0.04-2.58)
Uterus	15	2.67 (0.80-8.86)
Ovary	0	NA
Prostate	129	0.62 (0.34-1.13)
Bladder	83	1.55 (0.90-2.64)
Kidney	45	0.67 (0.25-1.76)
Thyroid	18	1.02 (0.27-3.84)
Hematologic	59	1.56 (0.80-3.03)
Other sites	63	0.65 (0.31-1.34)
All cancers	1416	1.05 (0.91-1.21)



B

Site of cancers	Pre-OP HR	Post-OP HR
Head and neck[a]	2.45 (1.17-5.14)	0.96 (0.50-1.87)
Esophagus	NA	0.69 (0.20-2.37)
Stomache	1.63 (0.64-4.18)	0.93 (0.45-1.91)
Liver	0.91 (0.39-2.09)	0.67 (0.39-1.16)
Pancreas	0.83 (0.11-6.34)	1.09 (0.36-3.28)
Lung	1.63 (0.96-2.77)	1.37 (0.98-1.93)
Bone	NA	1.25 (0.33-4.70)
Skin	3.36 (0.93-12.15)	1.64 (0.59-4.55)
Breast	0.88 (0.27-2.86)	0.70 (0.33-1.50)
Cervix	NA	0.41 (0.05-3.24)
Uterus	5.52 (1.03-29.65)	2.06 (0.51-8.37)
Ovary	NA	NA
Prostate	0.58 (0.18-1.86)	0.63 (0.32-1.22)
Bladder	0.59 (0.14-2.47)	1.84 (1.05-3.19)
Kidney	1.19 (0.28-5.14)	0.51 (0.15-1.71)
Thyroid	NA	1.37 (0.36-5.13)
Hematologic	1.53 (0.46-5.16)	1.60 (0.77-3.31)
Other sites	1.20 (0.42-3.42)	0.47 (0.19-1.2)
All cancers	1.20 (0.93-1.53)	1.01 (0.85-1.18)

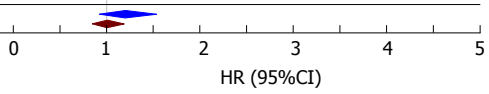


Figure 3 Secondary cancer site analysis for (A) with/without radiotherapy, and (B) preoperative and postoperative radiotherapy. Referenced by surgery-alone group, [a]$P < 0.05$; HR: Hazard ratio; NA: Not applicable.

of the radiotherapy effect. We found that the overall SPM risk did not increase in irradiated patients. Considering the diagnosis age of rectal cancer patients tends to be older, we would expect to find less radiation-induced cancers than in younger cancer patients[23]. In our sensitivity test, we added attained cancer year into the model and performed a subgroup analysis focused on long-term survivors. The absence of a radiotherapy effect was still in consistent in these analyses. Considering age is not an exclusive factor that affect surgical complication in colo-rectal cancer patients[24,25], we suggest irradiation should not be avoided either in the elderly rectal cancer patients.

In Berrington's SEER study, the relative risk of second lung cancer in irradiated rectal cancer patients was 1.27[9]. Additionally, second primary lung cancer has been reported to increase after irradiation in prostate cancer patients[26,27]. In our second cancer site analysis, lung cancer was the only increased SPM subsite that was associated with radiotherapy. One reason for this

relationship may be that lung cancer can be induced efficiently by relatively low doses of radiation, which has been shown in breast cancer and Hodgkin lymphoma survivors[28,29]. Another explanation is the possible uneven distribution of patients who smoke. Of the other specific solid tumor sites, both bladder carcinoma and uterus carcinoma showed non-significant increasing trend, which was broadly consistent with previous studies. We found that the risk of subsequent prostate cancer was decreased in irradiated patients, although again the difference was not statistically significant (HR = 0.62, 95%CI: 0.34-1.13). A recent meta-analysis supported this finding that radiotherapy for rectal cancer is associated with a decreased prostate cancer risk[30]. However, the mechanism is still unclear.

The strength of our study is that these data were derived from population-based registries, which permits a powerful evaluation of SPM risk according to a variety of relevant variables. By controlling for treatment and patient characteristics, we can minimize the potential for bias. We also performed a sensitivity analysis to test the robustness of our conclusions. Nonetheless, our study had several limitations. The main limitation was the relatively short mean follow-up. However, there were still more than 3000 patients followed up for more than 10 years. In the sensitivity analysis, the HR of radiotherapy in patients followed more than 5 or 10 years remained statistically insignificant. This conclusion is not likely to be altered after even longer follow-up periods. Second, the radiotherapy dose and volume were not available in the NHIRD, which made it impossible to analyze the radiotherapy dose response. Instead of the dose, we used radiation portals as a surrogate and applied strict criteria for the different radiotherapy regimens to ensure that the radiotherapy dose was consistent in each regimen group. Radiation techniques have evolved in the past decades, but we could not ascertain the radiotherapy technique information used for each patient. The use of the intensity-modulated radiation therapy (IMRT) technique may result in a greater volume of low-dose irradiated tissue and therefore more SPM[31]. However, the three-dimensional conformal radiation therapy (3DCRT) technique was still the standard treatment for rectal cancer during the study period. We also adjusted for the diagnosis year, which may have helped to eliminate this bias. Third, the lack of data on smoking and other lifestyle information likely suggests that there is residual confounding.

In the future, we advocate that study regards to SPM related to radiotherapy should carefully adjust comorbidities, chemotherapy, and use competing risk model to yield true effect of radiotherapy. Also studies should focused on the mechanisms by which radiation may produce carcinogenic changes, especially in SPM outside irradiation volume.

In conclusion, in this population-based study, no increased risk of developing SPM was found in rectal cancer patients who received pelvic radiotherapy in their initial treatment. The SPM risk remained the same among the preoperative long-course, preoperative short-course, and postoperative radiotherapy groups. Therefore, the SPM risk should not be a major consideration in treatment decisions. However, rectal cancer survivors, similarly to other cancer survivors, are burdened with an overall higher probability of developing a second primary cancer. Life-long follow-up is recommended.

ARTICLE HIGHLIGHTS

Research background

Previous literature on second primary malignancy (SPM) risk after radiotherapy in rectal cancer survivors yielded controversial results. Also, lack of comorbidities, chemotherapy, and competing risk adjustment may cause biased conclusion. In addition, whether different radiotherapy regimens results in different SPM risk has not been investigated. In this study, we meticulously collected and analyzed all factors may contribute in SPM, and yielded true radiotherapy effect.

Research motivation

The risk of developing an SPM has received greater attention in clinical practice. Although several studies have investigated the relationship between radiotherapy and SPM in rectal cancer patients, the conclusions have been diverse.

Research objectives

To analyze true radiotherapy effect on developing an SPM in rectal cancer patients.

Research methods

We used Taiwan's National Health Insurance Research Database to identify rectal cancer patients between 1996 and 2011. The cohort was composed of patients aged 20 years or older who were diagnosed with a first primary rectal cancer. SPM risk was analyzed by competing risk model. The overall and site-specific SPM incidence rates were compared among the radiotherapy groups by multivariate Cox regression, taking chemotherapy and comorbidities into account. Sensitivity tests were performed for attained-year adjustment and long-term survivor analysis.

Research results

In this large-scale population-based cohort study, we found no increase of SPM due to radiotherapy in rectal patients. Different radiotherapy regimens results in same SPM risk. Factors that were significantly associated with a higher risk for SPMs included male sex, age, liver cirrhosis, autoimmune disease, and COPD. Compared with the surgery-only group, a significantly increased HR for SPM in the radiotherapy group was only evident for lung cancer (HR = 1.42, 95%CI: 1.04-1.93; $P < 0.001$). The risk of bladder, uterus, skin, and hematologic cancer was elevated in irradiated patients, but the difference was not statistically significant.

Research conclusions

This study confirmed no increased risk of SPM due to radiotherapy in rectal patients. Many secondary malignancy may only reflect the patients' genetic backgrounds, cancer-related treatments, lifestyles, and environmental risk factors. After careful confounder adjustment and appropriate statistical analysis, no radiotherapy effect on SPM can be drawn. This is an important conclusion to both patients and physicians.

Research perspectives

Some comorbidities confounders have profound effects on developing secondary malignancy. Also, death is a strong competing risk need to handle. In future, we need to explore and investigate the mechanism of oncogenic effect of radiotherapy, especially in cancer outside radiation volume.

REFERENCES

1 **De Angelis R**, Sant M, Coleman MP, Francisci S, Baili P, Pierannunzio D, Trama A, Visser O, Brenner H, Ardanaz E, Bielska-Lasota M, Engholm G, Nennecke A, Siesling S, Berrino F, Capocaccia R; EUROCARE-5 Working Group. Cancer survival in Europe 1999-2007 by country and age: results of EUROCARE--5-a population-based study. *Lancet Oncol* 2014; **15**: 23-34 [PMID: 24314615 DOI: 10.1016/S1470-2045(13)70546-1]

2 **Siegel R**, Desantis C, Jemal A. Colorectal cancer statistics, 2014. *CA Cancer J Clin* 2014; **64**: 104-117 [PMID: 24639052 DOI: 10.3322/caac.21220]

3 **van Gijn W**, Marijnen CA, Nagtegaal ID, Kranenbarg EM, Putter H, Wiggers T, Rutten HJ, Påhlman L, Glimelius B, van de Velde CJ; Dutch Colorectal Cancer Group. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol* 2011; **12**: 575-582 [PMID: 21596621 DOI: 10.1016/S1470-2045(11)70097-3]

4 **Sebag-Montefiore D**, Stephens RJ, Steele R, Monson J, Grieve R, Khanna S, Quirke P, Couture J, de Metz C, Myint AS, Bessell E, Griffiths G, Thompson LC, Parmar M. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009; **373**: 811-820 [PMID: 19269519 DOI: 10.1016/S0140-6736(09)60484-0]

5 **Birgisson H**, Påhlman L, Gunnarsson U, Glimelius B. Late adverse effects of radiation therapy for rectal cancer - a systematic overview. *Acta Oncol* 2007; **46**: 504-516 [PMID: 17497318 DOI: 10.1080/02841860701348670]

6 **Phipps AI**, Chan AT, Ogino S. Anatomic subsite of primary colorectal cancer and subsequent risk and distribution of second cancers. *Cancer* 2013; **119**: 3140-3147 [PMID: 23856984 DOI: 10.1002/cncr.28076]

7 **Lee YT**, Liu CJ, Hu YW, Teng CJ, Tzeng CH, Yeh CM, Chen TJ, Lin JK, Lin CC, Lan YT, Wang HS, Yang SH, Jiang JK, Chen WS, Lin TC, Chang SC, Chen MH, Teng HW, Liu JH, Yen CC. Incidence of Second Primary Malignancies Following Colorectal Cancer: A Distinct Pattern of Occurrence Between Colon and Rectal Cancers and Association of Co-Morbidity with Second Primary Malignancies in a Population-Based Cohort of 98,876 Patients in Taiwan. *Medicine* (Baltimore) 2015; **94**: e1079 [PMID: 26131831 DOI: 10.1097/MD.0000000000001079]

8 **Wood ME**, Vogel V, Ng A, Foxhall L, Goodwin P, Travis LB. Second malignant neoplasms: assessment and strategies for risk reduction. *J Clin Oncol* 2012; **30**: 3734-3745 [PMID: 23008293 DOI: 10.1200/JCO.2012.41.8681]

9 **Berrington de Gonzalez A**, Curtis RE, Kry SF, Gilbert E, Lamart S, Berg CD, Stovall M, Ron E. Proportion of second cancers attributable to radiotherapy treatment in adults: a cohort study in the US SEER cancer registries. *Lancet Oncol* 2011; **12**: 353-360 [PMID: 21454129 DOI: 10.1016/S1470-2045(11)70061-4]

10 **Wiltink LM**, Nout RA, Fiocco M, Meershoek-Klein Kranenbarg E, Jürgenliemk-Schulz IM, Jobsen JJ, Nagtegaal ID, Rutten HJ, van de Velde CJ, Creutzberg CL, Marijnen CA. No Increased Risk of Second Cancer After Radiotherapy in Patients Treated for Rectal or Endometrial Cancer in the Randomized TME, PORTEC-1, and PORTEC-2 Trials. *J Clin Oncol* 2015; **33**: 1640-1646 [PMID: 25534376 DOI: 10.1200/JCO.2014.58.6693]

11 **Kendal WS**, Nicholas G. A population-based analysis of second primary cancers after irradiation for rectal cancer. *Am J Clin Oncol* 2007; **30**: 333-339 [PMID: 17762431 DOI: 10.1097/01.coc.0000258084.55036.9e]

12 **Birgisson H**, Påhlman L, Gunnarsson U, Glimelius B. Occurrence of second cancers in patients treated with radiotherapy for rectal cancer. *J Clin Oncol* 2005; **23**: 6126-6131 [PMID: 16135478 DOI: 10.1200/JCO.2005.02.543]

13 **Cheng TM**. Taiwan's new national health insurance program: genesis and experience so far. *Health Aff* (Millwood) 2003; **22**: 61-76 [PMID: 12757273 DOI: 10.1377/hlthaff.22.3.61]

14 **Travis LB**. The epidemiology of second primary cancers. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 2020-2026 [PMID: 17057028 DOI: 10.1158/1055-9965.EPI-06-0414]

15 **Colorectal Cancer Collaborative Group**. Adjuvant radiotherapy for rectal cancer: a systematic overview of 8,507 patients from 22 randomised trials. *Lancet* 2001; **358**: 1291-1304 [PMID: 11684209 DOI: 10.1016/S0140-6736(01)06409-1]

16 **Mohiuddin M**, Marks J, Marks G. Management of rectal cancer: short- vs. long-course preoperative radiation. *Int J Radiat Oncol Biol Phys* 2008; **72**: 636-643 [PMID: 19014778 DOI: 10.1016/j.ijrobp.2008.05.069]

17 **Martling A**, Smedby KE, Birgisson H, Olsson H, Granath F, Ekbom A, Glimelius B. Risk of second primary cancer in patients treated with radiotherapy for rectal cancer. *Br J Surg* 2017; **104**: 278-287 [PMID: 27802358 DOI: 10.1002/bjs.10327]

18 **Young RP**, Hopkins RJ, Christmas T, Black PN, Metcalf P, Gamble GD. COPD prevalence is increased in lung cancer, independent of age, sex and smoking history. *Eur Respir J* 2009; **34**: 380-386 [PMID: 19196816 DOI: 10.1183/09031936.00144208]

19 **Noordzij M**, Leffondré K, van Stralen KJ, Zoccali C, Dekker FW, Jager KJ. When do we need competing risks methods for survival analysis in nephrology? *Nephrol Dial Transplant* 2013; **28**: 2670-2677 [PMID: 23975843 DOI: 10.1093/ndt/gft355]

20 **Delwail V**, Jais JP, Colonna P, Andrieu JM. Fifteen-year secondary leukaemia risk observed in 761 patients with Hodgkin's disease prospectively treated by MOPP or ABVD chemotherapy plus high-dose irradiation. *Br J Haematol* 2002; **118**: 189-194 [PMID: 12100147 DOI: 10.1046/j.1365-2141.2002.03564.x]

21 **van Leeuwen FE**, Klokman WJ, Hagenbeek A, Noyon R, van den Belt-Dusebout AW, van Kerkhoff EH, van Heerde P, Somers R. Second cancer risk following Hodgkin's disease: a 20-year follow-up study. *J Clin Oncol* 1994; **12**: 312-325 [PMID: 8113838 DOI: 10.1200/JCO.1994.12.2.312]

22 **Curtis RE**, Boice JD Jr, Stovall M, Bernstein L, Greenberg RS, Flannery JT, Schwartz AG, Weyer P, Moloney WC, Hoover RN. Risk of leukemia after chemotherapy and radiation treatment for breast cancer. *N Engl J Med* 1992; **326**: 1745-1751 [PMID: 1594016 DOI: 10.1056/NEJM199206253262605]

23 **VanderWalde AM**, Hurria A. Second malignancies among elderly survivors of cancer. *Oncologist* 2011; **16**: 1572-1581 [PMID: 22042787 DOI: 10.1634/theoncologist.2011-0214]

24 **Grosso G**, Biondi A, Marventano S, Mistretta A, Calabrese G, Basile F. Major postoperative complications and survival for colon cancer elderly patients. *BMC Surg* 2012; **12** Suppl 1: S20 [PMID: 23173563 DOI: 10.1186/1471-2482-12-S1-S20]

25 **Biondi A**, Vacante M, Ambrosino I, Cristaldi E, Pietrapertosa G, Basile F. Role of surgery for colorectal cancer in the elderly. *World J Gastrointest Surg* 2016; **8**: 606-613 [PMID: 27721923 DOI: 10.4240/wjgs.v8.i9.606]

26 **Brenner DJ**, Curtis RE, Hall EJ, Ron E. Second malignancies in prostate carcinoma patients after radiotherapy compared with surgery. *Cancer* 2000; **88**: 398-406 [PMID: 10640974 DOI: 10.1002/(SICI)1097-0142(20000115)88:2<398::AID-CNCR22>3.0.CO;2-V]

27 **Joung JY**, Lim J, Oh CM, Jung KW, Cho H, Kim SH, Seo HK, Park WS, Chung J, Lee KH, Won YJ. Risk of Second Primary Cancer among Prostate Cancer Patients in Korea: A Population-Based Cohort Study. *PLoS One* 2015; **10**: e0140693 [PMID: 26469085 DOI: 10.1371/journal.pone.0140693]

28 **Travis LB**, Gospodarowicz M, Curtis RE, Clarke EA, Andersson M, Glimelius B, Joensuu T, Lynch CF, van Leeuwen FE, Holowaty E, Storm H, Glimelius I, Pukkala E, Stovall M, Fraumeni JF Jr, Boice JD Jr, Gilbert E. Lung cancer following chemotherapy and radiotherapy for Hodgkin's disease. *J Natl Cancer Inst* 2002; **94**: 182-192 [PMID: 11830608 DOI: 10.1093/jnci/94.3.182]

29 **Rubino C**, de Vathaire F, Shamsaldin A, Labbe M, Lê MG. Radiation dose, chemotherapy, hormonal treatment and risk of second cancer after breast cancer treatment. *Br J Cancer* 2003; **89**: 840-846 [PMID: 12942115 DOI: 10.1038/sj.bjc.6601138]

30 **Lee YC**, Hsieh CC, Li CY, Chuang JP, Lee JC. Secondary Cancers

After Radiation Therapy for Primary Prostate or Rectal Cancer. *World J Surg* 2016; **40**: 895-905 [PMID: 26711638 DOI: 10.1007/s00268-015-3324-x]

31 **Zwahlen DR**, Ruben JD, Jones P, Gagliardi F, Millar JL, Schneider U. Effect of intensity-modulated pelvic radiotherapy on second cancer risk in the postoperative treatment of endometrial and cervical cancer. *Int J Radiat Oncol Biol Phys* 2009; **74**: 539-545 [PMID: 19427555 DOI: 10.1016/j.ijrobp.2009.01.051]

P- Reviewer: Biondi A, Kin SH, Velenik V **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4596-4605

DOI: 10.3748/wjg.v24.i40.4596

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

ORIGINAL ARTICLE

Retrospective Study

Outcomes of furazolidone- and amoxicillin-based quadruple therapy for *Helicobacter pylori* infection and predictors of failed eradication

Ya-Wen Zhang, Wei-Ling Hu, Yuan Cai, Wen-Fang Zheng, Qin Du, John J Kim, John Y Kao, Ning Dai, Jian-Min Si

Ya-Wen Zhang, Wei-Ling Hu, Yuan Cai, Wen-Fang Zheng, Ning Dai, Jian-Min Si, Department of Gastroenterology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Ya-Wen Zhang, Wei-Ling Hu, Wen-Fang Zheng, Jian-Min Si, Institute of Gastroenterology, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Qin Du, Department of Gastroenterology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China

John J Kim, Division of Gastroenterology, Loma Linda University, Loma Linda, CA 92354, United States

John Y Kao, Division of Gastroenterology, Department of Internal Medicine, Michigan Medicine, University of Michigan, Ann Arbor, MI 48109, United States

ORCID number: Ya-Wen Zhang (0000-0002-6867-0399); Wei-Ling Hu (0000-0002-9145-7027); Yuan Cai (0000-0003-2356-1399); Wen-Fang Zheng (0000-0002-4646-0518); Qin Du (0000-0002-6840-8358); John J Kim (0000-0003-4341-8661); John Y Kao (0000-0001-5338-4587); Ning Dai (0000-0003-0749-9230); Jian-Min Si (0000-0002-1254-7949).

Author contributions: Hu WL, Dai N and Si JM designed the study; Zhang YW, Cai Y and Zheng WF performed the research; Du Q contributed to providing support to patients; Zhang YW and Kim JJ analyzed the data; Zhang YW wrote the paper; Hu WL, Kim JJ and Kao JY critically revised the manuscript; all authors had access to the study data and had reviewed and approved the final version of the article.

Supported by the Zhejiang Science and Technology Project, No. LGF18H160012.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Sir Run Run Shaw Hospital.

Informed consent statement: Patients were not required to provide informed consent to the study because the data were obtained retrospectively after completing treatment.

Conflict-of-interest statement: The authors received grants from Zhejiang Science and Technology Project during the conduct of the study and declare no other conflict of interest related to this study.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Wei-Ling Hu, MD, PhD, Doctor, Department of Gastroenterology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, No. 3, East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. huweiling@zju.edu.cn
Telephone: +86-571-86006181
Fax: +86-571-86006181

Received: July 1, 2018
Peer-review started: July 2, 2018
First decision: July 25, 2018
Revised: August 16, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

AIM

To evaluate the outcomes of furazolidone- and amoxicillin-based quadruple therapy for treatment of *Helicobacter pylori* (*H. pylori*) infection and identify predictors of failed eradication.

METHODS

Patients with *H. pylori* infection treated with furazolidone, amoxicillin, bismuth, and proton pump inhibitor therapy (January 2015 to December 2015) who received the ^{13}C-urea breath test > 4 wk after treatment were evaluated. Demographic and clinical data including prior *H. pylori* treatment attempts, medication adherence, alcohol and cigarette consumption during therapy, and treatment-related adverse events were recorded by reviewing medical records and telephone surveys. *H. pylori* eradication rates for overall and subgroups were evaluated. Multivariate analysis was performed to identify independent predictors of failed *H. pylori* eradication.

RESULTS

Of the 992 patients treated and retested for *H. pylori* infection, the overall eradication rate was 94.5% [95% confidence interval (CI): 94.1%-95.9%]. *H. pylori* eradication rate of primary therapy was 95.0% (95%CI: 93.5%-96.5%), while that of rescue therapy was 91.3% (95%CI: 86.8%-95.8%). Among the 859 patients who completed the study protocol, 144 (17%) reported treatment-related adverse events including 24 (3%) leading to premature discontinuation. On multivariate analysis, poor medication adherence [adjusted odds ratio (AOR) = 6.7, 95%CI: 2.8-15.8], two or more previous *H. pylori* treatments (AOR = 7.4, 95%CI: 2.2-24.9), alcohol consumption during therapy (AOR = 4.4, 95%CI: 1.5-12.3), and possibly smoking during therapy (AOR = 1.9, 95%CI: 0.9-4.3) were associated with failed *H. pylori* eradication.

CONCLUSION

Furazolidone- and amoxicillin-based quadruple therapy for *H. pylori* infection in an area with a high prevalence of clarithromycin resistance demonstrated high eradication rates as primary and rescue therapies with a favorable safety profile. Patient education targeting abstinence from alcohol during therapy and strict medication adherence may further optimize *H. pylori* eradication.

Key words: *Helicobacter pylori*; Furazolidone; Quadruple regimen; Side effects; Eradication

Core tip: This study examined the outcomes of furazolidone- and amoxicillin-based quadruple therapy as both primary and rescue therapies for *Helicobacter pylori* (*H. pylori*) infection in nearly a thousand patients. Detailed data on adverse events and factors associated with failed *H. pylori* eradication were evaluated. Furazolidone- and amoxicillin-based quadruple therapy demonstrated a high *H. pylori* eradication rate exceeding 90% with a favorable safety profile in a real-world setting. Abstinence from alcohol during therapy and strict medication adherence may further optimize eradication. The results validate updated guidelines recommending furazolidone-based quadruple therapy as a first-line treatment for *H. pylori* infection in areas with a high prevalence of clarithromycin resistance.

Zhang YW, Hu WL, Cai Y, Zheng WF, Du Q, Kim JJ, Kao JY, Dai N, Si JM. Outcomes of furazolidone- and amoxicillin-based quadruple therapy for *Helicobacter pylori* infection and predictors of failed eradication. *World J Gastroenterol* 2018; 24(40): 4596-4605 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4596.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4596

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a common pathogen associated with the development of peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. The prevalence of *H. pylori* infection exceeds 50% worldwide, with a higher prevalence in developing countries[1,2]. Effective eradication of *H. pylori* by a combination of antimicrobial and acid suppressive therapies reduces the risk of recurrent peptic ulcers and possible gastric cancer[3,4]. However, with the emergence of antibiotic-resistant *H. pylori* strains, traditional triple therapies have become increasingly ineffective, with some studies reporting eradication rates as low as 50%[5-8]. Selecting optimal therapies for antibiotic-resistant *H. pylori* infection has become a global public health priority.

Furazolidone is a monoamine oxidase inhibitor and nitrofurantoin-type antibiotic commonly used in Asia. Compared to high rates of resistance observed with clarithromycin, metronidazole, and levofloxacin, *H. pylori* strains resistant to furazolidone remain uncommon[9-11]. However, early animal studies demonstrating increased adverse events have limited widespread application of furazolidone in the treatment of *H. pylori* infection[12-16]. Given the high prevalence of *H. pylori* strains resistant to clarithromycin and metronidazole observed in recent studies, international guidelines recommend bismuth containing quadruple regimens that include amoxicillin, furazolidone or tetracycline for rescue therapies[17-19]. Furthermore, updated Chinese and international guidelines recommended furazolidone, amoxicillin, bismuth, and proton pump inhibitor (PPI) quadruple therapy as a first-line regimen option for *H. pylori* infection[20].

Although a number of studies with limited sample

size demonstrate high efficacy of furazolidone- and amoxicillin-based quadruple therapy for treatment of *H. pylori* infection, data on the adverse events, particularly impacting treatment course, are not well described[16]. Furthermore, predictors of failed *H. pylori* eradication other than the choice of regimen or poor medication adherence are largely unknown[16,21]. Given the high prevalence of clarithromycin-resistant *H. pylori* infection observed at our center, furazolidone- and amoxicillin-based quadruple therapy has been adopted as a first-line therapy for treatment of *H. pylori* since 2013. Therefore, we performed a retrospective study of patients who received furazolidone- and amoxicillin-based quadruple therapy for treatment of *H. pylori* at our center. The aim of our study was to evaluate the efficacy and safety of furazolidone- and amoxicillin-based quadruple therapy as primary and rescue therapies for *H. pylori* infection and also to identify predictors of failed *H. pylori* eradication.

MATERIALS AND METHODS

Study population

Patients diagnosed with *H. pylori* infection at Sir Run Run Shaw Hospital (Hangzhou, China) from January 2015 to December 2015 who received furazolidone- and amoxicillin-based quadruple therapy and had a follow-up ^{13}C-urea breath test (^{13}C-UBT) > 4 wk after the completion of therapy were evaluated. All patients who received one of two forms of direct *H. pylori* testing available at our center (^{13}C-UBT or gastric biopsy) were searched, and pharmacy records were examined to identify patients who received furazolidone- and amoxicillin-based quadruple therapy. All patients aged ⩾ 18 years who received repeat *H. pylori* breath test > 4 wk after treatment were eligible for the study. Patients who lacked repeat *H. pylori* testing to evaluate for eradication status or received therapies other than furazolidone- and amoxicillin-based quadruple therapy were excluded. Medical records including endoscopy, pathology, ^{13}C-UBT, and pharmacy records were reviewed to characterize the clinical course before and after treatment of *H. pylori* infection. After the follow-up breath test, all patients were seen in an outpatient visit and contacted for a detailed telephone survey. The protocol was approved by the Ethics Committee of Sir Run Run Shaw Hospital prior to initiating the study.

Treatment

Per hospital clinical pathway since 2013, all patients with *H. pylori* infection without contraindications to penicillin, furazolidone, bismuth, or proton pump inhibitor were treated with furazolidone, amoxicillin, bismuth, and PPI for 10-14 d unless specified by the clinician. Patients were treated with furazolidone 100 mg, amoxicillin 1 g, proton pump inhibitor (esomeprazole 20 mg, rabeprazole 10 mg, pantoprazole 40 mg, lansoprazole 30 mg, or omeprazole 20 mg), and colloidal bismuth pectin (200 mg to 400 mg); all were taken twice a day. Patients were instructed to take antibiotics immediately after meals but take PPI and bismuth 30 min before meals. Four weeks after the completion of treatment, all patients were recommended to obtain a follow-up ^{13}C-UBT and an outpatient consultation.

Data collection

Baseline data including age, gender, smoking status, alcohol status, and educational levels at the time of *H. pylori* testing as well as all prior *H. pylori* treatment attempts were recorded by reviewing medical records and telephone surveys. Endoscopy and *H. pylori* breath test reports were reviewed to obtain information on the date and indication and/or diagnosis for *H. pylori* testing. Data on *H. pylori* treatment regimens and duration were obtained by reviewing electronic pharmacy records. Data including medication adherence, potential treatment-related adverse events (dizziness, headache, fatigue, fever, anorexia, nausea, vomiting, diarrhea, constipation, abdominal discomfort or pain, bitter taste, skin rash/pruritus, weight loss, dysphagia, dyspnea, blurred vision, and myalgia), as well as smoking and tobacco status before and during treatment were collected at the time of repeat *H. pylori* testing, outpatient consultation, or by a follow-up phone survey. In order to evaluate *H. pylori* resistance pattern, available *H. pylori* culture and antibiotic susceptibility data at the center between January 2013 and December 2014 were also collected.

Definitions and outcomes

The primary endpoint of the study was *H. pylori* eradication rate. Secondary endpoints were treatment-related adverse events and predictors of eradication failure. The primary endpoint was also analyzed by subgroups by patients receiving primary or rescue therapy and those with or without adverse events. Potential treatment-related adverse events were expressed as proportion of individuals experiencing a specific side effect and any side effects. Predictors of eradication failure including demographic (age, gender, and educational level), clinical (number of previous treatment and indication/diagnosis of *H. pylori* testing), and treatment-related factors (PPI type, bismuth dose, treatment duration, medication adherence, smoking during treatment, and alcohol use during treatment) were evaluated. The number of previous *H. pylori* infection treatment was categorized as none, one, or ⩾ 2 prior treatment attempts. Smoking status was defined as non-smoker, abstinence during therapy, and smoking during therapy. Alcohol use was defined as non-alcohol user, abstinence during therapy, and alcohol use during therapy. Educational levels were categorized by years of education (< 7, 7-9, 10-12, 13-16, or > 16 years). Poor adherence to *H. pylori* treatment was defined as patient reporting < 80% adherence of prescribed therapy[22]. Severe adverse event was defined as treatment-related

Table 1 Baseline demographic and clinical characteristics (*n* = 992) *n* (%)

Variable	Information
Age (mean age ± SD)	46.7 ± 12.4
Gender	
Male	501 (50.5)
Female	491 (49.5)
Smoking history	199/859 (23.2)
Alcohol intake history	231/859 (26.9)
Educational level	
< 7 yr	164 (16.5)
7-9 yr	249 (25.1)
10-12 yr	197 (19.9)
13-16 yr	229 (23.1)
> 16 yr	20 (2.0)
Unknown	133 (13.4)
Diagnosis	
Functional dyspepsia	478 (48.2)
Peptic ulcers	259 (26.1)
Erosive esophagitis	69 (7.0)
Other sources of upper GI bleeding	5 (0.5)
Gastric tumors	6 (0.6)
Asymptomatic gastritis	75 (7.6)
^{13}C-UBT positive during health checkup	100 (10.1)
Number of previous *H. pylori* treatment(s)	
None	842 (84.9)
One	127 (12.8)
Two or more	23 (2.3)
PPI type	
Esomeprazole	264 (26.6)
Rabeprazole	224 (22.6)
Pantoprazole	435 (43.9)
Other PPIs	69 (7.0)
Bismuth dose	
400 mg per day	213 (21.5)
600 mg per day	391 (39.4)
800 mg per day	388 (39.1)
Duration of regimen	
14-d regimen	971 (97.9)
10-d regimen	21 (2.1)

GI: Gastrointestinal; PPI: Proton pump inhibitor; ^{13}C-UBT: ^{13}C-urea breath test.

adverse event necessitating discontinuation of therapy within 10 d.

Statistical analysis

Sociodemographic and outcome data were described using number and frequency for categorical variables and mean and standard deviation for continuous variables. Eradication rates between different groups were compared using the χ^2 test. Initially, potential factors associated with failed eradication was evaluated by using a χ^2 test or Fisher's exact test. Afterwards, variables associated with failed eradication were included in a multiple logistic regression model to evaluate for predictors of failed eradication. All statistical analyses were performed using IBM SPSS Statistics V22.0 software. Two-sided *P*-values < 0.05 were considered significant.

RESULTS

During the one-year study period, 992 patients were treated with furazolidone- and amoxicillin-based quadruple therapy and received the ^{13}C-UBT > 4 wk after eradication (Table 1). The mean age of the patients was 46.7 ± 12.4 years, 501 (50.5%) were male, and 259 (26.1%) were treated for indication of peptic ulcers. Furthermore, 842 (84.9%) patients had no prior *H. pylori* treatment, 127 (12.8%) had one prior treatment, and 23 (2.3%) had ⩾ 2 prior treatments. Nine hundred and seventy-one (97.9%) and 21 (2.1%) patients were prescribed a 14-d regimen and a 10-d regimen, respectively. *H. pylori* culture and antibiotic susceptibility study available from 2013-2014 (*n* = 52) showed clarithromycin-resistant strains in 9 (17.3%), levofloxacin-resistant strains in 20 (38.5%), metronidazole-resistant strains in 38 (73.1%), furazolidone-resistant strains in 2 (3.8%), and none with amoxicillin-resistant strains (Supplementary Table 1).

H. pylori eradication rate

Of the 992 patients, 859 completed the study protocol. The overall eradication rate was 94.5% (95%CI: 94.1%-95.9%). *H. pylori* eradication rates were 95.0% (95%CI: 93.5%-96.5%) and 91.3% (95%CI: 86.8%-95.8%) for primary and rescue therapies, respectively. Among those who completed the follow-up, patients who did not experience medication-related adverse events had a higher eradication rate (95.5% *vs* 90.3%, mean difference = 5.2%, 95%CI: 0.7%-11.7%) compared to those who experienced any reported adverse events (Table 2).

Treatment-related adverse events

Of the 859 patients who completed the study, 144 (16.8%) experienced one or more treatment-related adverse events (Table 3). The common adverse events including abdominal pain in 39 (4.5%), nausea in 20 (2.3%), dizziness in 11 (1.3%), fatigue in 11 (1.3%), anorexia in 13 (1.5%), and skin rash/pruritus in 18 (2.1%) were reported. Twenty-four (2.8%) patients experienced severe treatment-associated adverse events necessitating premature discontinuation of intended therapy including 10 (1.2%) who completed < 10 d of treatment. Skin rash/pruritus (*n* = 3, 0.4%) was the most common severe treatment-related adverse event.

Predictors of failed H. pylori eradication

On univariate analysis, the number of previous *H. pylori* treatment (78.3%-95.0%, *P* = 0.002), smoking status (88.2%-95.6%, *P* = 0.004), alcohol status (79.4%-95.5%, *P* < 0.001), and poor *H. pylori* treatment adherence (77.5% *vs* 96.2%, *P* < 0.001) were associated with failed *H. pylori* eradication (Table 4). Multivariate analysis demonstrated that ⩾ 2 prior *H. pylori* treatment attempts (AOR = 7.4; 95%CI: 2.2-24.9, *P* = 0.001) compared to no treatment, and poor adherence (AOR = 6.7; 95%CI: 2.8-15.8, *P* < 0.001) compared to acceptable adherence were associated with failed *H. pylori* eradication. Furthermore, alcohol use during treatment compared

Table 2 *Helicobacter pylori* eradication rates with furazolidone- and amoxicillin-based quadruple therapy: Overall and by subgroup % (95%CI)

Variable	*n*/N	Eradication rate
Overall	937/992	94.5 (94.1-95.9)
Primary	800/842	95.0 (93.5-96.5)
Rescue	137/150	91.3 (86.8-95.8)
Adverse events[1]		
Without	683/715	95.5 (94.0-97.0)[2]
With	130/144	90.3 (85.5-95.1)

[1]Patients who completed the study protocol were divided into two groups: without or with adverse events during therapy. Eradication rates of two groups were calculated and the difference between the two groups was analyzed. [2]Eradication rates were higher among patients without (mean difference = 5.2%, 95%CI: 0.7%-11.7%, P = 0.01) compared to those with adverse events during therapy. *n*: Number of successful eradication; N: Number of total patients.

to non-alcohol user (AOR = 4.4; 95%CI: 1.5-12.3, P = 0.008), but not alcohol users abstinent during treatment (AOR = 1.0; 95%CI: 0.4-2.3, P = 1.00), was associated with failed *H. pylori* eradication. Finally, smoking during treatment demonstrated a trend towards failed *H. pylori* eradication (AOR = 1.9; 95%CI: 0.9-4.3, P = 0.10) compared to non-smokers. Age, gender, educational level, PPI type, bismuth dose, therapy duration, and the indication for treatment were not associated with failed *H. pylori* eradication.

DISCUSSION

In this single-center study evaluating furazolidone- and amoxicillin-based quadruple therapy for *H. pylori* infection in an area with a high prevalence of clarithromycin resistance, the eradication rates were high at > 90% for both primary and rescue therapies. Furthermore, treatment-related adverse events were infrequent with fewer than 3% requiring treatment discontinuation. Poor adherence to prescribed therapy, two or more prior eradication attempts, and concurrent alcohol use during treatment were associated with failed eradication.

The rise in the prevalence of antibiotic-resistant *H. pylori* strains has led to increased treatment failure with traditional triple therapies[2,10,23]. In recognition of high global prevalence of clarithromycin- and/or metronidazole-resistant *H. pylori* infection, the updated Maastricht V/Florence Consensus Report emphasized that bismuth quadruple or non-bismuth quadruple, concomitant therapies (PPI, amoxicillin, clarithromycin, and a nitroimidazole) are now the treatment of choice in regions with high (> 15%) clarithromycin resistance while bismuth quadruple therapies are recommended in regions with high dual resistance to clarithromycin and metronidazole (> 15%)[18]. Furthermore, the guidelines recommended that clarithromycin should be avoided and a combination of antibiotics with high barrier to resistance (amoxicillin, tetracycline, furazolidone, and rifabutin) should be selected. The Fifth Chinese National Consensus Report recommended furazolidone, amoxicillin, bismuth, and PPI quadruple therapy as one of the first-line regimens for *H. pylori* therapy given that estimated resistance to clarithromycin and metronidazole exceeds 20% and 40%, respectively, in China[20].

Our results from a real-world experience demonstrated that furazolidone- and amoxicillin-based quadruple therapy achieved a 95% *H. pylori* eradication rate which is within the higher range of all eradication rates reported in the literature[16]. Although older studies mostly containing furazolidone as a component of substandard regiments (inadequate duration or absence of PPI) reported a low pooled-eradication rate of 76%, our findings are consistent with recent studies reporting high eradication rates of 85%-95% in combination with 14 d of amoxicillin[24-26]. For example, in a randomized study of 424 patients with *H. pylori* infection from Shanghai comparing four different bismuth-based quadruple therapies (amoxicillin, tetracycline, metronidazole, or furazolidone) as rescue therapies, furazolidone-containing regimens had a higher eradication rate (93.4% *vs* 85.9%; mean difference = 7.6%, 95%CI: 1.4%-13.8%) compared to non-furazolidone containing regimens per intent to treat (ITT)[24]. Furthermore, a multicenter prospective study that included 180 patients with *H. pylori*-positive duodenal ulcer allocated to amoxicillin 1 g, furazolidone 100 mg, rabeprazole 10 mg, and bismuth 220 mg twice a day for 10 d demonstrated an eradication rate of 86% per ITT[26]. In another randomized controlled study comparing different durations and doses of furazolidone, 40 patients receiving furazolidone 200 mg to 300 mg per day with amoxicillin, PPI, and bismuth for 2 wk as rescue therapies led to an eradication rate of 88% per ITT[25]. Finally, a retrospective study of 27 United States patients receiving furazolidone-containing non-bismuth quadruple therapy for 2 wk demonstrated a high eradication rate of 97% per ITT[9]. The eradication rate of 95% in our study is remarkable, especially given that 15% of patients have experienced prior treatment failure.

The high eradication rates of *H. pylori* with furazolidone- and amoxicillin-containing quadruple therapy in our study may be related to several factors. First, two antibiotics (furazolidone and amoxicillin) with the highest barrier to resistance were included in the treatment regimen. With the exception of Iran where furazolidone-resistant *H. pylori* is common (5% to 22%), the reported resistance rates in China, Vietnam, and United States are consistently < 5%[2,11,23,27-30]. A recent local study examining 545 *H. pylori* cultures obtained from children showed absence of furazolidone-resistant *H. pylori*, consistent with the low (4%) resistance rate shown at our center[29]. In addition to the low prevalence of furazolidone-resistant *H. pylori* (< 5%), amoxicillin as the backbone of eradication therapy continues to have the lowest prevalence of *H. pylori* resistance reported globally (< 1%-2%) and in China (< 5%). Second, bismuth

Table 3 Adverse events of furazolidone- and amoxicillin-based quadruple therapy (*n* = 859) *n* (%)

Adverse event	Number	Severe	Impact on treatment	Eradication
Abdominal discomfort	39 (4.5)	-	2 stopped prior to completion (10, 12 d)	38 (97.4)
Dizziness	11 (1.3)	2 (0.2)	4 stopped prior to completion (7, 10, 10, 12 d); 1 experienced dizziness after drinking alcohol and stopped prior to completion (10 d); 1 took 50% medicine	10 (90.9)
Nausea (with/without vomiting)	20 (2.3)	-	1 took 75% medicine	16 (80.0)
Fatigue	11 (1.3)	1 (0.1)	1 stopped prior to completion (12 d); 1 changed to traditional Chinese medicine during therapy (7 d)	9 (81.8)
Anorexia	13 (1.5)	-	1 took 80% medicine	13 (100)
Skin rash/pruritus	18 (2.1)	3 (0.4)	4 stopped prior to completion (4, 7, 10, 11 d); 2 changed to other regimens during therapy (2, 10 d); 1 took half of amoxicillin and all other drugs	15 (83.3)
Fever	2 (0.2)	2 (0.2)	2 stopped prior to completion (7, 9 d)	2 (100)
Diarrhea	9 (1.1)	1 (0.1)	1 stopped prior to completion (less than 7 d)	8 (88.9)
Constipation	3 (0.4)	-	-	3 (100)
Flatulence	2 (0.2)	-	-	2 (100)
Muscle pain or spasm (shoulder/back)	3 (0.4)	-	-	1 (33.3)
Acid regurgitation	1 (0.1)	-	-	1 (100)
Abdominal pain	4 (0.5)	1 (0.1)	2 stopped prior to completion (7,10 d)	4 (100)
Weight loss	3 (0.4)	-	-	3 (100)
Bitter taste/dry throat	2 (0.2)	-	1 took 75% medicine	2 (100)
Belching	1 (0.1)	-	-	1 (100)
Chest congestion	1 (0.1)	-	-	1 (100)
Heartburn	1 (0.1)	-	-	1 (100)
Total	144 (16.8)	10 (1.2)	24 (2.8)	130 (90.3)

that has been shown to improve treatment eradication rate by 30%-40% in areas with a high prevalence of *H. pylori* resistance was routinely added in our study[31]. Third, almost all (98%) patients received a 14-d regimen and none of the patients were prescribed < 10 d of intended therapy. Although the results are inconsistent, a systematical review of 75 studies demonstrated that longer duration of therapy improves eradication and 14 d of treatment have been recommended by updated guidelines[18,32,33]. Finally, selection bias favoring higher eradication rate is possible among population returning for confirmatory *H. pylori* testing.

Our study demonstrated that adverse events occurred in 17% (95%CI: 14.3%-19.3%) of the cohort with premature discontinuation of therapy occurring in 2.8% (95%CI: 1.7%-3.9%). The adverse events (abdominal discomfort, dizziness, nausea, fatigue, anorexia, rash, and pruritus) observed in our study were mild and non-specific, similar to other studies evaluating furazolidone-containing regimens[34]. Furthermore, all side effects resolved after the completion or withdrawal of therapy without any documented events of severe hepatotoxicity or kidney injury. Although the incidence of adverse events with furazolidone-containing *H. pylori* regimen is common (18%-33%)[16,25], the incidence of adverse events associated with furazolidone-containing regimen is not elevated compared to amoxicillin-based triple or tetracycline and metronidazole-based quadruple therapy[24]. A Chinese meta-analysis of 788 patients also demonstrated no difference in the incidence of adverse events between furazolidone-containing quadruple therapy compared to other quadruple therapy regimens as rescue therapies (14.1% *vs* 13.8%; OR = 1.04, 95%CI: 0.7-1.6)[35]. The incidence of furazolidone-associated adverse events is dose-dependent and severe among those treated with high- (400 mg per day) compared to low-dose furazolidone (200 mg per day), longer duration, and co-therapy with bismuth. Low-dose furazolidone studies generally demonstrate a low incidence of adverse events of < 20%[36-39]. Although the eradication rate in patients with adverse events was lower (90.3% *vs* 95.5%, mean difference = -5.2%, 95%CI: -0.7% to -11.7%) compared to those without adverse events in our study, the overall eradication rate remained high at > 90%.

Furazolidone is a synthetic nitrofuran that has been widely used as an antibiotic to treat enteric infections globally. The carcinogenetic effects of furazolidone suggested in early animal studies[12-15,40] have remained speculative in clinical settings. Furazolidone is a category 3 agent and considered unclassifiable in regards to carcinogenicity in humans[41]. Despite being a widely used antibiotic in Asia for more than two decades, teratogenicity or carcinogenicity in humans has yet to be reported despite close scrutiny[42]. Furazolidone is currently not available in the United States due to the lack of a commercial market[43]. The abandonment of furazolidone-based therapy of finite duration due to concerns of side effects may be misguided[43]. Our current study of nearly 1000 patients demonstrating a favorable safety profile supports the use of low-dose furazolidone-based quadruple therapy for *H. pylori* infection.

Multivariate analysis demonstrated that poor adherence (AOR = 6.7, 95%CI: 2.8-15.8), multiple treatment (AOR = 7.4, 95%CI: 2.2-24.9), alcohol

Table 4 Univariate and multivariate analyses for predictors of failed *Helicobacter pylori* eradication

Factor		Eradication rate	*P* value[1]	Multivariate[2]	
		n/N (%)		OR (95%CI)	*P* value
Age (yr)	< 60	781/827 (94.4)	0.96	-	-
	⩾ 60	156/165 (94.5)			
Gender	Male	469/501 (93.6)	0.24	-	-
	Female	468/491 (95.3)			
Education[2]	< 7 yr	150/164 (91.5)	0.29	-	-
	7-9 yr	237/249 (95.2)			
	10-12 yr	187/197 (94.9)			
	13-16 yr	219/229 (95.6)			
	> 16 yr	20/20 (100)			
Number of previous	None	800/842 (95.0)	0.002	Reference	-
H. pylori treatment(s)	One	119/127 (93.7)		1.2 (0.5-2.7)	0.73
	Two or more	18/23 (78.3)		7.4 (2.2-24.9)	0.001
Diagnosis	Functional dyspepsia	453/478 (94.8)	0.49	-	-
	Peptic ulcers	245/259 (94.6)			
	Erosive esophagitis	67/69 (97.1)			
	Other sources of upper GI bleeding	4/5 (80.0)			
	Gastric neoplasm	6/6 (100.0)			
	Asymptomatic gastritis	68/75 (90.7)			
	^{13}C-UBT positive during health checkup	94/100 (94.0)			
PPI type	Esomeprazole	253/264 (95.8)	0.42	-	-
	Rabeprazole	209/224 (93.3)			
	Pantoprazole	408/435 (93.8)			
	Other PPIs	67/69 (97.1)			
Bismuth	400 mg per day	204/213 (95.8)	0.4	-	-
	600 mg per day	371/391 (94.9)			
	800 mg per day	362/388 (93.3)			
Duration of regimen	10 d	19/21 (90.5)	0.33	-	-
	14 d	918/971 (94.5)			
Adherence[2]	Took 80% medicine or more	782/819 (95.5)	< 0.001	Reference	-
	Took less than 80% medicine	31/40 (77.5)		6.7 (2.8-15.8)	< 0.001
Smoking[2]	Non-smoker	631/660 (95.6)	0.004	Reference	-
	Abstinence during therapy	77/80 (96.3)		0.7 (0.2-2.7)	0.65
	Smoking during therapy	105/119 (88.2)		1.9 (0.9-4.3)	0.10
Alcohol[2]	Non-alcohol user	600/628 (95.5)	< 0.001	Reference	-
	Abstinence during therapy	186/197 (94.4)		1.0 (0.4-2.3)	1.00
	Alcohol use during therapy	27/34 (79.4)		4.4 (1.5-12.3)	0.008

[1]Univariate analysis; [2]Data analyzed only for patients who completed the study protocol (n = 859). n: Number of successful eradication; N: Number of total patients; GI: Gastrointestinal; PPI: Proton pump inhibitor; ^{13}C-UBT: ^{13}C-urea breath test.

use (AOR = 4.4, 95%CI: 1.5-12.3), and possibly smoking (AOR = 1.9, 95%CI: 0.9-4.3) during therapy were associated with failed *H. pylori* eradication. As expected and consistent with previous findings, poor adherence defined by taking < 80% of the prescribed therapy and history of multiple treatment failures defined by ⩾ 2 treatment attempts had more than 6-fold and 7-fold increased risks of treatment failure, respectively[44,45]. Concurrent alcohol, but not alcohol abstinence during therapy, compared to non-alcohol use increased the odds of treatment failure in our study. Although the reason is unclear, concurrent alcohol use with furazolidone may lead to increased adverse events that may impact adherence to therapy. Smoking has been previously associated with decreased *H. pylori* eradication rate with proposed reasons including adverse impact on adherence, decreased gastric mucosal blood flow, increased gastric acidity, and altered PPI metabolism[46,47].

Our findings have clinical implications. Rather than pathogen-associated factors, host-associated factors were primary determinants of successful eradication of *H. pylori* with furazolidone- and amoxicillin-containing quadruple therapy. Furthermore, excluding prior treatment failure, other predictors can potentially be modified during the treatment course to optimize the eradication rate. Our findings highlight the role of physician-patient communication, emphasizing the importance of adherence to prescribed therapy and alcohol cessation during therapy to optimize *H. pylori* eradication.

The strength of our study is the evaluation of a large patient population in a "real-world" setting examining furazolidone- and amoxicillin-containing quadruple therapy as both primary and rescue regimens. Furthermore, detailed data of adverse events as well as evaluation of factors associated with failed *H. pylori* eradication were analyzed. Finally, our study showed that furazolidone- and amoxicillin-based quadruple therapy led to a high eradication rate regardless of furazolidone dose (*i.e.,* 200 mg per day), bismuth dose, or PPI type previously raised as potential factors for successful *H.*

pylori eradication[48].

Our study has limitations. Our findings may not be generalizable in areas with highly variable *H. pylori*-resistant patterns or no access to furazolidone. Future studies evaluating the efficacy of furazolidone- and amoxicillin-based quadruple therapy in areas other than Iran or China may be invaluable. Furthermore, *H. pylori* culture and sensitivity were not performed in all enrolled patients. However, *H. pylori* antibiotic sensitivity data available in a subset of patients in our study paralleled findings from two recent large studies from the same region[29,49]. Finally, the analysis of patients who completed repeat evaluation of *H. pylori* after treatment may lead to bias in the interpretation of the results.

In conclusion, furazolidone- and amoxicillin-based quadruple therapy in a region with high clarithromycin resistance demonstrated high eradication rates as primary and rescue therapies with favorable safety profiles. Patient education targeting abstinence from alcohol and strict medication adherence may further optimize *H. pylori* eradication.

ARTICLE HIGHLIGHTS

Research background

With the increase of antibiotic resistance of *Helicobacter pylori* (*H. pylori*) worldwide, traditional triple therapies have become increasingly ineffective. Selecting optimal therapies for antibiotic-resistant *H. pylori* infection has become an important global public health priority.

Research motivation

Although studies with limited sample size demonstrate high efficacy of furazolidone-based quadruple therapy for treatment of *H. pylori*, data on the impact of adverse events and predictors of failed *H. pylori* eradication are not well described. Furthermore, evaluating efficacy and safety of furazolidone- and amoxicillin-based quadruple therapy for *H. pylori* and identifying predictors of failed eradication in a large patient population are lacking.

Research objectives

The aim of the study was to evaluate the outcomes of furazolidone- and amoxicillin-based quadruple therapy for treatment of *H. pylori* and identify predictors of failed eradication. Furazolidone- and amoxicillin-containing quadruple therapy demonstrated a high eradication rate exceeding 90% both as primary and rescue therapies with a favorable safety profile. Patient education targeting abstinence of alcohol use during therapy and strict medication adherence may further optimize *H. pylori* eradication. The results provided robust evidence for using furazolidone- and amoxicillin-containing quadruple therapy as a first-line therapy for *H. pylori* infection in areas with a high prevalence of clarithromycin resistance.

Research methods

Patients with *H. pylori* infection who were treated with furazolidone- and amoxicillin-based quadruple therapy and received ^{13}C-urea breath test > 4 wk after treatment from January 2015 to December 2015 were evaluated. Patient data including sociodemographic data, prior treatment attempts, medication adherence, and treatment-related adverse events were obtained by reviewing medical records and conducting telephone surveys. *H. pylori* eradication rates for overall and subgroups, treatment-related adverse events, and independent predictors of failed *H. pylori* eradication were evaluated.

Research results

Furazolidone- and amoxicillin-based quadruple therapy demonstrated a high eradication rate exceeding 90% as both primary and rescue therapies. Fewer than 3% of patients reported treatment-related adverse events leading to premature discontinuation. Poor medication adherence, previous *H. pylori* treatments, and alcohol consumption during therapy were associated with failed *H. pylori* eradication. These findings suggest that furazolidone- and amoxicillin-based quadruple therapy with proper patient education could optimize treatment of *H. pylori* infection in regions with high resistance to clarithromycin. Evaluating the efficacy of furazolidone- and amoxicillin-based quadruple therapy in areas other than China may be invaluable in future studies.

Research conclusions

Furazolidone- and amoxicillin-based quadruple therapy demonstrated high eradication rates as both primary and rescue therapies for *H. pylori* infection with a favorable safety profile in areas with a high rate of clarithromycin resistance. Abstinence from alcohol and strict medication adherence during therapy may further optimize *H. pylori* eradication. These findings validate updated guidelines recommending furazolidone-containing quadruple therapy as a first-line regimen for treatment of *H. pylori* infection in populations with a high rate of clarithromycin resistance.

Research perspectives

Selecting optimal treatment for *H. pylori* infection is important in regions with a high rate of resistance to clarithromycin. Targeted patient education may further optimize *H. pylori* eradication. Future studies confirming the high efficacy of furazolidone- and amoxicillin-based quadruple therapy in areas other than China may be invaluable.

REFERENCES

1 **Mandeville KL**, Krabshuis J, Ladep NG, Mulder CJ, Quigley EM, Khan SA. Gastroenterology in developing countries: issues and advances. *World J Gastroenterol* 2009; **15**: 2839-2854 [PMID: 19533805 DOI: 10.3748/wjg.15.2839]

2 **Hunt RH**, Xiao SD, Megraud F, Leon-Barua R, Bazzoli F, van der Merwe S, Vaz Coelho LG, Fock M, Fedail S, Cohen H, Malfertheiner P, Vakil N, Hamid S, Goh KL, Wong BC, Krabshuis J, Le Mair A; World Gastroenterology Organization. Helicobacter pylori in developing countries. World Gastroenterology Organisation Global Guideline. *J Gastrointestin Liver Dis* 2011; **20**: 299-304 [PMID: 21961099]

3 **Zhou L**, Lin S, Ding S, Huang X, Jin Z, Cui R, Meng L, Li Y, Zhang L, Guo C, Xue Y, Yan X, Zhang J. Relationship of Helicobacter pylori eradication with gastric cancer and gastric mucosal histological changes: a 10-year follow-up study. *Chin Med J* (Engl) 2014; **127**: 1454-1458 [PMID: 24762588]

4 **El-Nakeeb A**, Fikry A, Abd El-Hamed TM, Fouda el Y, El Awady S, Youssef T, Sherief D, Farid M. Effect of Helicobacter pylori eradication on ulcer recurrence after simple closure of perforated duodenal ulcer. *Int J Surg* 2009; **7**: 126-129 [PMID: 19138577 DOI: 10.1016/j.ijsu.2008.12.001]

5 **Venerito M**, Krieger T, Ecker T, Leandro G, Malfertheiner P. Meta-analysis of bismuth quadruple therapy versus clarithromycin triple therapy for empiric primary treatment of Helicobacter pylori infection. *Digestion* 2013; **88**: 33-45 [PMID: 23880479 DOI: 10.1159/000350719]

6 **Luther J**, Higgins PD, Schoenfeld PS, Moayyedi P, Vakil N, Chey WD. Empiric quadruple vs. triple therapy for primary treatment of Helicobacter pylori infection: Systematic review and meta-analysis of efficacy and tolerability. *Am J Gastroenterol* 2010; **105**: 65-73 [PMID: 19755966 DOI: 10.1038/ajg.2009.508]

7 **Malfertheiner P**, Bazzoli F, Delchier JC, Celiñski K, Giguère M, Rivière M, Mégraud F; Pylera Study Group. Helicobacter pylori eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open-label, non-inferiority, phase 3 trial. *Lancet* 2011; **377**: 905-913 [PMID: 21345487 DOI: 10.1016/S0140-6736(11)60020-2]

8 **Fallone CA**, Chiba N, van Zanten SV, Fischbach L, Gisbert JP, Hunt RH, Jones NL, Render C, Leontiadis GI, Moayyedi P, Marshall JK. The Toronto Consensus for the Treatment of Helicobacter pylori

Infection in Adults. *Gastroenterology* 2016; **151**: 51-69.e14 [PMID: 27102658 DOI: 10.1053/j.gastro.2016.04.006]
9 **Graham DY**, Osato MS, Hoffman J, Opekun AR, Anderson SY, El-Zimaity HM. Furazolidone combination therapies for Helicobacter pylori infection in the United States. *Aliment Pharmacol Ther* 2000; **14**: 211-215 [PMID: 10651662 DOI: 10.1046/j.1365-2036.2000.00640.x]
10 **Thung I**, Aramin H, Vavinskaya V, Gupta S, Park JY, Crowe SE, Valasek MA. Review article: the global emergence of Helicobacter pylori antibiotic resistance. *Aliment Pharmacol Ther* 2016; **43**: 514-533 [PMID: 26694080 DOI: 10.1111/apt.13497]
11 **Fakheri H**, Bari Z, Aarabi M, Malekzadeh R. Helicobacter pylori eradication in West Asia: a review. *World J Gastroenterol* 2014; **20**: 10355-10367 [PMID: 25132752 DOI: 10.3748/wjg.v20.i30.10355]
12 **Ahmed HH**, El-Aziem SH, Abdel-Wahhab MA. Potential role of cysteine and methionine in the protection against hormonal imbalance and mutagenicity induced by furazolidone in female rats. *Toxicology* 2008; **243**: 31-42 [PMID: 17964703 DOI: 10.1016/j.tox.2007.09.018]
13 **Madrigal-Bujaidar E**, Ibañez JC, Cassani M, Chamorro G. Effect of furazolidone on sister-chromatid exchanges, cell proliferation kinetics, and mitotic index in vivo and in vitro. *J Toxicol Environ Health* 1997; **51**: 89-96 [PMID: 9169063 DOI: 10.1080/00984109708984013]
14 **Ali BH**. Pharmacological, therapeutic and toxicological properties of furazolidone: some recent research. *Vet Res Commun* 1999; **23**: 343-360 [PMID: 10543364 DOI: 10.1023/A:1006333608012]
15 **Jin X**, Tang S, Chen Q, Zou J, Zhang T, Liu F, Zhang S, Sun C, Xiao X. Furazolidone induced oxidative DNA damage via up-regulating ROS that caused cell cycle arrest in human hepatoma G2 cells. *Toxicol Lett* 2011; **201**: 205-212 [PMID: 21195149 DOI: 10.1016/j.toxlet.2010.12.021]
16 **Zullo A**, Ierardi E, Hassan C, De Francesco V. Furazolidone-based therapies for Helicobacter pylori infection: a pooled-data analysis. *Saudi J Gastroenterol* 2012; **18**: 11-17 [PMID: 22249086 DOI: 10.4103/1319-3767.91729]
17 **Buzás GM**, Józan J. Nitrofuran-based regimens for the eradication of Helicobacter pylori infection. *J Gastroenterol Hepatol* 2007; **22**: 1571-1581 [PMID: 17845685 DOI: 10.1111/j.1440-1746.2007.05082.x]
18 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
19 **Heep M**, Kist M, Strobel S, Beck D, Lehn N. Secondary resistance among 554 isolates of Helicobacter pylori after failure of therapy. *Eur J Clin Microbiol Infect Dis* 2000; **19**: 538-541 [PMID: 10968325 DOI: 10.1007/s100960000288]
20 **Liu WZ**, Xie Y, Lu H, Cheng H, Zeng ZR, Zhou LY, Chen Y, Wang JB, Du YQ, Lu NH; Chinese Society of Gastroenterology, Chinese Study Group on Helicobacter pylori and Peptic Ulcer. Fifth Chinese National Consensus Report on the management of Helicobacter pylori infection. *Helicobacter* 2018; **23**: e12475 [PMID: 29512258 DOI: 10.1111/hel.12475]
21 **Fakheri H**, Bari Z, Sardarian H. A modified bismuth-containing quadruple therapy including a short course of furazolidone for Helicobacter pylori eradication after sequential therapy failure. *Helicobacter* 2012; **17**: 264-268 [PMID: 22759325 DOI: 10.1111/j.1523-5378.2012.00946.x]
22 **Song Z**, Suo B, Zhang L, Zhou L. Rabeprazole, Minocycline, Amoxicillin, and Bismuth as First-Line and Second-Line Regimens for Helicobacter pylori Eradication. *Helicobacter* 2016; **21**: 462-470 [PMID: 27060292 DOI: 10.1111/hel.12313]
23 **Safavi M**, Sabourian R, Foroumadi A. Treatment of Helicobacter pylori infection: Current and future insights. *World J Clin Cases* 2016; **4**: 5-19 [PMID: 26798626 DOI: 10.12998/wjcc.v4.i1.5]
24 **Liang X**, Xu X, Zheng Q, Zhang W, Sun Q, Liu W, Xiao S, Lu H. Efficacy of bismuth-containing quadruple therapies for clarithromycin-, metronidazole-, and fluoroquinolone-resistant Helicobacter pylori infections in a prospective study. *Clin Gastroenterol Hepatol* 2013; **11**: 802-7.e1 [PMID: 23376004 DOI: 10.1016/j.cgh.2013.01.008]
25 **Cheng H**, Hu FL. Furazolidone, amoxicillin, bismuth and rabeprazole quadruple rescue therapy for the eradication of Helicobacter pylori. *World J Gastroenterol* 2009; **15**: 860-864 [PMID: 19230048 DOI: 10.3748/wjg.15.860]
26 **Xie Y**, Zhu Y, Zhou H, Lu ZF, Yang Z, Shu X, Guo XB, Fan HZ, Tang JH, Zeng XP, Wen JB, Li XQ, He XX, Ma JH, Liu DS, Huang CB, Xu NJ, Wang NR, Lu NH. Furazolidone-based triple and quadruple eradication therapy for Helicobacter pylori infection. *World J Gastroenterol* 2014; **20**: 11415-11421 [PMID: 25170230 DOI: 10.3748/wjg.v20.i32.11415]
27 **Khademi F**, Poursina F, Hosseini E, Akbari M, Safaei HG. Helicobacter pylori in Iran: A systematic review on the antibiotic resistance. *Iran J Basic Med Sci* 2015; **18**: 2-7 [PMID: 25810869]
28 **Sun QJ**, Liang X, Zheng Q, Gu WQ, Liu WZ, Xiao SD, Lu H. Resistance of Helicobacter pylori to antibiotics from 2000 to 2009 in Shanghai. *World J Gastroenterol* 2010; **16**: 5118-5121 [PMID: 20976850 DOI: 10.3748/wjg.v16.i40.5118]
29 **Shu X**, Yin G, Liu M, Peng K, Zhao H, Jiang M. Antibiotics resistance of Helicobacter pylori in children with upper gastrointestinal symptoms in Hangzhou, China. *Helicobacter* 2018; **23**: e12481 [PMID: 29528162 DOI: 10.1111/hel.12481]
30 **Shiota S**, Reddy R, Alsarraj A, El-Serag HB, Graham DY. Antibiotic Resistance of Helicobacter pylori Among Male United States Veterans. *Clin Gastroenterol Hepatol* 2015; **13**: 1616-1624 [PMID: 25681693 DOI: 10.1016/j.cgh.2015.02.005]
31 **Dore MP**, Lu H, Graham DY. Role of bismuth in improving Helicobacter pylori eradication with triple therapy. *Gut* 2016; **65**: 870-878 [PMID: 26848181 DOI: 10.1136/gutjnl-2015-311019]
32 **Daghaghzadeh H**, Emami MH, Karimi S, Raeisi M. One-week versus two-week furazolidone-based quadruple therapy as the first-line treatment for Helicobacter pylori infection in Iran. *J Gastroenterol Hepatol* 2007; **22**: 1399-1403 [PMID: 17645463 DOI: 10.1111/j.1440-1746.2007.05029.x]
33 **Yuan Y**, Ford AC, Khan KJ, Gisbert JP, Forman D, Leontiadis GI, Tse F, Calvet X, Fallone C, Fischbach L, Oderda G, Bazzoli F, Moayyedi P. Optimum duration of regimens for Helicobacter pylori eradication. *Cochrane Database Syst Rev* 2013; : CD008337 [PMID: 24338763 DOI: 10.1002/14651858.CD008337.pub2]
34 **Altamirano A**, Bondani A. Adverse reactions to furazolidone and other drugs. A comparative review. *Scand J Gastroenterol Suppl* 1989; **169**: 70-80 [PMID: 2694347 DOI: 10.3109/00365528909091336]
35 **Xie LH.** Effect of furazolidone-containing quadruple remedial therapy for Helicobacter pylori eradication: a Meta-analysis. *Guangxi Yixue* 2015; **37**: 606-610.
36 **Hosseini V**, Mokhtare M, Gholami M, Taghvaei T, Maleki I, Valizadeh M, Bari Z, Fakheri H. A Comparison between Moderate- and High-dose Furazolidone in Triple Regimens for Helicobacterpylori Eradication in Iran. *Middle East J Dig Dis* 2014; **6**: 195-202 [PMID: 25349682]
37 **Roghani HS**, Massarrat S, Shirekhoda M, Butorab Z. Effect of different doses of furazolidone with amoxicillin and omeprazole on eradication of Helicobacter pylori. *J Gastroenterol Hepatol* 2003; **18**: 778-782 [PMID: 12795748 DOI: 10.1046/j.1440-1746.2003.03058.x]
38 **Mokhtare M**, Hosseini V, Tirgar Fakheri H, Maleki I, Taghvaei T, Valizadeh SM, Sardarian H, Agah S, Khalilian A. Comparison of quadruple and triple Furazolidone containing regimens on eradication of helicobacter pylori. *Med J Islam Repub Iran* 2015; **29**: 195 [PMID: 26157713]
39 **Fakheri H**, Merat S, Hosseini V, Malekzadeh R. Low-dose furazolidone in triple and quadruple regimens for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2004; **19**: 89-93 [PMID: 14687170 DOI: 10.1046/j.1365-2036.2003.01822.x]

40 **De Francesco V**, Ierardi E, Hassan C, Zullo A. Furazolidone therapy for Helicobacter pylori: is it effective and safe? *World J Gastroenterol* 2009; **15**: 1914-1915 [PMID: 19370795 DOI: 10.3748/wjg.15.1914]

41 **Talebi Bezmin Abadi A**. Furazolidone and Helicobacter pylori Treatment. *Middle East J Dig Dis* 2015; **7**: 110-111 [PMID: 26106473]

42 Some food additives, feed additives and naturally occurring substances. *IARC Monogr Eval Carcinog Risk Chem Hum* 1983; **31**: 1-291 [PMID: 6579000]

43 **Graham DY**, Lu H. Furazolidone in Helicobacter pylori therapy: misunderstood and often unfairly maligned drug told in a story of French bread. *Saudi J Gastroenterol* 2012; **18**: 1-2 [PMID: 22249084 DOI: 10.4103/1319-3767.91724]

44 **Lefebvre M**, Chang HJ, Morse A, van Zanten SV, Goodman KJ; CANHelp Working Group. Adherence and barriers to H. pylori treatment in Arctic Canada. *Int J Circumpolar Health* 2013; **72**: 22791 [PMID: 24416723 DOI: 10.3402/ijch.v72i0.22791]

45 **Shakya Shrestha S**, Bhandari M, Thapa SR, Shrestha R, Poudyal R, Purbey B, Gurung RB. Medication Adherence Pattern and Factors affecting Adherence in Helicobacter Pylori Eradication Therapy. *Kathmandu Univ Med J* (KUMJ) 2016; **14**: 58-64 [PMID: 27892443]

46 **Murthy SN**, Dinoso VP Jr, Clearfield HR, Chey WY. Simultaneous measurement of basal pancreatic, gastric acid secretion, plasma gastrin, and secretin during smoking. *Gastroenterology* 1977; **73**: 758-761 [PMID: 892379]

47 **Budzyński J**, Swiatkowski M. [Pathophysiology of the harmful influence of smoking on the course of gastric and duodenal ulcer diseases]. *Przegl Lek* 1996; **53**: 811-815 [PMID: 9173445]

48 **Mohammadi M**, Attaran B, Malekzadeh R, Graham DY. Furazolidone, an Underutilized Drug for H. pylori Eradication: Lessons from Iran. *Dig Dis Sci* 2017; **62**: 1890-1896 [PMID: 28577244 DOI: 10.1007/s10620-017-4628-5]

49 **Ji Z**, Han F, Meng F, Tu M, Yang N, Zhang J. The Association of Age and Antibiotic Resistance of Helicobacter Pylori: A Study in Jiaxing City, Zhejiang Province, China. *Medicine* (Baltimore) 2016; **95**: e2831 [PMID: 26937912 DOI: 10.1097/MD.0000000000002831]

P- Reviewer: Karatapanis S, Tarnawski AS **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4606-4614

DOI: 10.3748/wjg.v24.i40.4606

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

ORIGINAL ARTICLE

Observational Study

Long term outcome of antiviral therapy in patients with hepatitis B associated decompensated cirrhosis

Young-Cheol Ju, Dae-Won Jun, Jun Choi, Waqar Khalid Saeed, Hyo-Young Lee, Hyun-Woo Oh

Young-Cheol Ju, Dae-Won Jun, Department of Translational Medicine, Graduate school of Biomedical Science and Engineering, Hanyang University, Seoul 04763, South Korea

Dae-Won Jun, Waqar Khalid Saeed, Hyo-Young Lee, Hyun-Woo Oh, Department of Internal Medicine, School of Medicine, Hanyang University, Seoul 04763, South Korea

Jun Choi, Department of Industrial Management Engineering, Korea University, Seoul 02841, South Korea

ORCID number: Young-Cheol Ju (0000-0002-4559-2164); Dae-Won Jun (0000-0002-2875-6139); Jun Choi (0000-0001-8640-4011); Waqar Khalid Saeed (0000-0002-7888-9108); Hyo-Young Lee (0000-0001-9977-5591); Hyun-Woo Oh (0000-0003-4224-8808).

Author contributions: Jun DW designed and supervised the study; Lee HY, Oh HW independently validated the operational definitions; Choi J collected the HIRA data and analyzed the data; Ju YC wrote the manuscript.

Supported by The Research Supporting Program of The Korean Association for the Study of the Liver and The Korean Liver Foundation.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of Hanyang University Hospital (Seoul).

Informed consent statement: This study used insurance reimbursement claims data provided by the Health Insurance Review and Assessment (HIRA). It did not need to make sure of informed written consent prior to study enrollment because the data was already encrypted personal identifiable information.

Conflict-of-interest statement: There are no conflicts of interest to report

Data sharing statement: No additional data are available.

STROBE Statement: The items that should be included in reports of observational studies were checked and the file of STROBE statement was uploaded.

Manuscript source: Unsolicited manuscript

Correspondence to: Dae-Won Jun, MD, PhD, Professor, Department of Internal Medicine, School of Medicine, Hanyang University, 222 Wangsimni-ro, Seongdong-gu, Seoul 04763, South Korea. noshin@hanyang.ac.kr
Telephone: +82-2-22908338
Fax: +82-2-9720068

Received: July 23, 2018
Peer-review started: July 23, 2018
First decision: August 25, 2018
Revised: September 6, 2018
Accepted: October 5, 2018
Article in press: October 5, 2018
Published online: October 28, 2018

Abstract

AIM

To investigate survival rate and incidence of hepatocellular carcinoma (HCC) in patients with decompensated cirrhosis in the antiviral era.

METHODS

We used the Korean Health Insurance Review and Assessment. Korea's health insurance system is a public single-payer system. The study population consisted of 286871 patients who were prescribed hepatitis B antiviral therapy for the first time between 2007 and 2014 in accordance with the insurance guidelines.

Overall, 48365 antiviral treatment-naïve patients treated between 2008 and 2009 were included, and each had a follow-up period ⩾ 5 years. Data were analyzed for the 1st decompensated chronic hepatitis B (CHB) and treatment-naïve patients (n = 7166).

RESULTS

The mean patient age was 43.5 years. The annual mortality rates were 2.4%-19.1%, and 5-year cumulative mortality rate was 32.6% in 1st decompensated CHB treatment-naïve subjects. But the annual mortality rates sharply decreased to 3.4% (2.4%-4.9%, 2-5 year) after one year of antiviral treatment. Incidence of HCC at first year was 14.3%, the annual incidence of HCC decreased to 2.5% (1.8%-3.7%, 2-5 year) after one year. 5-year cumulative incidence of HCC was 24.1%. Recurrence rate of decompensated event was 46.9% at first year, but the annual incidence of second decompensation events in decompensated CHB treatment-naïve patients was 3.4% (2.1%-5.4%, 2-5 year) after one year antiviral treatment. 5-year cumulative recurrence rate of decompensated events was 60.6%. Meanwhile, 5-year cumulative mortality rate was 3.1%, and 5-year cumulative incidence of HCC was 11.5% in compensated CHB treatment-naïve patients.

CONCLUSION

Long term outcome of decompensated cirrhosis treated with antiviral agent improved much, and incidence of hepatocellular carcinoma and mortality sharply decreased after one year treatment.

Key words: Hepatitis B; Antiviral agent; Decompensated cirrhosis; Mortality; Hepatocellular carcinoma

Core tip: It is well known that antiviral treatment improves clinical outcomes of chronic hepatitis B-associated decompensated cirrhosis. However, long term and large scale clinical data regarding survival rate, and incidence of hepatocellular carcinoma in patients with decompensated cirrhosis in the antiviral era are lacking. We investigated the survival rate and incidence of hepatocellular carcinoma (HCC) in patients with decompensated cirrhosis by using the Health Insurance Review and Assessment database. Long term outcome of treating hepatitis B-associated decompensated cirrhosis using antiviral agents improved much compare to previous reports. Cumulative mortality rate and incidence of HCC was sharply decreased after one year antiviral treatment.

Ju YC, Jun DW, Choi J, Saeed WK, Lee HY, Oh HW. Long term outcome of antiviral therapy in patients with hepatitis B-associated decompensated cirrhosis. *World J Gastroenterol* 2018; 24(40): 4606-4614 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4606.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4606

INTRODUCTION

There are many studies on the natural course of chronic hepatitis B (CHB)[1,2]. A population-based cohort study reported an 11% annual incidence of decompensated complications in patients with compensated chronic liver disease[1]. In patients with decompensated liver disease, the risk of hepatocellular carcinoma (HCC) and mortality increases by 7%-8% and up to 20%-50% annually, respectively[2].

It is clear that antiviral therapy reduces liver disease progression and mortality in decompensated CHB patients[3-5]. However, clinical data for long-term survival rate, the incidence of HCC, and the recurrence of decompensated events in patients with decompensated cirrhosis receiving antiviral agents are still lacking in the antiviral era.

A landmark study of clinical outcomes of CHB-associated decompensated cirrhosis was recently published[6,7]. Jang *et al*[6] evaluated the effects of antiviral therapy on mortality rate in decompensated cirrhosis patients and reported a 5-year survival rate of 423 decompensated cirrhosis patients of 59.7%. However, the sample size was small, and the follow-up period was short. Moreover, all patients were treated in a tertiary hospital setting and the selection bias was unclear. There are several meta-analyses on the effects of antiviral agents in patients with CHB-associated decompensated cirrhosis[8-11]. However, only three studies to date have included > 100 patients for > 1 year[6,12,13], and few studies have used a highly potent viral nucleos(t)ide analog (entecavir or tenofovir).

Here we used a nationwide database to investigate the long-term mortality rate and incidence of HCC in patients with CHB-associated decompensated cirrhosis who received antiviral agents.

MATERIALS AND METHODS

Data source

This study used insurance reimbursement claims data provided by the Health Insurance Review and Assessment (HIRA). The health insurance claims data are generated when a health care provider submits a reimbursement claim to HIRA for payment of the portion of the medical services provided to the patient covered by the National Health Insurance. Korea's health insurance system is a public single-payer system. 97.2% of the total population in Korean people is enrolled in national insurance system. Health care providers are automatically eligible and obliged to treat patients for services covered under the system. The system is controlled by the government[14].

Study design

This retrospective cohort study used HIRA claims data from January 2007 to December 2014. The provided data were approved by the National Health Insurance Service following a review process, and the data were released with an encrypted number according to the disclosure principle. The study was conducted with

approval from the institutional review board (IRB approval: HYUH 2017-04-006).

Study population

The study population consisted of 286871 patients who were prescribed hepatitis B antiviral therapy for the first time between 2007 and 2014 in accordance with the insurance guidelines. Overall, 48365 antiviral treatment-naïve patients treated between 2008 and 2009 were included, and each had a follow-up period ⩾ 5 years.

Inclusion criteria

The inclusion criteria were as follows: (1) CHB patients who were prescribed nucleos(t)ide analogs for the first time under the national reimbursement policy. The HIRA reimbursement criteria were as follows: hepatitis B surface antigen (HBsAg)-positive for ⩾ 6 mo, aminotransferase activity ⩾ 80 U/L, and baseline hepatitis B e antigen (HBeAg)-positive with hepatitis B virus (HBV)-DNA ⩾ 100000 copies/mL or HBeAg-negative with HBV-DNA ⩾ 10000 copies/mL; (2) prescribed oral antiviral agents for > 90 d; and (3) an observational period for ⩾ 5 years after the initial use of an oral antiviral agent. All subjects were nucleos(t)ide analog-naïve patients.

Exclusion criteria

Patients meeting any of the following criteria were excluded: (1) prescription of oral antiviral agents (lamivudine, clevudine, adefovir, telbivudine, entecavir, or tenofovir) within the prior 1 year under the reimbursement system; (2) decompensated event (tense ascites, variceal bleeding, hepatorenal syndrome, or hepatic encephalopathy) using the operational definition within the prior 2 years before nucleos(t)ide analog treatment; (3) diagnosis of any type of cancer before antiviral treatment; and (4) observational period < 5 years after nucleos(t)ide analogs treatment.

Definitions

"Compensated CHB treatment-naïve" was defined as having never developed a decompensated event within the prior 2 years before starting an antiviral agent. Both CHB and compensated cirrhosis were included. "Decompensated CHB treatment-naïve" was defined as having never developed a decompensated event within 2 years before starting the antiviral agent but at the start of antiviral treatment. Diagnosis of HCC is strictly regulated by HIRA, because government pays 95% of the bill. HCC can be diagnosed, if the typical hallmark (hypervascularity in the arterial phase and washout in the portal or delayed phase) is identified on one or more (two or more) imaging techniques (dynamic computed tomography, dynamic magnetic resonance imaging, gadolinium-ethoxybenzyldiethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging magnetic resonance imaging)[15].

Operational definitions

The operational definitions were as follows. Naïve nucleos(t)ide analog treatment was defined as having no record of prescribed antiviral agents through insurance reimbursement 1 year before the first administration of an antiviral agent. Decompensated CHB treatment-naïve was defined as the removal of ⩾ 3 L on paracentesis, endoscopic band ligation (or sclerotherapy) or drug therapy for variceal bleeding, lactulose enema after admission for hepatic encephalopathy, and treatment after admission for hepatorenal syndrome. The first decompensated event was defined as a complication such as tense ascites, variceal bleeding, hepatic encephalopathy, and/or hepatorenal syndrome at the start of antiviral treatment, but there were no decompensated events within 2 years before antiviral treatment. Tense ascites was defined as the need for paracentesis (C8050, C8051, and Q2470) while using an antiviral agent. Variceal bleeding was defined as the need for sclerotherapy, variceal ligation (Q2430, Q2431, Q2432, Q2433, Q2434, Q2435, Q2436, Q2437, Q2438, Q7631, Q7632, Q7633, and Q7634), or drug therapy for esophageal variceal bleeding (vasopressin, terlipressin, somatostatin, or octreotide) while on antiviral therapy. Hepatic encephalopathy was defined as administration of a lactulose enema (M0076) while using an antiviral agent. Hepatorenal syndrome was defined as co-administration of terlipressin and albumin while under antiviral therapy. Death was defined as HIRA code (clinical outcome code DGRSLT_TP_CD "4: Death").

Validation of operational definitions

Based on the operational definitions, the appropriateness of the data extracted from the HIRA was assessed as follows. The databases from tertiary hospitals covering 6 years (January 2009 to December 2014) were used to review the patients' medical records corresponding to the operational definitions to confirm agreement with the operational definitions (IRB approval: HYUH 2015-09-017). Overall, 133 patients had data corresponding to the definition of decompensated cirrhosis; 120 patients, tense ascites; 40 patients, variceal bleeding; 51 patients, hepatic encephalopathy; and 5 patients, hepatorenal syndrome.

Primary and secondary endpoints

The primary endpoint was mortality rate. The secondary endpoint was the incidence of decompensated cirrhosis-associated complications and HCC.

Statistical analysis

The *t*-test and chi-square tests were used to assess demographic differences based on sex and differences in biochemical data. Kaplan-Meier analysis was used to assess survival rate and re-bleeding frequency. The statistical analysis was performed using SAS software (version 9.4; SAS Institute, Inc., Cary, NC, United States).

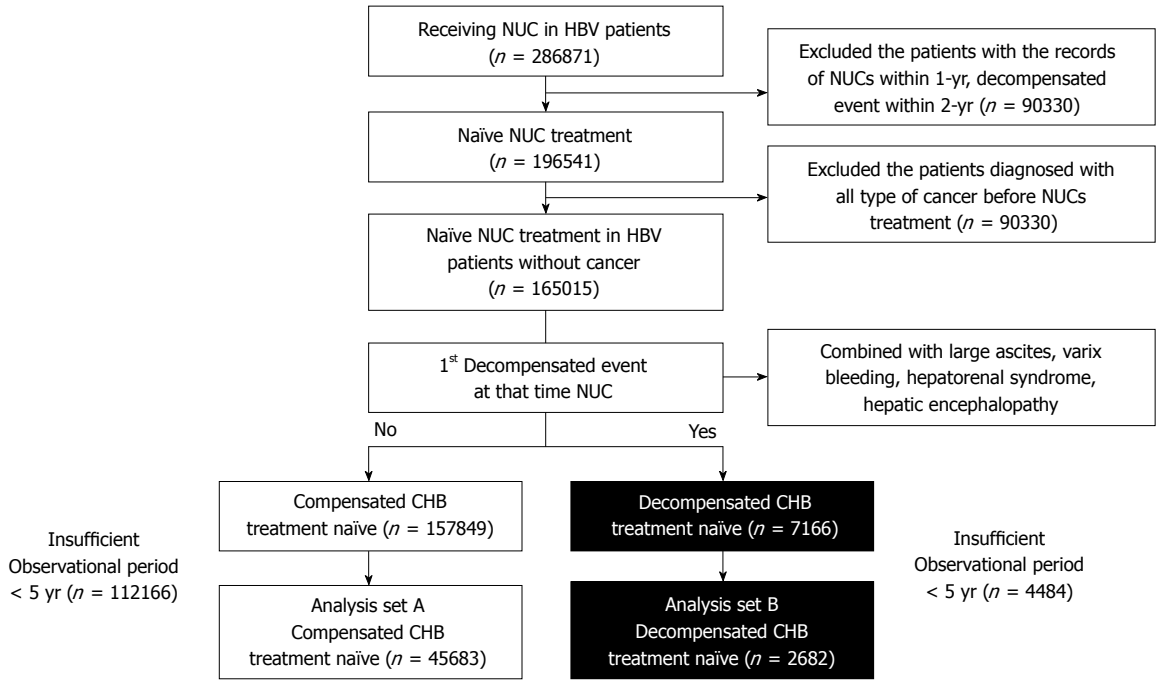


Figure 1 Flow chart of the study. NUC: Nucleos(t)ide; HBV: Hepatitis B virus; CHB: Chronic hepatitis B.

RESULTS

Patient selection

A total of 286871 CHB patients were prescribed nucleos(t)ide analogs under the reimbursement policy (HBsAg-positive, baseline HBeAg-positive with HBV-DNA ⩾ 100000 copies/mL or HBeAg-negative with HBV-DNA ⩾ 10000 copies/mL, and aminotransferase level ⩾ 80 IU/L) between 2007 and 2014 (Figure 1). A total of 196541 treatment-naïve patients who were never prescribed oral antiviral agents (lamivudine, clevudine, adefovir, telbivudine, entecavir, or tenofovir) within the previous 1 year were selected. Only those with an observation period ⩾ 5 years were included. Ultimately, 45683 compensated CHB treatment-naïve (of 157849) and 7166 decompensated CHB treatment-naïve subjects were selected (Figure 1). The 45683 patients included showed compensated liver disease, while 2682 patients had accompanying decompensated complications at initiation of nucleos(t)ide analog treatment (Table 1). Mean patient age was 43.5 years, and the patients took various antiviral agent (entecavir: 41.7%; lamivudine: 17.4%; clevudine: 6.3%; combination: 29.4%).

Validation of operational definitions

The validity of the operational definitions was tested as follows. The operational definitions were used to identify patients from the databases of tertiary hospitals (covering 6 years between 2009 and 2014), and their medical records were reviewed to assess the agreement with the operational definitions. Overall, 120 patients with tense ascites were identified according to the operational definitions. Among them, 107 (89.2%) had matching and 13 had non-matching medical records. Among the unmatched 13 patients, five underwent postoperative paracentesis, six underwent paracentesis due to multiple organ failure caused by sepsis, and two underwent paracentesis because of malignant condition. A total of 40 patients with variceal bleeding were extracted according to the operational definition; of them, 38 (95.0%) had matching and 2 had non-matching medical records. Both non-matching cases involved peptic ulcer disease being misdiagnosed as variceal bleeding. A total of 51 hepatic encephalopathy cases were extracted according to the operational definition; of them, 49 (96.0%) had matching and 2 had non-matching medical records. Finally, five patients with hepatorenal syndrome were extracted based on the operational definition, and all had matching medical records.

Clinical course of compensated CHB treatment-naïve patients treated with nucleos(t)ide analog

In compensated CHB treatment-naïve patients, the 5-year cumulative incidence of various complications was 7.4%, while the annual incidence of the first onset of decompensated complications after using an antiviral agent was 1.2%-2.0% (Table 2). The frequency of decompensated complications decreased significantly as the duration of antiviral agent use increased (P for trend, < 0.0001) (Figure 2A).

The annual incidence of HCC was 1.8%-3.5%, and it decreased significantly during the period in which an antiviral agent was used (P for trend, < 0.0001) (Figure 2B). In compensated CHB treatment-naïve patients, the 5-year cumulative incidence of HCC was 11.5%, which was higher than that of decompensated complications (7.4%) (P < 0.0001). Finally, the annual mortality rate was 0.6%-0.7% (Figure 2C), whereas the 5-year

Table 1 Patients' basic characteristics *n* (%)

	Total (*n* = 48365)	Compensated HBV group[1] (*n* = 45683)	Decompensated cirrhosis group (*n* = 2682)	[a]*P* value
Sex (male)	32691 (67.6)	30881 (67.6)	1810 (67.5)	0.9214
Age (yr)	43.5 ± 12.3	43.0 ± 12.2	51.7 ± 11.5	< 0.0001
Nucleos(t)ide analog				
Entecavir	20147 (41.7)	18644 (40.8)	1503 (56.0)	< 0.0001
Lamivudine	8411 (17.4)	7937 (17.4)	474 (17.7)	0.7105
Clevudine	3029 (6.3)	2898 (6.3)	131 (4.9)	0.0028
Lamivudine + adefovir	2525 (5.2)	2415 (5.3)	110 (4.1)	0.0084
Telbivudine	32 (0.1)	30 (0.1)	2 (0.1)	1.0000
Others	14221 (29.4)	13759 (30.1)	462 (17.2)	< 0.0001
Medication compliance				
< 25%	12793 (26.5)	12045 (26.4)	748 (27.9)	0.0700
25%-50%	6067 (12.5)	5769 (12.6)	298 (11.1)	0.0229
50%-75%	7075 (14.6)	6686 (14.6)	389 (14.5)	0.8735
> 75%	22430 (46.4)	21183 (46.4)	1247 (46.5)	0.9835
Type of first decompensation				
Refractory ascites	2022 (55.6)		2022 (55.6)	
Varix bleeding	711 (19.6)		711 (19.6)	
Hepatorenal syndrome	151 (4.2)		151 (4.2)	
Hepatic encephalopathy	752 (20.7)		752 (20.7)	

[1]Patients with chronic hepatitis and with early compensated cirrhosis. [a]$P < 0.05$ by Chi-square and Student's *t*-test. HBV: Hepatitis B virus.

cumulative mortality rate was 3.1% and the cumulative survival rate was 96.9%.

Clinical course of decompensated CHB treatment-naïve patients treated with nucleos(t)ide analog

The annual incidence of a second decompensation event in decompensated CHB treatment patients was 2.1%-46.9%: It was highest within the first year (46.9%) but decreased over the antiviral treatment period (*P* for trend, < 0.0001) (Figure 2D). The 5-year cumulative recurrence rate was 60.6%.

The annual incidence of HCC was 1.8%-14.3%, and it decreased significantly as antiviral treatment period increased (*P* for trend, < 0.0001) (Figure 2E). The 5-year cumulative incidence of HCC was 24.1%. The development of HCC was high within the first year after antiviral agent use (14.3%) and decreased thereafter, but the incidence remained steady even 1 year after the start of antiviral therapy, showing an annual incidence of 2.5% (1.8%-3.7%, 2-5 year). Finally, the annual mortality rate was approximately 2.4%-19.1%, whereas the 5-year cumulative mortality rate was 32.6% and the cumulative survival rate was 67.4%.

DISCUSSION

In compensated CHB treatment-naïve patients, the 5-year cumulative incidences of HCC and decompensated complications were 11.5% and 7.4%, respectively. In CHB-associated decompensated cirrhosis patients, the 5-year cumulative incidence of HCC was 24.1%, whereas the mortality rate was 32.6% after antiviral therapy (Figure 3).

This study used national database that was compiled from physician reimbursement claims for medical services. There is inherent weakness when someone uses such database which relies on local and regional practices in order to generate a reimbursement claim. Therefore, we tried to used performance or drug medication code rather than diagnosis code. Use of antiviral agents, albumin, vasoactive drugs (vasopressin, terlipressin, somatostatin, or octreotide) are strictly regulated by government. All above drugs have very narrow indication and regulated strictly, because of high price. All health care providers should submit a supporting laboratory data or documents that meet reimbursement criteria. Because government pays 95% of total medical expenses in case of HCC, diagnose of HCC verified by the government according to strict criteria.[15] So we tried to use diagnostic code (ICD-10) and popular medication, such as diuretics and oral lactulose relies on local and regional practices in order to generate reimbursement. Another challenge when use reimbursement data is baseline disease severity is quite heterogeneous, because laboratory data was not visible. To correct the disease severity of baseline only first decompensated cirrhosis patients and antiviral treatment-naïve patients were enrolled. Baseline alanine aminotransferase ⩾ 80 IU with HBV-DNA ⩾ 100000 copies/mL (in HBeAg-negative patients) or HBV-DNA ⩾ 10000 copies/mL (in HBeAg-positive patients) should be clarified by HIRA.

Several studies have examined the natural course of CHB in patients without antiviral therapy[2,16,17]. Previous reports showed 5-year survival rate of decompensated cirrhosis at 14%-35% under supportive treatment[5,18]. There are two Asian studies[19,20]. Five-year survival rate was 19% in 102 untreated decompensated cirrhosis patients[20]. In our study, 5-year cumulative survival rate was 67.4% in 1st decompensated CHB treatment-naïve subjects. Median survival was only 2 years, and the 2-year possibility of survival was 57% in patients with CHB-

Table 2 Rate of clinical progression of liver disease

	Compensated chronic hepatitis B (n = 45683)									Decompensated cirrhosis (n = 2682)								
	Newly decompensated incidence (n = 3401)			Hepatoma incidence (n = 5268)			Mortality incidence (n = 1415)			Second decompensated incidence (n = 1626)			Hepatoma incidence (n = 1295)			Mortality incidence (n = 874)		
	Event	Percent	Accumulation (%)	Event	Percent	Accumulation (%)	Event	Percent	Accumulation (%)	Event	Percent	Accumulation (%)	Event	Percent	Accumulation (%)	Event	Percent	Accumulation (%)
< 1 yr	923	2.02%	2.02%	1602	3.51%	3.51%	253	0.55%	0.55%	1257	46.87%	46.87%	765	14.26%	14.26%	513	19.13%	19.13%
1-2 yr	662	1.45%	3.47%	1053	2.31%	5.81%	249	0.55%	1.10%	144	5.37%	52.24%	200	3.73%	17.99%	132	4.92%	24.05%
2-3 yr	634	1.39%	4.86%	947	2.07%	7.88%	280	0.61%	1.71%	99	3.69%	55.93%	140	2.61%	20.60%	94	3.50%	27.55%
3-4 yr	631	1.38%	6.24%	802	1.76%	9.64%	324	0.71%	2.42%	71	2.65%	58.58%	94	1.75%	22.35%	65	2.42%	29.98%
4-5 yr	551	1.21%	7.44%	864	1.89%	11.53%	309	0.68%	3.10%	55	2.05%	60.63%	96	1.79%	24.14%	70	2.61%	32.59%
[a]P for trend		< 0.0001			< 0.0001			0.0004			< 0.0001			< 0.0001			< 0.0001	

associated decompensated cirrhosis in a meta-analysis[17]. The 1-year possibility of death of patients with clinical stage 3 disease (ascites ± varix) was 20%, while the 1-year death rate was 57% in patients with clinical stage 4 disease (variceal bleeding ± ascites)[16].

Data of the clinical course of CHB-associated decompensated cirrhosis with antiviral agent use are sparse. Antiviral treatment was effective in improving survival rate in decompensated cirrhosis[18]. Two-year survival rate of 70 decompensated patients was 83% in entecavir treatment group[21]. In our study, 2-year cumulative survival rate was 76.0% in 1st decompensated CHB treatment-naïve subjects. Das *et al*[20] showed the clinical course of patients with CHB-associated decompensated cirrhosis from 1998 to 2008. Of the 253 patients, 151 (59.7%) received antiviral treatment. The 5-year survival rate was 21% in the antiviral treatment group and 19% in the untreated group, quite low compared to our results (21% *vs* 67.4%, respectively). The reason might be differences in baseline characteristics and their use of low-potent antiviral agents. The dominant antiviral agent was lamivudine (88%) in Das's study, whereas most of our patients were prescribed entecavir (41.7%) or combination treatment (29.4%). Jang *et al*[6] evaluated the effects of antiviral therapy on mortality rate in decompensated cirrhosis patients and reported a 5-year survival rate of 423 decompensated cirrhosis patients of 59.7%. Although we used very strict operational definitions, the 5-year survival rate (61.4%) of decompensated CHB treatment-naïve patients was comparable to that of Jang's results (59.7%). The definition of tense ascites used in the present study was relatively restricted to cases in which paracentesis of > 3 L was performed. Thus, patients with mild-grade ascites, which can be controlled by diuretics, were excluded from our study. This is the largest cohort study to date to analyze the clinical outcomes of antiviral therapy in decompensated CHB patients. In our study, 7166 decompensated treatment-naïve patients were enrolled.

Until now, the annual incidence of HCC in patients with compensated cirrhosis was known to be 7%-8%[2]. Antiviral therapy reduced the incidence of HCC in compensated liver disease patients[3,4,22]. In the present study, in compensated CHB patients on antiviral therapy, the annual incidence and 5-year cumulative incidence of HCC were 1.7%-3.5% and 11.5%, respectively. It seems low compared to those of previous studies[3,4,22], but the incidence of HCC looked higher than that of first decompensated complications (7.4%). This means that, in compensated CHB patients, clinicians should be more alert to HCC than to newly developing decompensated complications.

There is little room for debate about whether antiviral therapy can reduce the incidence of HCC in patients with compensated CHB, but no studies have reported whether it can reduce the incidence of HCC in patients with decompensated cirrhosis. In present study, the 5-year cumulative incidence of HCC in patients with decompensated cirrhosis and on antiviral therapy was 24.1%. Interestingly, antiviral therapy after the onset of decompensated complications reduced the incidence of HCC from 14.26% in the first year to 3.73%, 2.61%, 1.75%, and 1.79% in the following years. However, additional studies are needed to determine whether such a drastic decrease in the incidence of HCC is a result of antiviral therapy itself or a result of modifying one's lifestyle, for example, abstaining from alcohol (Figure 2).

The limitations of the present study are as follows. First, the present study used data that were specific to insurance reimbursement claims; thus, the data lacked accurate information about the patients' disease severity and blood test results (HBV-DNA polymerase chain reaction, liver enzymes, alpha-fetoprotein, *etc.*). To minimize this problem, we included treatment-naïve patients (all baseline HBeAg-positive patients with HBV-DNA ⩾ 100000 copies/mL or HBeAg-negative patients with HBV-DNA ⩾ 10000 copies/mL and alanine aminotransferase or aspartate aminotransferase level ⩾ 80 IU/L). Second, mismatching operational definitions are possible. In our study, the concordance rate of the operational definition of uncontrolled ascites was 89.1%, which means that approximately 10% of cases did not match. We tried to validate the operational

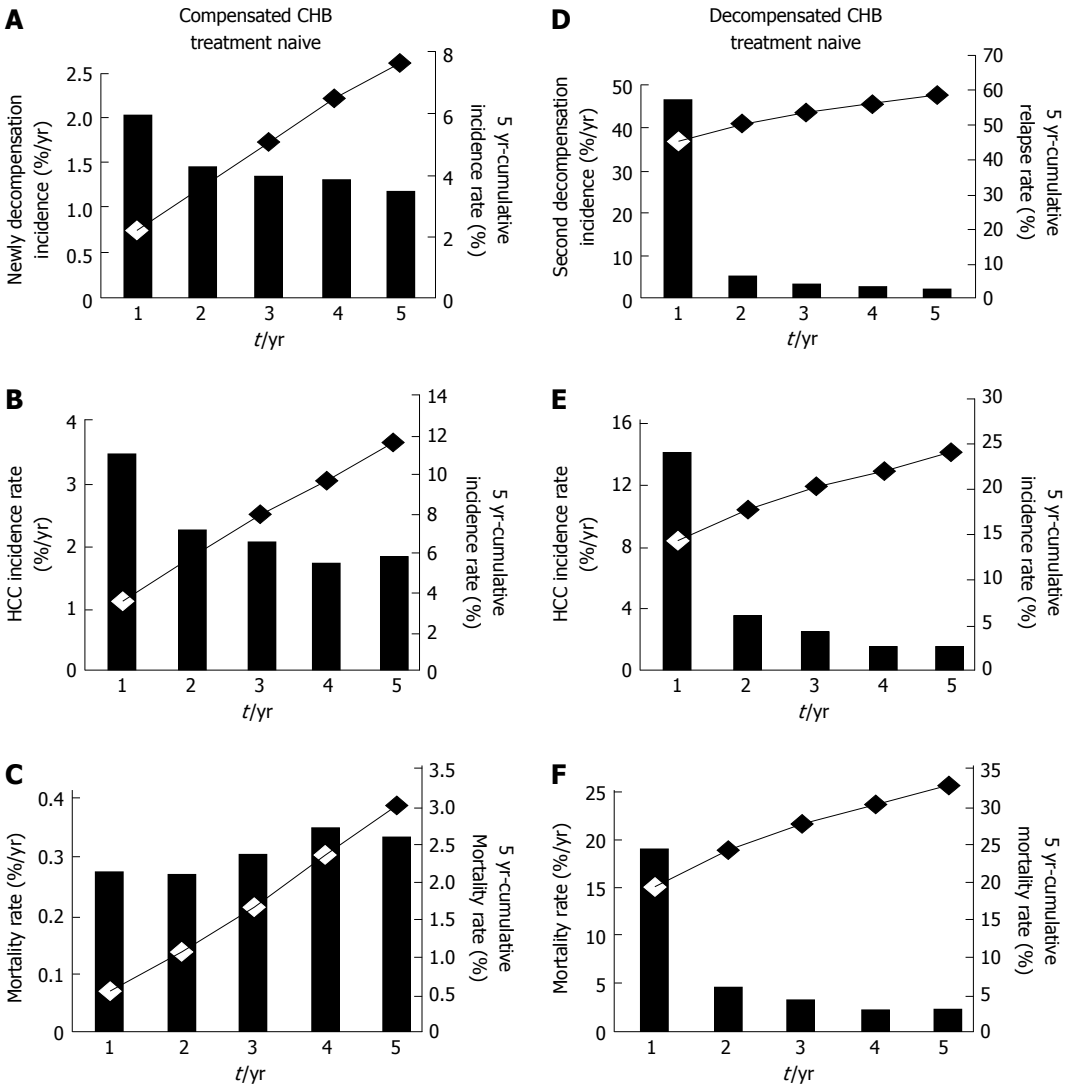


Figure 2 Clinical course of chronic hepatitis B patients receiving antiviral agents. CHB: Chronic hepatitis B. HCC: Hepatocellular carcinoma.

definitions in various clinical settings, because pattern of prescribing antiviral agent might be quite different depending on the clinical settings. We investigated two different sized hospitals; one is tertiary hospital and the other is secondary hospital. Although we tried to validate the operational definitions using real world data from two different hospitals, we thought 133 patients were not enough to validate for 7166 decompensated patients' data. Third, the definition of "compensated CHB" is more lenient as it included both CHB and early compensated cirrhosis patients, while "decompensated CHB" is very strict, as it only includes "patients with large amounts of ascites" but generally "ascites is a feature of decompensation." Patients with moderate ascites who used diuretics and had mild variceal bleeding with no drug therapy were excluded from the study. Consequently, the decompensated complications defined in the present study may have been underestimated compared to those in actual patients with decompensated cirrhosis. Fourth, the present study used diagnosis codes to diagnose HCC. Because the diagnosis of HCC was based on dynamic contrast-enhanced computed tomography or magnetic resonance imaging findings without a biopsy, the numbers may have been overestimated compared to the actual incidence of HCC. Fifth, given that it was impossible to check the medical and radiological records of all patients, those with both CHB and compensated cirrhosis were labeled compensated CHB treatment-naïve. As a result, some patients with compensated cirrhosis and only a small amount of ascites may have been included in our patients with compensated liver disease. Finally, this study did not consider the patients' alcohol consumption history. In patients with hepatitis B, alcohol consumption is known to increase the incidence of HCC. However, the present study was unable to identify the patients' alcohol consumption and drinking habits.

In conclusion, in compensated CHB patients, the 5-year cumulative incidences of decompensated complications and HCC were 7.4% and 11.5%, respectively. In decompensated CHB patients, the annual and 5-year cumulative incidences of HCC were

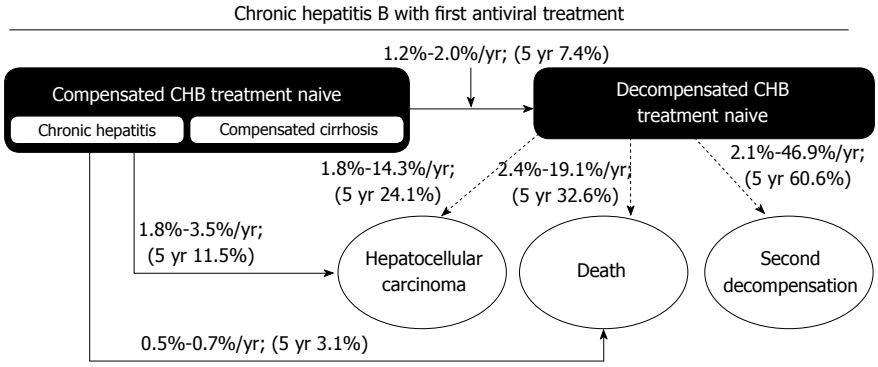


Figure 3 Shifting of clinical stage in patients receiving antiviral agents. CHB: Chronic hepatitis B.

1.8%-14.3% and 24.1%, respectively. Moreover, the annual and 5-year cumulative mortality rates were approximately 2.6%-19.1% and 32.6%, respectively.

ARTICLE HIGHLIGHTS

Research background

According to a population-based cohort study, the annual incidence of decompensated complications was 11% in patients with compensated chronic liver disease. It is clear that antiviral therapy reduces liver disease progression and mortality in decompensated chronic hepatitis B (CHB) patients. However, clinical data for long-term survival rate, the incidence of hepatocellular carcinoma (HCC), and the recurrence of decompensated events in patients with decompensated cirrhosis treated with antiviral agents are still lacking in the antiviral era

Research motivation

Several studies have examined the natural course of CHB in patients without antiviral therapy. Data of the clinical course of CHB-associated decompensated cirrhosis with antiviral agent use are sparse. In this study, we tried to investigate the survival rate and incidence of HCC in patients with decompensated cirrhosis in the antiviral era.

Research objectives

The primary objective was mortality rate and the secondary objectives were the incidence of decompensated cirrhosis-associated complications and HCC.

Research methods

The data source of this study was the insurance reimbursement claims data provided by the Korean Health Insurance Review and Assessment (HIRA). Overall, 48365 antiviral treatment-naïve patients treated between 2008 and 2009 were included, and each had a follow-up period $\geqslant$ 5 years. Naïve nucleos(t)ide analog treatment and the decompensated complications were defined with the operational definitions. The appropriateness of the data extracted from the HIRA was assessed and the validation the operational definitions was conducted in two different sized hospitals.

Research results

The 45683 patients included showed compensated liver disease, while 2682 patients had accompanying decompensated complications at initiation of nucleos(t)ide analog treatment. Mean patient age was 43.5 years. In compensated CHB treatment-naïve patients, the 5-year cumulative incidence of various complications was 7.4%, while the annual incidence of the first onset of decompensated complications after using an antiviral agent was 1.2%-2.0%. The 5-year cumulative incidence of HCC in compensated CHB treatment-naïve patients was 11.5%, which was higher than that of decompensated complications (7.4%). In decompensated CHB treatment-naïve patients, the annual incidence of a second decompensation event in decompensated CHB treatment patients was 2.1%-46.9%: It was highest within the first year (46.9%). The 5-year cumulative incidence of HCC in decompensated CHB treatment patients was 24.1%. The 5-year cumulative mortality rate was 32.6% and the cumulative survival rate was 67.4%.

Research conclusions

This study used national database that was compiled from physician reimbursement claims for medical services. There would be several limitations, but we proposed the new methodology when using a long term and large scale clinical database such as HIRA. According to the present study, we suggested that clinicians should be more alert to HCC than to newly developing decompensated complications. Interestingly, antiviral therapy after the onset of decompensated complications reduced the incidence of HCC from 14.26% in the first year to 3.73%, 2.61%, 1.75%, and 1.79% in the following years. However, additional studies are needed to determine. In conclusion, long term outcome of treating hepatitis B-associated decompensated cirrhosis using antiviral agents improved much compare to previous reports. Incidence of cumulative mortality rate and hepatocellular carcinoma was sharply decreased after one year antiviral treatment.

Research perspectives

We investigated the mortality rate and the incidence of decompensated cirrhosis-associated complications and HCC in the antiviral era. We hope the data suggested in this study would be helpful for the future study of comparing antiviral agents.

REFERENCES

1 **Fleming KM**, Aithal GP, Card TR, West J. The rate of decompensation and clinical progression of disease in people with cirrhosis: a cohort study. *Aliment Pharmacol Ther* 2010; **32**: 1343-1350 [PMID: 21050236 DOI: 10.1111/j.1365-2036.2010.04473.x]
2 **Fattovich G**, Giustina G, Schalm SW, Hadziyannis S, Sanchez-Tapias J, Almasio P, Christensen E, Krogsgaard K, Degos F, Carneiro de Moura M. Occurrence of hepatocellular carcinoma and decompensation in western European patients with cirrhosis type B. The EUROHEP Study Group on Hepatitis B Virus and Cirrhosis. *Hepatology* 1995; **21**: 77-82 [PMID: 7806171]
3 **Tada T**, Kumada T, Toyoda H, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Kitabtake S, Ito T. Long-term prognosis of patients with hepatitis B infection: causes of death and utility of nucleos(t)ide analogue therapy. *J Gastroenterol* 2015; **50**: 795-804 [PMID: 25376770 DOI: 10.1007/s00535-014-1011-6]
4 **Lim YS**, Han S, Heo NY, Shim JH, Lee HC, Suh DJ. Mortality, liver transplantation, and hepatocellular carcinoma among patients with chronic hepatitis B treated with entecavir vs lamivudine. *Gastroenterology* 2014; **147**: 152-161 [PMID: 24583062 DOI: 10.1053/j.gastro.2014.02.033]
5 **Chu CM**, Liaw YF. Hepatitis B virus-related cirrhosis: natural history and treatment. *Semin Liver Dis* 2006; **26**: 142-152 [PMID: 16673292 DOI: 10.1055/s-2006-939752]
6 **Jang JW**, Choi JY, Kim YS, Woo HY, Choi SK, Lee CH, Kim TY,

Sohn JH, Tak WY, Han KH. Long-term effect of antiviral therapy on disease course after decompensation in patients with hepatitis B virus-related cirrhosis. *Hepatology*2015; **61**: 1809-1820 [PMID: 25627342 DOI: 10.1002/hep.27723]
7 **Jang JW**, Choi JY, Kim YS, Yoo JJ, Woo HY, Choi SK, Jun CH, Lee CH, Sohn JH, Tak WY, Lee YR, Han KH. Effects of Virologic Response to Treatment on Short- and Long-term Outcomes of Patients With Chronic Hepatitis B Virus Infection and Decompensated Cirrhosis. *Clin Gastroenterol Hepatol* 2018 [PMID: 29753085 DOI: 10.1016/j.cgh.2018.04.063]
8 **Wang FY**, Li B, Li Y, Liu H, Qu WD, Xu HW, Qi JN, Qin CY. Entecavir for Patients with Hepatitis B Decompensated Cirrhosis in China: a meta-analysis. *Sci Rep* 2016; **6**: 32722 [PMID: 27601086 DOI: 10.1038/srep32722]
9 **Peng H**, Liu J, Yang M, Tong S, Yin W, Tang H, Hu P, Hu H, Ren H. Efficacy of lamivudine combined with adefovir dipivoxil versus entecavir monotherapy in patients with hepatitis B-associated decompensated cirrhosis: A meta-analysis. *J Clin Pharmacol* 2014; **54**: 189-200 [PMID: 24105676 DOI: 10.1002/jcph.181]
10 **Ye XG**, Su QM. Effects of entecavir and lamivudine for hepatitis B decompensated cirrhosis: meta-analysis. *World J Gastroenterol* 2013; **19**: 6665-6678 [PMID: 24151397 DOI: 10.3748/wjg.v19.i39.6665]
11 **Singal AK**, Fontana RJ. Meta-analysis: oral anti-viral agents in adults with decompensated hepatitis B virus cirrhosis. *Aliment Pharmacol Ther* 2012; **35**: 674-689 [PMID: 22257108 DOI: 10.1111/j.1365-2036.2011.04990.x]
12 **Fontana RJ**, Keeffe EB, Carey W, Fried M, Reddy R, Kowdley KV, Soldevila-Pico C, McClure LA, Lok AS; National Institutes of Health Hepatitis B Virus Orthotopic Liver Transplantation Study Group. Effect of lamivudine treatment on survival of 309 North American patients awaiting liver transplantation for chronic hepatitis B. *Liver Transpl* 2002; **8**: 433-439 [PMID: 12004342 DOI: 10.1053/jlts.2002.32983]
13 **Fontana RJ**, Hann HW, Perrillo RP, Vierling JM, Wright T, Rakela J, Anschuetz G, Davis R, Gardner SD, Brown NA. Determinants of early mortality in patients with decompensated chronic hepatitis B treated with antiviral therapy. *Gastroenterology* 2002; **123**: 719-727 [PMID: 12198698 DOI: 10.1053/gast.2002.35352]
14 **Health Insurance Review and Assessment**. Accessed July 22, 2018 Available from: URL: http://www.hira.or.kr/eng/main.do
15 **Korean Liver Cancer Study Group (KLCSG)**. National Cancer Center, Korea (NCC). 2014 KLCSG-NCC Korea Practice Guideline for the Management of Hepatocellular Carcinoma. *Gut Liver* 2015; **9**: 267-317 [PMID: 25918260 DOI: 10.5009/gnl14460]
16 **D'Amico G**, Pasta L, Morabito A, D'Amico M, Caltagirone M, Malizia G, Tinè F, Giannuoli G, Traina M, Vizzini G, Politi F, Luca A, Virdone R, Licata A, Pagliaro L. Competing risks and prognostic stages of cirrhosis: a 25-year inception cohort study of 494 patients. *Aliment Pharmacol Ther* 2014; **39**: 1180-1193 [PMID: 24654740 DOI: 10.1111/apt.12721]
17 **D'Amico G**, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol* 2006; **44**: 217-231 [PMID: 16298014 DOI: 10.1016/j.jhep.2005.10.013]
18 **Peng CY**, Chien RN, Liaw YF. Hepatitis B virus-related decompensated liver cirrhosis: benefits of antiviral therapy. *J Hepatol* 2012; **57**: 442-450 [PMID: 22504333 DOI: 10.1016/j.jhep.2012.02.033]
19 **Hui AY**, Chan HL, Leung NW, Hung LC, Chan FK, Sung JJ. Survival and prognostic indicators in patients with hepatitis B virus-related cirrhosis after onset of hepatic decompensation. *J Clin Gastroenterol* 2002; **34**: 569-572 [PMID: 11960072 DOI: 10.1097/00004836-200205000-00018]
20 **Das K**, Das K, Datta S, Pal S, Hembram JR, Dhali GK, Santra A, Chowdhury A. Course of disease and survival after onset of decompensation in hepatitis B virus-related cirrhosis. *Liver Int* 2010; **30**: 1033-1042 [PMID: 20492502 DOI: 10.1111/j.1478-3231.2010.02255.x]
21 **Shim JH**, Lee HC, Kim KM, Lim YS, Chung YH, Lee YS, Suh DJ. Efficacy of entecavir in treatment-naïve patients with hepatitis B virus-related decompensated cirrhosis. *J Hepatol* 2010; **52**: 176-182 [PMID: 20006394 DOI: 10.1016/j.jhep.2009.11.007]
22 **Hosaka T**, Suzuki F, Kobayashi M, Seko Y, Kawamura Y, Sezaki H, Akuta N, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H. Long-term entecavir treatment reduces hepatocellular carcinoma incidence in patients with hepatitis B virus infection. *Hepatology* 2013; **58**: 98-107 [PMID: 23213040 DOI: 10.1002/hep.26180]

P- Reviewer: Kanda T, Kim DJ, Niu ZS, Sirin G **S- Editor**: Ma RY
L- Editor: A **E- Editor**: Bian YN

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 October 28; 24(40): 4615-4616

DOI: 10.3748/wjg.v24.i40.4615

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

LETTERS TO THE EDITOR

To our readers: Important questions from the editors/ editorial board

Andrzej S Tarnawski

Andrzej S Tarnawski, Gastroenterology Research Department, University of California Irvine and the Veterans Administration Long Beach Healthcare System, Long Beach, CA 90822, United States

ORCID number: Andrzej S Tarnawski (0000-0002-6813-6698).

Author contributions: Tarnawski AS designed the overall concept, outline of this manuscript and was responsible for writing, and editing of the manuscript.

Conflict-of-interest statement: Tarnawski AS has nothing to disclose.

Manuscript source: Invited manuscript

Correspondence to: Andrzej S Tarnawski, MD, PhD, DSc, FACG, AGAF, Professor, Gastroenterology Research Department, University of California Irvine and the Veterans Administration Long Beach Healthcare System, 5901 E. Seventh Street, Long Beach, CA 90822, United States. atarnawski@yahoo.com
Telephone: +1-562-8264956
Fax: +1-562-8265675

Received: September 5, 2018
Peer-review started: September 5, 2018
First decision: October 8, 2018
Revised: October 12, 2018
Accepted: October 15, 2018
Article in press: October 15, 2018
Published online: October 28, 2018

Abstract

This letter proposes updating the *World Journal of Gastroenterology* (*WJG*) with several new items and subsections and seeks the readers' input for these suggested changes. In order to further enhance the scope of the *WJG*, to make it more interactive, and to accommodate our readers' interest, the editors ask the readers for their input regarding potential new proposed changes and new subsections listed below: (1) new subsection - selected highlights from other related Baishideng Publishing Group world series journals; (2) new subsection - challenging images of the week - a quiz with an answer and references on another page; (3) to re-introduce or restore for some issues the cover page on which selected illustration; (4) new subsection - new hypotheses, new concepts and revisiting old concepts in a short, commentary format; (5) preview of the forthcoming issue articles (title and authors); (6) suggestions submitted by the readers - new topics and initiatives; and (7) additional topic area - molecular basis of gastric/liver diseases as an additional area for articles submitted to the journal.

Key words: *World Journal of Gastroenterology*; Readers' input; Proposal; Changes; New subsections

Core tip: In this letter, we propose updating the *World Journal of Gastroenterology* (*WJG*) with several new items and sections, and seek the readers' input for these suggested changes. In order to further enhance the scope of the *WJG*, to make it more interactive, and to accommodate our readers' interest, the editors ask the readers for their input regarding potential new proposed changes including new subsections.

Tanawski AS. To our readers: Important questions from the editors/editorial board. *World J Gastroenterol* 2018; 24(40): 4615-4616 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i40/4615.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i40.4615

TO THE EDITOR

In order to further enhance the scope of the *World Journal of Gastroenterology* (*WJG*), to make it more interactive, and to accommodate our readers' interest, we ask the readers for their input regarding potential new proposed changes and new subsections listed below:

(1) New subsection - Selected highlights from other related Baishideng Publishing Group (BPG) world series journals (*World Journal of Gastrointestinal Oncology*, *World Journal of Gastrointestinal Endoscopy*, *World Journal of Gastrointestinal Pathophysiology*, *World Journal of Gastrointestinal Surgery*, and others) notable, important articles - title, authors, abstract and perhaps one key figure; a maximum 2-3 per 1 *WJG* Issue. This would provide not only a new information but also would facilitate exposure of the *WJG* readers to important papers in other BPG world series journals. The editors of these other BPG journals would select the best papers.

(2) New subsection - challenging images of the week - a quiz with an answer and references on another page. Two to three images per issue - endoscopic, computed tomography (CT)-scan, magnetic resonance imaging (MRI), histology, *etc*. Some of the images can be used from these other BPG journals. Some of the examples are in the "Welcome to the *World Journal of Gastroenterology*" sample issue (at https://www.wjgnet.com/1007-9327/) but we would introduce them to each or every second issue. The quiz could originate from the current or archival issues of other BPG journals, but the readers will be also welcome to submit a quiz with detailed labeling, explanation, and answers.

(3) To re-introduce or restore for some issues the cover page on which selected illustration and/or visual abstracts from current articles will be presented (when worthwhile) as well as the photos of the key authors of frontiers and major editorial articles.

(4) New subsection - new hypotheses, new concepts and revisiting old concepts in a short, commentary format.

(5) Preview of the forthcoming *WJG* issue articles (title and authors).

(6) Suggestions submitted by the readers - new topics and initiatives after careful selection by the editors for content that could improve the quality of the journal: the readers, their voice, initiative, and suggestions are very important and they count.

(7) Additional topic area - molecular basis of gastric/liver diseases as an additional area for articles submitted to the journal.

The above suggestions are for the sake of the discussion and exchanging ideas, and naturally, not all are doable at this time.

Thank you for your consideration.

P- Reviewer: Contini S, Ikura Y, Slomiany BL, Tai DI
S- Editor: Ma RY **L- Editor**: A **E- Editor**: Bian YN

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327