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

World Journal of *Gastroenterology*

World J Gastroenterol 2018 August 21; 24(31): 3469-3566

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 31 August 21, 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
August 21, 2018

World Journal of Gastroenterology

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

World J Gastroenterol 2018 August 21; 24(31): 3469-3471

DOI: 10.3748/wjg.v24.i31.3469

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

EDITORIAL

Locoregional therapy response in patients with hepatocellular cancer waiting for liver transplantation: Only selection or biological effect?

Quirino Lai, Michele Di Martino, Pierleone Lucatelli, Gianluca Mennini

Quirino Lai, Gianluca Mennini, Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Rome 00161, Italy

Michele Di Martino, Pierleone Lucatelli, Department of Radiology, Sapienza University of Rome, Rome 00161, Italy

ORCID number: Quirino Lai (0000-0003-1487-3235); Michele Di Martino (0000-0003-1504-1166); Pierleone Lucatelli (0000-0002-7448-1404); Gianluca Mennini (0000-0002-6412-6863).

Author contributions: Lai Q, Di Martino M, Lucatelli P and Mennini G 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: Quirino Lai, MD, PhD, Academic Fellow, Academic Research, Doctor, Senior Lecturer, Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Viale del Policlinico 155, Rome 00161, Italy. lai.quirino@libero.it
Telephone: +39-34-93020126
Fax: +39-6-499701

Received: May 18, 2018
Peer-review started: May 19, 2018
First decision: May 29, 2018
Revised: June 29, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: August 21, 2018

Abstract

Locoregional treatments (LRT) represent a broad strategy used for reducing the risk of drop-off and contextually improving the survivals in patients with hepatocellular cancer receiving a liver transplantation (LT). However, it is not sufficiently clear if LRT are only a surrogate of tumor aggressiveness or if they consent a real benefit in terms of tumor stabilization. A recent study by Pommergaard *et al* reported the results from the European Liver Transplant Registry. Patients receiving LRT before LT had better 5-year survival rates respect to no-LRT cases (69.7% *vs* 65.8%; $P < 0.001$). When the number of LRT was tested, one-to-two treatments were connected with improved survivals respect to no treatment [hazard ratio (HR) = 0.85 and 0.71, respectively]. The efficacy of LRT was also reported in the presence of larger tumors (HR = 0.78) and micro-macrovascular invasion (HR = 0.71). The results observed in the present study are partially in discordance with other analyses showing a detrimental effect of LRT. The main problem in the interpretation of these results is connected with the possible initial selection biases present in the studies. The most recent guidelines suggest to perform LRT before the transplant, but the level of evidence is typically low due to the absence of prospectively designed studies.

Key words: Allocation; Recurrence; Trans-arterial chemo-embolization; Radiofrequency ablation; Model for end-stage liver disease

Core tip: The role of locoregional treatments in the setting of hepatocellular cancer and liver transplantation is controversial. On one side, neoadjuvant approaches should consent a selection of tumor aggressiveness. On the other side, a real survival improvement thanks to the tumor ablation should be achieved. Recent evidences report an effective beneficial role of locoregional strategies in terms of survival and recurrence. However, several biases must be taken into account in these studies, due to the heterogeneous characteristics of treated *vs* untreated subjects. Further studies are need with the intent to clarify this important topic.

Lai Q, Di Martino M, Lucatelli P, Mennini G. Locoregional therapy response in patients with hepatocellular cancer waiting for liver transplantation: Only selection or biological effect? *World J Gastroenterol* 2018; 24(31): 3469-3471 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3469.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3469

INTRODUCTION

Liver transplantation (LT) represents the gold-standard treatment in patients with unresectable hepatocellular cancer (HCC) developed on underlying cirrhosis[1]. Unfortunately, LT represents a scarce resource, mainly due to the limited number of available donors[2]. Thus, in patients awaiting LT, disease burden may progress beyond the conventional LT criteria while on the waiting list[3]. Several strategies have been adopted with the intent to alleviate the risk of drop-out due to tumor progression: for example, Model for End-Stage Liver Disease (MELD) exception points are routinely used in several regions in the presence of T2 HCCs[4]. Another widespread strategy is the use of locoregional treatments (LRTs) as a neo-adjuvant strategy with the intent to bridge patients to LT[5] or downstage patients initially outside transplantation criteria[6].

Response to LRT has been correlated with improved post-LT survival rates in several studies[7,8]. However, it is not sufficiently clear if LRTs are only a surrogate of tumor aggressiveness, efficaciously selecting patients with favorable tumor biology, or if they are beneficial concerning tumor stabilization, mainly in case of complete or partial response.

STUDY ANALYSIS

In a recent Issue on Transplant International, Pommergaard *et al*[9] reported the results of a multicentric study based on the European Liver Transplant Registry (ELTR) and focused on the use of LRT in HCC patients undergoing LT. A total of 4978 patients (no LRT = 1406, 28.2%; LRT = 3572, 71.8%) were enrolled. As expected, the median waiting time was longer in the LRT group (4 mo *vs* 1.7 mo; $P < 0.001$) and the median MELD score was higher in the directly transplanted subjects (12 *vs* 10; $P < 0.001$). Overall, patients receiving LRT before LT had better 5-year survival rates respect to no-LRT cases (69.7% *vs* 65.8%; $P < 0.001$). When the different treatment types were investigated, the use of radiofrequency ablation (RFA) had the strongest association with an improved overall survival [hazard ratio (HR) = 0.51]. The beneficial effect was also observed in case of the combination of RFA and trans-arterial chemo-embolization (TACE) (HR = 0.74). Several sub-analyses were also done. As for the number of LRT performed, one-two treatments were connected with improved survivals respect to no treatment (HR = 0.85 and 0.71, respectively). On the opposite, three or more treatments showed no association (HR = 1.11).

When a subclass of HCCs being larger (> 3 cm) or with more nodules (> 5 lesions) was examined, LRT maintained their protective role for the risk of death (HR = 0.78). In this context, RFA, TACE or combined RFA + TACE all were significantly associated with improved survival (HR = 0.54, 0.81, and 0.60, respectively).

Stratifying the entire population according to the underlying liver status (cirrhosis *vs* non-cirrhotic liver), in case of HCC on cirrhosis LRT were also protective (HR = 0.86).

In the presence of pathological micro-macrovascular invasion, the effect of LRT was strong (HR = 0.71). Both RFA and TACE (HR = 0.54 and 0.69, respectively) were associated with improved survival.

PERSPECTIVE

The role of LRT in the setting of HCC and LT has not been fully clarified, mainly in light of the potential detrimental effects of repetitive treatments. For example, a recent meta-analysis performed on 1122 TACE patients showed an increased risk of post-LT hepatic artery complications (odds ratio = 1.57; $P = 0.02$)[10]. Another study from the US performed on 3601 patients all meeting the Milan Criteria, showed that the increasing number of LRT significantly predicted post-LT recurrence (3 LRTs: HR = 2.1; $P < 0.001$; 4 + LRTs: HR = 2.5; $P < 0.001$)[5]. Interestingly, LRT patients achieving complete response had superior 5-year recurrence-free survivals when compared with untreated cases or LRT subjects not achieving complete response (72% *vs* 69% *vs* 67%; respectively)[5]. The here described study performed on a large population of European HCC cases showed the beneficial role of LRT, mainly in case of RFA use. Moreover, the repetitive number of treatments was not connected with worse results. LRT maintained their protective role for the risk of death even when larger tumors or harmful clinical conditions like vascular invasion were investigated. It is difficult to definitively clarify if the LRT only select low-risk HCC, or if their ability of tumor burden zeroing should also have some impact regarding survival improvement. It is clear that

the response after LRT is a robust predictor of post-LT course. A recent large multicentric European study based on 2103 HCC patients identified the poor radiological response after LRT as one of the most important predictors for the risk of low intention-to-treat benefit after transplant[11]. Another multicentric European study performed on 276 cases all treated with LRT showed that an HCC-related remaining vital tissue in the main lesion ≥ 2 cm at pathological assessment after LT was a strong independent risk factor for post-LT recurrence (HR = 5.6; $P < 0.001$)[12]. All of these results have been positively recognized by the recent European Association for the Study of the Liver (EASL) guidelines, in which it is stated that "in LT candidates with HCC, the use of pre-transplant (neoadjuvant) loco-regional therapies is recommended if feasible, as it reduces the risk of pre-LT drop-out and aims at lowering post-LT recurrence - particularly when complete or partial tumour response are achieved"[13]. Unfortunately, although the strength of recommendation for this statement is strong, the scientific evidence is low, clearly underlining the lack of prospectively designed studies. More researchers are needed, with the intent to better explore the role of LRT concerning intention-to-treat survivals.

Moreover, we should remember the critical impact that local allocation rules and waiting time duration may play on the role and the effect of LRT. As an example, in the United States most HCC patients wait for at least six months from the diagnosis before having the opportunity to be transplanted. More studies also focused on these aspects are surely needed.

REFERENCES

1 **Lai Q**, Lerut JP. Hepatocellular cancer: how to expand safely inclusion criteria for liver transplantation. *Curr Opin Organ Transplant* 2014; **19**: 229-234 [PMID: 24811435 DOI: 10.1097/MOT.0000000000000085]

2 **Lai Q**, Melandro F, Levi Sandri GB, Mennini G, Corradini SG, Merli M, Berloco PB, Rossi M. Use of elderly donors for liver transplantation: has the limit been reached? *J Gastrointestin Liver Dis* 2011; **20**: 383-387 [PMID: 22187704]

3 **Mehta N**, Heimbach J, Lee D, Dodge JL, Harnois D, Burns J, Sanchez W, Roberts JP, Yao FY. Wait Time of Less Than 6 and Greater Than 18 Months Predicts Hepatocellular Carcinoma Recurrence After Liver Transplantation: Proposing a Wait Time "Sweet Spot". *Transplantation* 2017; **101**: 2071-2078 [PMID: 28353492 DOI: 10.1097/TP.0000000000001752]

4 **Marrero JA**, Kulik LM, Sirlin C, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; Epub ahead of print [PMID: 29624699 DOI: 10.1002/hep.29913]

5 **Agopian VG**, Harlander-Locke MP, Ruiz RM, Klintmalm GB, Senguttuvan S, Florman SS, Haydel B, Hoteit M, Levine MH, Lee DD, Taner CB, Verna EC, Halazun KJ, Abdelmessih R, Tevar AD, Humar A, Aucejo F, Chapman WC, Vachharajani N, Nguyen MH, Melcher ML, Nydam TL, Mobley C, Ghobrial RM, Amundsen B, Markmann JF, Langnas AN, Carney CA, Berumen J, Hemming AW, Sudan DL, Hong JC, Kim J, Zimmerman MA, Rana A, Kueht ML, Jones CM, Fishbein TM, Busuttil RW. Impact of Pretransplant Bridging Locoregional Therapy for Patients With Hepatocellular Carcinoma Within Milan Criteria Undergoing Liver Transplantation: Analysis of 3601 Patients From the US Multicenter HCC Transplant Consortium. *Ann Surg* 2017; **266**: 525-535 [PMID: 28654545 DOI: 10.1097/SLA.0000000000002381]

6 **Kulik L**, Heimbach JK, Zaiem F, Almasri J, Prokop LJ, Wang Z, Murad MH, Mohammed K. Therapies for patients with hepatocellular carcinoma awaiting liver transplantation: A systematic review and meta-analysis. *Hepatology* 2018; **67**: 381-400 [PMID: 28859222 DOI: 10.1002/hep.29485]

7 **Lai Q**, Nicolini D, Inostroza Nunez M, Iesari S, Goffette P, Agostini A, Giovagnoni A, Vivarelli M, Lerut J. A Novel Prognostic Index in Patients With Hepatocellular Cancer Waiting for Liver Transplantation: Time-Radiological-response-Alpha-fetoprotein-INflammation (TRAIN) Score. *Ann Surg* 2016; **264**: 787-796 [PMID: 27429025 DOI: 10.1097/SLA.0000000000001881]

8 **Lai Q**, Avolio AW, Graziadei I, Otto G, Rossi M, Tisone G, Goffette P, Vogel W, Pitton MB, Lerut J; European Hepatocellular Cancer Liver Transplant Study Group. Alpha-fetoprotein and modified response evaluation criteria in solid tumors progression after locoregional therapy as predictors of hepatocellular cancer recurrence and death after transplantation. *Liver Transpl* 2013; **19**: 1108-1118 [PMID: 23873764 DOI: 10.1002/lt.23706]

9 **Pommergaard HC**, Rostved AA, Adam R, Thygesen LC, Salizzoni M, Gómez Bravo MA, Cherqui D, De Simone P, Boudjema K, Mazzaferro V, Soubrane O, García-Valdecasas JC, Fabregat Prous J, Pinna AD, O'Grady J, Karam V, Duvoux C, Rasmussen A; European Liver and Intestine Transplant Association (ELITA). Locoregional treatments before liver transplantation for hepatocellular carcinoma: a study from the European Liver Transplant Registry. *Transpl Int* 2018; **31**: 531-539 [PMID: 29380442 DOI: 10.1111/tri.13123]

10 **Sneiders D**, Houwen T, Pengel LHM, Polak WG, Dor FJMF, Hartog H. Systematic Review and Meta-Analysis of Posttransplant Hepatic Artery and Biliary Complications in Patients Treated With Transarterial Chemoembolization Before Liver Transplantation. *Transplantation* 2018; **102**: 88-96 [PMID: 28885493 DOI: 10.1097/TP.0000000000001936]

11 **Lai Q**, Vitale A, Iesari S, Finkenstedt A, Mennini G, Spoletini G, Hoppe-Lotichius M, Vennarecci G, Manzia TM, Nicolini D, Avolio AW, Frigo AC, Graziadei I, Rossi M, Tsochatzis E, Otto G, Ettorre GM, Tisone G, Vivarelli M, Agnes S, Cillo U, Lerut J; European Hepatocellular Cancer Liver Transplant Study Group. Intention-to-treat survival benefit of liver transplantation in patients with hepatocellular cancer. *Hepatology* 2017; **66**: 1910-1919 [PMID: 28653750 DOI: 10.1002/hep.29342]

12 **Manzia TM**, Lai Q, Iesari S, Perera MTPR, Komuta M, Carvalheiro A, Shah T, Angelico R, Quaranta C, Nicolini D, Montalti R, Scarpelli M, Palmieri G, Orlacchio A, Vivarelli M, Angelico M, Lerut J, Tisone G. Impact of remnant vital tissue after locoregional treatment and liver transplant in hepatocellular cancer patients, a multicentre cohort study. *Transpl Int* 2018; Epub ahead of print [PMID: 29572974 DOI: 10.1111/tri.13153]

13 **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]

P- Reviewer: Bramhall S, Chiu KW, Inoue K, Koksal A, Ramsay MA, Therapondos G, Wang GY **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 August 21; 24(31): 3472-3487

DOI: 10.3748/wjg.v24.i31.3472

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

REVIEW

Helicobacter pylori: A foodborne pathogen?

Nicoletta C Quaglia, Angela Dambrosio

Nicoletta C Quaglia, Angela Dambrosio, Department of Emergency and Organ Transplantation, Section of Veterinary Clinic and Animal Production, University of Bari "Aldo Moro", Valenzano 70010, Italy

ORCID number: Nicoletta C Quaglia (0000-0003-4854-7390); Angela Dambrosio (0000-0002-4935-4331).

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

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

Manuscript source: Invited Manuscript

Correspondence to: Nicoletta C Quaglia, MDV, PhD, Professor, Department of Emergency and Organ Transplantation, Section of Veterinary Clinic and Animal Production, University of Bari "Aldo Moro", Strada Prov.le per Casamassima, Km 3, Valenzano 70010, Italy. nicolettacristiana.quaglia@uniba.it
Telephone: +39-080-5443849
Fax: +39-080-5443849

Received: May 2, 2018
Peer-review started: May 4, 2018
First decision: May 17, 2018
Revised: June 19, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: August 21, 2018

Abstract

Helicobacter pylori (*H. pylori*) is an organism that is widespread in the human population and is sometimes responsible for some of the most common chronic clinical disorders of the upper gastrointestinal tract in humans, such as chronic-active gastritis, duodenal and gastric ulcer disease, low-grade B-cell mucosa associated lymphoid tissue lymphoma of the stomach, and gastric adenocarcinoma, which is the third leading cause of cancer death worldwide. The routes of infection have not yet been firmly established, and different routes of transmission have been suggested, although the most commonly accepted hypothesis is that infection takes place through the faecal-oral route and that contaminated water and foods might play an important role in transmission of the microorganism to humans. Furthermore, several authors have considered *H. pylori* to be a foodborne pathogen because of some of its microbiological and epidemiological characteristics. *H. pylori* has been detected in drinking water, seawater, vegetables and foods of animal origin. *H. pylori* survives in complex foodstuffs such as milk, vegetables and ready-to-eat foods. This review article presents an overview of the present knowledge on the microbiological aspects in terms of phenotypic characteristics and growth requirements of *H. pylori*, focusing on the potential role that foodstuffs and water may play in the transmission of the pathogen to humans and the methods successfully used for the detection of this microorganism in foodstuffs and water.

Key words: *Helicobacter pylori*; Viable but nonculturable state; Foodborne pathogen; Food; Water; Animal reservoirs; Culture methods; Molecular methods

Core tip: To date, the transmission routes and reservoirs of *Helicobacter pylori* (*H. pylori*) are topics of debate. Epidemiological evidence and the occurrence of *H. pylori* in foods of animal origin, vegetables and water corroborate the hypothesis advanced by numerous authors that *H. pylori* may be a foodborne pathogen. The present review is focused on the

evidence supporting the role of foods and water in the transmission of *H. pylori* to humans and on the methods for detecting the pathogen in foodstuffs and water.

Quaglia NC, Dambrosio A. *Helicobacter pylori*: A foodborne pathogen? *World J Gastroenterol* 2018; 24(31): 3472-3487 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3472.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3472

INTRODUCTION

The first isolation of *Helicobacter pylori* (*H. pylori*) in 1982 by Marshall and Warren[1,2] marked a turning point in understanding gastrointestinal microbial ecology and disease[3]. Following the initial scepticism regarding the aetiologic importance of this organism, it is now recognized that infections with *H. pylori* are linked to some of the most common chronic clinical disorders of the upper gastrointestinal tract in humans[4]. In fact, *H. pylori* has been acknowledged as a major cause of chronic-active gastritis and is associated with duodenal and gastric ulcer disease, low-grade B-cell mucosa-associated lymphoid tissue lymphoma of the stomach (MALToma)[5], and gastric adenocarcinoma, which is the third leading cause of cancer death worldwide[4,6,7]. Furthermore, *H. pylori* has been linked to a variety of extra-gastric disorders, including coronary heart disease, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease, thrombocytopenic purpura, and iron deficiency anaemia[8].

Human infection by *H. pylori* is a great public health hazard because *H. pylori* colonizes the gastric mucosa of approximately half of the world's population[9-12]. The infection is usually acquired in infancy and early childhood, and it is long lasting, often remaining for the entire lifespan[13]. The prevalence of *H. pylori* shows large geographical variation, with infection rates much higher in developing countries (in some areas > 85%) than in Europe and North America (approximately 30%-40%)[14,15]. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages[16]. The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people. A comparison of prevalence rates by age suggests that the acquisition of *H. pylori* is decreasing in recent cohorts, and this finding is most apparent in developed countries and may be linked to improvements in hygiene practices. Furthermore, it has been estimated that between two and 20 percent of people infected with *H. pylori* develop peptic ulcer disease[17].

Although *H. pylori* can cause severe illnesses with a high rate of morbidity and mortality, the complex interactions between this microbe and humans, particularly its transmission pathways to humans and reservoirs, are largely unknown, although multiple routes of transmission have been suggested[3,18-20]. The current literature suggests that the transmission of *H. pylori* occurs from person to person *via* the oral-oral, faecal-oral, and gastric-oral routes and that the infection dose for humans is low[6,21]. *H. pylori* may be a sex-transmitted pathogen[22,23] and may lead to fibrocystic breast changes[24]. The oral cavity can be primarily colonized by *H. pylori*, and this can be linked to later gastric infection[25]. Faecal-oral transmission has more important implications than oral-oral transmission because *H. pylori* may occur in food and water supplies subsequent to faecal contamination[26]. Furthermore, several authors have considered *H. pylori* to be a foodborne pathogen because of its microbiological and epidemiological characteristics[6,10,27-31]. Information on the distribution of *H. pylori* in water, vegetables and foods of animal origin is critical in determining its potential transmission in foods.

This review article presents a brief overview of the present knowledge on the microbiological characteristics of *H. pylori* in terms of its phenotypic characteristics and growth requirements, focusing on the potential role that foodstuffs and water may play in the transmission of this pathogen to humans and the methods for isolating and detecting this microorganism in foodstuffs and water.

LITERATURE SEARCH

A PubMed search was conducted using the following keywords and phrases: "*Helicobacter pylori*, *Helicobacter pylori* and food, *Helicobacter pylori* and milk, *Helicobacter pylori* and water, VBNC, survival of *Helicobacter pylori*". In addition, we performed a manual review of the reference lists of the primary and review articles to ensure identification of all relevant articles.

MICROBIOLOGICAL CHARACTERISTICS

Phenotypic characteristics

H. pylori was originally thought to be a species belonging to the genus *Campylobacter* and was first named *Campylobacter pyloridis*, which was later corrected to *Campylobacter pylori* (*C. pylori*)[32]. Because subsequent 16S rRNA sequence analysis showed that the distance between the species belonging to the genus *Campylobacter* and *C. pylori* was sufficient to exclude *C. pylori* from this genus[33], it was renamed *Helicobacter pylori*[34], the first member of the new genus *Helicobacter*.

H. pylori organisms are spiral or curved bacilli ranging from 0.3 to 1.0 μm in width and 1.5 to 10.0 μm in length; they are gram-negative and assume a rod-like shape when cultured on solid medium[34]. Furthermore, after prolonged *in vitro* culture and under adverse environmental conditions, such as an insufficient supply of nutrients, desiccation, lack of protection against oxygen, and exposure to antimicrobial agents, *H. pylori* can survive entering the viable but non-culturable

(VBNC) state, changing its rod-like shape to a coccoid shape[35-37].

When this morphological change occurs, *H. pylori* is unable to grow on agar plates using conventional cultivation methods[38-40].

Bacteria in the VBNC state maintain their metabolic activity, pathogenicity and ability to return to active regrowth conditions[41,42]. For *H. pylori,* the ability to return to active regrowth conditions has not yet been proven. Nevertheless, the aptitude of *H. pylori* to overcome stressed conditions is very significant for public health[29], even if the role of VBNC in the transmission of *H. pylori*, especially by food and water, is still controversial.

H. pylori is motile and usually possesses four to six unipolar-sheathed flagella, which may be an adaptation to survive in gastric juices[3,43].

Growth requirements

Since the discovery of *H. pylori*, bacterial culture has been used as a routine diagnostic test and is considered the gold standard. *H. pylori* culture is recommended for performing antibiotic susceptibility testing if primary resistance to clarithromycin is higher than 20% or after failure of second-line treatment[44]. Despite the long use of bacterial culture, to date, there are no defined media for the selective culture of *H. pylori* because of its fastidious nature with particular growth requirements of atmosphere, nutrient-rich media, high humidity (98%), and long incubation time (5-7 d)[3,44].

H. pylori is a capnophilic organism that requires an atmosphere with a high level of CO_2 (from 5% to 10%). It has been considered a microaerophile, but the concentration of O_2 required for its growth is still a topic of discussion[44].

H. pylori requires a complex culture substrate (solid or liquid) with some forms of supplementation, such as whole sheep or horse blood, haemoglobin, serum, coal, yeast, or yolk emulsion[45,46], which may serve as nutritional substrates. These supplements also detoxify the medium and protect the microorganism[7].

Furthermore, if isolation is attempted from samples with basic microbial flora, it is necessary to make the media selective through supplementation of several antibiotics[47].

Growth in liquid media is enhanced by agitation, which allows gas dispersion and incubation in a CO_2-rich atmosphere[31,47].

H. pylori grows within a temperature range of 30 ℃ to 37 ℃, with optimum growth at 37 ℃, but is not able to grow at 25 ℃[43]. At 42 ℃, growth is variable[29].

Similar to *C. jejuni*, *H. pylori* survives longer at 4 ℃ than at room temperature, and it grows within a pH range of 4.5 to 7.3, with optimum at pH 5.5. *H. pylori* grows well in the presence of 0.5% and 1% NaCl but not of 2% NaCl. The minimum water activity (a_w) for growth is between 0.96 and 0.98. These data suggest that this microorganism is most likely not able to grow in many types of food[47].

H. pylori is catalase and oxidase positive; it is also characterized by strong urease activity and is negative for hippurate and nitrate reduction, characteristics that discriminate it from species belonging to the genus *Campylobacter*[29].

EVIDENCE SUPPORTING THE ROLE OF FOODS IN TRANSMISSION OF *H. PYLORI* TO HUMANS

Since 1997, when the transmission of *H. pylori* through water and foods was hypothesized for the first time, several studies have evaluated the survival and the presence of this microorganism in different foodstuffs (Tables 1 and 2).

One of the most important topics supporting this thesis was the phylogenetic proximity of *H. pylori* to *C. jejuni*, which led to the hypothesis that the transmission pathways described for the latter could also be applied to *H. pylori*[27]. Several investigators have also considered *H. pylori* a foodborne pathogen based on some of its epidemiological characteristics[27,28,30] such as the high prevalence of infection within closed family groups and among individuals living in institutions[48]. These aspects suggest that in addition to direct transmission, this bacterium may be transmitted indirectly through a common source, such as through consumption of the "same foods at the same table"[26]. The finding that the prevalence of *H. pylori* infection is greater in geographical areas in which the hygienic conditions of life are poor also supports this hypothesis[30,49].

Additional indirect evidence of the transmission of *H. pylori* to humans through foods of animal origin has been provided by epidemiological studies on the presence of antibodies in slaughterhouse workers and in veterinary workers. The incidence rates in these workers were positive and were greater than those in workers who had no direct contact with carcasses[50,51] (Figure 1).

Foods presenting intrinsic factors, such as a_w higher than 0.97 and pH ranging from 4.9 to 6.0, could theoretically provide conditions for *H. pylori* survival[30,52].

Therefore, data on survival ability may be more important than concerns about the growth of the microorganism in foods when determining the role of foods in *H. pylori* transmission to humans[53].

Survival of H. pylori in foodstuffs

Several studies have demonstrated the survival of *H. pylori* in water, milk, ready-to-eat foods, vegetables, pasteurized apple and orange juices, ground beef and dry fermented sausages[26,53-60] (Table 1).

H. pylori is able to survive in artificially contaminated milk stored at 4 ℃ for several days (from 5 to 9 d in pasteurized milk and from approximately 6 to 12 d in sterile milk)[26,53,54,56]. These findings corroborate the hypothesis that post-processed contaminated milk may play a more effective role than other foods in the transmission of *H. pylori* infection due to the intrinsic

Table 1 Studies evaluating the survival of *Helicobacter pylori* in artificially contaminated foods

Year	Food	Method	Observations	Ref.
1998	Sterilized milk	Bacterial count on chocolate agar	10 d at 4 ℃ 3 d at 25 ℃	Fan *et al*[54]
2001	Pasteurized milk water tofu, tofu, yogurt, lettuce and chicken	Bacterial count on tryptic soy agar, non-selective Wilkins-Chalgren Anaerobe blood agar and selective Wilkins-Chalgren Anaerobe blood agar	from 5 to 7 d in pasteurized milk, tofu and water tofu at 4 ℃ for up to 2 d in lettuce and raw chicken at 4 ℃ for up to 1 d in yogurt at 4 ℃	Poms *et al*[26]
2000	Ground beef packaged in vacuum and air	Bacterial count on *H. pylori* special peptone agar	6 d in ground beef packaged in air at 4 ℃ 3-6 d in ground beef packaged in vacuum at 4 ℃ 3 d in ground beef packaged in air and in vacuum at -18 ℃	Stevenson *et al*[55]
2002	Ground beef, sterile milk, and apple and orange juices	Bacterial count on brain heart infusion agar and horse serum	7 d in ground beef at 4 ℃ 11 d in irradiated ground beef at 4 ℃ 6 d in sterile milk at 4 ℃ 1 d in apple and orange juice at 4 ℃ and 25 ℃	Jiang *et al*[56]
2004	Lettuce and carrots	Bacterial count on *Helicobacter* special peptone agar and Columbia blood agar	3 d in lettuce at 8 ℃ 5 d in sterilized carrot at 8 ℃ 3 d in sanitized carrot at 8 ℃	Gomes *et al*[57]
2007	Sterile milk and pasteurized milk	Bacterial count on Wilkins-Chalgren anaerobe agar	12 d in sterile milk at 4 ℃ 9 d in pasteurized milk at 4 ℃	Quaglia *et al*[53]
2010	Spinach	Bacterial count on brucella blood agar, Wilkins-Chalgren anaerobe blood agar	6 d at 8 ℃	Buck *et al*[58]
2011	Traditional Turkish fermented sausage (*sucuk*)	Bacterial count on Wilkins-Chalgren anaerobe blood agar	7 d	Guner *et al*[59]
2017	Spring onion, cabbage, lettuce and spinach	Bacterial count on non-selective Blood base agar with 5% horse blood	3 d in spring onion, lettuce and spinach 4 d in cabbage stored at 4 ℃	Ng *et al*[60]

characteristics of this organism[53]. It is well known that *H. pylori*'s ability to survive in an acidic pH environment is urea dependent[3], and because urea is present in milk[61], the urea-dependent acid resistance of *H. pylori* may account for the long-term survival of *H. pylori* in this foodstuff[47]. Moreover, the microorganism is able to survive in milk for longer than the best-before date on an open milk package, making milk a possible source of transmission of this microorganism to humans. In fact, although the *H. pylori* load contaminating milk under natural conditions is unknown (although it is presumably lower than that used *in vitro*), the infection dose for humans is low; thus, even a small number of *H. pylori* cells surviving in foods may represent a potential health hazard for consumers[53].

Other studies have been conducted on the survival of microorganisms in other more complex foodstuffs. *H. pylori* survives for approximately 7 d in ground beef at 4 ℃, up to 3 d at -18 ℃[55,56] and for only 2 d in prepacked boneless, skinless chicken thighs[26]. Vacuum packaging has no impact on survival time. However, if the high level of background bacteria present in the ground beef is eliminated, survival time increases to an undetectable level (< 10 cfu/g) within 11 d[55]. The fate of *H. pylori* during the fermentation process of a traditional Turkish fermented sausage (*sucuk*) was investigated. The results of this study showed that the microorganism could survive and grow during the fermentation process of *sucuk* (22 ℃ for 7 d). A possible explanation is that some fermentation products, such as protein degradation compounds and CO_2, might have been used by this pathogen and that indigenous bacteria might have created a microenvironment suitable for *H. pylori* growth[59]. In contrast, *H. pylori* is not able to survive in yogurt[26] or pasteurized fruit juice[56] because its growth is hampered by the acidic pH and organic acids from lactic acid bacteria growth[62,63].

Survival time in vegetables is shorter: 3 d in sanitized lettuce and carrot stored at 8 ℃, 4 d in sterilized carrot and 5 d in carrot packaged in a modified atmosphere[57]. A possible explanation could be the not-robust nature of this bacterium that on the surface of vegetables is exposed to oxygen and desiccation as opposed to what happens in liquid food and the presence of a high load of natural bacterial flora. Moreover, *H. pylori* is able to survive in contaminated vegetables despite the abovementioned adverse conditions, as it is able to form biofilms[60]. However, a study on the survival of *H. pylori* in artificially contaminated spinach showed that this bacterium is able to survive for up to 6 d in VBNC forms that are still viable and can maintain its virulence factors despite its lack of cultivability[58].

Occurrence of H. pylori in foodstuffs

Based on these findings, several studies have attempted to prove the occurrence of *H. pylori* in foodstuffs (Table

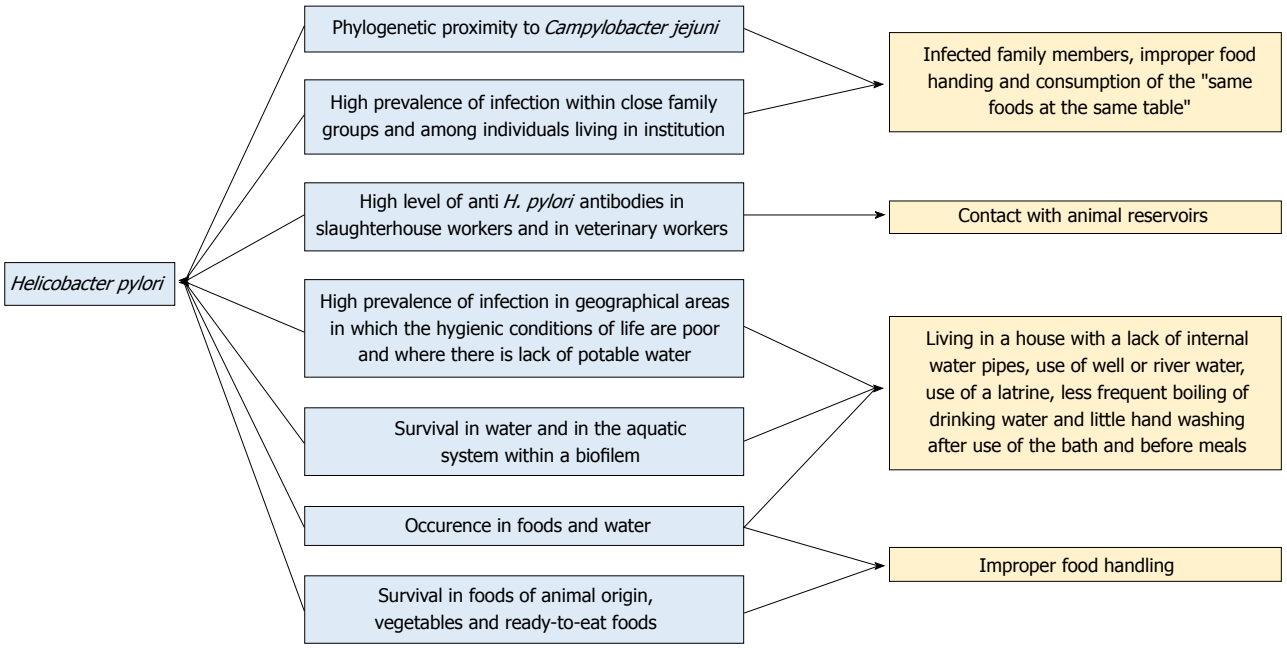


Figure 1 Evidence supporting the role of foods and water in the transmission of *Helicobacter pylori* to humans. In light blue is the epidemiological evidence supporting the hypothesis that *H. pylori* is a foodborne and/or a waterborne pathogen; in orange are the risks factors for *H. pylori* infection. *H. pylori*: *Helicobacter pylori*.

2). To the best of our knowledge, the first report about the presence of *H. pylori* in sheep milk was prompted by the observation that Sardinian shepherds with direct animal contact had a higher prevalence of infection than did their same-household siblings[64]. *H. pylori* was isolated in 1 out of 38 PCR-positive raw sheep milk samples and in one out of 6 PCR-positive sheep gastric tissue samples[65].

After these findings, *H. pylori* has rarely been isolated from raw milk samples[66-69]. Bacteriological isolation of *H. pylori* occurred in one sample of raw cow milk out of 13 PCR-positive samples during a survey conducted in Japan[66]. It was also isolated in 4 samples of raw cow milk out of 20 samples analysed in Greece[67] and in Iran in 2 samples of raw sheep milk out of 11 PCR-positive samples and in 1 raw buffalo milk out of 15 PCR-positive samples[68]. Afterward, Mousavi *et al*[69] and Saedi *et al*[70] reported a higher prevalence of *H. pylori* in raw cow, sheep, goat, buffalo and camel milks in Iran than that previously mentioned.

Furthermore, only a few studies have been carried out on the occurrence of *H. pylori* in dairy products other than milk. For example, in the survey of Mousavi *et al*[69], 30% of Iranian traditional cheese, 15% of cream, 5% of butter and 27% of ice cream samples all made from unpasteurized milk were positive for *H. pylori*.

Compared to the few bacteriological isolations, the prevalence of *H. pylori* DNA is higher depending on the sensitivity of the method employed and the target gene[65,66,68,71-73]. Conversely, these findings were not confirmed in the studies conducted by Jiang and Doyle[56], Turutoglu *et al*[74] and Bianchini *et al*[75], which failed to detect *H. pylori* in cow and sheep raw milk in the United States, Turkey and Italy, respectively, by PCR and bacteriological analysis.

The attempts to culture *H. pylori* from the majority of PCR-positive samples may have been unsuccessful for several reasons: the low number of contaminating bacteria in milk samples, the presence of VBNC forms that are not detectable by conventional microbiological culture-based protocols, and the relatively long period of storage before analysis, which could have affected the vitality of the few *H. pylori* cells present in the contaminated samples[37,67,71].

Studies on the detection of *H. pylori* in food products other than milk are quite rare. *H. pylori* was isolated in 25%, 37%, 22%, 28% and 14% of cow, sheep, goat, buffalo and camel meat samples[70] and in 1.42% and 12.5% of hamburger and minced beef samples, respectively[76]. *H. pylori* DNA was detected in 36% and 44% of raw chicken and ready-to-eat raw tuna meat samples, respectively[77]. Furthermore, Hemmatinezhad *et al*[78] analysed 550 samples of ready-to-eat foods, detecting *H. pylori* in 74% of samples; olive salad (36%), restaurant salad (30%), fruit salad (28%) and soup (22%) were the most commonly contaminated. Additionally, Ghorbani *et al*[79] recovered *H. pylori* in 60 out of 300 ready-to-eat food samples (20%), including ready-to-eat fish (15%), ham (8.33%), chicken sandwiches (5%), vegetable sandwiches (18%), meat sandwiches (10%), and minced meat (32%).

Few reports have addressed the occurrence of *H. pylori* in vegetables. In two surveys conducted in Iran, many of the vegetables analysed were positive for the presence of this microorganism: 13.72%[80]

Table 2 Studies evaluating the occurrence of *Helicobacter pylori* in foods

Year	Food	Method	Number of samples *n*	Observations	Ref.
2001	Raw sheep milk	Culture and PCR	63 raw sheep milk	60% PCR positive samples 2.6% culture positive samples	Dore *et al*[65]
2002	Raw and pasteurized cow milk	Semi nested PCR, culture method and electron microscopy	18 raw cow milk	Raw milk: 72.2% semi-nested PCR positive samples; 1 culture positive sample	Fujimura *et al*[66]
			20 pasteurized milk	Pasteurized milk: 55% semi-nested PCR positive samples	
2002	Raw sheep milk	Culture	440 raw sheep milk	0% positive samples	Turutoglu *et al*[74]
2008	Raw goat, sheep, and cow milks	Nested-PCR	160 raw goat milk	25.6% positive goat milk	Quaglia *et al*[71]
			130 raw sheep milk	33% positive sheep milk	
			110 raw cow milk	50% positive cow milk	
2008	Raw chicken and ready-to- eat raw tuna	Multiplex PCR	11 raw chicken	36% positive raw chicken	Meng *et al*[77]
			18 ready-to-eat raw tuna	44% positive ready-to-eat raw tuna	
2011	Raw cow milk	FISH	20	20% positive samples	Angelidis *et al*[67]
2012	Raw cow, sheep, goat, buffalo and camel milks	PCR	75 raw cow milk	16.00% positive cow milk	Rahimi *et al*[68]
			58 raw sheep milk	13.79% positive sheep milk	
			42 raw goat milk	4.76% positive goat milk	
			20 raw buffalo milk	13.33% positive camel milk	
			15 raw camel milk	20.00% positive buffalo milk	
2014	Milk and traditional dairy products	Culture and PCR	120 raw cow milk	16.6% positive cow milk	Mousavi *et al*[69]
			100 raw goat milk	28% positive goat milk	
			100 raw sheep milk	35% positive sheep milk	
			80 raw buffalo milk	15% positive buffalo milk	
			60 raw camel milk	13.3% positive camel milk	
			60 raw donkey milk	0% positive donkey milk	
			100 cheese	30% positive cheese	
			100 butter	15% positive cream	
			100 cream	5% positive butter	
			100 ice cream	27% positive ice cream	
2014	Vegetables and salad	Culture and PCR	60 salad	16.6% positive salad	Atapoor *et al*[81]
			40 basil	12.5% positive basil	
			40 radish	7.5% positive radish	
			40 leek	20% positive leek	
			80 spinach	6.25% positive spinach	
			80 lettuce	13.75% positive lettuce	
			120 parsley	6.6% positive parsley	
2014	Washed and unwashed vegetables	Culture and PCR	430 washed and unwashed vegetable	13.72% positive vegetables and salads	Yahaghi *et al*[80]
2015	Raw cow, sheep, goat, buffalo and camel milks	PCR	75 raw cow milk	16.00% positive cow milk	Talaei *et al*[72]
			58 raw sheep milk	13.79% positive sheep milk	
			42 raw goat milk	4.76% positive goat milk	
			20 raw buffalo milk	13.33% positive camel milk	
			15 raw camel milk	20.00% positive buffalo milk	
2015	Raw cow milk	Culture and nested PCR	50 raw cow milk	22% positive cow milk	Osman *et al*[73]
2015	Raw cow milk	Culture and nested PCR	163 raw cow milk	0% positive cow milk	Bianchini *et al*[75]
2016	Raw cow, sheep, goat, buffalo and camel milks and meats	Culture and PCR	420 raw milk	21.90% positive raw milk	Saedi *et al*[70]
			400 raw meat	26.25% positive meat	
2016	Ready-to-eat food	Culture and PCR	550 ready-to-eat food	13.45% positive ready-to-eat food	Hemmatinezhad *et al*[78]
2016	Ready-to-eat food and minced meat	Culture and PCR	60 ready-to-eat fish	15% positive ready-to-eat fish	Ghorbani *et al*[79]
			60 ham	8.33% positive ham	
			40 chicken sandwich	5% positive chicken sandwich	
			40 vegetable sandwich	45% positive vegetable sandwich	
			50 meat sandwich	20% positive meat sandwich	
			50 minced meat	32% positive minced meat	
2017	Hamburger and minced meat	Culture and nested PCR	80 hamburger	1.42% positive hamburger	Gilani *et al*[76]
			70 minced-meat	12.5% positive minced-meat	

and 9.56%[81] of the vegetables and traditional salads analysed.

The high prevalence of *H. pylori* in ready-to-eat foods, meats, milks and vegetables could be due to post-

processing contamination. In fact, the high prevalence of *H. pylori* in healthy human carriers[11] suggests that foodstuff contamination due to poor hygiene management during milking, chilling and storage and during the handling, preparation and packaging of ready-to-eat foods may occur. Furthermore, *H. pylori* strains isolated from foods showed genotypes of *vac*A alleles similar to those in isolates from human clinical samples, endorsing the hypothesis that foods can be the source of *H. pylori* transmission to humans[76,78-80].

However, the existence of animal reservoirs of the microorganism cannot be excluded[65,66]. In addition, the histopathology of lesions by *H. pylori* in humans differs from that of many other gastric helicobacters, causing mild or absent inflammatory responses in their natural hosts[82]. These data suggest that *H. pylori* may have not originally evolved as a human pathogen but was likely introduced into the human population from a mammalian reservoir sometime in the distant past[65]. This hypothesis is further supported by evidence of *H. pylori* in the gastric mucosa of calves, pigs and horses[83] and its isolation from sheep gastric tissue and milk[65,84]. Furthermore, in the studies of Papiez *et al*[85] and Dore *et al*[65], *H. pylori* prevalence was higher in shepherds with direct animal contact than in controls without contact with sheep. Considering the 100% positive 13C-urea breath test in sheep, it may be reasonable to assume that these animal species may act as reservoirs and spreaders of *H. pylori*[85,86]. However, further epidemiological and experimental studies are needed to corroborate these few data.

EVIDENCE SUPPORTING THE ROLE OF WATER IN THE TRANSMISSION OF *H. PYLORI* TO HUMANS

In the last Joint Monitoring Report (JMP) of 2017, "Progress on Drinking Water, Sanitation and Hygiene" by WHO and UNICEF[87], the first global assessment of safe drinking water and sanitation services was reported. In 2015, approximately 2.1 billion people did not manage water safely, and among them, 844 million did not even have basic drinking water services, spending more than 30 min per trip to collect water from external sources, and some of them still drank untreated water from surface water sources such as streams and lakes. Globally, at least 2 billion people use a stool-contaminated source of drinking water. Contaminated water can transmit diseases such as diarrhoea, cholera, dysentery, typhoid and polio[87].

It has been estimated that *H. pylori* colonizes more than half of the world's population, and contaminated water is mentioned as one of major causes[60,88-91].

Bellack *et al*[92] developed a conceptual model of the role of water in *H. pylori* transmission. The hypothesis is that both humans and animals are long-term hosts and that water is a relatively short-term reservoir. *H. pylori* may survive in water for a period before it is ingested as drinking water, accidentally during bathing, or through other pathways involving food. The infected person will spread *H. pylori* through faeces; through direct faecal-oral transmission, an infected person can infect another person or contaminate water bodies through direct contamination with faeces or indirectly with wastewater that comes into contact with the water used to drink. Animal contamination of water reserves may occur by defecating directly in surface waters or by faeces penetrating groundwater. The type of soil and heavy rain events can play an important role because they can facilitate the penetrability of manure containing bacteria in groundwater[92].

Several epidemiological studies have been conducted on the transmission of *H. pylori* through water, and several risk factors have been highlighted, such as living in a house with a lack of internal water pipes, the use of well or river water, the use of a latrine, less frequent boiling of drinking water and little hand washing after use of the bath and before meals[93-96] (Figure 1).

In 1991, a survey carried out on 407 Peruvian Lima children aged 2 mo to 12 years from families with different socioeconomic statuses showed an overall *H. pylori* prevalence of 48%. The children underwent the 13C-urea breath test, and the results showed a higher incidence among the children of low-income families than among those of high-income families (56% *vs* 32%). An important risk factor was the water supply; incidence increased three-fold when the water sources were outside the home compared to those whose homes had internal water sources. Furthermore, the municipal water supply seemed to be an important source of infection among Lima children from families of both low and high socioeconomic status because children from high-income families whose homes had municipal water were 12 times more likely to be infected than were those from high-income families whose water supplies came from community wells[97].

The poor basic hygiene conditions and the lack of potable water have been reported as the cause of *H. pylori* infection in an epidemiological study of a population in a rural area of the state of Mato Grosso in Brazil. The survey was conducted on 40 children and adolescents and 164 adults. *H. pylori* antibodies were detected in 31 (77.5%) children and adolescents and in 139 (84.7%) adults. The most important identified risk factor is using untreated water that could be contaminated by wastewater due to the lack of a sewage system[98].

Nurgalieva *et al*[49] conducted a similar study on 233 adults and 55 children in Kazakhstan. The overall prevalence of *H. pylori* was 86% among adults and 64% among children. The prevalence of *H. pylori* infection was inversely correlated with the index of clean water (CWI) (boil water before consumption, frequency of recovery and reuse of water and frequency of bath and shower). Infection was significantly lower among those

with a high CWI (56%) than those with a moderate (79%) or low (95%) CWI. Moreover, the prevalence of *H. pylori* was inversely related to socioeconomic status. Those living in a family in which the levels of education and study were low had a higher rate of *H. pylori* infection (90%) than did those from a higher socioeconomic group (69%)[49].

In another epidemiological survey conducted in Germany on 3347 children from cities and rural areas, 179 children (119 from cities and 60 from rural areas) were infected by *H. pylori*. Among the children from rural areas, positivity significantly increased with the consumption of water from non-municipal sources[99].

Fujimura *et al*[100] studied the presence of *H. pylori* in 4 Japanese rivers and in 224 children who lived near one river using the stool antigen test for *H. pylori* prevalence.

The results of this study showed that *H. pylori* DNA was frequently present in river water from the middle and downstream reaches in which the human biosphere is embedded. The author concluded that river water in the natural environment could be a risk factor for *H. pylori* transmission.

Occurrence of H. pylori in water

Despite the several epidemiological studies that support the hypothesis that *H. pylori* is a waterborne pathogen, the real role of water in the spread of the pathogen remains a topic of discussion. As with foodstuffs, the fastidious nature of the bacterium and the difficulties in isolating it from environmental sources do not provide unequivocal evidence about the role of water as a source of transmission of this microorganism.

Culture methods, immunological methods and molecular methods have been employed to detect *H. pylori* in the aquatic environment.

Several studies on the occurrence of *H. pylori* in sewage and drinking water samples have been carried out worldwide using molecular methods. In many of these surveys, it was not possible to isolate the bacterium using culture methods (Table 3).

Based on the findings that there is an association between water sources and the prevalence of *H. pylori* infection in Peruvian children[97], *H. pylori* DNA was detected in drinking water samples from different locations near Lima (Peru) in two different surveys using molecular methods. These results provided evidence of the presence of *H. pylori* DNA in drinking water in Peru and were consistent with conclusions from a previous epidemiological study of the same population[101,102]. In addition, other studies have highlighted the presence of *H. pylori* DNA in samples of tap water, well water[103], aquatic systems located in Mexico City[104], trucks for water transport and lake water[105], further supporting the hypothesis of the transmissibility of *H. pylori* through water.

Despite the high incidence of *H. pylori* DNA in water, only a few studies have reported bacteriological isolation of this microorganism. Bacteriological isolation of *H. pylori* occurred in the study of Lu *et al*[106], who analysed untreated municipal wastewater samples using a series of steps beginning with immunomagnetic separation and cell culture.

In a survey carried out in Iraq, out of 198 samples of treated municipal drinking water, 10 strains of *H. pylori* were isolated and identified. The low concentration of chlorine in the water samples and the ability to form biofilms in water pipes[107,108] were the reasons that *H. pylori* was isolated[109].

H. pylori was also isolated in tap water, dental unit water, and bottled mineral water in Iran. Out of 200 water samples collected in Iran, 5 cultures were positive. Two out of 50 tap water samples (4%), 2 out of 35 dental unit water samples (5.8%), and 1 out of 40 samples (2.5%) from water coolers in public places were found to be contaminated with *H. pylori*[110]. Ranjbar *et al*[111] examined 450 bottled mineral water samples and confirmed the presence of *H. pylori* in bottled mineral water.

Survival studies in water samples showed that *H. pylori* could be cultured from 48 h up to 20 d in autoclaved distilled water. An increase in survival occurs at low temperatures; in fact, high temperature causes loss of culturability[89,91,112-118]. Shahamat *et al*[118] used an autoradiographic method to detect the metabolic activity of *H. pylori* VBNC in water. Four *H. pylori* strains were studied using 72-h cultures in water and incubated with [3H] thymidine for 24-72 h. After being exposed to the Kodak NTB2 emulsion for 3-28 d, the organism was vital and culturable under these conditions for up to 48 h and, in some cases, 20 to 30 d (Table 3).

One factor in support of *H. pylori* infection being waterborne or related to poor health practices is the association, which some authors claim, of *H. pylori* with free-living amoebae (FLA), such as *Acanthamoeba*, *Naegleria*, *Vermamoeba* or *Balamuthia*, which are ubiquitous protozoa commonly found in water[119-122].

Several studies have also shown that *H. pylori* can be present as a biofilm on the pipes of the drinking water system with the ability to adhere to different hydraulic materials, such as copper and stainless steel[121,123,124]. As a result, *H. pylori* is likely to survive in an aquatic system within a biofilm rather than in the planktonic state[39,125].

H. pylori cells were able to survive for short periods in chlorinated drinking water in the VBNC form, which would allow them to reach final consumption points and, at the same time, enable them to be undetectable by culture methods[115].

Moreover, in biofilms, the resistance of *H. pylori* to chlorine increases significantly[120,126]. Therefore, it is possible that if the organism enters a distribution system, it may survive disinfection treatment within the biofilm matrix[115]. This characteristic may be based on the lack of isolation in some surveys of *H. pylori*. In fact, in water samples treated with suitable potabilization

Table 3 Studies evaluating the occurrence and survival of *Helicobacter pylori* in water

Year	Water and study type	Method	Observations	Ref.
1993	Survival of *H. pylori* in artificially contaminated sterile river water	Culture Autoradiography	Culture up to 48 h	Shahamat *et al*[118]
1996	Occurrence of *H. pylori* in 48 water samples: 30 from municipal water system, 14 from community taps, 4 from brick tanks or plastic barrels of different households	IMS and PCR	50% PCR positive samples	Hulten *et al*[101]
1997	Study on *H. pylori* resistance to chlorination	Culture	*H. pylori* were readily inactivated by free chlorine	Johnson *et al*[117]
1999	Occurrence of *H. pylori* in water from rivers and ponds	IMS and nested PCR	*H. pylori*-specific DNA was detected in samples	Sasaki *et al*[103]
1999	Occurrence of *H. pylori* in water from delivery truck and two lakes	Nested PCR and Southern blot hybridization	PCR positive samples from truck PCR positive samples from two lakes	McKeown *et al*[105]
2001	Occurrence of *H. pylori* in 10 seawater samples, 10 river water samples, 10 tap water samples, 6 well water samples	IMS, real-time PCR and nested PCR	2 PCR positive samples of well water	Horiuchi *et al*[127]
2001	Occurrence of *H. pylori* in 139 ground water samples	PCR and Southern blot hybridization	69% positive samples	Mazari-Hiriart *et al*[104]
2002	Occurrence of *H. pylori* in raw municipal wastewater	IMS, culture and PCR	23 out of 37 isolated strains were confirmed to be *H. pylori* 11 out of 23 strains of *H. pylori* demonstrated *vac*A gene heterogeneity	Lu *et al*[106]
2002	Study on the susceptibility of *H. pylori* to chlorine, monochloramine, and ozone compared to that of *Escherichia coli*	Culture	*H. pylori* was more resistant than *E. coli* to chlorine and ozone but not monochloramine	Baker *et al*[116]
2004	Occurrence of *H. pylori* in water and biofilms: 11 samples from domestic proprieties, 7 samples from educational properties and from hydrants, and samples from reservoirs and water meters of 3 water utilities	Culture, IMS and PCR	All cultures were negative 26% PCR positive sample with the highest frequency in biofilm	Watson *et al*[120]
2004	Occurrence of *H. pylori* in seawater	Nested-PCR	*H. pylori* DNA only detected in fractionated water samples containing zooplanktonic organisms	Cellini *et al*[130]
2005	Occurrence of *H. pylori* in seawater	Filtration (200 mm, filter), culture and PCR	*H. pylori* was only isolated from fractionated water samples containing large zooplanktonic organisms	Cellini *et al*[131]
2005	Occurrence of *H. pylori* in 36 seawater samples	Culture and PCR	30 positive samples	Carbone *et al*[132]
2006	Study on the ability of *H. pylori* to adhere on different water-exposed abiotic surfaces	Scanning electron microscope	*H. pylori* was able to adhere to all substrates tested	Azevedo *et al*[123]
2007	Study on the ability of *H. pylori* to adhere to stainless steel 304 in different environmental conditions	Epifluorescence microscopy	*H. pylori* was able to adhere to stainless steel 304	Azevedo *et al*[124]
2007	Study on the resistance of *H. pylori* to chlorination	Culture, FISH, PCR and RT-PCR	Culture until 5 min FISH viable cells until 3 h PCR samples positive after 24 h RT-PCR positive after 24 h	Moreno *et al*[115]
2007	Survival of *H. pylori* in spiked bottled mineral water (drinking water)	Culture epifluorescence microscopy and PCR	Culture until 5 d Cell viability until 14 d	Queralt *et al*[114]
2007	Survival of *H. pylori* in spiked chlorinated filtered water (drinking water)	Culture, FISH and PCR	Culture until 5 min FISH viable cells until 3 h PCR positive after 24 h RT-PCR after 24 h	Monero-Mesonero *et al*[115]
2009	Occurrence of *H. pylori* in 75 drinking and environmental water samples and 21 natural water biofilms samples	Real-time PCR	0% positive samples	Janzon *et al* [129]
2010	Occurrence of *H. pylori* in 198 drinking water samples	Culture	10 out of 469 isolated strains were confirmed *H. pylori*	Al-Sulami *et al*[109]
2011	Occurrence of *H. pylori* in 137 seawater samples	PCR	21% of the samples were positive for *H. pylori*	Twing *et al*[134]

2013	Occurrence of *H. pylori* in 50 tap water samples, 35 dental units' water samples, and 40 bottled mineral water samples	Culture and PCR	2 positive tap water samples 2 positive water from dental unit samples 1 positive water coolers sample	Bahrami *et al*[110]
2013	Occurrence of *H. pylori* in 31 seawater samples	Culture and PCR	4 positive samples	Holman *et al*[135]
2016	Occurrence of *H. pylori* in 450 bottled mineral water samples	Culture and PCR	8 positive samples	Ranjbar *et al*[111]
2018	Occurrence of *H. pylori* in 241 drinking water samples	PCR	49 positive samples	Boehnke *et al*[102]

H. pylori: *Helicobacter pylori*.

systems, the lack of isolation can be caused by the formation of the biofilm or by the conversion of *H. pylori* in the form of VBNC[127-129].

Some important clues have emerged from Italian research on the presence of bacteria in seawater. Work by Cellini *et al*[130,131] and subsequent investigations[132] suggest that a significant reservoir for the microorganism is seawater, in which *H. pylori* can occur both in a free-living form and in association with plankton. Plankton-related *H. pylori* cells were detected in both summer and winter months depending on the flowering of Copepods and Cladocerans. The authors supposed that zooplankton organisms represent a sort of protected niche for survival of the microorganism. The finding of *H. pylori* attached to planktonic organisms is particularly interesting for the role of the latter in the seafood chain and its subsequent potential role in the spread of *H. pylori* infection[130-132]. More generally, the presence of *H. pylori* in seawater could also be a health hazard for swimmers and others using those waters for work or pleasure[133].

Moreover, *H. pylori* DNA has been isolated in 21% of samples from freshwater, estuarine and beach sites in Delaware (United States)[134] and in seawater sampled from 31 locations in Georgia, Trinidad and Puerto Rico[135]. In both reports, no correlation between the occurrence of *H. pylori* and faecal indicator bacteria was found, suggesting that standard water quality tests are ineffective in predicting the presence of this pathogen in natural waters, confirming the potential risk for *H. pylori* presence in marine waters.

METHODS FOR THE DETECTION OF *H. PYLORI* IN FOODS AND WATER

The isolation of *H. pylori* from food samples, particularly when they present high loads of accompanying microflora, is demanding and time consuming because it requires the use of selective media with numerous antibiotics, microaerophilic conditions and long incubation periods (7 d)[55,71]. The detection of *H. pylori* in food samples and water by means of conventional microbiological techniques generally employed for clinical specimens, which are unable to detect the VBNC, may yield false negative results and thus underestimate the presence of the bacterium in food; furthermore, the presence of *H. pylori* in VBNC state in food and water represents a potential microbiological risk for consumers, especially as a source of virulence factors[37,136,137].

Several solid and liquid culture media for the selective isolation of *H. pylori* from foods have been tested. The culture media most suitable for *H. pylori* growth often contain defibrinated horse or sheep blood acting as a reducing agent[29]. Furthermore, to achieve replication of *H. pylori* in broth culture, agitation is mandatory to provide good dispersion of gases throughout the liquid[31].

Brain heart infusion broth (BHIB) with growth supplement and selective agents has been evaluated[26,56]. BHIB with horse serum supplemented with porcine stomach mucin (0.3%), ferrous sulphate and sodium pyruvate (5%) or urea, along with the adjustment of the pH to 5.5 or 4.5, enhances the survival and possibly enables the growth of *H. pylori* in enrichment medium with fresh ground beef. In particular, pH 5.5 greatly enhances the growth and detectability of *H. pylori* in foods and should be considered an important factor for the detection of *H. pylori* in enrichment culture[56]. Stevenson *et al*[55] compared the growth of *H. pylori* in several liquid and solid media. None of the media tested presented an outstanding performance; only *H. pylori* special peptone agar offered the advantage of allowing the formation of the largest *H. pylori* colonies, and it was suitable for recovering *H. pylori* from environmental samples likely to be contaminated with large numbers of competing microorganisms[31,55].

Poms and Tatini[26] evaluated the efficacy for the *in vitro* isolation of *H. pylori* from foods of two solid media containing tryptic soy agar and Wilkins-Chalgren anaerobe agar supplemented with 5% defibrinated horse blood. The latter, to which several antibiotics (30 mg/L colistinmethanesulphonate, 100 mg/L cycloheximide, 30 mg/L nalidixic acid, 30 mg/L trimethoprim, and 10 mg/L vancomycin) were added, was found to be highly selective for the recovery of *H. pylori* from foods, but it lacked sufficient sensitivity to detect very low numbers of the bacterium.

Many authors have successfully used Wilkins-Chalgren anaerobe-agar or the broth developed by Poms *et al*[26] for the isolation of *H. pylori* in foods, both supplemented as described above[70,76,78-80].

However, there are still no standardized isolation protocols that are able to isolate the few *H. pylori* cells

present in samples rich in microbial flora such as food. Furthermore, the pathogen is able to enter a VBNC state that remains metabolically active but fails to develop into colonies when cultured on routine media[40]. Consequently, molecular assays have been conducted to detect *H. pylori* DNA in water and foodstuffs.

Immuno-separation (IMS) followed by PCR has been successfully used by several investigators[138-140]. The advantage of using this protocol is that it offers excellent specificity using the IMS able to concentrate the pathogen from foods, followed by high sensitivity of the molecular methods[29]. Nevertheless, it appears expensive, exacting and time consuming. Autoradiography and ATP bioluminescence have been successfully used for the detection of *H. pylori* from water, human stools and pure cultures but have never been tested on food[29,118]. In addition, the ATP bioluminescence assay does not allow for distinguishing among ATP from different cell sources when applied to a complex system such as a foodstuff[141].

A multiplex touchdown PCR (MT-PCR) method for the identification (16S rRNA gene) and genotyping (*vac*A- s1/m1, s1/m2, and s2/m2- and *cag*A genes) of *H. pylori* directly from artificially contaminated sheep milk was developed[142]. The characterization of the genes encoding virulence factors provides important information with respect to the sanitary assessment of food items because of the greater pathogenicity of certain *H. pylori* genotypes. Hence, for public health purposes, the evaluation of a food containing *H. pylori* will thus have to include the genotyping of isolates. This rapid, sensitive (15 cfu/mL) and specific molecular method presents the advantage of detecting and genotyping *H. pylori* from microbiologically complex foodstuffs in a single step[142]. A nested PCR approach has been employed for the detection of the *H. pylori glm*M gene from seawater and sheep, goat and cow milks[71,130,141]. The sensitivity of the nested PCR technique was 3 cfu/mL in all types of milk and 62 cfu/mL in seawater samples, and therefore, compared to the previously described MT-PCR, it was more sensitive for the detection of *H. pylori* from foods (3 cfu/mL *vs* 15 cfu/mL) with the same specificity[141]. Osman *et al*[73] employed nested PCR in 50 cow milk samples, and 22% were positive for the presence of the *H. pylori glm*M gene.

H. pylori, as with many other bacteria, is able to form biofilms within which it can survive due to the different protection mechanisms that the biofilm offers. Quantitative real-time PCR was developed for the detection of *H. pylori* in drinking water biofilms of different ages. The target gene was the *ure*A subunit of the *H. pylori urea* gene, which showed high specificity and sensitivity[143].

As is well known, the main limit of PCR assays is their inability to distinguish live organisms from dead organisms. PCR techniques can, however, be used to screen water and foodstuffs, thus making it necessary to use conventional isolation methods only on those samples that test positive by PCR.

In a study by Buck *et al*[58], mRNA of known virulence factor (*vac*A) was detected in VBNC *H. pylori* cells using RT-PCR. This method exploits the unstable nature of bacterial mRNA to infer pathogenic viability when *H. pylori* becomes non-cultivable[144]. The half-life of mRNA of *E. coli* and *Vibrio vulnificus* cells is approximately 3-8 min and less than 60 min, respectively[145,146]. Thus, mRNA can be an excellent indicator of viability when *H. pylori* occurs in the VBNC state. Moreover, detection of transcripts from the *vac*A virulence gene may deduce continued virulence activity of *H. pylori* when present in the VBNC state[58]. This molecular technique offers significant promise for the detection of microorganisms in water and foodstuff and is a valid alternative to culture methods.

The fluorescence in situ hybridization (FISH) assay with the rRNA-direct molecular method has been applied for the specific detection of *H. pylori* in river and wastewater samples[147] and in raw bulk tank bovine milk[67] and for the assessment of its survival in chlorinated drinking water[115,148]. The authors concluded that FISH was a rapid method for the direct detection and specific identification of viable bacteria in food[67].

CONCLUSION

Several studies report the presence and survival of *H. pylori* in foods and water, especially in milk and in ready-to-eat products, suggesting that they can be sources of infection for humans.

Although many of the findings reported in the literature are based on indirect evidence of *H. pylori* in food and water through molecular methods and there are only in a few cases on the bacteriological isolation of this microorganism, the possibility that food and water can be routes of transmission among others cannot be ruled out.

Most of the bacteriological isolations of the pathogen in foods and water have been obtained in work conducted in Iran; a possible explanation could be the greater prevalence of the disease in this geographical area than in other areas. Moreover, the discrepancy in the prevalence of *H. pylori* in the different surveys could be related to the type and number of samples tested, sampling method, experimental methodology and climate differences in the regions from which the samples were collected.

However, to confirm a definite foodborne and waterborne role of *H. pylori* transmission, more surveys are needed on the presence of *H. pylori* in other foods of animal origin, particularly in seafood, and on the survival ability of this microorganism in dry fermented sausages and dairy products. Further investigations on the possible role of humans and animals as reservoirs of the microorganism are also required to clarify the faecal-oral route of transmission and the method of food and water contamination. Finally, the development of molecular biology methods and, above all, bacteriological isolation methods of *H. pylori* from

water and food would add provide data that could confirm or deny the role of *H. pylori* as a foodborne and waterborne pathogen.

REFERENCES

1 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]
2 **Marshall BJ.** History of the discovery of Campylobacter pylori. In: Blaser MJ. Campylobacter pylori in gastritis and peptic ulcer disease. New York: Igaku Shoin Publishers, 1989: 7-24
3 **Dunn BE**, Cohen H, Blaser MJ. Helicobacter pylori. *Clin Microbiol Rev* 1997; **10**: 720-741 [PMID: 9336670]
4 **Kao CY**, Sheu BS, Wu JJ. Helicobacter pylori infection: An overview of bacterial virulence factors and pathogenesis. *Biomed J* 2016; **39**: 14-23 [PMID: 27105595 DOI: 10.1016/j.bj.2015.06.002]
5 **Leja M**, Axon A, Brenner H. Epidemiology of Helicobacter pylori infection. *Helicobacter* 2016; **21** Suppl 1: 3-7 [PMID: 27531531 DOI: 10.1111/hel.12332]
6 **Kusters JG**, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* 2006; **19**: 449-490 [PMID: 16847081 DOI: 10.1128/CMR.00054-05]
7 **Hatakeyama M**, Brzozowski T. Pathogenesis of Helicobacter pylori infection. *Helicobacter* 2006; **11** Suppl 1: 14-20 [PMID: 16925606 DOI: 10.1111/j.1478-405X.2006.00424.x]
8 **Kuo CH**, Chen YH, Goh KL, Chang LL. Helicobacter pylori and Systemic Disease. *Gastroenterol Res Pract* 2014; **2014**: 358494 [PMID: 24772167 DOI: 10.1155/2014/358494]
9 **Torres J**, Pérez-Pérez G, Goodman KJ, Atherton JC, Gold BD, Harris PR, la Garza AM, Guarner J, Muñoz O. A comprehensive review of the natural history of Helicobacter pylori infection in children. *Arch Med Res* 2000; **31**: 431-469 [PMID: 11179581 DOI: 10.1016/S0188-4409(00)00099-0]
10 **Frenck RW Jr**, Clemens J. Helicobacter in the developing world. *Microbes Infect* 2003; **5**: 705-713 [PMID: 12814771 DOI: 10.1016/S1286-4579(03)00112-6]
11 **Olivares D**, Gisbert JP. Factors involved in the pathogenesis of Helicobacter pylori infection. *Rev Esp Enferm Dig* 2006; **98**: 374-386 [PMID: 16944998]
12 **Axon A**. How to influence health providers. *Helicobacter* 2007; **12** Suppl 2: 80-84 [PMID: 17991182 DOI: 10.1111/j.1523-5378.2007.00569.x]
13 **Singh M**, Prasad KN, Yachha SK, Saxena A, Krishnani N. Helicobacter pylori infection in children: prevalence, diagnosis and treatment outcome. *Trans R Soc Trop Med Hyg* 2006; **100**: 227-233 [PMID: 16260014 DOI: 10.1016/j.trstmh.2005.03.009]
14 **Ozaydin N**, Turkyilmaz SA, Cali S. Prevalence and risk factors of Helicobacter pylori in Turkey: a nationally-representative, cross-sectional, screening with the ^{13}C-Urea breath test. *BMC Public Health* 2013; **13**: 1215 [PMID: 24359515 DOI: 10.1186/1471-2458-13-1215]
15 **Khedmat H**, Karbasi-Afshar R, Agah S, Taheri S. Helicobacter pylori Infection in the general population: A Middle Eastern perspective. *Caspian J Intern Med* 2013; **4**: 745-753 [PMID: 24294467]
16 **Perez-Perez GI**, Rothenbacher D, Brenner H. Epidemiology of Helicobacter pylori infection. *Helicobacter* 2004; **9** Suppl 1: 1-6 [PMID: 15347299 DOI: 10.1111/j.1083-4389.2004.00248.x]
17 **Breckan RK**, Paulssen EJ, Asfeldt AM, Kvamme JM, Straume B, Florholmen J. The All-Age Prevalence of Helicobacter pylori Infection and Potential Transmission Routes. A Population-Based Study. *Helicobacter* 2016; **21**: 586-595 [PMID: 27172105 DOI: 10.1111/hel.12316]
18 **Goodman KJ**, Correa P. The transmission of Helicobacter pylori. A critical review of the evidence. *Int J Epidemiol* 1995; **24**: 875-887 [PMID: 8557443 DOI: 10.1093/ije/24.5.875]
19 **Brown LM**. Helicobacter pylori: epidemiology and routes of transmission. *Epidemiol Rev* 2000; **22**: 283-297 [PMID: 11218379]
20 **Allaker RP**, Young KA, Hardie JM, Domizio P, Meadows NJ. Prevalence of helicobacter pylori at oral and gastrointestinal sites in children: evidence for possible oral-to-oral transmission. *J Med Microbiol* 2002; **51**: 312-317 [PMID: 11926736 DOI: 10.1099/0022-1317-51-4-312]
21 **Mitchell H**, Katelaris P. Epidemiology, clinical impacts and current clinical management of Helicobacter pylori infection. *Med J Aust* 2016; **204**: 376-380 [PMID: 27256648 DOI: 10.5694/mja16.00104]
22 **Singh V**, Trikha B, Vaiphei K, Nain CK, Thennarasu K, Singh K. Helicobacter pylori: evidence for spouse-to-spouse transmission. *J Gastroenterol Hepatol* 1999; **14**: 519-522 [PMID: 10385058 DOI: 10.1046/j.1440-1746.1999.01908.x]
23 **Schütze K**, Hentschel E, Dragosics B, Hirschl AM. Helicobacter pylori reinfection with identical organisms: transmission by the patients' spouses. *Gut* 1995; **36**: 831-833 [PMID: 7615268 DOI: 10.1136/gut.36.6.831]
24 **Kast RE**. Some fibrocystic breast change may be caused by sexually transmitted H. pylori during oral nipple contact: supporting literature and case report of resolution after gut H. pylori eradication treatment. *Med Hypotheses* 2007; **68**: 1041-1046 [PMID: 17113238 DOI: 10.1016/j.mehy.2006.09.050]
25 **Yee JKC**. Are the view of Helicobacter pylori colonized in the oral cavity an illusion? *Exp Mol Med* 2017; **49**: e397 [PMID: 29170474 DOI: 10.1038/emm.2017.225]
26 **Poms RE**, Tatini SR. Survival of Helicobacter pylori in ready-to-eat foods at 4 degrees C. *Int J Food Microbiol* 2001; **63**: 281-286 [PMID: 11246912 DOI: 10.1016/S0168-1605(00)00441-4]
27 **Wesley IV**. Helicobacter and Arcobacter: Potential human foodborne pathogens? *Trends Food Sci Technol* 1997; **8**: 293-299
28 **Meng J**, Doyle MP. Emerging issues in microbiological food safety. *Annu Rev Nutr* 1997; **17**: 255-275 [PMID: 9240928 DOI: 10.1146/annurev.nutr.17.1.255]
29 **Velázquez M**, Feirtag JM. Helicobacter pylori: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. *Int J Food Microbiol* 1999; **53**: 95-104 [PMID: 10634701 DOI: 10.1016/S0168-1605(99)00160-9]
30 **van Duynhoven YT**, de Jonge R. Transmission of Helicobacter pylori: a role for food? *Bull World Health Organ* 2001; **79**: 455-460 [PMID: 11417041]
31 **Gomes BC**, De Martinis ECP. The significance of Helicobacter pylori in water, food and environmental samples. *Food Control* 2004; **15**: 397-403 [DOI: 10.1016/S0956-7135(03)00106-3]
32 **Solnick JV**, Schauer DB. Emergence of diverse Helicobacter species in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev* 2001; **14**: 59-97 [PMID: 11148003 DOI: 10.1128/CMR.14.1.59-97.2001]
33 **Romaniuk PJ**, Zoltowska B, Trust TJ, Lane DJ, Olsen GJ, Pace NR, Stahl DA. Campylobacter pylori, the spiral bacterium associated with human gastritis, is not a true Campylobacter sp. *J Bacteriol* 1987; **169**: 2137-2141 [PMID: 3571163 DOI: 10.1128/jb.169.5.2137-2141.1987]
34 **Goodwin CS**, Armstrong JA. Microbiological aspects of Helicobacter pylori (Campylobacter pylori). *Eur J Clin Microbiol Infect Dis* 1990; **9**: 1-13 [PMID: 2406141 DOI: 10.1007/BF01969526]
35 **Pinto D**, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. *Crit Rev Microbiol* 2015; **41**: 61-76 [PMID: 23848175 DOI: 10.3109/1040841X.2013.794127]
36 **Percival SL**, Yates MV, Williams DW, Chalmers RM, Gray NF. Helicobacter pylori . In: Microbiology of waterborne diseases. Microbiological aspects and risks. 2nd ed. London: Elsevier, 2014: 119-153. ISBN: 978-0-12-415846-7.
37 **Cellini L**. Helicobacter pylori: a chameleon-like approach to life. *World J Gastroenterol* 2014; **20**: 5575-5582 [PMID: 24914317 DOI: 10.3748/wjg.v20.i19.5575]
38 **Cellini L**, Robuffo I, Di Campli E, Di Bartolomeo S, Taraborelli T, Dainelli B. Recovery of Helicobacter pylori ATCC43504 from

a viable but not culturable state: regrowth or resuscitation? *APMIS* 1998; **106**: 571-579 [PMID: 9674895]
39 **Andersen LP**, Rasmussen L. Helicobacter pylori-coccoid forms and biofilm formation. *FEMS Immunol Med Microbiol* 2009; **56**: 112-115 [PMID: 19453756 DOI: 10.1111/j.1574-695X.2009.00556.x]
40 **Li L**, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* 2014; **5**: 258 [PMID: 24917854 DOI: 10.3389/fmicb.2014.00258]
41 **Lleò MM**, Bonato B, Tafi MC, Signoretto C, Boaretti M, Canepari P. Resuscitation rate in different enterococcal species in the viable but non-culturable state. *J Appl Microbiol* 2001; **91**: 1095-1102 [PMID: 11851818]
42 **Senoh M**, Ghosh-Banerjee J, Ramamurthy T, Colwell RR, Miyoshi S, Nair GB, Takeda Y. Conversion of viable but nonculturable enteric bacteria to culturable by co-culture with eukaryotic cells. *Microbiol Immunol* 2012; **56**: 342-345 [PMID: 22537150 DOI: 10.1111/j.1348-0421.2012.00440.x]
43 **Versalovic J**, Fox JG. Helicobacter. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Manual of Clinical Microbiology 7h edition 1999: 727-736.
44 **Lopes AI**, Vale FF, Oleastro M. Helicobacter pylori infection - recent developments in diagnosis. *World J Gastroenterol* 2014; **20**: 9299-9313 [PMID: 25071324 DOI: 10.3748/wjg.v20.i28.9299]
45 **Hachem CY**, Clarridge JE, Evans DG, Graham DY. Comparison of agar based media for primary isolation of Helicobacter pylori. *J Clin Pathol* 1995; **48**: 714-716 [PMID: 7560195 DOI: 10.1136/jcp.48.8.714]
46 **Henriksen TH**, Brorson O, Schöyen R, Thoresen T, Setegn D, Madebo T. Rapid growth of Helicobacter pylori. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 1008-1011 [PMID: 8654438 DOI: 10.1007/BF01691385]
47 **Jiang X**, Doyle MP. Effect of environmental and substrate factors on survival and growth of Helicobacter pylori. *J Food Prot* 1998; **61**: 929-933 [PMID: 9713749 DOI: 10.4315/0362-028X-61.8.929]
48 **Roma-Giannikou E**, Karameris A, Balatsos B, Panayiotou J, Manika Z, Van-Vliet C, Rokkas T, Skandalis N, Kattamis C. Intrafamilial spread of Helicobacter pylori (H. pylori): A genetic analysis. European Helicobacter Study Group, Abstract from Athens Workshop 2002. XV International Workshop, September 11-14.
49 **Nurgalieva ZZ**, Malaty HM, Graham DY, Almuchambetova R, Machmudova A, Kapsultanova D, Osato MS, Hollinger FB, Zhangabylov A. Helicobacter pylori infection in Kazakhstan: effect of water source and household hygiene. *Am J Trop Med Hyg* 2002; **67**: 201-206 [PMID: 12389948 DOI: 10.4269/ajtmh.2002.67.201]
50 **Vaira D**, D'Anastasio C, Holton J, Dowsett J, Londei M, Salmon P, Gandolfi L. Is Campylobacter pylori a zoonosis? *Lancet* 1988; **2**: 1149 [PMID: 2903369]
51 **Husson MO**, Vincent P, Grabiaud MH, Furon D, Leclerc H. Anti-Helicobacter pylori IgG levels in abattoir workers. *Gastroenterol Clin Biol* 1991; **15**: 723-726 [PMID: 1816012]
52 **Beuchat LR**. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect* 2002; **4**: 413-423 [PMID: 11932192 DOI: 10.1016/S1286-4579(02)01555-1]
53 **Quaglia NC**, Dambrosio A, Normanno G, Parisi A, Firinu A, Lorusso V, Celano GV. Survival of Helicobacter pylori in artificially contaminated ultrahigh temperature and pasteurized milk. *Food Microbiol* 2007; **24**: 296-300 [PMID: 17188208 DOI: 10.1016/j.fm.2006.04.008]
54 **Fan XG**, Chua A, Li TG, Zeng QS. Survival of Helicobacter pylori in milk and tap water. *J Gastroenterol Hepatol* 1998; **13**: 1096-1098 [PMID: 9870794 DOI: 10.1111/j.1440-1746.1998.tb00582.x]
55 **Stevenson TH**, Bauer N, Lucia LM, Acuff GR. Attempts to isolate Helicobacter from cattle and survival of Helicobacter pylori in beef products. *J Food Prot* 2000; **63**: 174-178 [PMID: 10678420 DOI: 10.4315/0362-028X-63.2.174]
56 **Jiang X**, Doyle MP. Optimizing enrichment culture conditions for detecting Helicobacter pylori in foods. *J Food Prot* 2002; **65**: 1949-1954 [PMID: 12495015 DOI: 10.4315/0362-028X-65.12.1949]
57 **Gomes BC**, De Martinis ECP. Fate of Helicobacter pylori artificially inoculated in lettuce and carrot samples. *Braz J Microbiol* 2004; **35**: 145-150 [DOI: 10.1590/S1517-83822004000100024]
58 **Buck A**, Oliver JD. Survival of spinach-associated Helicobacter pylori in the viable but nonculturable state. *Food Control* 2010; **21**: 1150-1154 [DOI: 10.1016/j.foodcont.2010.01.010]
59 **Guner A**, Kav K, Tekinsen KK, Dogruer Y, Telli N. Survival of Helicobacter pylori in Turkish fermented sucuk and heat-treated sucuk during production. *J Food Prot* 2011; **74**: 2055-2061 [PMID: 22186045 DOI: 10.4315/0362-028X.JFP-11-019]
60 **Ng CG**, Loke MF, Goh KL, Vadivelu J, Ho B. Biofilm formation enhances Helicobacter pylori survivability in vegetables. *Food Microbiol* 2017; **62**: 68-76 [PMID: 27889168 DOI: 10.1016/j.fm.2016.10.010]
61 **Verma N**, Singh M. A disposable microbial based biosensor for quality control in milk. *Biosens Bioelectron* 2003; **18**: 1219-1224 [PMID: 12835039 DOI: 10.1016/S0956-5663(03)00085-X]
62 **West AP**, Millar MR, Tompkins DS. Effect of physical environment on survival of Helicobacter pylori. *J Clin Pathol* 1992; **45**: 228-231 [PMID: 1556231]
63 **Midolo PD**, Lambert JR, Hull R, Luo F, Grayson ML. In vitro inhibition of Helicobacter pylori NCTC 11637 by organic acids and lactic acid bacteria. *J Appl Bacteriol* 1995; **79**: 475-479 [PMID: 7592140 DOI: 10.1111/j.1365-2672.1995.tb03164.x]
64 **Dore MP**, Bilotta M, Vaira D, Manca A, Massarelli G, Leandro G, Atzei A, Pisanu G, Graham DY, Realdi G. High prevalence of Helicobacter pylori infection in shepherds. *Dig Dis Sci* 1999; **44**: 1161-1164 [PMID: 10389689 DOI: 10.1023/A:1026676223825]
65 **Dore MP**, Sepulveda AR, El-Zimaity H, Yamaoka Y, Osato MS, Mototsugu K, Nieddu AM, Realdi G, Graham DY. Isolation of Helicobacter pylori from sheep-implications for transmission to humans. *Am J Gastroenterol* 2001; **96**: 1396-1401 [PMID: 11374673 DOI: 10.1111/j.1572-0241.2001.03772.x]
66 **Fujimura S**, Kawamura T, Kato S, Tateno H, Watanabe A. Detection of Helicobacter pylori in cow's milk. *Lett Appl Microbiol* 2002; **35**: 504-507 [PMID: 12460433 DOI: 10.1046/j.1472-765X.2002.01229.x]
67 **Angelidis AS**, Tirodimos I, Bobos M, Kalamaki MS, Papageorgiou DK, Arvanitidou M. Detection of Helicobacter pylori in raw bovine milk by fluorescence in situ hybridization (FISH). *Int J Food Microbiol* 2011; **151**: 252-256 [PMID: 21974980 DOI: 10.1016/j.ijfoodmicro.2011.09.007]
68 **Rahimi E**, Kheirabadi EK. Detection of Helicobacter pylori in bovine, buffalo, camel, ovine, and caprine milk in Iran. *Foodborne Pathog Dis* 2012; **9**: 453-456 [PMID: 22458716 DOI: 10.1089/fpd.2011.1060]
69 **Mousavi S**, Dehkordi FS, Rahimi E. Virulence factors and antibiotic resistance of Helicobacter pylori isolated from raw milk and unpasteurized dairy products in Iran. *J Venom Anim Toxins Incl Trop Dis* 2014; **20**: 51 [PMID: 25873940 DOI: 10.1186/1678-9199-20-51]
70 **Saeidi E**, Sheikhshahrokh A. vacA Genotype Status of Helicobacter pylori Isolated from Foods with Animal Origin. *Biomed Res Int* 2016; **2016**: 8701067 [PMID: 27088092 DOI: 10.1155/2016/8701067]
71 **Quaglia NC**, Dambrosio A, Normanno G, Parisi A, Patrono R, Ranieri G, Rella A, Celano GV. High occurrence of Helicobacter pylori in raw goat, sheep and cow milk inferred by glmM gene: a risk of food-borne infection? *Int J Food Microbiol* 2008; **124**: 43-47 [PMID: 18372071 DOI: 10.1016/j.ijfoodmicro.2008.02.011]
72 **Talaei R**, Souod N, Momtaz H, Dabiri H. Milk of livestock as a possible transmission route of Helicobacter pylori infection. *Gastroenterol Hepatol Bed Bench* 2015; **8**: S30-S36 [PMID: 26171135 DOI: 10.22037/ghfbb.v8iSupplement.764]
73 **Osman EY**, El-Eragi AM, Musa AM, El-Magboul SB, A/Rahman MB, Abdo AE. Detection of Helicobacter pylori glmM gene in bovine milk using Nested polymerase chain reaction.

Vet World 2015; **8**: 913-917 [PMID: 27047175 DOI: 10.14202/vetworld.2015.913-917]
74 **Turutoglu H**, Mudul S. Investigation of Helicobacter pylori in raw sheep milk samples. *J Vet Med B Infect Dis Vet Public Health* 2002; **49**: 308-309 [PMID: 12241034 DOI: 10.1046/j.1439-0450.2002.00557.x]
75 **Bianchini V**, Recordati C, Borella L, Gualdi V, Scanziani E, Selvatico E, Luini M. Helicobacteraceae in Bulk Tank Milk of Dairy Herds from Northern Italy. *Biomed Res Int* 2015; **2015**: 639521 [PMID: 26090429 DOI: 10.1155/2015/639521]
76 **Gilani A**, Razavilar V, Rokni N, Rahimi E. *VacA* and *cagA* genotypes status and antimicrobial resistance properties of *Helicobacter pylori* strains isolated from meat products in Isfahan province, Iran. *Iran J Vet Res* 2017; **18**: 97-102 [PMID: 28775748 DOI: 10.22099/ijvr.2017.4088]
77 **Meng X**, Zhang H, Law J, Tsang R, Tsang T. Detection of Helicobacter pylori from food sources by a novel multiplex PCR assay. *J Food Saf* 2008; **28**: 609-619 [DOI: 10.1111/j.1745-4565.2008.00135.x]
78 **Hemmatinezhad B**, Momtaz H, Rahimi E. VacA, cagA, iceA and oipA genotypes status and antimicrobial resistance properties of Helicobacter pylori isolated from various types of ready to eat foods. *Ann Clin Microbiol Antimicrob* 2016; **15**: 2 [PMID: 26792758 DOI: 10.1186/s12941-015-0115-z]
79 **Ghorbani F**, Gheisari E, Dehkordi FS. Genotyping of vacA alleles of Helicobacter pylori strains recovered from some Iranian food items. *Trop J Pharm Res* 2016; **15**: 1631-1636 [DOI: 10.4314/tjpr.v15i8.5]
80 **Yahaghi E**, Khamesipour F, Mashayekhi F, Safarpoor Dehkordi F, Sakhaei MH, Masoudimanesh M, Khameneie MK. Helicobacter pylori in vegetables and salads: genotyping and antimicrobial resistance properties. *Biomed Res Int* 2014; **2014**: 757941 [PMID: 25184146 DOI: 10.1155/2014/757941]
81 **Atapoor S**, Safarpoor Dehkordi F, Rahimi E. Detection of Helicobacter pylori in Various Types of Vegetables and Salads. *Jundishapur J Microbiol* 2014; **7**: e10013 [PMID: 25147709 DOI: 10.5812/jjm.10013]
82 **Thomson MA**, Storey P, Greer R, Cleghorn GJ. Canine-human transmission of Gastrospirillum hominis. *Lancet* 1994; **343**: 1605-1607 [PMID: 7911923 DOI: 10.1016/S0140-6736(94)93060-0]
83 **Dimola S**, Caruso ML. Helicobacter pylori in animals affecting the human habitat through the food chain. *Anticancer Res* 1999; **19**: 3889-3894 [PMID: 10628327]
84 **Quaglia NC**, Dambrosio A, Normanno G, Alberti F, Rella A, Tamborrino C, Celano GV. Detection of Helicobacter pylori in gastric mucosa of sheep: preliminary results. *Rivista dell' Associazione Italiana Veterinari Igienisti* 2009; **3**: 45-48 [DOI: 10.4081/ijfs.2009.1420]
85 **Papiez D**, Konturek PC, Bielanski W, Plonka M, Dobrzanska M, Kaminska A, Szczyrk U, Bochenek A, Wierzchos E. Prevalence of Helicobacter pylori infection in Polish shepherds and their families. *Dig Liver Dis* 2003; **35**: 10-15 [PMID: 12725601 DOI: 10.1016/S1590-8658(02)00004-X]
86 **Mladenova-Hristova I**, Grekova O, Patel A. Zoonotic potential of Helicobacter spp. *J Microbiol Immunol Infect* 2017; **50**: 265-269 [PMID: 28655571 DOI: 10.1016/j.jmii.2016.11.003]
87 World Health Organization (WHO) and the United Nations Children's Fund (UNICEF). Progress on drinking water, sanitation and hygiene: 2017 update and Sustainable Development Goal baselines. The Joint Monitoring Programme (JMP) report. 2018. Available from: URL: https://www.unicef.org/publications/index_96611.html
88 **Lambert JR**, Lin SK, Aranda-Michel J. Helicobacter pylori. *Scand J Gastroenterol Suppl* 1995; **208**: 33-46 [PMID: 7777803 DOI: 10.3109/00365529509107760]
89 **Vu C**, Ng YY. Prevalence of Helicobacter pylori in peptic ulcer disease in a Singapore hospital. *Singapore Med J* 2000; **41**: 478-481 [PMID: 11281437]
90 **Adams BL**, Bates TC, Oliver JD. Survival of Helicobacter pylori in a natural freshwater environment. *Appl Environ Microbiol* 2003; **69**: 7462-7466 [PMID: 14660399 DOI: 10.1128/AEM.69.12.7462-7466.2003]
91 **Aziz RK**, Khalifa MM, Sharaf RR. Contaminated water as a source of Helicobacter pylori infection: A review. *J Adv Res* 2015; **6**: 539-547 [PMID: 26199743 DOI: 10.1016/j.jare.2013.07.007]
92 **Bellack NR**, Koehoorn MW, MacNab YC, Morshed MG. A conceptual model of water's role as a reservoir in Helicobacter pylori transmission: a review of the evidence. *Epidemiol Infect* 2006; **134**: 439-449 [PMID: 16512966 DOI: 10.1017/S0950268806006005]
93 **Lee YY**, Ismail AW, Mustaffa N, Musa KI, Majid NA, Choo KE, Mahendra Raj S, Derakhshan MH, Malaty HM, Graham DY. Sociocultural and dietary practices among Malay subjects in the north-eastern region of Peninsular Malaysia: a region of low prevalence of Helicobacter pylori infection. *Helicobacter* 2012; **17**: 54-61 [PMID: 22221617 DOI: 10.1111/j.1523-5378.2011.00917.x]
94 **Porras C**, Nodora J, Sexton R, Ferreccio C, Jimenez S, Dominguez RL, Cook P, Anderson G, Morgan DR, Baker LH, Greenberg ER, Herrero R. Epidemiology of Helicobacter pylori infection in six Latin American countries (SWOG Trial S0701). *Cancer Causes Control* 2013; **24**: 209-215 [PMID: 23263777 DOI: 10.1007/s10552-012-0117-5]
95 **Syam AF**, Miftahussurur M, Makmun D, Nusi IA, Zain LH, Zulkhairi, Akil F, Uswan WB, Simanjuntak D, Uchida T, Adi P, Utari AP, Rezkitha YA, Subsomwong P, Nasronudin, Suzuki R, Yamaoka Y. Risk Factors and Prevalence of Helicobacter pylori in Five Largest Islands of Indonesia: A Preliminary Study. *PLoS One* 2015; **10**: e0140186 [PMID: 26599790 DOI: 10.1371/journal.pone.0140186]
96 **Awuku YA**, Simpong DL, Alhassan IK, Tuoyire DA, Afaa T, Adu P. Prevalence of helicobacter pylori infection among children living in a rural setting in Sub-Saharan Africa. *BMC Public Health* 2017; **17**: 360 [PMID: 28438158 DOI: 10.1186/s12889-017-4274-z]
97 **Klein PD**, Graham DY, Gaillour A, Opekun AR, Smith EO. Water source as risk factor for Helicobacter pylori infection in Peruvian children. Gastrointestinal Physiology Working Group. *Lancet* 1991; **337**: 1503-1506 [PMID: 1675369 DOI: 10.1016/0140-6736(91)93196-G]
98 **Souto FJ**, Fontes CJ, Rocha GA, de Oliveira AM,Mendes EM, Queiroz DM. Prevalence of Helicobacter pylori Infection in a Rural Area of the State of Mato Grosso, Brazil. *Memorias do Instituto Oswaldo Cruz* 1998; **93**: 171-174 [DOI: 10.1590/S0074-02761998000200006]
99 **Herbarth O**, Krumbiegel P, Fritz GJ, Richter M, Schlink U, Müller DM, Richter T. Helicobacter pylori prevalences and risk factors among school beginners in a German urban center and its rural county. *Environ Health Perspect* 2001; **109**: 573-577 [PMID: 11445510]
100 **Fujimura S**, Kato S, Kawamura T. Helicobacter pylori in Japanese river water and its prevalence in Japanese children. *Lett Appl Microbiol* 2004; **38**: 517-521 [PMID: 15130149 DOI: 10.1111/j.1472-765X.2004.01529.x]
101 **Hulten K**, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, Evans DG, Engstrand L, Graham DY, El-Zaatari FA. Helicobacter pylori in the drinking water in Peru. *Gastroenterology* 1996; **110**: 1031-1035 [PMID: 8612990 DOI: 10.1053/gast.1996.v110.pm8612990]
102 **Boehnke KF**, Brewster RK, Sánchez BN, Valdivieso M, Bussalleu A, Guevara M, Saenz CG, Alva SO, Gil E, Xi C. An assessment of drinking water contamination with Helicobacter pylori in Lima, Peru. *Helicobacter* 2018; **23**: e12462 [PMID: 29316052]
103 **Sasaki K**, Tajiri Y, Sata M, Fujii Y, Matsubara F, Zhao M, Shimizu S, Toyonaga A, Tanikawa K. Helicobacter pylori in the natural environment. *Scand J Infect Dis* 1999; **31**: 275-279 [PMID: 10482057]
104 **Mazari-Hiriart M**, López-Vidal Y, Calva JJ. Helicobacter pylori in water systems for human use in Mexico City. *Water Sci Technol* 2001; **43**: 93-98 [PMID: 11464777]
105 **McKeown I**, Orr P, Macdonald S, Kabani A, Brown R, Coghlan G, Dawood M, Embil J, Sargent M, Smart G, Bernstein CN.

Helicobacter pylori in the Canadian arctic: seroprevalence and detection in community water samples. *Am J Gastroenterol* 1999; **94**: 1823-1829 [PMID: 10406242 DOI: 10.1111/j.1572-0241.1999.01212.x]
106 **Lu Y**, Redlinger TE, Avitia R, Galindo A, Goodman K. Isolation and genotyping of Helicobacter pylori from untreated municipal wastewater. *Appl Environ Microbiol* 2002; **68**: 1436-1439 [PMID: 11872498 DOI: 10.1128/AEM.68.3.1436-1439.2002]
107 **Al-Taee MR**. Assessment of water quality due to microbial growth in drinking water distribution systems in Basrah city. *Marina Mesopotamica* 2001; **16**: 37-46
108 **Momba MNB**, Makala N. Comparing the effect of various pipe materials on biofilm formation in chlorinated and combined chlorine-chloraminated water systems. *Water SA* 2003; **30**: 175-182 [DOI: 10.4314/wsa.v30i2.5061]
109 **Al-Sulami AA**, Al-Taee AM, Juma'a MG. Isolation and identification of Helicobacter pylori from drinking water in Basra governorate, Iraq. *East Mediterr Health J* 2010; **16**: 920-925 [PMID: 21218716]
110 **Bahrami AR**, Rahimi E, Ghasemian Safaei H. Detection of Helicobacter pylori in city water, dental units' water, and bottled mineral water in Isfahan, Iran. *ScientificWorldJournal* 2013; **2013**: 280510 [PMID: 23606812 DOI: 10.1155/2013/280510]
111 **Ranjbar R**, Khamesipour F, Jonaidi-Jafari N, Rahimi E. Helicobacter pylori in bottled mineral water: genotyping and antimicrobial resistance properties. *BMC Microbiol* 2016; **16**: 40 [PMID: 26970903 DOI: 10.1186/s12866-016-0647-1]
112 **Vale FF**, Vítor JM. Transmission pathway of Helicobacter pylori: does food play a role in rural and urban areas? *Int J Food Microbiol* 2010; **138**: 1-12 [PMID: 20122750 DOI: 10.1016/j.ijfoodmicro.2010.01.016]
113 **Azevedo NF**, Almeida C, Cerqueira L, Dias S, Keevil CW, Vieira MJ. Coccoid form of Helicobacter pylori as a morphological manifestation of cell adaptation to the environment. *Appl Environ Microbiol* 2007; **73**: 3423-3427 [PMID: 17400788 DOI: 10.1128/AEM.00047-07]
114 **Queralt N**, Araujo R. Analysis of the survival of H. pylori within a laboratory-based aquatic model system using molecular and classical techniques. *Microb Ecol* 2007; **54**: 771-777 [PMID: 17401696 DOI: 10.1007/s00248-007-9242-1]
115 **Moreno Y**, Piqueres P, Alonso JL, Jiménez A, González A, Ferrús MA. Survival and viability of Helicobacter pylori after inoculation into chlorinated drinking water. *Water Res* 2007; **41**: 3490-3496 [PMID: 17585990 DOI: 10.1016/j.watres.2007.05.020]
116 **Baker KH**, Hegarty JP, Redmond B, Reed NA, Herson DS. Effect of oxidizing disinfectants (chlorine, monochloramine, and ozone) on Helicobacter pylori. *Appl Environ Microbiol* 2002; **68**: 981-984 [PMID: 11823249 DOI: 10.1128/AEM.68.2.981-984.2002]
117 **Johnson CH**, Rice EW, Reasoner DJ. Inactivation of Helicobacter pylori by chlorination. *Appl Environ Microbiol* 1997; **63**: 4969-4970 [PMID: 9406419]
118 **Shahamat M**, Mai U, Paszko-Kolva C, Kessel M, Colwell RR. Use of autoradiography to assess viability of Helicobacter pylori in water. *Appl Environ Microbiol* 1993; **59**: 1231-1235 [PMID: 8489232]
119 **Winiecka-Krusnell J**, Wreiber K, von Euler A, Engstrand L, Linder E. Free-living amoebae promote growth and survival of Helicobacter pylori. *Scand J Infect Dis* 2002; **34**: 253-256 [PMID: 12064686 DOI: 10.1080/00365540110080052]
120 **Watson CL**, Owen RJ, Said B, Lai S, Lee JV, Surman-Lee S, Nichols G. Detection of Helicobacter pylori by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. *J Appl Microbiol* 2004; **97**: 690-698 [PMID: 15357718 DOI: 10.1111/j.1365-2672.2004.02360.x]
121 **Percival SL**, Thomas JG. Transmission of Helicobacter pylori and the role of water and biofilms. *J Water Health* 2009; **7**: 469-477 [PMID: 19491497 DOI: 10.2166/wh.2009.070]
122 **Moreno-Mesonero L**, Moreno Y, Alonso JL, Ferrús MA. Detection of viable Helicobacter pylori inside free-living amoebae in wastewater and drinking water samples from Eastern Spain. *Environ Microbiol* 2017; **19**: 4103-4112 [PMID: 28707344 DOI: 10.1111/1462-2920.13856]
123 **Azevedo NF**, Pacheco AP, Keevil CW, Vieira MJ. Adhesion of water stressed Helicobacter pylori to abiotic surfaces. *J Appl Microbiol* 2006; **101**: 718-724 [PMID: 16907822 DOI: 10.1111/j.1365-2672.2006.03029.x]
124 **Azevedo NF**, Pinto AR, Reis NM, Vieira MJ, Keevil CW. Shear stress, temperature, and inoculation concentration influence the adhesion of water-stressed Helicobacter pylori to stainless steel 304 and polypropylene. *Appl Environ Microbiol* 2006; **72**: 2936-2941 [PMID: 16598000 DOI: 10.1128/AEM.72.4.2936-2941.2006]
125 **Costerton JW**, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; **284**: 1318-1322 [PMID: 10334980 DOI: 10.1126/science.284.5418.1318]
126 **Yu FP**, McFeters GA. Physiological responses of bacteria in biofilms to disinfection. *Appl Environ Microbiol* 1994; **60**: 2462-2466 [PMID: 8074525]
127 **Horiuchi T**, Ohkusa T, Watanabe M, Kobayashi D, Miwa H, Eishi Y. Helicobacter pylori DNA in drinking water in Japan. *Microbiol Immunol* 2001; **45**: 515-519 [PMID: 11529557 DOI: 10.1111/j.1348-0421.2001.tb02652.x]
128 **Böckelmann U**, Dörries HH, Ayuso-Gabella MN, Salgot de Marçay M, Tandoi V, Levantesi C, Masciopinto C, Van Houtte E, Szewzyk U, Wintgens T, Grohmann E. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl Environ Microbiol* 2009; **75**: 154-163 [PMID: 19011075 DOI: 10.1128/AEM.01649-08]
129 **Janzon A**, Sjöling A, Lothigius A, Ahmed D, Qadri F, Svennerholm AM. Failure to detect Helicobacter pylori DNA in drinking and environmental water in Dhaka, Bangladesh, using highly sensitive real-time PCR assays. *Appl Environ Microbiol* 2009; **75**: 3039-3044 [PMID: 19304824]
130 **Cellini L**, Del Vecchio A, Di Candia M, Di Campli E, Favaro M, Donelli G. Detection of free and plankton-associated Helicobacter pylori in seawater. *J Appl Microbiol* 2004; **97**: 285-292 [PMID: 15239694 DOI: 10.1111/j.1365-2672.2004.02307.x]
131 **Cellini L**, Grand R, Prenna M, Pasquantonio M, Pane L. Detection of Helicobacter pylori associated with zooplankton. *Aquat Microb Ecol* 2005; **40**: 115-120 [DOI: 10.3354/ame040115]
132 **Carbone M**, Maugeri TL, Gugliandolo C, La Camera E, Biondo C, Fera MT. Occurrence of Helicobacter pylori DNA in the coastal environment of southern Italy (Straits of Messina). *J Appl Microbiol* 2005; **98**: 768-774 [PMID: 15715881 DOI: 10.1111/j.1365-2672.2004.02517.x]
133 **Dixon B**. Helicobacter from seas? ASM news 2005; **71**: 4-5
134 **Twing KI**, Kirchman DL, Campbell BJ. Temporal study of Helicobacter pylori presence in coastal freshwater, estuary and marine waters. *Water Res* 2011; **45**: 1897-1905 [PMID: 21193216 DOI: 10.1016/j.watres.2010.12.013]
135 **Holman CB**, Bachoon DS, Otero E, Ramsubhag A. Detection of Helicobacter pylori in the coastal waters of Georgia, Puerto Rico and Trinidad. *Mar Pollut Bull* 2014; **79**: 354-358 [PMID: 24332757 DOI: 10.1016/j.marpolbul.2013.11.021]
136 **Bode G**, Mauch F, Malfertheiner P. The coccoid forms of Helicobacter pylori. Criteria for their viability. *Epidemiol Infect* 1993; **111**: 483-490 [PMID: 8270008]
137 **Cao J**, Li ZQ, Borch K, Petersson F, Mårdh S. Detection of spiral and coccoid forms of Helicobacter pylori using a murine monoclonal antibody. *Clin Chim Acta* 1997; **267**: 183-196 [PMID: 9469252 DOI: 10.1016/S0009-8981(97)00134-4]
138 **Cohen AE**. A PCR method for the detection of Helicobacter pylori in food products: US Food and Drug Administration (FDA) Study. Proceedings of the 16th Food Microbiology Symposium "Current concepts in foodborne pathogens and rapid and automated methods in food microbiology"; 1996 University of Wisconsin, River Falls
139 **Enroth H**, Engstrand L. Immunomagnetic separation and PCR for detection of Helicobacter pylori in water and stool specimens. *J Clin Microbiol* 1995; **33**: 2162-2165 [PMID: 7559969]
140 **Hultén K**, Enroth H, Nyström T, Engstrand L. Presence

of Helicobacter species DNA in Swedish water. *J Appl Microbiol* 1998; **85**: 282-286 [PMID: 9750301 DOI: 10.1046/j.1365-2672.1998.00500.x]

141 **Quaglia NC**, Dambrosio A, Normanno G, Celano GV. Evaluation of a Nested-PCR assay based on the phosphoglucosamine mutase gene (glmM) for the detection of Helicobacter pylori from raw milk. *Food Control* 2009; **20**: 119-123

142 **Quaglia NC**, Normanno G, Dambrosio A, Celano GV, Parisi A, Firinu A, Buonavoglia C. Multiplex-touchdown PCR assay for the detection and genotyping of Helicobacter pylori from artificially contaminated sheep milk. *J Food Prot* 2005; **68**: 2136-2139 [PMID: 16245719 DOI: 10.4315/0362-028X-68.10.2136]

143 **Linke S**, Lenz J, Gemein S, Exner M, Gebel J. Detection of Helicobacter pylori in biofilms by real-time PCR. *Int J Hyg Environ Health* 2010; **213**: 176-182 [PMID: 20427237 DOI: 10.1016/j.ijheh.2010.03.006]

144 **Lahtinen SJ**, Ahokoski H, Reinikainen JP, Gueimonde M, Nurmi J, Ouwehand AC, Salminen SJ. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. *Lett Appl Microbiol* 2008; **46**: 693-698 [PMID: 18444975 DOI: 10.1111/j.1472-765X.2008.02374.x]

145 **Sheridan GE**, Masters CI, Shallcross JA, MacKey BM. Detection of mRNA by reverse transcription-PCR as an indicator of viability in Escherichia coli cells. *Appl Environ Microbiol* 1998; **64**: 1313-1318 [PMID: 9546166]

146 **Smith B**, Oliver JD. In situ and in vitro gene expression by Vibrio vulnificus during entry into, persistence within, and resuscitation from the viable but nonculturable state. *Appl Environ Microbiol* 2006; **72**: 1445-1451 [PMID: 16461698 DOI: 10.1128/AEM.72.2.1445-1451.2006]

147 **Moreno Y**, Ferrús MA, Alonso JL, Jiménez A, Hernández J. Use of fluorescent in situ hybridization to evidence the presence of Helicobacter pylori in water. *Water Res* 2003; **37**: 2251-2256 [PMID: 12691913 DOI: 10.1016/S0043-1354(02)00624-3]

148 **Piqueres P**, Moreno Y, Alonso JL, Ferrús MA. A combination of direct viable count and fluorescent in situ hybridization for estimating Helicobacter pylori cell viability. *Res Microbiol* 2006; **157**: 345-349 [PMID: 16380234 DOI: 10.1016/j.resmic.2005.09.003]

P- Reviewer: Hoff DA, El-Shabrawi MH, Chiba T, Chmiela M, Balaban YH, Reyes VE, Ding SZ, Chen XZ **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 August 21; 24(31): 3488-3499

DOI: 10.3748/wjg.v24.i31.3488

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

REVIEW

Hepatitis B virus infection: Defective surface antigen expression and pathogenesis

Chun-Chen Wu, Ying-Shan Chen, Liang Cao, Xin-Wen Chen, Meng-Ji Lu

Chun-Chen Wu, Ying-Shan Chen, Liang Cao, Xin-Wen Chen, State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, Hubei Province, China

Liang Cao, Department of Microbiology and Immunology, Feinberg School of Medicine Northwestern University, Chicago, IL 60611, United States

Meng-Ji Lu, Institute of Virology, University Hospital of Essen, Essen 45122, Germany

ORCID number: Chun-Chen Wu (0000-0002-6888-213X); Ying-Shan Chen (0000-0002-2276-4496); Liang Cao (0000-0001-5974-9815); Xin-Wen Chen (0000-0002-4052-8155); Meng-Ji Lu (0000-0003-4287-9941).

Author contributions: Wu CC contributed to analysis and interpretation of the data and drafting the article; Chen YS and Cao L contributed to revising the article for important intellectual content; Chen XW and Lu MJ contributed to conception and design, analysis and interpretation of data, and drafting and revising the article for important intellectual content.

Supported by the National Nature Science Foundation of China, No. 31770180; and the Youth Innovation Promotion Association CAS, No. 2016303.

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

Manuscript source: Invited manuscript

Correspondence to: Meng-Ji Lu, PhD, Professor, Institute of Virology, University Hospital Essen, Hufelandstrasse 55, Essen 45122, Germany. mengji.lu@uni-due.de
Telephone: +49-201-7233530
Fax: +49-201-7235929

Received: April 27, 2018
Peer-review started: May 4, 2018
First decision: May 23, 2018
Revised: June 1, 2018
Accepted: June 25, 2018
Article in press: June 25, 2018
Published online: August 21, 2018

Abstract

Hepatitis B virus (HBV) infection is a global public health concern. HBV causes chronic infection in patients and can lead to liver cirrhosis, hepatocellular carcinoma, and other severe liver diseases. Thus, understanding HBV-related pathogenesis is of particular importance for prevention and clinical intervention. HBV surface antigens are indispensable for HBV virion formation and are useful viral markers for diagnosis and clinical assessment. During chronic HBV infection, HBV genomes may acquire and accumulate mutations and deletions, leading to the expression of defective HBV surface antigens. These defective HBV surface antigens have been found to play important roles in the progression of HBV-associated liver diseases. In this review, we focus our discussion on the nature of defective HBV surface antigen mutations and their contribution to the pathogenesis of fulminant hepatitis B. The relationship between defective surface antigens and occult HBV infection are also discussed.

Key words: Hepatitis B surface protein; Defective surface antigen mutants; Endoplasmic reticulum stress; Fulminant hepatitis B; Occult hepatitis B virus infection; Pathogenesis

Core tip: Defective surface antigen mutation is a type of mutation with great clinical relevance. Many previous publications have explored the association of defective surface antigen mutation with the development of hepatitis B virus (HBV)-associated hepatocellular carcinoma. However, there are no reviews available that elaborate on the relationship between defective surface antigen mutation and HBV-associated fulminant hepatitis (FH), as well as occult hepatitis B virus infection (OBI). This review will focus on these two aspects to discuss the nature of defective HBV surface antigen mutations and their contribution to the pathogenesis of FH. The relationship between defective surface antigens and OBI are also discussed.

Wu CC, Chen YS, Cao L, Chen XW, Lu MJ. Hepatitis B virus infection: Defective surface antigen expression and pathogenesis. *World J Gastroenterol* 2018; 24(31): 3488-3499 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3488.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3488

INTRODUCTION

Hepatitis B virus (HBV) is an important human pathogen that has caused chronic infections worldwide[1]. Recent data obtained from a modeling study has shown that the global prevalence of hepatitis B surface antigen (HBsAg) was 3.9% in 2016, corresponding to an estimated 290 million infections worldwide[2]. HBV mainly infects hepatocytes and causes a wide spectrum of clinical manifestations, ranging from an asymptomatic carrier state to acute or chronic hepatitis, with progression to liver cirrhosis, hepatocellular carcinoma (HCC), and other severe liver diseases[3,4]. Currently, interferon-α and nucleotide analogs are used to treat chronic HBV (CHB) infections; however, the outcome is far from satisfactory[5,6]. Prophylaxis using the current HBV vaccines has no impact on existing infections. Therapeutic vaccines of chronic HBV infection are under investigation, but further development is still required[7]. Therefore, understanding the molecular pathogenesis of HBV infection will provide opportunities for the development of better therapies and vaccines.

HBV belongs to the family *Hepadnaviridae* and is a small, enveloped virus with a partially double-stranded DNA genome approximately 3.2 kb in size[8]. During the life cycle of HBV, pre-genomic RNA (pgRNA) is transcribed from covalently closed circular DNA (cccDNA) and serves as the template for HBV DNA replication through a viral polymerase-mediated reverse transcription[9,10]. Because viral polymerase lacks a proof-reading function, the HBV genome evolves with an estimated rate of nucleotide substitutions of 1×10^{-3} to 1×10^{-6} per replication cycle, according to various investigators[11]. Although HBV genome replication involves a step of reverse transcription, which is similar to retroviral replication, the complex HBV genome structure with overlapping open reading frames and regulatory sequences apparently limits the spectrum and rate of mutations[3,12]. Nevertheless, this unique replication strategy leads to the great diversity of HBV genomes, thus resulting in the occurrence of various genotypes, subtypes, mutants, recombinants, and even viral quasi-species in the context of long-term HBV evolution[13,14]. Several reports have suggested that the emergence of HBV variants plays important roles in the progression of HBV-associated liver diseases[11,15-18]. Defective surface antigen mutation is a type of mutation with great clinical relevance[11,15,19]. In this review, we report the current information on HBV surface antigen mutations. Further, we focus our discussion on the contribution of defective surface antigen mutations on the pathogenesis of HBV-associated liver diseases.

BIOLOGY OF HBV SURFACE ANTIGEN

Three viral envelope/surface proteins - large surface antigens (LHBs), middle surface antigens (MHBs), and small surface antigens (SHBs) - are expressed from a single open reading frame (S-ORF)[20,21], but they are translated from two different mRNAs. LHBs are encoded by the 2.4 kb subgenomic RNA, and MHBs and SHBs are encoded by the 2.1 kb subgenomic RNA[3]. Subgenomic RNAs of 2.4 kb and 2.1 kb are driven by preS1 and preS2/S promoters, respectively, allowing variable regulation of protein expression[3]. The preS1 promoter is situated within the upstream region of the S-ORF, whereas the preS2 promoter corresponds to the preS1 domain[21]. Therefore, the transcription of the 2.1 kb subgenomic RNA is also regulated by the preS1 domain[11] (Figure 1).

The three surface proteins share the same carboxy-terminal region and only differ in length due to their amino-terminal regions. As a result, the LHBs contain the preS1 + preS2 + S [389 or 400 amino acid (aa) residues], MHBs contain the preS2 + S (281 aa residues), and SHBs contain the S domain (226 aa residues) alone[3,20,22] (Figure 1). Additionally, a truncated and mutated preS2/S (the LHBs and truncated MHBs) can be produced by integrated viral sequences that are defective for replication[23,24]. LHBs, MHBs, and SHBs are important for HBV structure and life cycle. Besides mediating HBV entry through binding to HBV receptors, the sodium taurocholate co-transporting polypeptide (NTCP) on hepatocytes, via the preS1 2-48 aa domain (numbering for HBV-genotype D) and subsequent infection, LHBs are indispensable for the formation and budding of virions[3,25-29]. It has been proposed that LHBs rearrange their structure during the maturation of HBV virions and thereby regulate the release and infectivity of virions[30-32]. The exact role of MHBs in the HBV life cycle remains an enigma.

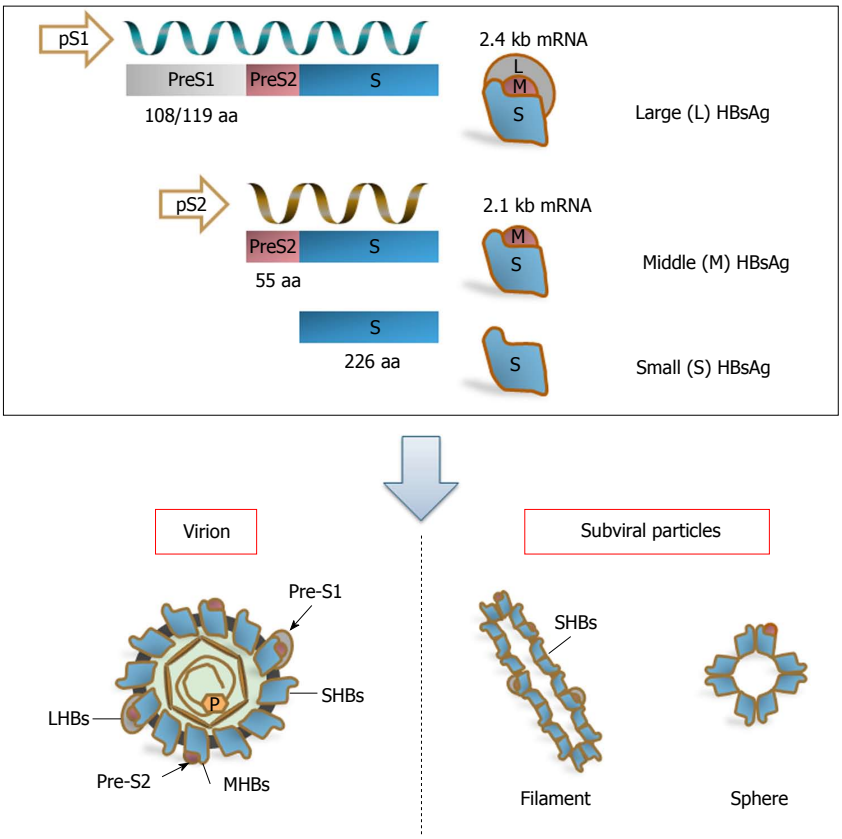


Figure 1 The transcription and expression of hepatitis B virus surface proteins. The three HBV surface proteins, LHBs, MHBs, and SHBs, are translated from two different mRNAs: LHBs are encoded by the preS1 promoter-initiated 2.4 kb subgenomic RNA; MHBs and SHBs are encoded by the preS2 promoter-initiated 2.1 kb subgenomic RNA. The 2.4 and 2.1 kb subgenomic RNAs share the same 3' end and only differ in length due to differences at the 5' end, which lead to different amino-terminal but identical carboxy-terminal regions of the three surface antigens. Therefore, LHBs contain preS1 + preS2 + S (389 or 400 aa residues), MHBs contain preS2 + S (281 aa residues), and SHBs contain the S domain (226 aa residues) alone. For mature/infectious virions, LHBs, MHBs, and SHBs are present in the envelopes at a ratio of approximately 1:1:4. In addition, the major fraction of SHBs forms subviral particles (filaments and spheres) together with the minor parts of LHBs and/or MHBs. HBV: Hepatitis B virus; LHBs: Large surface antigens; MHBs: Middle surface antigens; SHBs: Small surface antigens.

Early reports indicated that MHBs might be dispensable for HBV replication and virion formation; however, our data and those of other groups have shown that MHBs play a role in virion secretion[33-36]. Recently, MHBs were found to interact with ceruloplasmin and influence the production of extracellular virions[34]. As the predominant component of viral particles, including infectious virions and noninfectious subviral particles, SHBs are necessary for the production of virions and subviral particles[35]. For mature/infectious virions, LHBs, MHBs, and SHBs are present in the envelopes at a ratio of approximately 1:1:4[20]. Disturbance of this proportion impairs the production and release of virions[33]. For subviral particles, their amount outnumbers virions by 10000- to 1000000-fold, and the particles are detected serologically as HBsAg[11,37]. The secretion of subviral particles can also be suppressed by LHBs in a dose-dependent manner[38-41], thus promoting the S protein toward virion formation.

In addition, preS1, preS2, and S domains contain various B- and T-cell epitopes, which play an important role in inducing the host immune response[42,43]. The major hydrophilic region (MHR) between aa 100-169 of SHBs, especially the a-determinant located at aa 124-147, serves as the most important antigenic determinant in HBV surface proteins and is essential for HBsAg detection and HBV vaccine development[44,45]. Plasma-derived and recombinant HBsAg have been used for vaccine preparations and have induced strong specific and protective antibody responses in vaccines[46-48]. The presence of anti-HBs antibodies is considered to confer immunity against HBV infection. In contrast, a high quantity of circulating HBsAg in chronically HBV-infected patients is proposed as a factor leading to immune disturbance. Defective peripheral HBsAg-specific T cell responses in chronically infected patients were found to be correlated with serum HBsAg titers[49,50], suggesting that HBsAg overproduction influences the host's immune system in a way that is advantageous for the virus. *In vitro*, HBsAg can interfere with Toll-like receptor functions and trigger interleukin (IL)-10 production in Kuppfer cells[51-54]. Recently, published data has suggested that HBsAg may facilitate the induction of myeloid-derived suppressor cells in chronically HBV-infected patients[55]. HBsAg is also associated with the induction of regulatory T cells, as shown in HBV mouse models[56,57]. Thus, HBsAg is not only a structural component of virions and subviral

particles, but it also serves as an important immune modulator.

DEFECTIVE SURFACE ANTIGEN MUTATION AND HBV BIOLOGY

HBsAg mutants were first identified in individuals vaccinated against HBV but who were infected despite the presence of protective anti-HBs antibodies[58]. Those "immune escape" mutants with aa substitutions within a-determinants were found in different clinical settings, including vaccines, transplant patients receiving hyperimmunoglobulins, and immunocompromised patients with HBV reactivation[59-61]. Such mutant HBsAg commonly showed reduced binding to anti-HBs antibodies and decreased reactivity in established HBsAg detection assays[59-65]. The most widely known mutation is the sG145R mutation, which has been shown to be replication competent, may persist stably over time, and may be transmitted vertically or horizontally[66-69]. The sG145R mutation induces a strongly impaired anti-HBs antibody response, which could not efficiently clear HBsAg in an HBV hydrodynamic injection mouse model[70]. A similar result was also observed for another immune escape mutation, sK122I, indicating that such a defective surface antigen mutation may impair HBsAg neutralization and clearance during HBV infection. In addition, sG145R, sK122I, and other immune escape mutants occurring in the a-determinant of SHBs, such as the sT123N mutation, could affect HBsAg secretion[70-73].

Recently, chronically HBV-infected patients routinely received antiviral therapy based on nucleotide analogs[74]. Treatment with first-generation drugs, such as famciclovir and adeforvir, resulted in the emergence of drug-resistant HBV mutants, with aa substitutions within the HBV polymerase domain[75]. Some drug-resistant mutations occurring in the viral polymerase may lead to a stop codon mutation in the overlapping surface gene, cause intracellular retention of surface proteins, and result in secretion defects of viral particles, such as rtA181T/sW172*, rtM204I/sW196*, and rtV191I/sW182*, as shown in previous reports[76-78] and in our unpublished data. The primary sW182* mutation has also been identified in CHB patients. It was found to induce retention of the truncated S protein in the perinuclear endoplasmic reticulum (ER) and was associated with lower HBV transcript levels owing to decreased stability, but without impact on HBV replication[79].

Defective surface antigen mutations have been frequently detected in chronic HBV infection[16,71-73,80,81], in which deletions in the preS domains are the most common mutations[80,81]. Deletions in the preS domains are often clustered at the 3′ end of preS1 and the 5′ end of preS2[11,19,81-83]. Given that the preS2/S promoter is situated within the preS1 domain[11], deletions at the 3′ end of the preS1 may reduce MHBs and SHBs expression at the transcriptional level. Deletions at the 5′ end of the preS2 may remove the N-terminal preS2 domain, including the start codon of preS2 in the MHBs protein, leading to an impaired or complete loss of MHBs expression[84]. These changes may disrupt the proper LHBs, MHBs, and SHBs ratio in the envelopes of virions. In addition, the junction between the preS1 and preS2 domain is required for virion formation[32]. For these reasons, preS deletions may potentially affect virion assembly, stability, or infectivity.

A large amount of evidence has demonstrated that DHBV envelope proteins can regulate cccDNA formation and amplification[85,86]. Infection of envelope protein-deficient recombinant DHBV results in more cccDNA accumulation[85,87,88]. Similarly, deficiencies in HBV envelope proteins can modestly increase the cccDNA level and result in a dramatic accumulation of deproteinized rcDNA[89-91]. It has been demonstrated that preS/S mutants with surface antigen secretion deficiency isolated from patients can lead to an increased accumulation of cccDNA molecules in the nuclei[79]. Therefore, defective surface antigen mutation may affect cccDNA synthesis and amplification.

DEFECTIVE SURFACE ANTIGEN MUTATIONS AND THE HOST

Defective surface antigen mutations have been found in acute hepatitis B infection, chronic hepatitis B infection, and occult HBV infection and are associated with advanced liver disease, including liver cirrhosis, fulminant hepatitis B, and HCC[15,82,92-105]. It has been questioned whether HBV mutants arise due to viral adaptation to inflammation and decreased liver function or, alternatively, causally contribute to liver pathogenesis. The mechanism of defective surface antigen mutations to HCC development has been widely elucidated[11,23,41,106-111]. Here, we will emphasize in our discussion the relationship between defective surface antigen mutations and fulminant hepatitis B, as well as occult HBV infection.

Defective surface antigen expression and fulminant hepatitis

There is increasing evidence that defective surface antigen expression may play a role in the pathogenesis of fulminant hepatitis (FH). preS deletions, particularly those unable to synthesize the MHBs protein, have been associated with cases of FH[16,71,112,113], suggesting the potential pathogenic role of preS deletions. A mutation in the CAAT element of the S promoter has been found in the HBV genome isolated from an FH patient. This mutation led to excessive LHBs expression over MHBs and SHBs proteins and resulted in virus retention and misassembly[114-116]. Obviously, the accumulation of LHBs may be due to hepatocyte injury, as shown in transgenic mice with LHBs expression[41]. One of our previous studies also identified deletions within the preS

regions from HBV strains isolated from a patient with HBV-associated FH[84]. In addition, a hepatitis B immune globulin (HBIG)-escape mutant sG145R on the HBsAg, causing 30% inhibition of virion secretion, has been identified from a study on FH strains, suggesting the potential role of defective surface antigen expression in the fulminant clinical course of HBV infection[71].

Mechanistically, defective surface antigen expression, such as specific mutations in the preS/S gene, may lead to secretion defects of viral proteins and particles, resulting in an accumulation of viral products in the ER of hepatocytes and causing ER stress and hepatocyte injury[16]. Subsequently, autophagy may be triggered[117-125] and thus enhance HBV replication[126,127]. Consistent with this speculation, it has been demonstrated that defective surface antigen expression may increase the replication capability of HBV, albeit the mechanism is still undefined[71,84]. In addition, the deficiency of hepadnavirus envelope proteins can result in accumulation of cccDNA[85,87,88] or deproteinized rcDNA[89-91] and may ultimately cause death of the infected hepatocytes by a direct cytopathic effect[85,87,88]. Meanwhile, the increase of the cccDNA level may facilitate HBV replication. Both the defect in viral particle secretion and enhanced replication competence may contribute to the severity of fulminant hepatitis[128].

The adaptive immune response, particularly the cytotoxic T lymphocyte (CTL) response, plays a crucial role in viral clearance and disease pathogenesis of HBV infection[129-131]. Intracellular retention of HBV surface proteins was found to be associated with FH in a transgenic mouse model showing panlobular necrosis and hepatic failure by inducing the indirect cytotoxic activity of CTLs[132]. In this setting, intracellular accumulation of viral products due to defective surface antigen expression mutations may cause liver damage through abnormal activation of the CTL response. Consistently, we also observed significantly stronger intrahepatic CTL responses and antibody responses specific to secretion-deficient HBsAg due to preS deletions[84]. A preS deletion mutant was found in a patient with acute exacerbation of liver diseases, along with wild-type HBV genomes. The co-existence of deletion mutants and wild-type HBV apparently allows the complementation and enhancement of HBV genome replication in hepatocytes. In an HBV mouse model, co-replication of a deletion mutant and wild-type HBV induced higher cellular and humoral immunity. Our findings further suggested the proposed role of HBV variants in the immunopathogenesis of HBV infection. Moreover, the mutations associated with defective surface antigen expression, such as deletion or missense mutation of the preS2 ATG codon, can cause deletions or alterations of B- and T-cell epitopes located in preS1 and preS2 proteins. Considering that M protein-specific T- and B-cell immunities are important early events in the host immune response to HBV infection[43], these mutations may lead to an immune evasion and thus likely favor a more severe clinical course of infection[14,133]. In chronic HBV infection, high HBV replication levels were found to be associated with lower cellular immune responses to HBV; however, massive infiltration of unspecific immune cells occurred within the liver, accompanied by severe liver damage[134-136]. Thus, the presence of these mutations, including aa substitutions at the immunodominant epitopes for B or T cell recognition, may contribute to the spread of highly replicative escape mutants. It may also facilitate the development of fulminant hepatitis in chronically HBV-infected patients and heavily immunocompromised patients, like those with human immunodeficiency virus (HIV) co-infection[137] (Figure 2).

Defective surface antigen expression and occult hepatitis B virus infection

Occult HBV infection (OBI) is characterized by the presence of very low levels of HBV DNA in the plasma and/or liver of individuals negative for HBV surface antigen (HBsAg) and positive/negative for antibodies to the hepatitis core antigen (anti-HBc)[45,138,139]. OBI harbors the potential risk of HBV transmission through blood transfusion, organ transplantation, and hemodialysis as well as from occult infection or HBsAg-positive mothers to newborns[45]. The persistence of OBI may lead to the development of cirrhosis and HCC[45,140-145]. The reactivation of OBI can occur in patients following chemotherapy, immunosuppressive therapy, and after transplantation as well as in patients co-infected with HIV or hepatitis C virus (HCV)[45,146,147], which can result in the development of fulminant hepatitis and death[139,148-153].

Defective surface antigen expression mutations may be associated with OBI. Point mutations and deletions as well as insertion mutations are commonly encountered in OBI, in which mutations in the *preS/S* gene are the most extensively studied[45]. High frequencies of MHR mutations, including those mutations within and outside of the a-determinant, have been observed in OBI strains of individuals[154-158]. *In vitro* and *in vivo* experiments have demonstrated that these MHR mutations can significantly decrease the detection sensitivity of commercial HBsAg immunoassays and impair virion and/or S protein secretion[156]. preS/S mutations with deletions covering the preS1 and preS2/S promoters, preS1 region, and preS2 region have been frequently reported in OBI. This can alter the transcription of 2.4 kb and 2.1 kb HBV RNAs, expression of three envelope proteins, and the ratio of LHBs/MHBs/SHBs proteins[45]. preS/S insertions, such as 2-8 aa insertions between codons 121 and 124 located upstream of the a-determinant, have also been observed in OBI patients[159].

On one hand, these mutations associated with defective surface antigen expression can directly decrease the levels of surface antigens. On the other hand, these mutations can cause the retention and

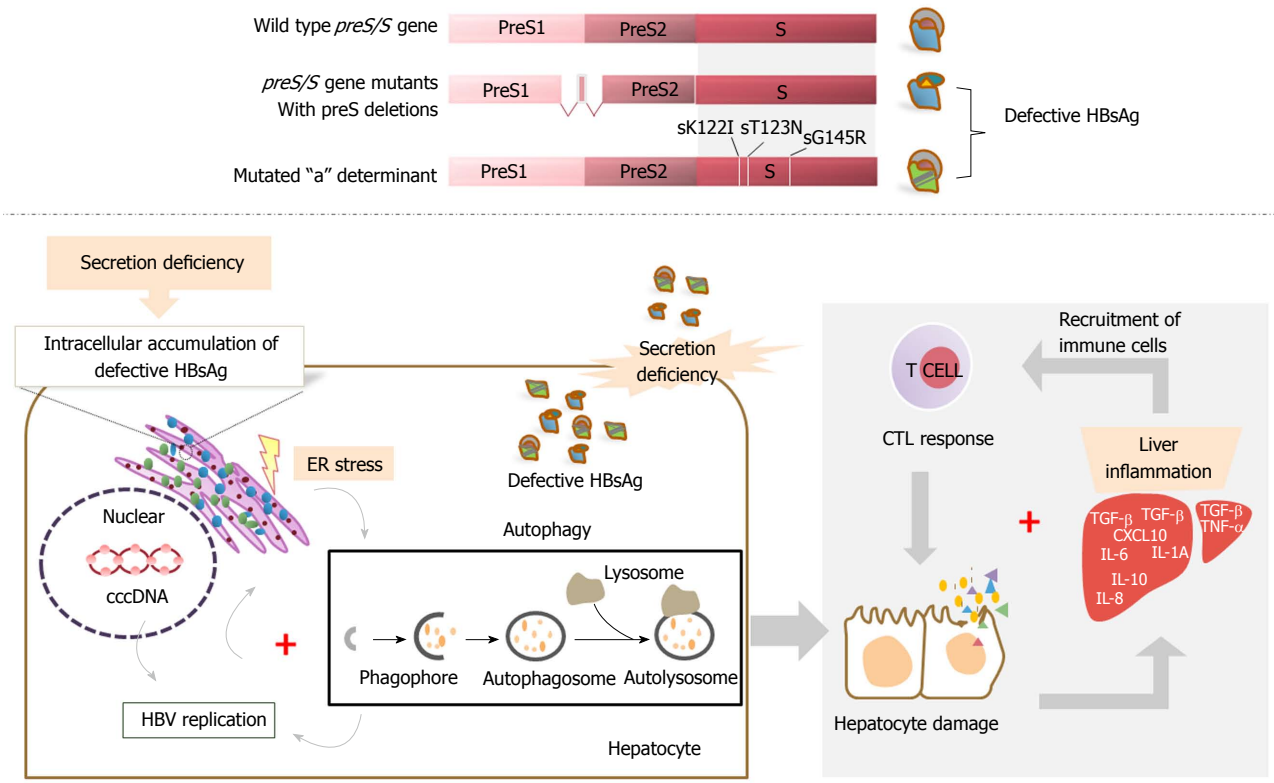


Figure 2 The proposed pathogenic role of mutated secretion-defective hepatitis B surface antigen in fulminant hepatitis. Defective surface antigens, such as preS deletions and mutations within the "a" determinant, may lead to secretion deficiency of HBsAg. Defective HBsAg can promote covalently closed circular DNA (cccDNA) synthesis and amplification, thus facilitating HBV replication. The intracellular accumulation of defective HBsAg can cause endoplasmic reticulum (ER) stress, subsequently trigger autophagy, and may further enhance HBV replication. The enhanced HBV replication, in turn, leads to accumulation of more defective HBsAg, possibly resulting in a positive feedback with unfavorable outcomes and hepatocyte damages. Inflammation may occur in the liver by recruiting immune cells. Cytotoxic T lymphocyte (CTL) response may be abnormally activated and damage infected hepatocytes, contributing to the progression of fulminant hepatitis. HBsAg: Hepatitis B surface antigen.

accumulation of HBsAg within cells and impair the secretion of HBsAg by altering the ratio of LHBs/MHBs/SHBs proteins[72,73,160,161]. Therefore, circulating HBsAg levels are low in the peripheral blood. Moreover, it is well documented that neutralizing antibodies produced during natural infection, or following active or passive immunization against HBV, are targeted to the conformational epitopes of the a-determinant[162]. Hence, single or multiple mutations occurring within this region can lead to conformational changes with impaired antigenicity[72,160]. A recent report has identified novel SHBs mutations outside the MHR from untreated CHB patients. These mutations impaired virion secretion and caused lower binding affinity to antibodies used for HBsAg immunoassays[163]. For these reasons, the mutations can render HBsAg undetectable or poorly detected by immunoassays based on monoclonal antibodies against wild-type virus[60,62,65,164], contributing to some cases of OBI[165-170] (Figure 3).

PERSPECTIVES

Defective surface antigen expression has been well documented to be relevant for the progression of HBV-associated liver diseases, such as HCC. However, the role of defective surface antigen expression in FH still needs to be clarified in future research, particularly, using *in vivo* models and in patients. The exact molecular mechanisms of how defective HBV surface antigens cause damage to hepatocytes and induce liver injury and subsequent pathogenic processes should be investigated. A deep understanding of the molecular mechanisms of HBV pathogenesis related to defective surface antigens is crucial to designing future therapeutic approaches. A critical question would be whether currently used nucleotide analogues (NAs) and interferon-based therapies can prevent such pathogenic processes. NAs are able to efficiently inhibit HBV DNA synthesis but not gene expression. Thus, HBV proteins, including surface antigens, are continuously produced under NA therapies. Another problem is the production of mutated HBV proteins from integrated HBV DNA, which are not controlled by NA therapies at all. Thus, specific interventions may be required to block the pathogenic potential of HBV proteins, besides efficient inhibition of HBV DNA synthesis. RNA silencing may be a suitable choice to achieve this goal[5,6,171,172].

An additional issue to be addressed is whether the defective surface antigen-related mutations may represent novel biomarkers of OBI. With improvement of HBV antigen and DNA detection assays, OBI will likely be easier to diagnose in the future. However,

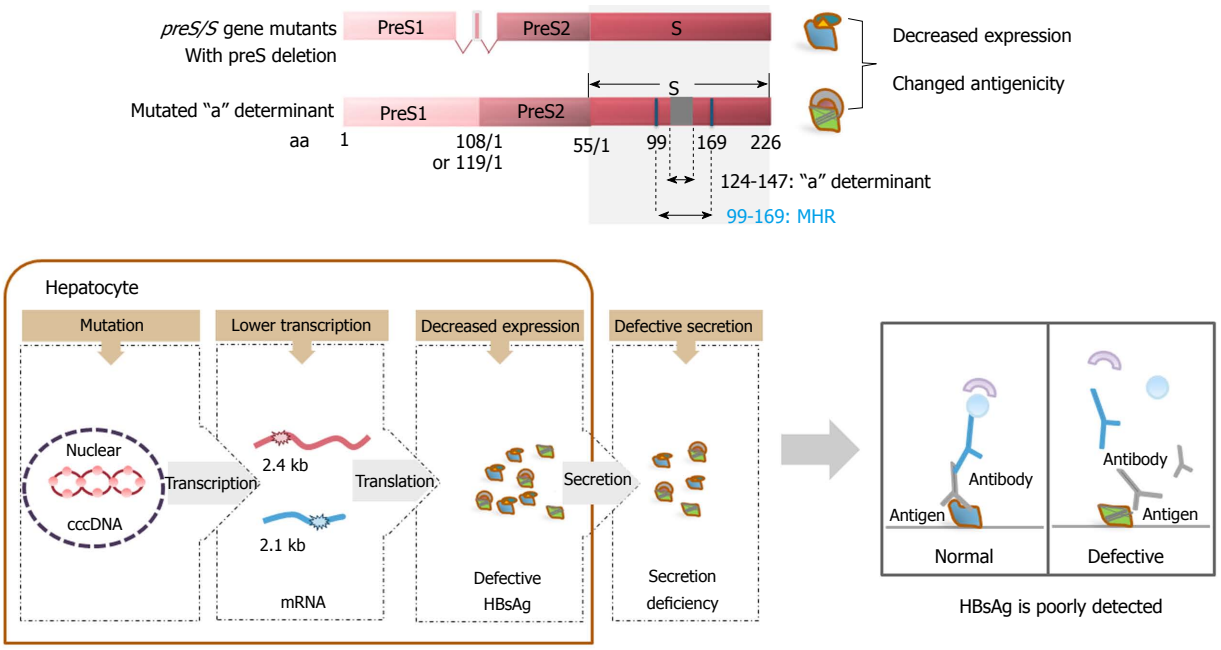


Figure 3 The relationship between the expression of defective surface antigens and occult hepatitis B virus infection. Surface antigen mutations, such as preS deletions, can impair the transcription of 2.4 and 2.1 kb HBV RNAs, leading to decreased levels of three HBV surface proteins. In addition, defective surface antigens with preS deletions and mutations within the "a" determinant are secretion deficient. Single or multiple mutations occurring within the MHR between the aa residues 99-169 of SHBs, especially those within the "a" determinant between aa 124-147, can lead to conformational changes of HBsAg. Mutated HBsAg is poorly detected by immunoassays based on monoclonal antibodies, contributing to some cases of OBI. OBI: Occult hepatitis B virus infection; HBsAg: Hepatitis B surface antigen.

the question remains whether OBI may be related to significant HBV pathogenesis and require therapeutic interventions, such as prophylaxis and antiviral therapy, to prevent HBV reactivation[173].

REFERENCES

1 **Ott JJ**, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 2012; **30**: 2212-2219 [PMID: 22273662 DOI: 10.1016/j.vaccine.2011.12.116]

2 **Polaris Observatory Collaborators.** Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol* 2018; **3**: 383-403 [PMID: 29599078 DOI: 10.1016/S2468-1253(18)30056-6]

3 **Glebe D**, Bremer CM. The molecular virology of hepatitis B virus. *Semin Liver Dis* 2013; **33**: 103-112 [PMID: 23749666 DOI: 10.1055/s-0033-1345717]

4 **Bernal W**, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet* 2010; **376**: 190-201 [PMID: 20638564 DOI: 10.1016/S0140-6736(10)60274-7]

5 **Lok AS**, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: From discovery to regulatory approval. *Hepatology* 2017; **66**: 1296-1313 [PMID: 28762522 DOI: 10.1002/hep.29323]

6 **Lok AS**, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: From discovery to regulatory approval. *J Hepatol* 2017; **67**: 847-861 [PMID: 28778687 DOI: 10.1016/j.jhep.2017.05.008]

7 **Kosinska AD**, Liu J, Lu M, Roggendorf M. Therapeutic vaccination and immunomodulation in the treatment of chronic hepatitis B: preclinical studies in the woodchuck. *Med Microbiol Immunol* 2015; **204**: 103-114 [PMID: 25535101 DOI: 10.1007/s00430-014-0379-5]

8 **Seeger C**, Mason WS. Molecular biology of hepatitis B virus infection. *Virology* 2015; **479-480**: 672-686 [PMID: 25759099 DOI: 10.1016/j.virol.2015.02.031]

9 **Nassal M**. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut* 2015; **64**: 1972-1984 [PMID: 26048673 DOI: 10.1136/gutjnl-2015-309809]

10 **Seeger C**. Control of viral transcripts as a concept for future HBV therapies. *Curr Opin Virol* 2018; **30**: 18-23 [PMID: 29453098 DOI: 10.1016/j.coviro.2018.01.009]

11 **Tong S**, Revill P. Overview of hepatitis B viral replication and genetic variability. *J Hepatol* 2016; **64**: S4-S16 [PMID: 27084035 DOI: 10.1016/j.jhep.2016.01.027]

12 **Gerlich WH**. Medical virology of hepatitis B: how it began and where we are now. *Virol J* 2013; **10**: 239 [PMID: 23870415 DOI: 10.1186/1743-422X-10-239]

13 **Lin CL**, Kao JH. Hepatitis B virus genotypes and variants. *Cold Spring Harb Perspect Med* 2015; **5**: a021436 [PMID: 25934462 DOI: 10.1101/cshperspect.a021436]

14 **Kay A**, Zoulim F. Hepatitis B virus genetic variability and evolution. *Virus Res* 2007; **127**: 164-176 [PMID: 17383765 DOI: 10.1016/j.virusres.2007.02.021]

15 **Kao JH**, Chen PJ, Chen DS. Recent advances in the research of hepatitis B virus-related hepatocellular carcinoma: epidemiologic and molecular biological aspects. *Adv Cancer Res* 2010; **108**: 21-72 [PMID: 21034965 DOI: 10.1016/B978-0-12-380888-2.00002-9]

16 **Pollicino T**, Cacciola I, Saffioti F, Raimondo G. Hepatitis B virus PreS/S gene variants: pathobiology and clinical implications. *J Hepatol* 2014; **61**: 408-417 [PMID: 24801416 DOI: 10.1016/j.jhep.2014.04.041]

17 **Shen T**, Yan XM. Hepatitis B virus genetic mutations and evolution in liver diseases. *World J Gastroenterol* 2014; **20**: 5435-5441 [PMID: 24833874 DOI: 10.3748/wjg.v20.i18.5435]

18 **Zhang ZH**, Wu CC, Chen XW, Li X, Li J, Lu MJ. Genetic variation of hepatitis B virus and its significance for pathogenesis. *World J Gastroenterol* 2016; **22**: 126-144 [PMID: 26755865 DOI: 10.3748/wjg.v22.i1.126]

19 **Chen BF**, Liu CJ, Jow GM, Chen PJ, Kao JH, Chen DS. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. *Gastroenterology* 2006; **130**: 1153-1168 [PMID: 16618410 DOI: 10.1053/j.gastro.2006.01.011]

20 **Heermann KH**, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH. Large surface proteins of hepatitis B virus containing the pre-s sequence. *J Virol* 1984; **52**: 396-402 [PMID: 6492255]

21 **Cattaneo R**, Will H, Hernandez N, Schaller H. Signals regulating hepatitis B surface antigen transcription. *Nature* 1983; **305**: 336-338 [PMID: 6621688]

22 **Stibbe W**, Gerlich WH. Structural relationships between minor and major proteins of hepatitis B surface antigen. *J Virol* 1983; **46**: 626-628 [PMID: 6842680]

23 **Schlüter V**, Meyer M, Hofschneider PH, Koshy R, Caselmann WH. Integrated hepatitis B virus X and 3' truncated preS/S sequences derived from human hepatomas encode functionally active transactivators. *Oncogene* 1994; **9**: 3335-3344 [PMID: 7936659]

24 **Li YW**, Yang FC, Lu HQ, Zhang JS. Hepatocellular carcinoma and hepatitis B surface protein. *World J Gastroenterol* 2016; **22**: 1943-1952 [PMID: 26877602 DOI: 10.3748/wjg.v22.i6.1943]

25 **Le Seyec J**, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P. Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. *J Virol* 1999; **73**: 2052-2057 [PMID: 9971786]

26 **Schulze A**, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology* 2007; **46**: 1759-1768 [PMID: 18046710 DOI: 10.1002/hep.21896]

27 **Glebe D**, Urban S, Knoop EV, Cag N, Krass P, Grün S, Bulavaite A, Sasnauskas K, Gerlich WH. Mapping of the hepatitis B virus attachment site by use of infection-inhibiting preS1 lipopeptides and tupaia hepatocytes. *Gastroenterology* 2005; **129**: 234-245 [PMID: 16012950]

28 **Glebe D**, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH. Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* 2003; **77**: 9511-9521 [PMID: 12915565]

29 **Yan H**, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012; **1**: e00049 [PMID: 23150796 DOI: 10.7554/eLife.00049]

30 **Ostapchuk P**, Hearing P, Ganem D. A dramatic shift in the transmembrane topology of a viral envelope glycoprotein accompanies hepatitis B viral morphogenesis. *EMBO J* 1994; **13**: 1048-1057 [PMID: 8131739]

31 **Bruss V**, Lu X, Thomssen R, Gerlich WH. Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein. *EMBO J* 1994; **13**: 2273-2279 [PMID: 8194518]

32 **Bruss V**. Hepatitis B virus morphogenesis. *World J Gastroenterol* 2007; **13**: 65-73 [PMID: 17206755]

33 **Garcia T**, Li J, Sureau C, Ito K, Qin Y, Wands J, Tong S. Drastic reduction in the production of subviral particles does not impair hepatitis B virus virion secretion. *J Virol* 2009; **83**: 11152-11165 [PMID: 19706705 DOI: 10.1128/JVI.00905-09]

34 **Zhao K**, Wu C, Yao Y, Cao L, Zhang Z, Yuan Y, Wang Y, Pei R, Chen J, Hu X, Zhou Y, Lu M, Chen X. Ceruloplasmin inhibits the production of extracellular hepatitis B virions by targeting its middle surface protein. *J Gen Virol* 2017; **98**: 1410-1421 [PMID: 28678687 DOI: 10.1099/jgv.0.000794]

35 **Bruss V**, Ganem D. The role of envelope proteins in hepatitis B virus assembly. *Proc Natl Acad Sci USA* 1991; **88**: 1059-1063 [PMID: 1992457]

36 **Fernholz D**, Galle PR, Stemler M, Brunetto M, Bonino F, Will H. Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier. *Virology* 1993; **194**: 137-148 [PMID: 8480417 DOI: 10.1006/viro.1993.1243]

37 **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129 [PMID: 15014185 DOI: 10.1056/NEJMra031087]

38 **Persing DH**, Varmus HE, Ganem D. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science* 1986; **234**: 1388-1391 [PMID: 3787251]

39 **Ou JH**, Rutter WJ. Regulation of secretion of the hepatitis B virus major surface antigen by the preS-1 protein. *J Virol* 1987; **61**: 782-786 [PMID: 3806798]

40 **Chisari FV**, Filippi P, McLachlan A, Milich DR, Riggs M, Lee S, Palmiter RD, Pinkert CA, Brinster RL. Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. *J Virol* 1986; **60**: 880-887 [PMID: 3783819]

41 **Chisari FV**, Filippi P, Buras J, McLachlan A, Popper H, Pinkert CA, Palmiter RD, Brinster RL. Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. *Proc Natl Acad Sci USA* 1987; **84**: 6909-6913 [PMID: 3477814]

42 **Nayersina R**, Fowler P, Guilhot S, Missale G, Cerny A, Schlicht HJ, Vitiello A, Chesnut R, Person JL, Redeker AG, Chisari FV. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *J Immunol* 1993; **150**: 4659-4671 [PMID: 7683326]

43 **Chisari FV**, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; **13**: 29-60 [PMID: 7612225 DOI: 10.1146/annurev.iy.13.040195.000333]

44 **Coppola N**, Onorato L, Minichini C, Di Caprio G, Starace M, Sagnelli C, Sagnelli E. Clinical significance of hepatitis B surface antigen mutants. *World J Hepatol* 2015; **7**: 2729-2739 [PMID: 26644816 DOI: 10.4254/wjh.v7.i27.2729]

45 **Zhu HL**, Li X, Li J, Zhang ZH. Genetic variation of occult hepatitis B virus infection. *World J Gastroenterol* 2016; **22**: 3531-3546 [PMID: 27053845 DOI: 10.3748/wjg.v22.i13.3531]

46 **Maupas P**, Chiron JP, Barin F, Coursaget P, Goudeau A, Perrin J, Denis F, Mar ID. Efficacy of hepatitis B vaccine in prevention of early HBsAg carrier state in children. Controlled trial in an endemic area (Senegal). *Lancet* 1981; **1**: 289-292 [PMID: 6109938]

47 **Szmuness W**, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, Sadovsky R, Morrison JM, Kellner A. Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. *N Engl J Med* 1980; **303**: 833-841 [PMID: 6997738 DOI: 10.1056/NEJM198010093031501]

48 **Valenzuela P**, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 1982; **298**: 347-350 [PMID: 7045698]

49 **Reignat S**, Webster GJ, Brown D, Ogg GS, King A, Seneviratne SL, Dusheiko G, Williams R, Maini MK, Bertoletti A. Escaping high viral load exhaustion: CD8 cells with altered tetramer binding in chronic hepatitis B virus infection. *J Exp Med* 2002; **195**: 1089-1101 [PMID: 11994415]

50 **Guidotti LG**, Isogawa M, Chisari FV. Host-virus interactions in hepatitis B virus infection. *Curr Opin Immunol* 2015; **36**: 61-66 [PMID: 26186123 DOI: 10.1016/j.coi.2015.06.016]

51 **Wang S**, Chen Z, Hu C, Qian F, Cheng Y, Wu M, Shi B, Chen J, Hu Y, Yuan Z. Hepatitis B virus surface antigen selectively inhibits TLR2 ligand-induced IL-12 production in monocytes/macrophages by interfering with JNK activation. *J Immunol* 2013; **190**: 5142-5151 [PMID: 23585678 DOI: 10.4049/jimmunol.1201625]

52 **Liu J**, Yu Q, Wu W, Huang X, Broering R, Werner M, Roggendorf M, Yang D, Lu M. TLR2 Stimulation Strengthens Intrahepatic Myeloid-Derived Cell-Mediated T Cell Tolerance through Inducing Kupffer Cell Expansion and IL-10 Production. *J Immunol* 2018; **200**: 2341-2351 [PMID: 29459406 DOI: 10.4049/jimmunol.1700540]

53 **Jiang M**, Broering R, Trippler M, Poggenpohl L, Fiedler M, Gerken G, Lu M, Schlaak JF. Toll-like receptor-mediated immune responses are attenuated in the presence of high levels of hepatitis B virus surface antigen. *J Viral Hepat* 2014; **21**: 860-872 [PMID: 24498958 DOI: 10.1111/jvh.12216]

54 **Wu J**, Meng Z, Jiang M, Pei R, Trippler M, Broering R, Bucchi A, Sowa JP, Dittmer U, Yang D, Roggendorf M, Gerken G, Lu M, Schlaak JF. Hepatitis B virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells. *Hepatology* 2009; **49**: 1132-1140

[PMID: 19140219 DOI: 10.1002/hep.22751]
55 **Fang Z**, Li J, Yu X, Zhang D, Ren G, Shi B, Wang C, Kosinska AD, Wang S, Zhou X, Kozlowski M, Hu Y, Yuan Z. Polarization of Monocytic Myeloid-Derived Suppressor Cells by Hepatitis B Surface Antigen Is Mediated via ERK/IL-6/STAT3 Signaling Feedback and Restrains the Activation of T Cells in Chronic Hepatitis B Virus Infection. *J Immunol* 2015; **195**: 4873-4883 [PMID: 26416274 DOI: 10.4049/jimmunol.1501362]
56 **Dietze KK**, Schimmer S, Kretzmer F, Wang J, Lin Y, Huang X, Wu W, Wang B, Lu M, Dittmer U, Yang D, Liu J. Characterization of the Treg Response in the Hepatitis B Virus Hydrodynamic Injection Mouse Model. *PLoS One* 2016; **11**: e0151717 [PMID: 26986976 DOI: 10.1371/journal.pone.0151717]
57 **Kosinska AD**, Pishraft-Sabet L, Wu W, Fang Z, Lenart M, Chen J, Dietze KK, Wang C, Kemper T, Lin Y, Yeh SH, Liu J, Dittmer U, Yuan Z, Roggendorf M, Lu M. Low hepatitis B virus-specific T-cell response in males correlates with high regulatory T-cell numbers in murine models. *Hepatology* 2017; **66**: 69-83 [PMID: 28295453 DOI: 10.1002/hep.29155]
58 **Zanetti AR**, Tanzi E, Manzillo G, Maio G, Sbreglia C, Caporaso N, Thomas H, Zuckerman AJ. Hepatitis B variant in Europe. *Lancet* 1988; **2**: 1132-1133 [PMID: 2460710]
59 **Carman WF**, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC. Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325-329 [PMID: 1697396]
60 **Cooreman MP**, van Roosmalen MH, te Morsche R, Sünnen CM, de Ven EM, Jansen JB, Tytgat GN, de Wit PL, Paulij WP. Characterization of the reactivity pattern of murine monoclonal antibodies against wild-type hepatitis B surface antigen to G145R and other naturally occurring "a" loop escape mutations. *Hepatology* 1999; **30**: 1287-1292 [PMID: 10534351 DOI: 10.1002/hep.510300508]
61 **Cooreman MP**, Leroux-Roels G, Paulij WP. Vaccine- and hepatitis B immune globulin-induced escape mutations of hepatitis B virus surface antigen. *J Biomed Sci* 2001; **8**: 237-247 [PMID: 11385295 DOI: 10.1159/000054039]
62 **Chiou HL**, Lee TS, Kuo J, Mau YC, Ho MS. Altered antigenicity of 'a' determinant variants of hepatitis B virus. *J Gen Virol* 1997; **78** (Pt 10): 2639-2645 [PMID: 9349486 DOI: 10.1099/0022-1317-78-10-2639]
63 **Günther S**, Fischer L, Pult I, Sterneck M, Will H. Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999; **52**: 25-137 [PMID: 10384235]
64 **Lu M**, Lorentz T. De novo infection in a renal transplant recipient caused by novel mutants of hepatitis B virus despite the presence of protective anti-hepatitis B surface antibody. *J Infect Dis* 2003; **187**: 1323-1326 [PMID: 12696014 DOI: 10.1086/373902]
65 **Seddigh-Tonekaboni S**, Waters JA, Jeffers S, Gehrke R, Ofenloch B, Horsch A, Hess G, Thomas HC, Karayiannis P. Effect of variation in the common "a" determinant on the antigenicity of hepatitis B surface antigen. *J Med Virol* 2000; **60**: 113-121 [PMID: 10596008]
66 **Ghany MG**, Ayola B, Villamil FG, Gish RG, Rojter S, Vierling JM, Lok AS. Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. *Hepatology* 1998; **27**: 213-222 [PMID: 9425940 DOI: 10.1002/hep.510270133]
67 **Hsu HY**, Chang MH, Ni YH, Lin HH, Wang SM, Chen DS. Surface gene mutants of hepatitis B virus in infants who develop acute or chronic infections despite immunoprophylaxis. *Hepatology* 1997; **26**: 786-791 [PMID: 9303514 DOI: 10.1002/hep.510260336]
68 **Okamoto H**, Yano K, Nozaki Y, Matsui A, Miyazaki H, Yamamoto K, Tsuda F, Machida A, Mishiro S. Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. *Pediatr Res* 1992; **32**: 264-268 [PMID: 1383917 DOI: 10.1203/00006450-199209000-00002]
69 **Protzer-Knolle U**, Naumann U, Bartenschlager R, Berg T, Hopf U, Meyer zum Büschenfelde KH, Neuhaus P, Gerken G. Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology* 1998; **27**: 254-263 [PMID: 9425945 DOI: 10.1002/hep.510270138]
70 **Wu C**, Deng W, Deng L, Cao L, Qin B, Li S, Wang Y, Pei R, Yang D, Lu M, Chen X. Amino acid substitutions at positions 122 and 145 of hepatitis B virus surface antigen (HBsAg) determine the antigenicity and immunogenicity of HBsAg and influence in vivo HBsAg clearance. *J Virol* 2012; **86**: 4658-4669 [PMID: 22301154 DOI: 10.1128/JVI.06353-11]
71 **Kalinina T**, Riu A, Fischer L, Will H, Sterneck M. A dominant hepatitis B virus population defective in virus secretion because of several S-gene mutations from a patient with fulminant hepatitis. *Hepatology* 2001; **34**: 385-394 [PMID: 11481624 DOI: 10.1053/jhep.2001.26516]
72 **Wu C**, Zhang X, Tian Y, Song J, Yang D, Roggendorf M, Lu M, Chen X. Biological significance of amino acid substitutions in hepatitis B surface antigen (HBsAg) for glycosylation, secretion, antigenicity and immunogenicity of HBsAg and hepatitis B virus replication. *J Gen Virol* 2010; **91**: 483-492 [PMID: 19812261 DOI: 10.1099/vir.0.012740-0]
73 **Li S**, Zhao K, Liu S, Wu C, Yao Y, Cao L, Hu X, Zhou Y, Wang Y, Pei R, Lu M, Chen X. HBsAg sT123N mutation induces stronger antibody responses to HBsAg and HBcAg and accelerates in vivo HBsAg clearance. *Virus Res* 2015; **210**: 119-125 [PMID: 26260331 DOI: 10.1016/j.virusres.2015.08.004]
74 **Zoulim F**, Durantel D. Antiviral therapies and prospects for a cure of chronic hepatitis B. *Cold Spring Harb Perspect Med* 2015; **5** [PMID: 25833942 DOI: 10.1101/cshperspect.a021501]
75 **Zoulim F**, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009; **137**: 1593-1608.e1-2 [PMID: 19737565 DOI: 10.1053/j.gastro.2009.08.063]
76 **Warner N**, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. *Hepatology* 2008; **48**: 88-98 [PMID: 18537180 DOI: 10.1002/hep.22295]
77 **Ahn SH**, Park YK, Park ES, Kim JH, Kim DH, Lim KH, Jang MS, Choe WH, Ko SY, Sung IK, Kwon SY, Kim KH. The impact of the hepatitis B virus polymerase rtA181T mutation on replication and drug resistance is potentially affected by overlapping changes in surface gene. *J Virol* 2014; **88**: 6805-6818 [PMID: 24696492 DOI: 10.1128/JVI.00635-14]
78 **Yeh CT**. Development of HBV S gene mutants in chronic hepatitis B patients receiving nucleotide/nucleoside analogue therapy. *Antivir Ther* 2010; **15**: 471-475 [PMID: 20516567 DOI: 10.3851/IMP1552]
79 **Pollicino T**, Amaddeo G, Restuccia A, Raffa G, Alibrandi A, Cutroneo G, Favaloro A, Maimone S, Squadrito G, Raimondo G. Impact of hepatitis B virus (HBV) preS/S genomic variability on HBV surface antigen and HBV DNA serum levels. *Hepatology* 2012; **56**: 434-443 [PMID: 22271491 DOI: 10.1002/hep.25592]
80 **Fernholz D**, Stemler M, Brunetto M, Bonino F, Will H. Replicating and virion secreting hepatitis B mutant virus unable to produce preS2 protein. *J Hepatol* 1991; **13** Suppl 4: S102-S104 [PMID: 1822500]
81 **Gerken G**, Kremsdorf D, Capel F, Petit MA, Dauguet C, Manns MP, Meyer zum Büschenfelde KH, Brechot C. Hepatitis B defective virus with rearrangements in the preS gene during chronic HBV infection. *Virology* 1991; **183**: 555-565 [PMID: 1853561]
82 **Sugauchi F**, Ohno T, Orito E, Sakugawa H, Ichida T, Komatsu M, Kuramitsu T, Ueda R, Miyakawa Y, Mizokami M. Influence of hepatitis B virus genotypes on the development of preS deletions and advanced liver disease. *J Med Virol* 2003; **70**: 537-544 [PMID: 12794715 DOI: 10.1002/jmv.10428]
83 **Chen CH**, Changchien CS, Lee CM, Hung CH, Hu TH, Wang JH, Wang JC, Lu SN. Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. *J Infect Dis* 2008; **198**: 1634-1642 [PMID: 18939932 DOI: 10.1086/592990]
84 **Cao L**, Wu C, Shi H, Gong Z, Zhang E, Wang H, Zhao K, Liu S, Li S, Gao X, Wang Y, Pei R, Lu M, Chen X. Coexistence of hepatitis

B virus quasispecies enhances viral replication and the ability to induce host antibody and cellular immune responses. *J Virol* 2014; **88**: 8656-8666 [PMID: 24850745 DOI: 10.1128/JVI.01123-14]
85 **Summers J**, Smith PM, Horwich AL. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; **64**: 2819-2824 [PMID: 2335817]
86 **Lenhoff RJ**, Summers J. Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. *J Virol* 1994; **68**: 4565-4571 [PMID: 8207830]
87 **Lenhoff RJ**, Luscombe CA, Summers J. Acute liver injury following infection with a cytopathic strain of duck hepatitis B virus. *Hepatology* 1999; **29**: 563-571 [PMID: 9918936 DOI: 10.1002/hep.510290236]
88 **Summers J**, Smith PM, Huang MJ, Yu MS. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. *J Virol* 1991; **65**: 1310-1317 [PMID: 1995945]
89 **Gao W**, Hu J. Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. *J Virol* 2007; **81**: 6164-6174 [PMID: 17409153 DOI: 10.1128/JVI.02721-06]
90 **Guo H**, Jiang D, Zhou T, Cuconati A, Block TM, Guo JT. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. *J Virol* 2007; **81**: 12472-12484 [PMID: 17804499 DOI: 10.1128/JVI.01123-07]
91 **Lentz TB**, Loeb DD. Roles of the envelope proteins in the amplification of covalently closed circular DNA and completion of synthesis of the plus-strand DNA in hepatitis B virus. *J Virol* 2011; **85**: 11916-11927 [PMID: 21900164 DOI: 10.1128/JVI.05373-11]
92 **Mu SC**, Lin YM, Jow GM, Chen BF. Occult hepatitis B virus infection in hepatitis B vaccinated children in Taiwan. *J Hepatol* 2009; **50**: 264-272 [PMID: 19070923 DOI: 10.1016/j.jhep.2008.09.017]
93 **Chirara MM**, Chetsanga CJ. Variant of hepatitis B virus isolated in Zimbabwe. *J Med Virol* 1994; **42**: 73-78 [PMID: 8308523]
94 **Bowyer SM**, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; **78** (Pt 7): 1719-1729 [PMID: 9225049 DOI: 10.1099/0022-1317-78-7-1719]
95 **Owiredu WK**, Kramvis A, Kew MC. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. *J Med Virol* 2001; **65**: 485-492 [PMID: 11596083]
96 **Garfein RS**, Bower WA, Loney CM, Hutin YJ, Xia GL, Jawanda J, Groom AV, Nainan OV, Murphy JS, Bell BP. Factors associated with fulminant liver failure during an outbreak among injection drug users with acute hepatitis B. *Hepatology* 2004; **40**: 865-873 [PMID: 15382123 DOI: 10.1002/hep.20383]
97 **Liaw YF**, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, Guan R, Lau GK, Locarnini S; Chronic Hepatitis B Guideline Working Party of the Asian-Pacific Association for the Study of the Liver. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263-283 [PMID: 19669255 DOI: 10.1007/s12072-008-9080-3]
98 **Shen T**, Yan XM, Zou YL, Gao JM, Dong H. Virologic characteristics of hepatitis B virus in patients infected via maternal-fetal transmission. *World J Gastroenterol* 2008; **14**: 5674-5682 [PMID: 18837083]
99 **Shen FC**, Su IJ, Wu HC, Hsieh YH, Yao WJ, Young KC, Chang TC, Hsieh HC, Tsai HN, Huang W. A pre-S gene chip to detect pre-S deletions in hepatitis B virus large surface antigen as a predictive marker for hepatoma risk in chronic hepatitis B virus carriers. *J Biomed Sci* 2009; **16**: 84 [PMID: 19751529 DOI: 10.1186/1423-0127-16-84]
100 **Shen T**, Yan XM, Zhang JP, Wang JL, Zuo RX, Li L, Wang LP. Evolution of Hepatitis B Virus in a Chronic HBV-Infected Patient over 2 Years. *Hepat Res Treat* 2011; **2011**: 939148 [PMID: 21785721 DOI: 10.1155/2011/939148]
101 **Heo NY**, Lee HC, Park YK, Park JW, Lim YS, Kim KM, Shim JH, Lee YJ. Lack of association between hepatitis B virus pre-S mutations and recurrence after surgical resection in hepatocellular carcinoma. *J Med Virol* 2013; **85**: 589-596 [PMID: 23296476 DOI: 10.1002/jmv.23502]
102 **Hung CH**, Chen CH, Lee CM, Hu TH, Lu SN, Wang JH, Huang CM. Role of viral genotypes and hepatitis B viral mutants in the risk of hepatocellular carcinoma associated with hepatitis B and C dual infection. *Intervirology* 2013; **56**: 316-324 [PMID: 23838434 DOI: 10.1159/000350738]
103 **Fan YF**, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, Lei HY, Shiau AL, Su IJ. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. *Hepatology* 2001; **33**: 277-286 [PMID: 11124846 DOI: 10.1053/jhep.2001.21163]
104 **Chen CH**, Hung CH, Lee CM, Hu TH, Wang JH, Wang JC, Lu SN, Changchien CS. Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. *Gastroenterology* 2007; **133**: 1466-1474 [PMID: 17915220 DOI: 10.1053/j.gastro.2007.09.002]
105 **Ghosh S**, Mondal RK, Banerjee P, Nandi M, Sarkar S, Das K, Santra A, Banerjee S, Chowdhury A, Datta S. Tracking the naturally occurring mutations across the full-length genome of hepatitis B virus of genotype D in different phases of chronic e-antigen-negative infection. *Clin Microbiol Infect* 2012; **18**: E412-E418 [PMID: 22827722 DOI: 10.1111/j.1469-0691.2012.03975.x]
106 **Xu Z**, Yen TS. Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles. *J Virol* 1996; **70**: 133-140 [PMID: 8523517]
107 **Su IJ**, Wang LH, Hsieh WC, Wu HC, Teng CF, Tsai HW, Huang W. The emerging role of hepatitis B virus pre-S2 deletion mutant proteins in HBV tumorigenesis. *J Biomed Sci* 2014; **21**: 98 [PMID: 25316153 DOI: 10.1186/s12929-014-0098-7]
108 **Arbuthnot P**, Kew M. Hepatitis B virus and hepatocellular carcinoma. *Int J Exp Pathol* 2001; **82**: 77-100 [PMID: 11454100]
109 **Hildt E**, Hofschneider PH. The PreS2 activators of the hepatitis B virus: activators of tumour promoter pathways. *Recent Results Cancer Res* 1998; **154**: 315-329 [PMID: 10027012]
110 **Caselmann WH**, Meyer M, Kekulé AS, Lauer U, Hofschneider PH, Koshy R. A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA. *Proc Natl Acad Sci USA* 1990; **87**: 2970-2974 [PMID: 2158099]
111 **Lauer U**, Weiss L, Lipp M, Hofschneider PH, Kekulé AS. The hepatitis B virus preS2/St transactivator utilizes AP-1 and other transcription factors for transactivation. *Hepatology* 1994; **19**: 23-31 [PMID: 8276360]
112 **Sterneck M**, Günther S, Gerlach J, Naoumov NV, Santantonio T, Fischer L, Rogiers X, Greten H, Williams R, Will H. Hepatitis B virus sequence changes evolving in liver transplant recipients with fulminant hepatitis. *J Hepatol* 1997; **26**: 754-764 [PMID: 9126786]
113 **Pollicino T**, Zanetti AR, Cacciola I, Petit MA, Smedile A, Campo S, Sagliocca L, Pasquali M, Tanzi E, Longo G, Raimondo G. Pre-S2 defective hepatitis B virus infection in patients with fulminant hepatitis. *Hepatology* 1997; **26**: 495-499 [PMID: 9252165 DOI: 10.1002/hep.510260235]
114 **Bock CT**, Tillmann HL, Maschek HJ, Manns MP, Trautwein C. A preS mutation isolated from a patient with chronic hepatitis B infection leads to virus retention and misassembly. *Gastroenterology* 1997; **113**: 1976-1982 [PMID: 9394738]
115 **Bock CT**, Kubicka S, Manns MP, Trautwein C. Two control elements in the hepatitis B virus S-promoter are important for full promoter activity mediated by CCAAT-binding factor. *Hepatology* 1999; **29**: 1236-1247 [PMID: 10094970 DOI: 10.1002/hep.510290426]
116 **Bock CT**, Tillmann HL, Manns MP, Trautwein C. The pre-S region determines the intracellular localization and appearance of hepatitis B virus. *Hepatology* 1999; **30**: 517-525 [PMID: 10421662 DOI: 10.1002/hep.510300206]
117 **Ogata M**, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* 2006;

26: 9220-9231 [PMID: 17030611 DOI: 10.1128/MCB.01453-06]
118 **Høyer-Hansen M**, Jäättelä M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ* 2007; **14**: 1576-1582 [PMID: 17612585 DOI: 10.1038/sj.cdd.4402200]
119 **Kouroku Y**, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ* 2007; **14**: 230-239 [PMID: 16794605 DOI: 10.1038/sj.cdd.4401984]
120 **Li J**, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ* 2008; **15**: 1460-1471 [PMID: 18551133 DOI: 10.1038/cdd.2008.81]
121 **Qin L**, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* 2010; **6**: 239-247 [PMID: 20104019]
122 **Lépine S**, Allegood JC, Park M, Dent P, Milstien S, Spiegel S. Sphingosine-1-phosphate phosphohydrolase-1 regulates ER stress-induced autophagy. *Cell Death Differ* 2011; **18**: 350-361 [PMID: 20798685 DOI: 10.1038/cdd.2010.104]
123 **Sakaki K**, Wu J, Kaufman RJ. Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. *J Biol Chem* 2008; **283**: 15370-15380 [PMID: 18356160 DOI: 10.1074/jbc.M710209200]
124 **Yorimitsu T**, Klionsky DJ. Endoplasmic reticulum stress: a new pathway to induce autophagy. *Autophagy* 2007; **3**: 160-162 [PMID: 17204854]
125 **Yorimitsu T**, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* 2006; **281**: 30299-30304 [PMID: 16901900 DOI: 10.1074/jbc.M607007200]
126 **Sir D**, Tian Y, Chen WL, Ann DK, Yen TS, Ou JH. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. *Proc Natl Acad Sci USA* 2010; **107**: 4383-4388 [PMID: 20142477 DOI: 10.1073/pnas.0911373107]
127 **Li J**, Liu Y, Wang Z, Liu K, Wang Y, Liu J, Ding H, Yuan Z. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. *J Virol* 2011; **85**: 6319-6333 [PMID: 21507968 DOI: 10.1128/JVI.02627-10]
128 **Naoumov NV**, Eddleston AL. Host immune response and variations in the virus genome: pathogenesis of liver damage caused by hepatitis B virus. *Gut* 1994; **35**: 1013-1017 [PMID: 7926896]
129 **Bertoletti A**, Ferrari C. Adaptive immunity in HBV infection. *J Hepatol* 2016; **64**: S71-S83 [PMID: 27084039 DOI: 10.1016/j.jhep.2016.01.026]
130 **Guidotti LG**, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006; **1**: 23-61 [PMID: 18039107 DOI: 10.1146/annurev.pathol.1.110304.100230]
131 **Peeridogaheh H**, Meshkat Z, Habibzadeh S, Arzanlou M, Shahi JM, Rostami S, Gerayli S, Teimourpour R. Current concepts on immunopathogenesis of hepatitis B virus infection. *Virus Res* 2018; **245**: 29-43 [PMID: 29273341 DOI: 10.1016/j.virusres.2017.12.007]
132 **Ando K**, Moriyama T, Guidotti LG, Wirth S, Schreiber RD, Schlicht HJ, Huang SN, Chisari FV. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. *J Exp Med* 1993; **178**: 1541-1554 [PMID: 8228807]
133 **Mina T**, Amini Bavil Olyaee S, Tacke F, Maes P, Van Ranst M, Pourkarim MR. Genomic Diversity of Hepatitis B Virus Infection Associated With Fulminant Hepatitis B Development. *Hepat Mon* 2015; **15**: e29477 [PMID: 26288637 DOI: 10.5812/hepatmon.29477v2]
134 **Bertoletti A**, Maini M, Williams R. Role of hepatitis B virus specific cytotoxic T cells in liver damage and viral control. *Antiviral Res* 2003; **60**: 61-66 [PMID: 14638399]
135 **Maini MK**, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, King AS, Herberg J, Gilson R, Alisa A, Williams R, Vergani D, Naoumov NV, Ferrari C, Bertoletti A. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* 2000; **191**: 1269-1280 [PMID: 10770795]
136 **Bertoletti A**, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Curr Opin Immunol* 2000; **12**: 403-408 [PMID: 10899021]
137 **Bagaglio S**, Albarello L, Biswas P, Uberti-Foppa C, Fortis C, Morsica G. Virological pattern of hepatitis B infection in an HIV-positive man with fatal fulminant hepatitis B: a case report. *J Med Case Rep* 2009; **3**: 110 [PMID: 19946588 DOI: 10.1186/1752-1947-3-110]
138 **Raimondo G**, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, Craxì A, Donato F, Ferrari C, Gaeta GB, Gerlich WH, Levrero M, Locarnini S, Michalak T, Mondelli MU, Pawlotsky JM, Pollicino T, Prati D, Puoti M, Samuel D, Shouval D, Smedile A, Squadrito G, Trépo C, Villa E, Will H, Zanetti AR, Zoulim F. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* 2008; **49**: 652-657 [PMID: 18715666 DOI: 10.1016/j.jhep.2008.07.014]
139 **Makvandi M**. Update on occult hepatitis B virus infection. *World J Gastroenterol* 2016; **22**: 8720-8734 [PMID: 27818588 DOI: 10.3748/wjg.v22.i39.8720]
140 **Chemin I**, Trépo C. Clinical impact of occult HBV infections. *J Clin Virol* 2005; **34** Suppl 1: S15-S21 [PMID: 16461218]
141 **Hashemi SJ**, Hajiani E, Masjedizadeh A, Makvandi M, Shayesteh AA, Alavinejad SP, Kadkhodaei A, Shahbazian H, Jasemi F, Karimi M. Occult hepatitis B infection in patients with cryptogenic liver cirrhosis in southwest of Iran. *Jundishapur J Microbiol* 2015; **8**: e16873 [PMID: 25861432 DOI: 10.5812/jjm.16873]
142 **Saitta C**, Tripodi G, Barbera A, Bertuccio A, Smedile A, Ciancio A, Raffa G, Sangiovanni A, Navarra G, Raimondo G, Pollicino T. Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma. *Liver Int* 2015; **35**: 2311-2317 [PMID: 25677098 DOI: 10.1111/liv.12807]
143 **Chemin I**, Zoulim F, Merle P, Arkhis A, Chevallier M, Kay A, Cova L, Chevallier P, Mandrand B, Trépo C. High incidence of hepatitis B infections among chronic hepatitis cases of unknown aetiology. *J Hepatol* 2001; **34**: 447-454 [PMID: 11322208]
144 **Pollicino T**, Squadrito G, Cerenzia G, Cacciola I, Raffa G, Craxi A, Farinati F, Missale G, Smedile A, Tiribelli C, Villa E, Raimondo G. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology* 2004; **126**: 102-110 [PMID: 14699492]
145 **Wong DK**, Huang FY, Lai CL, Poon RT, Seto WK, Fung J, Hung IF, Yuen MF. Occult hepatitis B infection and HBV replicative activity in patients with cryptogenic cause of hepatocellular carcinoma. *Hepatology* 2011; **54**: 829-836 [PMID: 21809355 DOI: 10.1002/hep.24551]
146 **Altfeld M**, Rockstroh JK, Addo M, Kupfer B, Pult I, Will H, Spengler U. Reactivation of hepatitis B in a long-term anti-HBs-positive patient with AIDS following lamivudine withdrawal. *J Hepatol* 1998; **29**: 306-309 [PMID: 9722213]
147 **Kidd-Ljunggren K**, Simonsen O. Reappearance of hepatitis B 10 years after kidney transplantation. *N Engl J Med* 1999; **341**: 127-128 [PMID: 10409028 DOI: 10.1056/NEJM199907083410213]
148 **Westhoff TH**, Jochimsen F, Schmittel A, Stoffler-Meilicke M, Schafer JH, Zidek W, Gerlich WH, Thiel E. Fatal hepatitis B virus reactivation by an escape mutant following rituximab therapy. *Blood* 2003; **102**: 1930 [PMID: 12930732 DOI: 10.1182/blood-2003-05-1403]
149 **Kubo S**, Tamori A, Ohba K, Shuto T, Yamamoto T, Tanaka H, Nishiguchi S, Wakasa K, Hirohashi K, Kinoshita H. Previous or occult hepatitis B virus infection in hepatitis C virus-associated hepatocellular carcinoma without hepatic fibrosis. *Dig Dis Sci* 2001; **46**: 2408-2414 [PMID: 11713944]
150 **Lalazar G**, Rund D, Shouval D. Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. *Br J Haematol* 2007; **136**: 699-712 [PMID: 17338776 DOI: 10.1111/j.1365-2141.2006.06465.x]
151 **García-Fulgueiras A**, García-Pina R, Morant C, García-Ortuzar V,

Génova R, Alvarez E. Hepatitis C and hepatitis B-related mortality in Spain. *Eur J Gastroenterol Hepatol* 2009; **21**: 895-901 [PMID: 19357523 DOI: 10.1097/MEG.0b013e328313139d]

152 **Marcellin P**, Pequignot F, Delarocque-Astagneau E, Zarski JP, Ganne N, Hillon P, Antona D, Bovet M, Mechain M, Asselah T, Desenclos JC, Jougla E. Mortality related to chronic hepatitis B and chronic hepatitis C in France: evidence for the role of HIV coinfection and alcohol consumption. *J Hepatol* 2008; **48**: 200-207 [PMID: 18086507 DOI: 10.1016/j.jhep.2007.09.010]

153 **Xie M**, Rao W, Yang T, Deng Y, Zheng H, Pan C, Liu Y, Shen Z, Jia J. Occult hepatitis B virus infection predicts de novo hepatitis B infection in patients with alcoholic cirrhosis after liver transplantation. *Liver Int* 2015; **35**: 897-904 [PMID: 24750566 DOI: 10.1111/liv.12567]

154 **Candotti D**, Grabarczyk P, Ghiazza P, Roig R, Casamitjana N, Iudicone P, Schmidt M, Bird A, Crookes R, Brojer E, Miceli M, Amiri A, Li C, Allain JP. Characterization of occult hepatitis B virus from blood donors carrying genotype A2 or genotype D strains. *J Hepatol* 2008; **49**: 537-547 [PMID: 18602718 DOI: 10.1016/j.jhep.2008.04.017]

155 **Allain JP**, Belkhiri D, Vermeulen M, Crookes R, Cable R, Amiri A, Reddy R, Bird A, Candotti D. Characterization of occult hepatitis B virus strains in South African blood donors. *Hepatology* 2009; **49**: 1868-1876 [PMID: 19434719 DOI: 10.1002/hep.22879]

156 **Huang CH**, Yuan Q, Chen PJ, Zhang YL, Chen CR, Zheng QB, Yeh SH, Yu H, Xue Y, Chen YX, Liu PG, Ge SX, Zhang J, Xia NS. Influence of mutations in hepatitis B virus surface protein on viral antigenicity and phenotype in occult HBV strains from blood donors. *J Hepatol* 2012; **57**: 720-729 [PMID: 22634131 DOI: 10.1016/j.jhep.2012.05.009]

157 **Saha D**, Pal A, Sarkar N, Das D, Blackard JT, Guha SK, Saha B, Chakravarty R. Occult hepatitis B virus infection in HIV positive patients at a tertiary healthcare unit in eastern India. *PLoS One* 2017; **12**: e0179035 [PMID: 28591184 DOI: 10.1371/journal.pone.0179035]

158 **Ye Q**, Shang SQ, Li W. A new vaccine escape mutant of hepatitis B virus causes occult infection. *Hum Vaccin Immunother* 2015; **11**: 407-410 [PMID: 25692622 DOI: 10.4161/21645515.2014.994461]

159 **Hou J**, Wang Z, Cheng J, Lin Y, Lau GK, Sun J, Zhou F, Waters J, Karayiannis P, Luo K. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology* 2001; **34**: 1027-1034 [PMID: 11679975 DOI: 10.1053/jhep.2001.28708]

160 **Kwei K**, Tang X, Lok AS, Sureau C, Garcia T, Li J, Wands J, Tong S. Impaired virion secretion by hepatitis B virus immune escape mutants and its rescue by wild-type envelope proteins or a second-site mutation. *J Virol* 2013; **87**: 2352-2357 [PMID: 23221548 DOI: 10.1128/JVI.02701-12]

161 **Biswas S**, Candotti D, Allain JP. Specific amino acid substitutions in the S protein prevent its excretion in vitro and may contribute to occult hepatitis B virus infection. *J Virol* 2013; **87**: 7882-7892 [PMID: 23658444 DOI: 10.1128/JVI.00710-13]

162 **Zuckerman JN**, Zuckerman AJ. Mutations of the surface protein of hepatitis B virus. *Antiviral Res* 2003; **60**: 75-78 [PMID: 14638401]

163 **Xiang KH**, Michailidis E, Ding H, Peng YQ, Su MZ, Li Y, Liu XE, Dao Thi VL, Wu XF, Schneider WM, Rice CM, Zhuang H, Li T. Effects of amino acid substitutions in hepatitis B virus surface protein on virion secretion, antigenicity, HBsAg and viral DNA. *J Hepatol* 2017; **66**: 288-296 [PMID: 27650283 DOI: 10.1016/j.jhep.2016.09.005]

164 **Waters JA**, Kennedy M, Voet P, Hauser P, Petre J, Carman W, Thomas HC. Loss of the common "A" determinant of hepatitis B surface antigen by a vaccine-induced escape mutant. *J Clin Invest* 1992; **90**: 2543-2547 [PMID: 1281839 DOI: 10.1172/JCI116148]

165 **Shahmoradi S**, Yahyapour Y, Mahmoodi M, Alavian SM, Fazeli Z, Jazayeri SM. High prevalence of occult hepatitis B virus infection in children born to HBsAg-positive mothers despite prophylaxis with hepatitis B vaccination and HBIG. *J Hepatol* 2012; **57**: 515-521 [PMID: 22617152 DOI: 10.1016/j.jhep.2012.04.021]

166 **Jeantet D**, Chemin I, Mandrand B, Tran A, Zoulim F, Merle P, Trepo C, Kay A. Cloning and expression of surface antigens from occult chronic hepatitis B virus infections and their recognition by commercial detection assays. *J Med Virol* 2004; **73**: 508-515 [PMID: 15221893 DOI: 10.1002/jmv.20119]

167 **Minuk GY**, Sun DF, Uhanova J, Zhang M, Caouette S, Nicolle LE, Gutkin A, Doucette K, Martin B, Giulivi A. Occult hepatitis B virus infection in a North American community-based population. *J Hepatol* 2005; **42**: 480-485 [PMID: 15763333 DOI: 10.1016/j.jhep.2004.11.037]

168 **Wands JR**, Marciniak RA, Isselbacher KJ, Varghese M, Don G, Halliday JW, Powell LW. Demonstration of previously undetected hepatitis B viral determinants in an Australian Aboriginal population by monoclonal anti-hbs antibody radioimmunoassays. *Lancet* 1982; **1**: 977-980 [PMID: 6176820]

169 **Weinberger KM**, Bauer T, Böhm S, Jilg W. High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. *J Gen Virol* 2000; **81**: 1165-1174 [PMID: 10769057 DOI: 10.1099/0022-1317-81-5-1165]

170 **Zaaijer HL**, Torres P, Ontañón A, Ponte LG, Koppelman MH, Lelie PN, Hemert FJ, Boot HJ. Multiple surface antigen mutations in five blood donors with occult hepatitis B virus infection. *J Med Virol* 2008; **80**: 1344-1349 [PMID: 18551607 DOI: 10.1002/jmv.21233]

171 **Gish RG**, Yuen MF, Chan HL, Given BD, Lai CL, Locarnini SA, Lau JY, Wooddell CI, Schluep T, Lewis DL. Synthetic RNAi triggers and their use in chronic hepatitis B therapies with curative intent. *Antiviral Res* 2015; **121**: 97-108 [PMID: 26129970 DOI: 10.1016/j.antiviral.2015.06.019]

172 **Schluep T**, Lickliter J, Hamilton J, Lewis DL, Lai CL, Lau JY, Locarnini SA, Gish RG, Given BD. Safety, Tolerability, and Pharmacokinetics of ARC-520 Injection, an RNA Interference-Based Therapeutic for the Treatment of Chronic Hepatitis B Virus Infection, in Healthy Volunteers. *Clin Pharmacol Drug Dev* 2017; **6**: 350-362 [PMID: 27739230 DOI: 10.1002/cpdd.318]

173 **Zobeiri M**. Occult hepatitis B: clinical viewpoint and management. *Hepat Res Treat* 2013; **2013**: 259148 [PMID: 23533738 DOI: 10.1155/2013/259148]

P- Reviewer: Farshadpour F, Zhao HT **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 August 21; 24(31): 3500-3512

DOI: 10.3748/wjg.v24.i31.3500

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

REVIEW

Immunometabolism: A novel perspective of liver cancer microenvironment and its influence on tumor progression

Qi Zhang, Yu Lou, Xue-Li Bai, Ting-Bo Liang

Qi Zhang, Yu Lou, Xue-Li Bai, Ting-Bo Liang, Department of Hepatobiliary and Pancreatic Surgery, the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China

Qi Zhang, Yu Lou, Xue-Li Bai, Ting-Bo Liang, Zhejiang Provincial Key Laboratory of Pancreatic Disease, Hangzhou 310009, Zhejiang Province, China

ORCID number: Qi Zhang (0000-0002-6096-0690); Yu Lou (0000-0003-2862-423X); Xue-Li Bai (0000-0001-6005-8384); Ting-Bo Liang (0000-0003-0143-3353).

Author contributions: Zhang Q and Lou Y contributed equally to this work; Liang TB and Bai XL designed the research; Zhang Q and Lou Y performed the research; Zhang Q and Lou Y wrote the paper; Liang TB and Bai XL revised the paper.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Manuscript source: Invited manuscript

Correspondence to: Ting-Bo Liang, MD, PhD, Attending Doctor, Chief Doctor, Doctor, Full Professor, Research Fellow, Surgeon, Department of Hepatobiliary and Pancreatic Surgery, the Second Affiliated Hospital, Zhejiang University School of Medicine, No. 88 Jiefang Road, Hangzhou 310009, Zhejiang Province, China. liangtingbo@zju.edu.cn
Telephone: +86-571-87783510
Fax: +86-571-87783510

Received: April 16, 2018
Peer-review started: April 16, 2018
First decision: May 16, 2018
Revised: May 29, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: August 21, 2018

Abstract

The initiation and progression of liver cancer, including hepatocellular carcinoma and intrahepatic cholangiocarcinoma, are dependent on its tumor microenvironment. Immune cells are key players in the liver cancer microenvironment and show complicated crosstalk with cancer cells. Emerging evidence has shown that the functions of immune cells are closely related to cell metabolism. However, the effects of metabolic changes of immune cells on liver cancer progression are largely undefined. In this review, we summarize the recent findings of immunometabolism and relate these findings to liver cancer progression. We also explore the translation of the understanding of immunometabolism for clinical use.

Key words: Cholangiocarcinoma; Hepatocellular carcinoma; Tumor microenvironment; Local immune status; Metabolite

Core tip: The liver microenvironment provides a special place for initiation and progression of liver cancer, in which immune cells play a vital role. On the one hand, immunosuppression leads to tumor survival and progression; on the other hand, the instigation of tumor metabolites and signal molecules to immune cells makes the state of immunosuppression further strengthened. Intensive studies of the metabolic state of immune cells in the tumor microenvironment is beneficial to our understanding of the regulation of pro-tumor patterns, and to provide theoretical basis

and guidance for immunometabolic therapies for liver cancer.

Zhang Q, Lou Y, Bai XL, Liang TB. Immunometabolism: A novel perspective of liver cancer microenvironment and its influence on tumor progression. *World J Gastroenterol* 2018; 24(31): 3500-3512 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3500.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3500

INTRODUCTION

Liver cancer is one of the most common malignancies and has the third leading mortality rate and seventh leading incidence rate worldwide[1]. To date, with over 42000 cases diagnosed and 30000 deaths in the United States per year, a continuous increase in both morbidity and mortality is observed in liver cancer[2]. Unfortunately, the prognosis of advanced liver cancer is still poor despite the many treatments that have been developed in the past few decades. Thus, novel approaches to treat liver cancer are urgently needed.

The tumor microenvironment (TME) plays critical roles in tumor development and is characterized by complicated components, including various types of non-tumoral cells and non-cellular materials[3]. TMEs can be largely distinct among different types of cancers and among different patients with the same type of cancer. Within a patient, the TME can consistently change as the tumor progresses and is influenced by the physiopathological conditions of the patient. Thus, it is theoretically impossible to identify the precise state of the TME. However, under certain conditions, the TME can be specialized to show typical traits, and in particular, this specialized TME may affect tumor progression. Understanding these particular TMEs, if not all of them, can outline the crosstalk between the TME and tumor cells and facilitate the development of novel strategies for tumor treatment[4].

Pathologically, liver cancer mainly includes hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). The two types of liver cancers share a similar hepatic microenvironment, but may have different TMEs due to the various biological characteristics of the tumor cells. For instance, in a considerable proportion of cases, ICC harbors isocitrate dehydrogenase (IDH) 1 and IDH2 mutations[5], which are rare in HCC[6]. IDH1 and IDH2 are important enzymes involved in cellular metabolism. Therefore, IDH1/2 mutations can substantially influence the metabolite profiles in cells and TMEs. In this case, the roles of the TME in liver cancer progression may be greatly altered. However, knowledge in this field is poor and scattered.

Immune cells are key players in liver cancer progression, and their recruitment and functions are profoundly affected by the TME[7]. Other molecules in the TME, such as fatty acids and glucose, are able to regulate the metabolism, phenotype and function of immune cells[8,9]. The term "immunometabolism" has recently been proposed and indicates the functional intracellular metabolic alterations that occur within immune cells[10]. These affected immune cells, in turn, could have significant effects on adjacent tumor cells. In this minireview, we collected evidence of TME heterogeneity resulting from the different biological features of cancer cells and functional changes of immune cells resulting from an altered TME and how the TME affects tumor progression *via* metabolically regulated immune cells.

CHARACTERISTICS OF IMMUNE CELLS IN LIVER CANCER MICROENVIRONMENT

With genetic and epigenetic changes, hepatoma cells express specific tumor-associated antigens, such as α-fetoprotein (AFP), glypican-3 (GPC3) and melanoma-associated gene-A1 (MAGE-A1), which can be taken up by antigen-presenting cells and presented to T cells, resulting in a cytotoxic reaction to eliminate cancerous cells[11,12]. However, immunosuppressive factors and immune-inhibitory checkpoint molecules inhibit anti-tumor reactions and create a special microenvironment to facilitate tumor progression[12]. Almost all types of immune cells are deeply involved in the TME of liver cancer (Figure 1), including macrophages, Kupffer cells, neutrophils, T cells, B cells, innate lymphoid cells (ILCs), dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, and myeloid-derived suppressor cells (MDSCs)[13-18].

Macrophages and neutrophils

Macrophages display remarkable heterogeneity in liver cancer for various reasons, such as the cell origin (resident Kupffer cells and recruited monocyte-derived macrophages), stimulating signals (*i.e*., microbes, cell debris) and functional phenotype (*i.e*., inflammatory, anti-inflammatory). Notably, macrophages can play two or more contradictory roles in a particular TME. As guardians, macrophages are normally activated by inflammatory signals and together with $CD4^+$ T cells eliminate precancerous senescent cells[19]. Kupffer cells directly show cytotoxicity against tumor cells[20-22]. In addition, macrophage-derived interleukin (IL)-12 hampers tumor progression by activating NK and NKT cells[23,24]. As an accomplice, however, tumor-associated macrophages (TAMs, most of which are M2-polarized macrophages), induced by IL-4 and tumor growth factor (TGF)-β, accumulate in liver cancer and are correlated with the poor prognosis of patients[25,26]. Importantly, these macrophages express the immune checkpoint protein programmed cell death-ligand 1 (PD-L1, also known as B7-H1), which inactivates $CD8^+$ T cells[13,27,28]. *Via* other immunosuppressive signals,

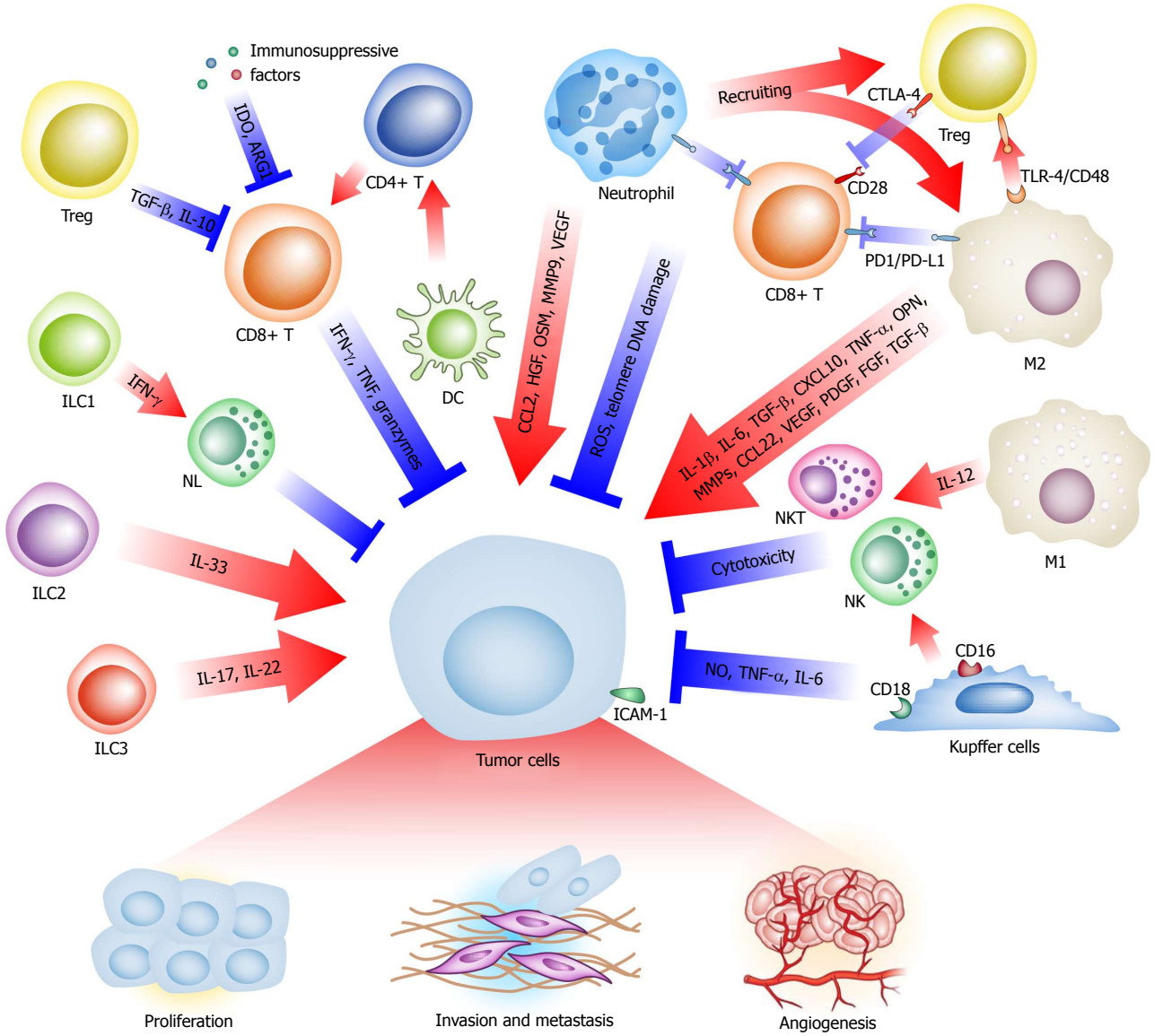


Figure 1 The immune cells in the tumor microenvironment regulate liver cancer progression. Many types of immune cells in the TME show pro- or anti-tumoral effects on the liver cancer cells by cell-specific mechanisms. Complicated crosstalk between immune cells is also common. TME: Tumor microenvironment; ILC: Innate lymphoid cell; NKT: Natural killer T.

such as Toll-like receptor (TLR) 4 and CD48/2B4, M2-polarized macrophages promote the recruitment of regulatory T cells (Tregs) and suppress the activity of NK cells[29-31]. Moreover, these macrophages can secrete various tumor proliferation-promoting cytokines, such as IL-1β, IL-6, TGF-β, C-X-C motif chemokine (CXCL) 10, invasion and metastasis-promoting factors like tumor necrosis factor (TNF)-α, osteopontin (OPN), matrix metalloproteinases (MMPs), C-C Motif chemokine ligand (CCL) 22, and proangiogenic growth factors, like vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and TGF-β, to build a tumor-prone inflammatory microenvironment[3,26,32-35].

Similar to macrophages, neutrophils also have diverse functions at different stages of liver cancer progression. In the case of a hepatic infection or injury, neutrophils gather at the wound site together with macrophages to eliminate pathogens and necrotic materials. Additionally, neutrophils stimulate reactive oxygen species (ROS) and telomere DNA damage in hepatocytes, mediating neoplasia and progression[36]. Mirroring macrophage plasticity, a pro-tumoral phenotype of tumor-associated neutrophils (TANs) is proposed[37,38]. Despite biomarkers of this subtype, immunosuppression is the most central function of TANs. The immunosuppressive molecule PD-L1 is regularly displayed in TANs[39] and recruits macrophages and Treg cells to the liver cancer TME and induces impaired anti-tumoral immunity[14]. The infiltrating TAN density and neutrophil-lymphocyte ratio is reported to be a predictor of outcome, chemotherapy resistance, and recurrence risk[40-42]. Furthermore, neutrophils promote tumor progression by secreting cytokines and other functional molecules, such as CCL2

for tumor growth, hepatocyte growth factor (HGF) and oncostatin M (OSM) for metastasis, and MMP9 and VEGF for angiogenesis[38,43-47].

T cells

$CD8^+$ T cells are the most important executors of adaptive immunity against neoplasms, including liver cancer. Unfortunately, the TME transforms these 'warriors' into 'servants'. Compared with the normal liver, tumor tissue has a lower density of $CD8^+$ T cells and a higher density of Tregs. The ratio of $CD8^+$ T cells to Tregs typically indicates a poor prognosis[48-50]. Recent studies suggest that interferon (IFN)-γ, TNF and granzyme secretion by $CD8^+$ cytotoxic T lymphocytes (CTLs) represent a common cytotoxic reaction against tumors[51,52]. Tregs, characterized by CD4, CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and forkhead box P3 (FoxP3) expression, can eliminate IL-2 *via* its receptor subunit CD25, downregulate CD80 and CD86 and conjugate to the co-stimulatory molecule CD28 competitively with CTLA-4 to suppress immune responses. In addition, Tregs secrete TGF-β and IL-10 into the TME to suppress T effector cells[52]. *Via* a complicated regulatory network, several subtypes of T cells contribute to the immunosuppressive TME.

ILCs

ILCs are recently identified innate immune cells that lack a specific antigen receptor. These cells originate from mucosal-associated lymphoid tissues and act as a sentry of the rapid immune response and regulator of immune homeostasis and inflammation[53]. Mirroring the classification of helper T cells (Th), ILCs are divided into three classes. ILC1s produce Th1-associated cytokines, ILC2s are associated with Th2-associated cytokine release, and ILC3s secrete Th17-associated cytokines[54]. As these three components simultaneously exist in the liver, they are likely to be involved in hepatocarcinogenesis and progression. ILC1s produce IFN-γ to activate NK cells and indirectly participate in cancer immunosurveillance, while ILC3s release IL-17 and IL-22, which may promote tumor growth and angiogenesis[16,55,56]. Moreover, we observed an increasing number of ILC2 in HCC tissue (unpublished data) and an elevated level of IL-33 in both serum and tumor tissue samples from HCC patients[57]. IL-33 can stimulate rapid growth and metastatic progression in breast cancer and cholangiocarcinoma[58,59]. Therefore, the initiation and growth of liver cancer promoted by the ILC2-related IL-33/ST2 axis might be theoretically tenable[60].

ALTERED METABOLISM OF LIVER CANCER AND ITS INFLUENCE ON THE TUMOR MICROENVIRONMENT

With their rapid growth and limited nutrition supply, the survival of tumor cells seems to be in question. However, the tumor is able to reprogram the cellular metabolism for neoplastic proliferation. The abnormal metabolism induced by cancer provides energy and metabolites for cell activity and additionally modifies many related pathways that influence various biological processes[61].

General alterations

The liver is a metabolic organ, and metabolic disruption of the liver can lead to spontaneous hepatocarcinogenesis[62]. The principal metabolic alterations in the liver cancer were profiled by metabolomics analysis, which showed elevated glycolysis, gluconeogenesis and β-oxidation, with reduced tricarboxylic acid cycle (TCA cycle) activity[63,64]. Aerobic glycolysis, also known as the "Warburg effect", is frequently observed in various tumors. This theory indicates that tumor cells predominantly use glycolysis, even in the presence of sufficient oxygen[65]. For liver cancer, glycolysis also plays a dominating role in glucose metabolism[63]. Accumulating studies have revealed that glycolysis is associated with genetic and epigenetic changes. Activated oncogenes and mutant tumor suppressors are associated with glycolysis. Glucose transporters (GLUTs), glycolysis-related enzyme hexokinase2 (HK2), pyruvate kinase M2 (PKM2), and lactate dehydrogenase (LDH) A are also overexpressed in liver cancer tissue, suggesting an increased glycolysis activity[66]. Moreover, the hypoxic TME and overexpression of β-catenin in liver cancer stabilize hypoxia inducible factor (HIF)-1α to activate glycolytic enzymes[67-70].

Fatty acid β-oxidation is enhanced in liver cancer to overcome the energy shortage and reduce tumor dependence on glucose. Metabolic alteration increases FA biosynthesis and glycerolipid metabolism, leading to fatty acid and lipid accumulation[71,72]. However, the alterations of various types of fatty acids are complicated. Additionally, almost all amino acids are increased in liver cancer due to the reduction of amino acid catabolism[63]. Although these general changes were identified, many other factors may influence the levels of metabolites in TME.

Involvement of specific mutations

Some specific mutations profoundly change the metabolism of liver cancer. The Tumor Cell Genomic Atlas (TCGA) project of HCC demonstrated that IDH and fibroblast growth factor receptor (FGFR) are meaningful mutations in HCC. IDHs are critical enzymes for cell metabolism, particularly the TCA cycle. IDH1/2 mutations convert α-ketogluterate (α-KG) into 2-hydrogluterate (2-HG)[73]. The unusually decreased ratio of α-KG/2-HG can significantly influence tumor cell biology by competing with α-KG and regulating epigenetic expression[74]. However, overproduced 2-HG by tumor cells can also be released into the TME since elevated serum and urinary levels of 2-HG can

be detected in patients with IDH1/2-mutated solid tumors[75,76]. Although 2-HG seems to be impermeable, this enzyme acts on stromal cells within the bone marrow microenvironment through an ROS/extracellular signal-regulated kinase (ERK) signaling pathway[77], suggesting that 2-HG may influence immune cells in the TME of liver cancer. This finding is important since IDH-like HCCs (particularly those containing IDH1/2 mutations and those with IDH-like gene expression) are indicative of worse clinical outcomes for undefined reasons[6]. In addition to 2-HG, IDH-like HCCs and IDH1/2 mutated ICCs have other significant metabolic alterations, such as gliomas due to pseudohypoxia, an interrupted Krebs cycle, and epigenetic changes[78]. In addition, IDH gain-of-function mutations also induce the reprogramming of pyruvate and lipid metabolism to maintain cell proliferation and clonogenicity[79,80]. Therefore, the TMEs of these tumors likely have distinct features, and the immune cells within such TMEs may be reprogrammed to have different functions.

FGFR genetic aberrations include mutations, amplifications, and gene fusions, which have been observed in over 10% of liver cancer[81-83]. FGFR2 fusions are active kinases with the highest incidence[84]. These genetic aberrations indirectly affect glucose and lipid metabolism by activating the kinase network[85]. Additionally, the oncogenic FGFR3-transforming acidic coiled-coil containing protein (TACC) 3 fusion activates oxidative phosphorylation and mitochondrial biogenesis, causing a mitochondrial respiration-dependent subtype of tumor cell[86].

Influence of hepatic viruses

Hepatitis viruses, particularly HBV in eastern countries and HCV in western countries, cause specific metabolic alterations during hepatocarcinogenesis and tumor progression. Hepatic virus infections activate many abnormal signaling pathways, which cause aberrant functions and expression of metabolism related enzymes[87]. As a consequence, HBV infection in patients is associated with increased free fatty acids (FFAs) and acyl-carnitines and decreased triglycerides, phospholipids, and sphingomyelins[88]. Thus, in the TME of HBV-related liver cancer, the increased FFAs and decreased glucose may result from increased β-oxidation and glycolysis, respectively. HBV-related inflammation can stimulate the expression of HIF-1α through the PI3K/AKT and mitogen-activated protein kinase (MAPK)-Ras-Raf pathways. The latter up-regulates GLUT1 and glycolytic enzymes, increases the glycolytic flow, and induces ROS accumulation. These alterations eventually cause DNA oxidative damage and malignant transformation[89-92]. HBx activates the adenosine 5'-monophosphate-activated protein kinase (AMPK) and fatty acid oxidation (FAO) pathways as well as the HBx-LXRα-SREBP1/FAS pathway, which allows HCC cells to survive under metabolic stress[93-95]. HCV establishes an insulin-resistant TME in the liver through several mechanisms[96,97]. For instance, HCV protein can induce the overexpression of protein phosphatase 2A (PP2A), which dysregulates hepatic glucose homeostasis by inhibiting AKT and the dephosphorylation of FoxO1[98]. However, the molecular mechanism of viral hepatitis-related metabolic alternations is not well understood. HBV integration into the tumor suppressor gene region causes metabolic alterations. Signaling molecules, such as TGF-β, mammalian target of rapamycin (mTOR), Smad3, and c-Myc, are activated by HBV and HCV, which is closely correlated with tumor progression[87,96].

Participation of other concomitant diseases

Liver cancer has a higher incidence in people with chronic non-infectious liver diseases. Therefore, the metabolic changes of liver cancer are related to cirrhosis and nonalcoholic fatty liver disease (NAFLD), which typically includes lipid anomalies, particularly dysregulated *de novo* lipogenesis[99]. For instance, peripheral insulin resistance together with enhanced mitochondrial β-oxidation and oxidative stress are the most prominent features of NAFLD and nonalcoholic steatohepatitis (NASH)[100]. Thus, NAFLD-related liver cancer is associated with elevated oxidative metabolism and amplified anaplerosis/cataplerosis[101]. With the increasing content of hepatic FFAs, liver cells may endure a mild respiratory dysfunction. The increasing import of FFAs into the mitochondria is accompanied by an elevated rate of β-oxidation. The overloaded fatty acid β-oxidation triggers the subsequent accumulation of ROS, which further leads to lipid peroxidation and severe mitochondrial oxidative damage[102,103]. These strong oxidizing products promote inflammation, fibrosis, and even carcinogenesis. Lipid metabolism shows an alteration from β-oxidation to ω-oxidation during liver cirrhosis[104]. Intriguingly, a metabolic switch from oxidative phosphorylation to glycolysis in hepatocytes in early-stage cirrhosis may satisfy the extreme energy requirements under such conditions[105]. This evidence demonstrates that concomitant liver diseases or even systemic metabolic diseases, such as diabetes mellitus, can influence the function of immune cells and their functions in promoting tumor progression.

Metabolic reprogramming of local immune cells in liver cancer

The role of metabolism in regulating immune cells has recently aroused general concerns. Evidence collected in several types of solid tumors indicated the importance of tumor immunometabolic reprogramming and suggested a novel and crucial area for future research of liver cancer[106]. The complicated crosstalk between metabolically reprogrammed immune cells and liver cancer cells has been suggested, but the molecular mechanisms need further exploration (Figure 2).

Because they exist at a considerable quantity, macrophages play a leading role in the crosstalk between liver cancer cells and immune cells. The

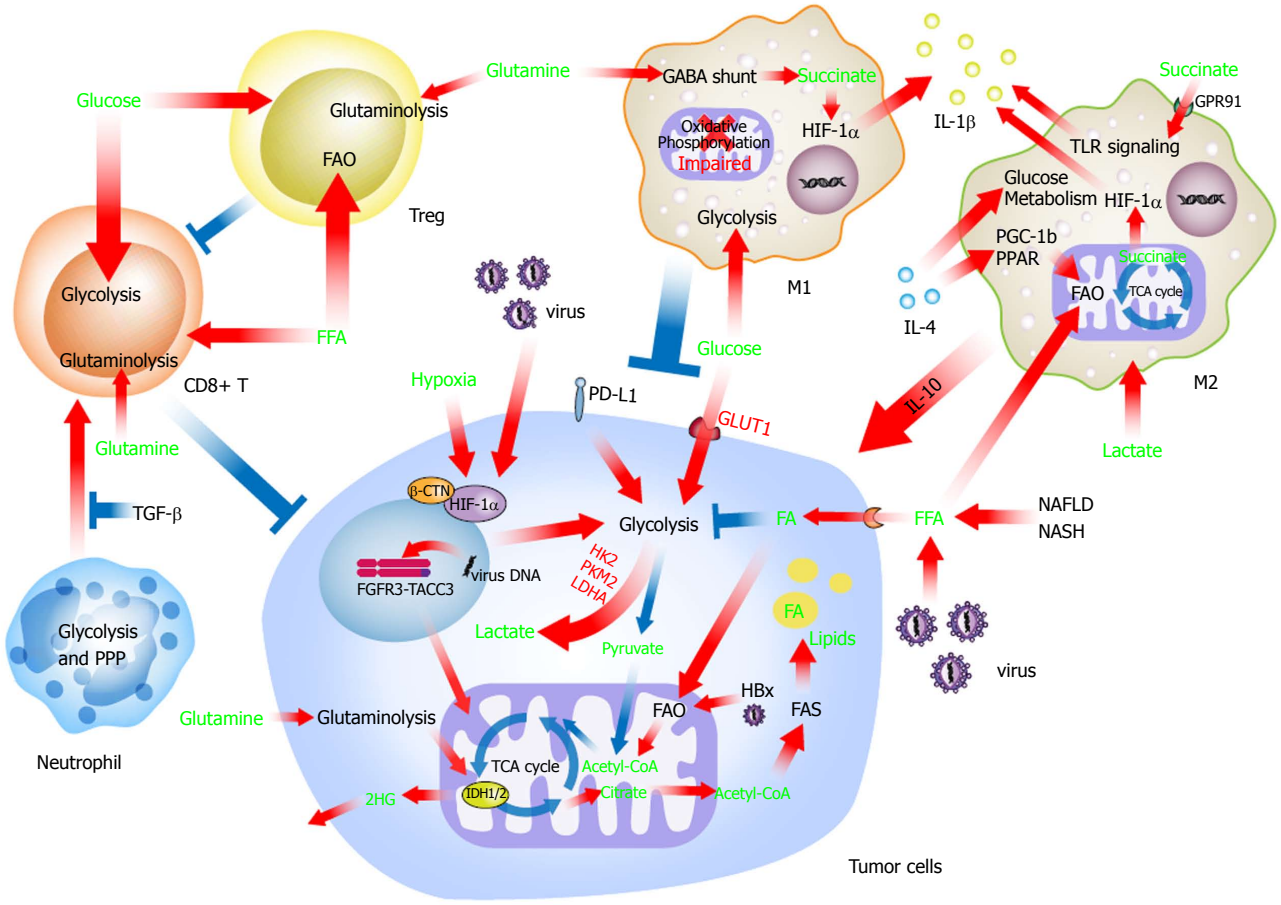


Figure 2 Metabolites in the tumor microenvironment affect the anti-/pro-tumoral functions of immune cells. Energy resources including glucose, glutamine, free fatty acids, and other metabolites such as succinate largely alter the functions of macrophages, neutrophils, and T cells. These metabolically reprogrammed immune cells then have a differed influence on the liver cancer cells compared to the original immune cells. FAO: Fatty acid oxidation.

phenotypes and functions of macrophages are hot topics in the field of immunometabolism. Professor O' Neill's group and other investigations have conducted several pioneering and important studies to decipher macrophage immunometabolism. Recent evidence has suggested that the energy source (*e.g.*, glucose and fatty acids) participates in the determination of macrophage polarization[107,108]. However, the role of FAO in the alternative activation of macrophages is still under debate. Huang *et al*[108] initially demonstrated an essential role for FAO in the alternative activation of mouse macrophages, while two groups in Germany and the United States subsequently provided convincing evidence that FAO was indispensable for the M2 polarization of human and mouse macrophages, respectively[109,110]. This discrepancy may partially reflect differences between mouse and human macrophages and the potential off-target effects of etomoxir (a carnitine palmitoyltransferase I A, CPT1A inhibitor used to inhibit FAO by some researchers). Although the role of FAO in macrophage activation has not yet been determined, FAO enhances the functions of M2 polarized macrophages[111].

Generally, M2 polarized macrophages use oxidative phosphorylation, FAO in particular, to obtain the required energy and exert pro-tumor function in various scenarios, including liver cancer[108,112,113]. Mechanistically, IL-4 induces the expression of peroxisome proliferator-activated receptors (PPAR) and PPAR gamma coactivator 1-beta (PGC-1b), which orchestrate alteration of FAO as well as mitochondrial respiration[114]. Similar to liver cancer cells in a background of chronic fatty liver disease, FFA accumulation attenuates mitochondrial respiration and increases FFA uptake *via* CD36, resulting in a predominant FAO mode in macrophages[115,116]. In addition, IL-4 signaling also increases glucose metabolism by the AKT/mTOR pathway[117-119]. Lactic acid produced by liver cancer cells through glycolysis is another non-negligible issue when considering the metabolic reprogramming of liver cancer-associated macrophages. The polarization of macrophages to an immunosuppressive and pro-tumoral phenotype is mediated by lactic acid *via* a different, as yet unidentified pathway[119-121] in liver cancer. M2 polarized macrophages also take up insulin growth factor 1 for a self-immunometabolic reprogramming, leading to reduced phagocytosis and lower energy expenditure[122], which may consequently regulate liver cancer progression.

However, M1 polarized macrophages have enhanced glycolytic metabolism and impaired oxidative phosph-

orylation through the AKT/mTOR/HIF-1α pathway[123]. This association is consistent with the fact that many glycolytic enzymes facilitate inflammatory cytokine production. For example, PKM2, which is critical for glycolysis, activates HIF-1α and induces IL-1β production[124]. Although under some conditions, FAO was also found to support inflammasome activation in M1 polarized macrophages by regulating CPT1A activity[125,126].

Other metabolites in the TME can also influence the phenotype and function of macrophages. Succinate is a known inflammation inducer of macrophages. Succinate enhances IL-1β secretion *via* succinate receptor 1 (SUCNR1, also known as GPR91)-mediated amplification of TLR signaling[127]. In addition, intracellular succinate, derived from the γ-aminobutyric acid (GABA) shunt and anerplerosis of α-KG upon stimulation of TLR4, enhances IL-1β production through the repurposing of mitochondrial function from ATP production to ROS generation[128,129].

Neutrophils are metabolically similar to M1 polarized macrophages and rely on aerobic glycolysis and the pentose phosphate pathway (PPP) as their principal mode of energy metabolism, by which the formation of neutrophil extracellular traps produces biological effects[130].

During differentiation, a switch from FAO to glycolysis and glutaminolysis triggers the maturation of T effector cells[131]. The rapid supply of ATP helps CTLs to meet their increasing bioenergetic and biosynthetic requirements[132]. Nevertheless, glucose shortage and lactic acid abundance limit the function of CTLs, and the enhancement of FAO preserves the cytotoxicity of CTLs in tumors[133]. By contrast, Treg cells favor FAO rather than glycolysis, by which these cells survive in the persistent low-glucose and hypoxic tumor microenvironment and suppress the tumor-killing function of CTLs[131,134]. Moreover, Treg cells rely on oxidative phosphorylation for energy supply, on FAO for cell differentiation, and on glutaminolysis for cell proliferation[134,135]. Taken together, these observations demonstrate that environment-related metabolism regulates the anti- and pro-tumor functions of tumor-infiltrating lymphocytes.

INFLUENCE OF REPROGRAMMED IMMUNE CELLS ON LIVER CANCER PROGRESSION

Immunometabolic reprogramming has a dual-function in tumor progression. Typically, M1 polarized macrophages facilitate inflammation and the antitumor response *via* elevated glycolysis, while M2 polarized macrophages play an FAO predominant role, secreting IL-10 to suppress the immune reaction. The switch in the metabolism of TAMs leads to an ample signaling transition by which these cells suppress immune reactions (by presenting PD-1, *etc.*), accelerate tumor proliferation (by releasing TNF, Wnt signals, *etc.*), promote angiogenesis (by secreting VEGF, FGF2, *etc.*), and enhance tumor invasion and metastasis (by MMP9, TGF-β, *etc.*)[26,136]. We further revealed that IL-1β facilitated epithelial-to-mesenchymal transition and subsequent metastasis in liver cancer, and this effect was mediated by a group of pro-inflammatory M2-like TAMs with an up-regulated level of glycolysis[137]. Interestingly, the function of TANs is opposite that of TAMs under different circumstances. For instance, the absence of TGF-β leads to a TANs-induced anti-tumor response by CTLs, whereas this situation is completely different in the presence of TGF-β[37].

The influence of metabolic reprogramming on lymphocytes differs in the presence of high functional heterogeneity. CTLs enhance glycolysis, glutaminolysis, and even FAO to exert anti-tumoral cytotoxicity, while $CD4^+$ T cells develop into two phenotypes with contrary functions. With a similar switch characterized by up-regulated glycolysis as well as increased glutaminolysis and PPP, Th1 cells induce macrophage- and NK cell-related anti-tumoral responses, whereas Th2 cells induce immunosuppressive reactions[113].

PERSPECTIVES OF LIVER CANCER THERAPY FROM IMMUNOMETABOLISM

Theoretically, it is feasible to target metabolic enzymes or metabolites in immune cells for therapeutic purposes. Indeed, several cancer-related immunometabolic molecules are suitable as potential targets for drug development (Table 1).

Considering carbohydrate metabolism, glycolysis is important to activated immune cells. Inhibition of HIF-1α can attenuate TAM/TAN-mediated IL-1β secretion, reduce hypoxic adaptation of tumor cells, and regulate the differentiation and function of lymphocytes[137,138]. The regulation of PI3K signaling by PTEN in immune cells may also show some effects on macrophage activation and Treg cell function with a concurrent influence on glycolysis[139,140].

The mTOR pathway may be a suitable target to regulate immune cells by manipulating cellular lipid metabolism. Inhibition of mTOR by rapamycin can block the development of macrophages and $CD4^+$ T cells in several scenarios, including liver cancer[141,142]. Moreover, FAO plays a predominant role in the metabolism of M2 polarized macrophages. Hence, inhibition of FAO by etomoxir or other methods (*e.g.*, targeted delivery of *CPT1A* siRNA/shRNA) may limit the immunosuppressive function of M2 macrophages. However, in a resource-deficient microenvironment, CTLs also take up FFA and exert cytotoxicity; thus, the selective increase of FFA import to CTLs enhances the anti-tumoral capacity of these cells[133]. It is also feasible to target proteins that play central roles in metabolic pathways (*i.e.*, iNOS, PKM2, Foxp3, *etc.*). The difficulty of this strategy is that targeting should be cell specific because the same metabolic pathway can have diverse effects on different

Table 1 Immunometabolic therapies for cancers

Targets	Agents	Mechanisms	Developments
PKM2	DASA, TEPP46	Inhibition of HIF1α	Preclinical
HIF1α	PX-478, RO7070179, EZN-2968	Inhibition of HIF1α	Phase 1
PTEN	VO-Ohpic, SF1670	Inhibition of PI3K	Preclinical
PDK	Dichloroacetate	Inhibition of glycolysis	Phase 2
GLUT1	Ritonavir	Inhibition of glycolysis	FDA-Approved
LDH	Gossypol (AT-101), FX11, Galloflavin	Inhibition of glycolysis	Phase 3
CPT1A	Etomoxir	Inhibition of FAO	Preclinical
CTLA-4	Ipilimumab	Checkpoint blockade; Inhibition of FAO	FDA-Approved
PD-1/PD-L1	Nivolumab, Pembrolizumab, Atezolizumab	Checkpoint blockade; Inhibition of FAO	FDA-Approved
AMPK	Metformin	Increased FAO; Inhibition of Complex I; Decreased mitochondrial ROS	FDA-Approved
mTOR	Temsirolimus, Everolimus	Inhibition of HIF-1α translation	FDA-Approved
IDO	Epacadostat	Regulation of tryptophan metabolism; Inhibition of mTORC1	FDA-Approved
IDH1/2 mutations	Ivosidenib (AG-120), IDH305, AG-881, DS-1001b	Inhibition of 2-HG production	Phase 3
FGFR	Regorafenib, Sunitinib, TAS120	Inhibition multi-targeted kinase	Phase 2
iNOS	L-NMMA, 1400W	Inhibition of NO production	Phase 2
FOXP3	P60	suppress NF-κB and NFAT	Preclinical

PKM2: Pyruvate kinase M2; HIF: Hypoxia inducible factor; LDH: Lactate dehydrogenase; FAO: Fatty acid oxidation; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; mTOR: Mammalian target of rapamycin; FGFR: Fibroblast growth factor receptor; FoxP3: Forkhead box P3; ROS: Reactive oxygen species.

immune cells.

Furthermore, drugs targeting tumor specific metabolic changes can also regulate the function of immune cells by altering the metabolites of the tumor. For instance, IDH1/2 inhibitors specifically reduce 2-HG production leading to destabilization of HIF-1α in immune cells, which can cause reduction of IL-1β secretion by macrophages.

CONCLUSION

Immunometabolism has increasingly become a component of immunology in the past decade. The current understanding suggests that a complicated metabolic network regulates the various functions of immune cells. This network can either be functionally oriented or environmentally adapted, but together, these complex interactions lead to immune microenvironment homeostasis, which affects the progression of liver cancer. However, numerous findings indicate that almost all types of immune cells present a self-contradictory function under different conditions, although the mechanisms are still unknown. In the future, more subtypes among immune cells will be defined as single cell detection systems are further developed, which may provide a key for functional heterogeneity. Therefore, liver cancer therapy that targets immunometabolism is a promising approach. Further work is urgently needed to explore the utility of targeting specific immunometabolic events in the liver cancer microenvironment for therapeutic gain.

REFERENCES

1 Fact sheets by Population-Globocan-IARC. IARC 2012
2 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]
3 **Quail DF**, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013; **19**: 1423-1437 [PMID: 24202395 DOI: 10.1038/nm.3394]
4 **Fang H**, Declerck YA. Targeting the tumor microenvironment: from understanding pathways to effective clinical trials. *Cancer Res* 2013; **73**: 4965-4977 [PMID: 23913938 DOI: 10.1158/0008-5472.CAN-13-0661]
5 **Farshidfar F**, Zheng S, Gingras MC, Newton Y, Shih J, Robertson AG, Hinoue T, Hoadley KA, Gibb EA, Roszik J, Covington KR, Wu CC, Shinbrot E, Stransky N, Hegde A, Yang JD, Reznik E, Sadeghi S, Pedamallu CS, Ojesina AI, Hess JM, Auman JT, Rhie SK, Bowlby R, Borad MJ; Cancer Genome Atlas Network, Zhu AX, Stuart JM, Sander C, Akbani R, Cherniack AD, Deshpande V, Mounajjed T, Foo WC, Torbenson MS, Kleiner DE, Laird PW, Wheeler DA, McRee AJ, Bathe OF, Andersen JB, Bardeesy N, Roberts LR, Kwong LN. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles. *Cell Rep* 2017; **18**: 2780-2794 [PMID: 28297679 DOI: 10.1016/j.celrep.2017.02.033]
6 **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]
7 **Gajewski TF**, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* 2013; **14**: 1014-1022 [PMID: 24048123 DOI: 10.1038/ni.2703]
8 **Yaqoob P**. Fatty acids as gatekeepers of immune cell regulation. *Trends Immunol* 2003; **24**: 639-645 [PMID: 14644137]
9 **Lawless SJ**, Kedia-Mehta N, Walls JF, McGarrigle R, Convery O, Sinclair LV, Navarro MN, Murray J, Finlay DK. Glucose represses dendritic cell-induced T cell responses. *Nat Commun* 2017; **8**: 15620 [PMID: 28555668 DOI: 10.1038/ncomms15620]
10 **O'Neill LA**, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol* 2016; **16**: 553-565 [PMID: 27396447 DOI: 10.1038/nri.2016.70]
11 **Flecken T**, Schmidt N, Hild S, Gostick E, Drognitz O, Zeiser R, Schemmer P, Bruns H, Eiermann T, Price DA, Blum HE, Neumann-Haefelin C, Thimme R. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+

T-cell responses in hepatocellular carcinoma. *Hepatology* 2014; **59**: 1415-1426 [PMID: 24002931 DOI: 10.1002/hep.26731]
12 **Prieto J**, Melero I, Sangro B. Immunological landscape and immunotherapy of hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 681-700 [PMID: 26484443 DOI: 10.1038/nrgastro.2015.173]
13 **Wu K**, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res* 2009; **69**: 8067-8075 [PMID: 19826049 DOI: 10.1158/0008-5472.can-09-0901]
14 **Zhou SL**, Zhou ZJ, Hu ZQ, Huang XW, Wang Z, Chen EB, Fan J, Cao Y, Dai Z, Zhou J. Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib. *Gastroenterology* 2016; **150**: 1646-1658.e17 [PMID: 26924089 DOI: 10.1053/j.gastro.2016.02.040]
15 **Shi JY**, Gao Q, Wang ZC, Zhou J, Wang XY, Min ZH, Shi YH, Shi GM, Ding ZB, Ke AW, Dai Z, Qiu SJ, Song K, Fan J. Margin-infiltrating CD20(+) B cells display an atypical memory phenotype and correlate with favorable prognosis in hepatocellular carcinoma. *Clin Cancer Res* 2013; **19**: 5994-6005 [PMID: 24056784 DOI: 10.1158/1078-0432.ccr-12-3497]
16 **Dadi S**, Chhangawala S, Whitlock BM, Franklin RA, Luo CT, Oh SA, Toure A, Pritykin Y, Huse M, Leslie CS, Li MO. Cancer Immunosurveillance by Tissue-Resident Innate Lymphoid Cells and Innate-like T Cells. *Cell* 2016; **164**: 365-377 [PMID: 26806130 DOI: 10.1016/j.cell.2016.01.002]
17 **Gonzalez-Carmona MA**, Lukacs-Kornek V, Timmerman A, Shabani S, Kornek M, Vogt A, Yildiz Y, Sievers E, Schmidt-Wolf IG, Caselmann WH, Sauerbruch T, Schmitz V. CD40ligand-expressing dendritic cells induce regression of hepatocellular carcinoma by activating innate and acquired immunity in vivo. *Hepatology* 2008; **48**: 157-168 [PMID: 18537185 DOI: 10.1002/hep.22296]
18 **Schrader J**. The role of MDSCs in hepatocellular carcinoma--in vivo veritas? *J Hepatol* 2013; **59**: 921-923 [PMID: 23958935 DOI: 10.1016/j.jhep.2013.08.003]
19 **Eggert T**, Wolter K, Ji J, Ma C, Yevsa T, Klotz S, Medina-Echeverz J, Longerich T, Forgues M, Reisinger F, Heikenwalder M, Wang XW, Zender L, Greten TF. Distinct Functions of Senescence-Associated Immune Responses in Liver Tumor Surveillance and Tumor Progression. *Cancer Cell* 2016; **30**: 533-547 [PMID: 27728804 DOI: 10.1016/j.ccell.2016.09.003]
20 **Aono K**, Isobe K, Nakashima I, Kondo S, Miyachi M, Nimura Y. Kupffer cells cytotoxicity against hepatoma cells is related to nitric oxide. *Biochem Biophys Res Commun* 1994; **201**: 1175-1181 [PMID: 8024559 DOI: 10.1006/bbrc.1994.1829]
21 **Saito H**, Kurose I, Ebinuma H, Fukumura D, Higuchi H, Atsukawa K, Tada S, Kimura H, Yonei Y, Masuda T, Miura S, Ishii H. Kupffer cell-mediated cytotoxicity against hepatoma cells occurs through production of nitric oxide and adhesion via ICAM-1/CD18. *Int Immunol* 1996; **8**: 1165-1172 [PMID: 8757962]
22 **Li XY**, Wu L, Li SW, Zhou WB, Wang MY, Zuo GQ, Liu CA, Ding X. Effect of CD16a, the surface receptor of Kupffer cells, on the growth of hepatocellular carcinoma cells. *Int J Mol Med* 2016; **37**: 1465-1474 [PMID: 27082928 DOI: 10.3892/ijmm.2016.2561]
23 **Rushfeldt C**, Sveinbjørnsson B, Seljelid R, Smedsrød B. Early events of hepatic metastasis formation in mice: role of Kupffer and NK-cells in natural and interferon-gamma-stimulated defense. *J Surg Res* 1999; **82**: 209-215 [PMID: 10090831 DOI: 10.1006/jsre.1998.5532]
24 **Seki S**, Nakashima H, Nakashima M, Kinoshita M. Antitumor immunity produced by the liver Kupffer cells, NK cells, NKT cells, and CD8 CD122 T cells. *Clin Dev Immunol* 2011; **2011**: 868345 [PMID: 22190974 DOI: 10.1155/2011/868345]
25 **Ding T**, Xu J, Wang F, Shi M, Zhang Y, Li SP, Zheng L. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. *Hum Pathol* 2009; **40**: 381-389 [PMID: 18992916 DOI: 10.1016/j.humpath.2008.08.011]
26 **Yeung OW**, Lo CM, Ling CC, Qi X, Geng W, Li CX, Ng KT, Forbes SJ, Guan XY, Poon RT, Fan ST, Man K. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *J Hepatol* 2015; **62**: 607-616 [PMID: 25450711 DOI: 10.1016/j.jhep.2014.10.029]
27 **Umemoto Y**, Okano S, Matsumoto Y, Nakagawara H, Matono R, Yoshiya S, Yamashita Y, Yoshizumi T, Ikegami T, Soejima Y, Harada M, Aishima S, Oda Y, Shirabe K, Maehara Y. Prognostic impact of programmed cell death 1 ligand 1 expression in human leukocyte antigen class I-positive hepatocellular carcinoma after curative hepatectomy. *J Gastroenterol* 2015; **50**: 65-75 [PMID: 24509608 DOI: 10.1007/s00535-014-0933-3]
28 **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]
29 **Zhou J**, Ding T, Pan W, Zhu LY, Li L, Zheng L. Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. *Int J Cancer* 2009; **125**: 1640-1648 [PMID: 19569243 DOI: 10.1002/ijc.24556]
30 **Wu Y**, Kuang DM, Pan WD, Wan YL, Lao XM, Wang D, Li XF, Zheng L. Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions. *Hepatology* 2013; **57**: 1107-1116 [PMID: 23225218 DOI: 10.1002/hep.26192]
31 **Yang J**, Zhang JX, Wang H, Wang GL, Hu QG, Zheng QC. Hepatocellular carcinoma and macrophage interaction induced tumor immunosuppression via Treg requires TLR4 signaling. *World J Gastroenterol* 2012; **18**: 2938-2947 [PMID: 22736917 DOI: 10.3748/wjg.v18.i23.2938]
32 **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]
33 **Wan S**, Zhao E, Kryczek I, Vatan L, Sadovskaya A, Ludema G, Simeone DM, Zou W, Welling TH. Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. *Gastroenterology* 2014; **147**: 1393-1404 [PMID: 25181692 DOI: 10.1053/j.gastro.2014.08.039]
34 **Wan S**, Kuo N, Kryczek I, Zou W, Welling TH. Myeloid cells in hepatocellular carcinoma. *Hepatology* 2015; **62**: 1304-1312 [PMID: 25914264 DOI: 10.1002/hep.27867]
35 **Krenkel O**, Tacke F. Liver macrophages in tissue homeostasis and disease. *Nat Rev Immunol* 2017; **17**: 306-321 [PMID: 28317925 DOI: 10.1038/nri.2017.11]
36 **Wilson CL**, Jurk D, Fullard N, Banks P, Page A, Luli S, Elsharkawy AM, Gieling RG, Chakraborty JB, Fox C, Richardson C, Callaghan K, Blair GE, Fox N, Lagnado A, Passos JF, Moore AJ, Smith GR, Tiniakos DG, Mann J, Oakley F, Mann DA. NFκB1 is a suppressor of neutrophil-driven hepatocellular carcinoma. *Nat Commun* 2015; **6**: 6818 [PMID: 25879839 DOI: 10.1038/ncomms7818]
37 **Fridlender ZG**, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009; **16**: 183-194 [PMID: 19732719 DOI: 10.1016/j.ccr.2009.06.017]
38 **Tsuda Y**, Fukui H, Asai A, Fukunishi S, Miyaji K, Fujiwara S, Teramura K, Fukuda A, Higuchi K. An immunosuppressive subtype of neutrophils identified in patients with hepatocellular carcinoma. *J Clin Biochem Nutr* 2012; **51**: 204-212 [PMID: 23170048 DOI: 10.3164/jcbn.12-32]
39 **He G**, Zhang H, Zhou J, Wang B, Chen Y, Kong Y, Xie X, Wang X, Fei R, Wei L, Chen H, Zeng H. Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling

pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res* 2015; **34**: 141 [PMID: 26581194 DOI: 10.1186/s13046-015-0256-0]
40 **Xiao WK**, Chen D, Li SQ, Fu SJ, Peng BG, Liang LJ. Prognostic significance of neutrophil-lymphocyte ratio in hepatocellular carcinoma: a meta-analysis. *BMC Cancer* 2014; **14**: 117 [PMID: 24559042 DOI: 10.1186/1471-2407-14-117]
41 **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]
42 **Motomura T**, Shirabe K, Mano Y, Muto J, Toshima T, Umemoto Y, Fukuhara T, Uchiyama H, Ikegami T, Yoshizumi T, Soejima Y, Maehara Y. Neutrophil-lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. *J Hepatol* 2013; **58**: 58-64 [PMID: 22925812 DOI: 10.1016/j.jhep.2012.08.017]
43 **Imai Y**, Kubota Y, Yamamoto S, Tsuji K, Shimatani M, Shibatani N, Takamido S, Matsushita M, Okazaki K. Neutrophils enhance invasion activity of human cholangiocellular carcinoma and hepatocellular carcinoma cells: an in vitro study. *J Gastroenterol Hepatol* 2005; **20**: 287-293 [PMID: 15683434 DOI: 10.1111/j.1440-1746.2004.03575.x]
44 **He M**, Peng A, Huang XZ, Shi DC, Wang JC, Zhao Q, Lin H, Kuang DM, Ke PF, Lao XM. Peritumoral stromal neutrophils are essential for c-Met-elicited metastasis in human hepatocellular carcinoma. *Oncoimmunology* 2016; **5**: e1219828 [PMID: 27853643 DOI: 10.1080/2162402x.2016.1219828]
45 **Li XF**, Chen DP, Ouyang FZ, Chen MM, Wu Y, Kuang DM, Zheng L. Increased autophagy sustains the survival and pro-tumourigenic effects of neutrophils in human hepatocellular carcinoma. *J Hepatol* 2015; **62**: 131-139 [PMID: 25152203 DOI: 10.1016/j.jhep.2014.08.023]
46 **Kuang DM**, Zhao Q, Wu Y, Peng C, Wang J, Xu Z, Yin XY, Zheng L. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J Hepatol* 2011; **54**: 948-955 [PMID: 21145847 DOI: 10.1016/j.jhep.2010.08.041]
47 **Xu R**, Huang H, Zhang Z, Wang FS. The role of neutrophils in the development of liver diseases. *Cell Mol Immunol* 2014; **11**: 224-231 [PMID: 24633014 DOI: 10.1038/cmi.2014.2]
48 **Guo CL**, Yang HC, Yang XH, Cheng W, Dong TX, Zhu WJ, Xu Z, Zhao L. Associations between infiltrating lymphocyte subsets and hepatocellular carcinoma. *Asian Pac J Cancer Prev* 2012; **13**: 5909-5913 [PMID: 23317279]
49 **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]
50 **Kalathil S**, Lugade AA, Miller A, Iyer R, Thanavala Y. Higher frequencies of GARP(+)CTLA-4(+)Foxp3(+) T regulatory cells and myeloid-derived suppressor cells in hepatocellular carcinoma patients are associated with impaired T-cell functionality. *Cancer Res* 2013; **73**: 2435-2444 [PMID: 23423978 DOI: 10.1158/0008-5472.can-12-3381]
51 **Garnelo M**, Tan A, Her Z, Yeong J, Lim CJ, Chen J, Lim KH, Weber A, Chow P, Chung A, Ooi LL, Toh HC, Heikenwalder M, Ng IO, Nardin A, Chen Q, Abastado JP, Chew V. Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. *Gut* 2017; **66**: 342-351 [PMID: 26669617 DOI: 10.1136/gutjnl-2015-310814]
52 **Ringelhan M**, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. *Nature Immunology* 2018; **19**: 222-232 [DOI: 10.1038/s41590-018-0044-z]
53 **Yang S**, Tian Z, Wu Y, van Velkinburgh JC, Ni B. Pivotal roles of ILCs in hepatic diseases. *Int Rev Immunol* 2015; **34**: 509-522 [PMID: 25730441 DOI: 10.3109/08830185.2015.1008631]
54 **Artis D**, Spits H. The biology of innate lymphoid cells. *Nature* 2015; **517**: 293-301 [PMID: 25592534 DOI: 10.1038/nature14189]
55 **Abe H**, Kimura A, Tsuruta S, Fukaya T, Sakaguchi R, Morita R, Sekiya T, Shichita T, Chayama K, Fujii-Kuriyama Y, Yoshimura A. Aryl hydrocarbon receptor plays protective roles in ConA-induced hepatic injury by both suppressing IFN-γ expression and inducing IL-22. *Int Immunol* 2014; **26**: 129-137 [PMID: 24150244 DOI: 10.1093/intimm/dxt049]
56 **Kirchberger S**, Royston DJ, Boulard O, Thornton E, Franchini F, Szabady RL, Harrison O, Powrie F. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med* 2013; **210**: 917-931 [PMID: 23589566 DOI: 10.1084/jem.20122308]
57 **Zhang P**, Liu XK, Chu Z, Ye JC, Li KL, Zhuang WL, Yang DJ, Jiang YF. Detection of interleukin-33 in serum and carcinoma tissue from patients with hepatocellular carcinoma and its clinical implications. *J Int Med Res* 2012; **40**: 1654-1661 [PMID: 23206447 DOI: 10.1177/030006051204000504]
58 **Li J**, Razumilava N, Gores GJ, Walters S, Mizuochi T, Mourya R, Bessho K, Wang YH, Glaser SS, Shivakumar P, Bezerra JA. Biliary repair and carcinogenesis are mediated by IL-33-dependent cholangiocyte proliferation. *J Clin Invest* 2014; **124**: 3241-3251 [PMID: 24892809 DOI: 10.1172/jci73742]
59 **Jovanovic IP**, Pejnovic NN, Radosavljevic GD, Pantic JM, Milovanovic MZ, Arsenijevic NN, Lukic ML. Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells. *Int J Cancer* 2014; **134**: 1669-1682 [PMID: 24105680 DOI: 10.1002/ijc.28481]
60 **McHedlidze T**, Waldner M, Zopf S, Walker J, Rankin AL, Schuchmann M, Voehringer D, McKenzie AN, Neurath MF, Pflanz S, Wirtz S. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity* 2013; **39**: 357-371 [PMID: 23954132 DOI: 10.1016/j.immuni.2013.07.018]
61 **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]
62 **Kettner NM**, Voicu H, Finegold MJ, Coarfa C, Sreekumar A, Putluri N, Katchy CA, Lee C, Moore DD, Fu L. Circadian Homeostasis of Liver Metabolism Suppresses Hepatocarcinogenesis. *Cancer Cell* 2016; **30**: 909-924 [PMID: 27889186 DOI: 10.1016/j.ccell.2016.10.007]
63 **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]
64 **Gingold JA**, Zhu D, Lee DF, Kaseb A, Chen J. Genomic Profiling and Metabolic Homeostasis in Primary Liver Cancers. *Trends Mol Med* 2018; **24**: 395-411 [PMID: 29530485 DOI: 10.1016/j.molmed.2018.02.006]
65 **Ngo H**, Tortorella SM, Ververis K, Karagiannis TC. The Warburg effect: molecular aspects and therapeutic possibilities. *Mol Biol Rep* 2015; **42**: 825-834 [PMID: 25253100 DOI: 10.1007/s11033-014-3764-7]
66 **Jones RG**, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev* 2009; **23**: 537-548 [PMID: 19270154 DOI: 10.1101/gad.1756509]
67 **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]
68 **Zhang Q**, Bai X, Chen W, Ma T, Hu Q, Liang C, Xie S, Chen C, Hu L, Xu S, Liang T. Wnt/β-catenin signaling enhances hypoxia-induced epithelial-mesenchymal transition in hepatocellular carcinoma via crosstalk with hif-1α signaling. *Carcinogenesis* 2013; **34**: 962-973 [PMID: 23358852 DOI: 10.1093/carcin/bgt027]
69 **Kitamura K**, Hatano E, Higashi T, Narita M, Seo S, Nakamoto Y, Yamanaka K, Nagata H, Taura K, Yasuchika K, Nitta T, Uemoto S. Proliferative activity in hepatocellular carcinoma is closely correlated with glucose metabolism but not angiogenesis.

J Hepatol 2011; **55**: 846-857 [PMID: 21334407 DOI: 10.1016/j.jhep.2011.01.038]
70 **Jia YY**, Zhao JY, Li BL, Gao K, Song Y, Liu MY, Yang XJ, Xue Y, Wen AD, Shi L. miR-592/WSB1/HIF-1α axis inhibits glycolytic metabolism to decrease hepatocellular carcinoma growth. *Oncotarget* 2016; **7**: 35257-35269 [PMID: 27153552 DOI: 10.18632/oncotarget.9135]
71 **Jiang J**, Nilsson-Ehle P, Xu N. Influence of liver cancer on lipid and lipoprotein metabolism. *Lipids Health Dis* 2006; **5**: 4 [PMID: 16515689 DOI: 10.1186/1476-511x-5-4]
72 **Budhu A**, Roessler S, Zhao X, Yu Z, Forgues M, Ji J, Karoly E, Qin LX, Ye QH, Jia HL, Fan J, Sun HC, Tang ZY, Wang XW. Integrated metabolite and gene expression profiles identify lipid biomarkers associated with progression of hepatocellular carcinoma and patient outcomes. *Gastroenterology* 2013; **144**: 1066-1075.e1 [PMID: 23376425 DOI: 10.1053/j.gastro.2013.01.054]
73 **Yang H**, Ye D, Guan KL, Xiong Y. IDH1 and IDH2 mutations in tumorigenesis: mechanistic insights and clinical perspectives. *Clin Cancer Res* 2012; **18**: 5562-5571 [PMID: 23071358 DOI: 10.1158/1078-0432.CCR-12-1773]
74 **Turkalp Z**, Karamchandani J, Das S. IDH mutation in glioma: new insights and promises for the future. *JAMA Neurol* 2014; **71**: 1319-1325 [PMID: 25155243 DOI: 10.1001/jamaneurol.2014.1205]
75 **Borger DR**, Goyal L, Yau T, Poon RT, Ancukiewicz M, Deshpande V, Christiani DC, Liebman HM, Yang H, Kim H, Yen K, Faris JE, Iafrate AJ, Kwak EL, Clark JW, Allen JN, Blaszkowsky LS, Murphy JE, Saha SK, Hong TS, Wo JY, Ferrone CR, Tanabe KK, Bardeesy N, Straley KS, Agresta S, Schenkein DP, Ellisen LW, Ryan DP, Zhu AX. Circulating oncometabolite 2-hydroxyglutarate is a potential surrogate biomarker in patients with isocitrate dehydrogenase-mutant intrahepatic cholangiocarcinoma. *Clin Cancer Res* 2014; **20**: 1884-1890 [PMID: 24478380 DOI: 10.1158/1078-0432.CCR-13-2649]
76 **Fathi AT**, Nahed BV, Wander SA, Iafrate AJ, Borger DR, Hu R, Thabet A, Cahill DP, Perry AM, Joseph CP, Muzikansky A, Chi AS. Elevation of Urinary 2-Hydroxyglutarate in IDH-Mutant Glioma. *Oncologist* 2016; **21**: 214-219 [PMID: 26834160 DOI: 10.1634/theoncologist.2015-0342]
77 **Chen JY**, Lai YS, Tsai HJ, Kuo CC, Yen BL, Yeh SP, Sun HS, Hung WC. The oncometabolite R-2-hydroxyglutarate activates NF-κB-dependent tumor-promoting stromal niche for acute myeloid leukemia cells. *Sci Rep* 2016; **6**: 32428 [PMID: 27577048 DOI: 10.1038/srep32428]
78 **Zhang C**, Moore LM, Li X, Yung WK, Zhang W. IDH1/2 mutations target a key hallmark of cancer by deregulating cellular metabolism in glioma. *Neuro Oncol* 2013; **15**: 1114-1126 [PMID: 23877318 DOI: 10.1093/neuonc/not087]
79 **Izquierdo-Garcia JL**, Viswanath P, Eriksson P, Cai L, Radoul M, Chaumeil MM, Blough M, Luchman HA, Weiss S, Cairncross JG, Phillips JJ, Pieper RO, Ronen SM. IDH1 Mutation Induces Reprogramming of Pyruvate Metabolism. *Cancer Res* 2015; **75**: 2999-3009 [PMID: 26045167 DOI: 10.1158/0008-5472.can-15-0840]
80 **Bogdanovic E**. IDH1, lipid metabolism and cancer: Shedding new light on old ideas. *Biochim Biophys Acta* 2015; **1850**: 1781-1785 [PMID: 25960387 DOI: 10.1016/j.bbagen.2015.04.014]
81 **Arai Y**, Totoki Y, Hosoda F, Shirota T, Hama N, Nakamura H, Ojima H, Furuta K, Shimada K, Okusaka T, Kosuge T, Shibata T. Fibroblast growth factor receptor 2 tyrosine kinase fusions define a unique molecular subtype of cholangiocarcinoma. *Hepatology* 2014; **59**: 1427-1434 [PMID: 24122810 DOI: 10.1002/hep.26890]
82 **Cheng AL**, Shen YC, Zhu AX. Targeting fibroblast growth factor receptor signaling in hepatocellular carcinoma. *Oncology* 2011; **81**: 372-380 [PMID: 22269894 DOI: 10.1159/000335472]
83 **Churi CR**, Shroff R, Wang Y, Rashid A, Kang HC, Weatherly J, Zuo M, Zinner R, Hong D, Meric-Bernstam F, Janku F, Crane CH, Mishra L, Vauthey JN, Wolff RA, Mills G, Javle M. Mutation profiling in cholangiocarcinoma: prognostic and therapeutic implications. *PLoS One* 2014; **9**: e115383 [PMID: 25536104 DOI: 10.1371/journal.pone.0115383]
84 **Sia D**, Tovar V, Moeini A, Llovet JM. Intrahepatic cholangiocarcinoma: pathogenesis and rationale for molecular therapies. *Oncogene* 2013; **32**: 4861-4870 [PMID: 23318457 DOI: 10.1038/onc.2012.617]
85 **Wu YM**, Su F, Kalyana-Sundaram S, Khazanov N, Ateeq B, Cao X, Lonigro RJ, Vats P, Wang R, Lin SF, Cheng AJ, Kunju LP, Siddiqui J, Tomlins SA, Wyngaard P, Sadis S, Roychowdhury S, Hussain MH, Feng FY, Zalupski MM, Talpaz M, Pienta KJ, Rhodes DR, Robinson DR, Chinnaiyan AM. Identification of targetable FGFR gene fusions in diverse cancers. *Cancer Discov* 2013; **3**: 636-647 [PMID: 23558953 DOI: 10.1158/2159-8290.cd-13-0050]
86 **Frattini V**, Pagnotta SM, Tala, Fan JJ, Russo MV, Lee SB, Garofano L, Zhang J, Shi P, Lewis G, Sanson H, Frederick V, Castano AM, Cerulo L, Rolland DCM, Mall R, Mokhtari K, Elenitoba-Johnson KSJ, Sanson M, Huang X, Ceccarelli M, Lasorella A, Iavarone A. A metabolic function of FGFR3-TACC3 gene fusions in cancer. *Nature* 2018; **553**: 222-227 [PMID: 29323298 DOI: 10.1038/nature25171]
87 **Chiang CH**, Huang KC. Association between metabolic factors and chronic hepatitis B virus infection. *World J Gastroenterol* 2014; **20**: 7213-7216 [PMID: 24966591 DOI: 10.3748/wjg.v20.i23.7213]
88 **Schoeman JC**, Hou J, Harms AC, Vreeken RJ, Berger R, Hankemeier T, Boonstra A. Metabolic characterization of the natural progression of chronic hepatitis B. *Genome Med* 2016; **8**: 64 [PMID: 27286979 DOI: 10.1186/s13073-016-0318-8]
89 **Agani F**, Jiang BH. Oxygen-independent regulation of HIF-1: novel involvement of PI3K/AKT/mTOR pathway in cancer. *Curr Cancer Drug Targets* 2013; **13**: 245-251 [PMID: 23297826]
90 **Kasuno K**, Takabuchi S, Fukuda K, Kizaka-Kondoh S, Yodoi J, Adachi T, Semenza GL, Hirota K. Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAPK and phosphatidylinositol 3-kinase signaling. *J Biol Chem* 2004; **279**: 2550-2558 [PMID: 14600153 DOI: 10.1074/jbc.M308197200]
91 **Tanaka H**, Yamamoto M, Hashimoto N, Miyakoshi M, Tamakawa S, Yoshie M, Tokusashi Y, Yokoyama K, Yaginuma Y, Ogawa K. Hypoxia-independent overexpression of hypoxia-inducible factor 1alpha as an early change in mouse hepatocarcinogenesis. *Cancer Res* 2006; **66**: 11263-11270 [PMID: 17145871 DOI: 10.1158/0008-5472.can-06-1699]
92 **Xia L**, Mo P, Huang W, Zhang L, Wang Y, Zhu H, Tian D, Liu J, Chen Z, Zhang Y, Chen Z, Hu H, Fan D, Nie Y, Wu K. The TNF-α/ROS/HIF-1-induced upregulation of FoxMI expression promotes HCC proliferation and resistance to apoptosis. *Carcinogenesis* 2012; **33**: 2250-2259 [PMID: 22831955 DOI: 10.1093/carcin/bgs249]
93 **Wang MD**, Wu H, Huang S, Zhang HL, Qin CJ, Zhao LH, Fu GB, Zhou X, Wang XM, Tang L, Wen W, Yang W, Tang SH, Cao D, Guo LN, Zeng M, Wu MC, Yan HX, Wang HY. HBx regulates fatty acid oxidation to promote hepatocellular carcinoma survival during metabolic stress. *Oncotarget* 2016; **7**: 6711-6726 [PMID: 26744319 DOI: 10.18632/oncotarget.6817]
94 **Kim K**, Kim KH, Kim HH, Cheong J. Hepatitis B virus X protein induces lipogenic transcription factor SREBP1 and fatty acid synthase through the activation of nuclear receptor LXRalpha. *Biochem J* 2008; **416**: 219-230 [PMID: 18782084 DOI: 10.1042/bj20081336]
95 **Kim SY**, Kim JK, Kim HJ, Ahn JK. Hepatitis B virus X protein sensitizes UV-induced apoptosis by transcriptional transactivation of Fas ligand gene expression. *IUBMB Life* 2005; **57**: 651-658 [PMID: 16203685 DOI: 10.1080/15216540500239697]
96 **Dai CY**, Yeh ML, Huang CF, Hou CH, Hsieh MY, Huang JF, Lin IL, Lin ZY, Chen SC, Wang LY, Chuang WL, Yu ML, Tung HD. Chronic hepatitis C infection is associated with insulin resistance and lipid profiles. *J Gastroenterol Hepatol* 2015; **30**: 879-884 [PMID: 23808794 DOI: 10.1111/jgh.12313]
97 **Kawaguchi Y**, Mizuta T. Interaction between hepatitis C virus and metabolic factors. *World J Gastroenterol* 2014; **20**: 2888-2901 [PMID: 24659880 DOI: 10.3748/wjg.v20.i11.2888]

98 **Bernsmeier C**, Calabrese D, Heim MH, Duong HT. Hepatitis C virus dysregulates glucose homeostasis by a dual mechanism involving induction of PGC1α and dephosphorylation of FoxO1. *J Viral Hepat* 2014; **21**: 9-18 [PMID: 24329853 DOI: 10.1111/jvh.12208]

99 **Lee S**, Mardinoglu A, Zhang C, Lee D, Nielsen J. Dysregulated signaling hubs of liver lipid metabolism reveal hepatocellular carcinoma pathogenesis. *Nucleic Acids Res* 2016; **44**: 5529-5539 [PMID: 27216817 DOI: 10.1093/nar/gkw462]

100 **Sanyal AJ**, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192 [PMID: 11266382 DOI: 10.1053/gast.2001.23256]

101 **Satapati S**, Kucejova B, Duarte JA, Fletcher JA, Reynolds L, Sunny NE, He T, Nair LA, Livingston KA, Fu X, Merritt ME, Sherry AD, Malloy CR, Shelton JM, Lambert J, Parks EJ, Corbin I, Magnuson MA, Browning JD, Burgess SC. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest* 2015; **125**: 4447-4462 [PMID: 26571396 DOI: 10.1172/JCI82204]

102 **Nassir F**, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. *Int J Mol Sci* 2014; **15**: 8713-8742 [PMID: 24837835 DOI: 10.3390/ijms15058713]

103 **Fromenty B**, Robin MA, Igoudjil A, Mansouri A, Pessayre D. The ins and outs of mitochondrial dysfunction in NASH. *Diabetes Metab* 2004; **30**: 121-138 [PMID: 15223984]

104 **Fujisawa K**, Takami T, Matsumoto T, Yamamoto N, Sakaida I. Profiling of the circadian metabolome in thioacetamide-induced liver cirrhosis in mice. *Hepatol Commun* 2017; **1**: 704-718 [PMID: 29404487 DOI: 10.1002/hep4.1075]

105 **Nishikawa T**, Bellance N, Damm A, Bing H, Zhu Z, Handa K, Yovchev MI, Sehgal V, Moss TJ, Oertel M, Ram PT, Pipinos II, Soto-Gutierrez A, Fox IJ, Nagrath D. A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease. *J Hepatol* 2014; **60**: 1203-1211 [PMID: 24583248 DOI: 10.1016/j.jhep.2014.02.014]

106 **Andrejeva G**, Rathmell JC. Similarities and Distinctions of Cancer and Immune Metabolism in Inflammation and Tumors. *Cell Metab* 2017; **26**: 49-70 [PMID: 28683294 DOI: 10.1016/j.cmet.2017.06.004]

107 **Haschemi A**, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, Knapp B, Haas R, Schmid JA, Jandl C, Amir S, Lubec G, Park J, Esterbauer H, Bilban M, Brizuela L, Pospisilik JA, Otterbein LE, Wagner O. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab* 2012; **15**: 813-826 [PMID: 22682222 DOI: 10.1016/j.cmet.2012.04.023]

108 **Huang SC**, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, Beatty W, Love-Gregory L, Lam WY, O'Neill CM, Yan C, Du H, Abumrad NA, Urban JF Jr, Artyomov MN, Pearce EL, Pearce EJ. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol* 2014; **15**: 846-855 [PMID: 25086775 DOI: 10.1038/ni.2956]

109 **Namgaladze D**, Brüne B. Fatty acid oxidation is dispensable for human macrophage IL-4-induced polarization. *Biochim Biophys Acta* 2014; **1841**: 1329-1335 [PMID: 24960101 DOI: 10.1016/j.bbalip.2014.06.007]

110 **Nomura M**, Liu J, Rovira II, Gonzalez-Hurtado E, Lee J, Wolfgang MJ, Finkel T. Fatty acid oxidation in macrophage polarization. *Nat Immunol* 2016; **17**: 216-217 [PMID: 26882249 DOI: 10.1038/ni.3366]

111 **Zhang Q**, Wang HR, Mao CY, Sun M, Dominah G, Chen L, Zhuang Z. Fatty acid oxidation contributes to IL-1β secretion in M2 macrophages and promotes macrophage-mediated tumor cell migration. *Mol Metab* 2018; **94**: 27-35 [DOI: 10.1016/j.molimm.2017.12.011]

112 **Vats D**, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, Wagner RA, Greaves DR, Murray PJ, Chawla A. Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell Metab* 2006; **4**: 13-24 [PMID: 16814729 DOI: 10.1016/j.cmet.2006.05.011]

113 **Biswas SK**. Metabolic Reprogramming of Immune Cells in Cancer Progression. *Immunity* 2015; **43**: 435-449 [PMID: 26377897 DOI: 10.1016/j.immuni.2015.09.001]

114 **Covarrubias AJ**, Aksoylar HI, Yu J, Snyder NW, Worth AJ, Iyer SS, Wang J, Ben-Sahra I, Byles V, Polynne-Stapornkul T, Espinosa EC, Lamming D, Manning BD, Zhang Y, Blair IA, Horng T. Akt-mTORC1 signaling regulates Acly to integrate metabolic input to control of macrophage activation. *Elife* 2016; **5** [PMID: 26894960 DOI: 10.7554/eLife.11612]

115 **Lim JE**, Chung E, Son Y. A neuropeptide, Substance-P, directly induces tissue-repairing M2 like macrophages by activating the PI3K/Akt/mTOR pathway even in the presence of IFNγ. *Sci Rep* 2017; **7**: 9417 [PMID: 28842601 DOI: 10.1038/s41598-017-09639-7]

116 **Zhou L**, Wang Q, Yin P, Xing W, Wu Z, Chen S, Lu X, Zhang Y, Lin X, Xu G. Serum metabolomics reveals the deregulation of fatty acids metabolism in hepatocellular carcinoma and chronic liver diseases. *Anal Bioanal Chem* 2012; **403**: 203-213 [PMID: 22349331 DOI: 10.1007/s00216-012-5782-4]

117 **Nath A**, Li I, Roberts LR, Chan C. Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma. *Sci Rep* 2015; **5**: 14752 [PMID: 26424075 DOI: 10.1038/srep14752]

118 **Feingold KR**, Shigenaga JK, Kazemi MR, McDonald CM, Patzek SM, Cross AS, Moser A, Grunfeld C. Mechanisms of triglyceride accumulation in activated macrophages. *J Leukoc Biol* 2012; **92**: 829-839 [PMID: 22753953 DOI: 10.1189/jlb.1111537]

119 **Colegio OR**, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, Cyrus N, Brokowski CE, Eisenbarth SC, Phillips GM, Cline GW, Phillips AJ, Medzhitov R. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 2014; **513**: 559-563 [PMID: 25043024 DOI: 10.1038/nature13490]

120 **Ruan GX**, Kazlauskas A. Lactate engages receptor tyrosine kinases Axl, Tie2, and vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis. *J Biol Chem* 2013; **288**: 21161-21172 [PMID: 23754286 DOI: 10.1074/jbc.M113.474619]

121 **Ohashi T**, Aoki M, Tomita H, Akazawa T, Sato K, Kuze B, Mizuta K, Hara A, Nagaoka H, Inoue N, Ito Y. M2-like macrophage polarization in high lactic acid-producing head and neck cancer. *Cancer Sci* 2017; **108**: 1128-1134 [PMID: 28370718 DOI: 10.1111/cas.13244]

122 **Spadaro O**, Camell CD, Bosurgi L, Nguyen KY, Youm YH, Rothlin CV, Dixit VD. IGF1 Shapes Macrophage Activation in Response to Immunometabolic Challenge. *Cell Rep* 2017; **19**: 225-234 [PMID: 28402847 DOI: 10.1016/j.celrep.2017.03.046]

123 **Pearce EL**, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity* 2013; **38**: 633-643 [PMID: 23601682 DOI: 10.1016/j.immuni.2013.04.005]

124 **Palsson-McDermott EM**, Curtis AM, Goel G, Lauterbach MA, Sheedy FJ, Gleeson LE, van den Bosch MW, Quinn SR, Domingo-Fernandez R, Johnston DG, Jiang JK, Israelsen WJ, Keane J, Thomas C, Clish C, Vander Heiden M, Xavier RJ, O'Neill LA. Pyruvate kinase M2 regulates Hif-1α activity and IL-1β induction and is a critical determinant of the warburg effect in LPS-activated macrophages. *Cell Metab* 2015; **21**: 65-80 [PMID: 25565206 DOI: 10.1016/j.cmet.2014.12.005]

125 **Moon JS**, Nakahira K, Chung KP, DeNicola GM, Koo MJ, Pabón MA, Rooney KT, Yoon JH, Ryter SW, Stout-Delgado H, Choi AM. NOX4-dependent fatty acid oxidation promotes NLRP3 inflammasome activation in macrophages. *Nat Med* 2016; **22**: 1002-1012 [PMID: 27455510 DOI: 10.1038/nm.4153]

126 **Moon JS**, Hisata S, Park MA, DeNicola GM, Ryter SW, Nakahira K, Choi AMK. mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. *Cell Rep* 2015; **12**: 102-115 [PMID: 26119735 DOI: 10.1016/j.celrep.2015.05.046]

127 **Littlewood-Evans A**, Sarret S, Apfel V, Loesle P, Dawson J, Zhang

J, Muller A, Tigani B, Kneuer R, Patel S, Valeaux S, Gommermann N, Rubic-Schneider T, Junt T, Carballido JM. GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis. *J Exp Med* 2016; **213**: 1655-1662 [PMID: 27481132 DOI: 10.1084/jem.20160061]

128 **Mills EL**, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, Tourlomousis P, Däbritz JHM, Gottlieb E, Latorre I, Corr SC, McManus G, Ryan D, Jacobs HT, Szibor M, Xavier RJ, Braun T, Frezza C, Murphy MP, O'Neill LA. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* 2016; **167**: 457-470.e13 [PMID: 27667687 DOI: 10.1016/j.cell.2016.08.064]

129 **Tannahill GM**, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH, Zheng L, Gardet A, Tong Z, Jany SS, Corr SC, Haneklaus M, Caffrey BE, Pierce K, Walmsley S, Beasley FC, Cummins E, Nizet V, Whyte M, Taylor CT, Lin H, Masters SL, Gottlieb E, Kelly VP, Clish C, Auron PE, Xavier RJ, O'Neill LA. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* 2013; **496**: 238-242 [PMID: 23535595 DOI: 10.1038/nature11986]

130 **Azevedo EP**, Rochael NC, Guimarães-Costa AB, de Souza-Vieira TS, Ganilho J, Saraiva EM, Palhano FL, Foguel D. A Metabolic Shift toward Pentose Phosphate Pathway Is Necessary for Amyloid Fibril- and Phorbol 12-Myristate 13-Acetate-induced Neutrophil Extracellular Trap (NET) Formation. *J Biol Chem* 2015; **290**: 22174-22183 [PMID: 26198639 DOI: 10.1074/jbc.M115.640094]

131 **Siska PJ**, Rathmell JC. T cell metabolic fitness in antitumor immunity. *Trends Immunol* 2015; **36**: 257-264 [PMID: 25773310 DOI: 10.1016/j.it.2015.02.007]

132 **Chang CH**, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O' Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 2013; **153**: 1239-1251 [PMID: 23746840 DOI: 10.1016/j.cell.2013.05.016]

133 **Zhang Y**, Kurupati R, Liu L, Zhou XY, Zhang G, Hudaihed A, Filisio F, Giles-Davis W, Xu X, Karakousis GC, Schuchter LM, Xu W, Amaravadi R, Xiao M, Sadek N, Krepler C, Herlyn M, Freeman GJ, Rabinowitz JD, Ertl HCJ. Enhancing CD8+ T Cell Fatty Acid Catabolism within a Metabolically Challenging Tumor Microenvironment Increases the Efficacy of Melanoma Immunotherapy. *Cancer Cell* 2017; **32**: 377-391.e9 [PMID: 28898698 DOI: 10.1016/j.ccell.2017.08.004]

134 **Wawman RE**, Bartlett H, Oo YH. Regulatory T Cell Metabolism in the Hepatic Microenvironment. *Front Immunol* 2018; **8**: 1889 [PMID: 29358934 DOI: 10.3389/fimmu.2017.01889]

135 **Beier UH**, Angelin A, Akimova T, Wang L, Liu Y, Xiao H, Koike MA, Hancock SA, Bhatti TR, Han R, Jiao J, Veasey SC, Sims CA, Baur JA, Wallace DC, Hancock WW. Essential role of mitochondrial energy metabolism in Foxp3^{+} T-regulatory cell function and allograft survival. *FASEB J* 2015; **29**: 2315-2326 [PMID: 25681462 DOI: 10.1096/fj.14-268409]

136 **Shirabe K**, Mano Y, Muto J, Matono R, Motomura T, Toshima T, Takeishi K, Uchiyama H, Yoshizumi T, Taketomi A, Morita M, Tsujitani S, Sakaguchi Y, Maehara Y. Role of tumor-associated macrophages in the progression of hepatocellular carcinoma. *Surg Today* 2012; **42**: 1-7 [PMID: 22116397 DOI: 10.1007/s00595-011-0058-8]

137 **Zhang J**, Zhang Q, Lou Y, Fu Q, Chen Q, Wei T, Yang J, Tang J, Wang J, Chen Y, Zhang X, Zhang J, Bai X, Liang T. Hypoxia-inducible factor-1α/interleukin-1β signaling enhances hepatoma epithelial-mesenchymal transition through macrophages in a hypoxic-inflammatory microenvironment. *Hepatology* 2018; **67**: 1872-1889 [PMID: 29171040 DOI: 10.1002/hep.29681]

138 **Clambey ET**, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, de Zoeten EF, Cambier JC, Stenmark KR, Colgan SP, Eltzschig HK. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proc Natl Acad Sci* 2012; **109**: E2784-E2793 [PMID: 22988108 DOI: 10.1073/pnas.1202366109]

139 **Chang M**, Hamilton JA, Scholz GM, Elsegood CL. Glycolytic control of adjuvant-induced macrophage survival: role of PI3K, MEK1/2, and Bcl-2. *J Leukoc Biol* 2009; **85**: 947-956 [PMID: 19270084 DOI: 10.1189/jlb.0908522]

140 **Sharma MD**, Shinde R, McGaha TL, Huang L, Holmgaard RB, Wolchok JD, Mautino MR, Celis E, Sharpe AH, Francisco LM, Powell JD, Yagita H, Mellor AL, Blazar BR, Munn DH. The PTEN pathway in Tregs is a critical driver of the suppressive tumor microenvironment. *Sci Adv* 2015; **1**: e1500845 [PMID: 26601142 DOI: 10.1126/sciadv.1500845]

141 **Chen W**, Ma T, Shen XN, Xia XF, Xu GD, Bai XL, Liang TB. Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway. *Cancer Res* 2012; **72**: 1363-1372 [PMID: 22287548 DOI: 10.1158/0008-5472.can-11-2684]

142 **Zeng H**, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* 2013; **499**: 485-490 [PMID: 23812589 DOI: 10.1038/nature12297]

P- Reviewer: Ooi LL, Rodríguez-Perálvarez M, Yamasaki 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 August 21; 24(31): 3513-3520

DOI: 10.3748/wjg.v24.i31.3513

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

MINIREVIEWS

Osteoporosis in primary biliary cholangitis

Christopher J Danford, Hirsh D Trivedi, Konstantinos Papamichael, Elliot B Tapper, Alan Bonder

Christopher J Danford, Hirsh D Trivedi, Konstantinos Papamichael, Alan Bonder, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, Unites States

Elliot B Tapper, Department of Hepatology, University of Michigan, Ann Arbor, MI 48109, Unites States

ORCID number: Christopher J Danford (0000-0003-0322-7705); Hirsh D Trivedi (0000-0002-8328-9211); Konstantinos Papamichael (0000-0003-1497-0254); Elliot B Tapper (0000-0002-0839-1515); Alan Bonder (0000-0001-6821-0416)

Author contributions: Danford CJ and Trivedi HD performed the literature search and drafting of the manuscript; all authors participated in the conceptual design of the paper and revision of the manuscript.

Supported by NIH T32 training grant, No. 4T32GM103702-04 to Trivedi HD.

Conflict-of-interest statement: There are no other financial disclosures or conflicts of interest in the production of this manuscript.

Manuscript source: Invited manuscript

Correspondence to: Alan Bonder, MD, Attending Doctor, Division of Gastroenterology and Hepatology, Beth Israel Deaconess Medical Center, 110 Francis St. Suite 8E, Boston, MA 02215, United States. abonder@bidmc.harvard.edu
Telephone: +1-617-6321070
Fax: +1-617-6231065

Received: June 1, 2018
Peer-review started: June 1, 2018
First decision: July 6, 2018
Revised: July 11, 2018
Accepted: July 22, 2018
Article in press: July 22, 2018
Published online: August 21, 2018

Abstract

Primary biliary cholangitis (PBC) is an autoimmune cholestatic liver disease with multiple debilitating complications. Osteoporosis is a common complication of PBC resulting in frequent fractures and leading to significant morbidity in this population, yet evidence for effective therapy is lacking. We sought to summarize our current understanding of the pathophysiology of osteoporosis in PBC, as well as current and emerging therapies in order to guide future research directions. A complete search with a comprehensive literature review was performed with studies from PubMed, EMBASE, Web of Science, Cochrane database, and the Countway Library. Osteoporosis in PBC is driven primarily by decreased bone formation, which differs from the increased bone resorption seen in postmenopausal osteoporosis. Despite this fundamental difference, current treatment recommendations are based primarily on experience with postmenopausal osteoporosis. Trials specific to PBC-related osteoporosis are small and have not consistently demonstrated a benefit in this population. As it stands, prevention of osteoporosis in PBC relies on the mitigation of risk factors such as smoking and alcohol use, as well as encouraging a healthy diet and weight-bearing exercise. The primary medical intervention for the treatment of osteoporosis in PBC remains bisphosphonates though a benefit in terms of fracture reduction has never been shown. This review outlines what is known regarding the pathogenesis of bone disease in PBC and summarizes current and emerging therapies.

Key words: Biliary cirrhosis; Cholestatic liver disease; Osteopenia; Hepatic osteodystrophy; Bisphosphonates; Hormone replacement therapy

Core tip: This article reviews the available literature on the pathophysiology and management of osteoporosis in primary biliary cholangitis (PBC). PBC-related osteoporosis is driven mainly by decreased bone formation as opposed to the increased bone resorption seen in postmenopausal osteoporosis. Despite this and a lack of evidence of efficacy, bisphosphonates remain the cornerstone of treatment. Future attention should be given to the use of anabolic bone agents in the treatment of PBC-related osteoporosis.

Danford CJ, Trivedi HD, Papamichael K, Tapper EB, Bonder A. Osteoporosis in primary biliary cholangitis. *World J Gastroenterol* 2018; 24(31): 3513-3520 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3513.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3513

INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic, progressive cholestatic liver disease associated with numerous extrahepatic complications. Osteoporosis, a disease of decreased bone density and strength, is a common complication of PBC[1-3]. The prevalence of osteoporosis in patients with PBC is roughly 30%[1-3] and higher in advanced stages of liver disease, up to 44% in those awaiting liver transplantation[4]. Osteoporosis is a major risk factor for fractures[3,5,6]. The incidence of fractures in PBC ranges from 0-14% over a two-year period[6-9]. The prevalence of fractures is reported to be 10%-20%[3,5,6], increasing to 22% in transplant waitlisted patients[4]. Serious injuries such as fractures are more difficult to manage in patients with advanced liver disease with perioperative morbidity and mortality approaching 80% and 60% respectively in cirrhotics undergoing emergent total hip arthroplasty[10]. Patients would therefore benefit from a timely diagnosis, effective risk factor modification, and treatment of PBC-related osteoporosis.

While effective treatments exist for postmenopausal osteoporosis, management of osteoporosis in PBC is limited by an incomplete understanding of pathophysiology specific to this disease process and a paucity of studies evaluating potential treatment. The current American Association for the Study of Liver Diseases guidelines recommend vitamin D and calcium supplementation based on experience in postmenopausal women as well as alendronate based on a single RCT in PBC[11]. An overreliance on postmenopausal osteoporosis data may lead to relatively ineffective treatment of PBC-related osteoporosis. To this end, we aim to summarize our current understanding of the pathophysiology behind bone disease in PBC as well as the evidence behind current and emerging therapies for osteoporosis in PBC.

The term hepatic osteodystrophy is sometimes used to refer to all metabolic bone disease seen in chronic liver disease. It refers to both osteomalacia, or decreased bone mineralization, and osteoporosis, or decreased bone mass, which can both be seen in advanced liver disease. Osteomalacia was once thought to be common in cholestatic liver disease; theoretically due dietary vitamin D malabsorption in the setting of severe cholestasis and impaired hepatic 25-hydroxylation[12,13]. However, it appears osteomalacia is actually quite rare in PBC and cholestatic liver disease and early studies were plagued by selection bias and a loose definition of osteomalacia[14]. It is now widely accepted that osteoporosis is the primary metabolic bone disease in PBC[15] and this review will focus on the management of osteoporosis in PBC.

Pathogenesis

The pathogenesis of osteoporosis in PBC appears to be largely driven by decreased bone formation, though increased bone resorption may play a role in certain scenarios. Several studies evaluating bone histomorphometry have shown decreased tetracycline double-labeling, decreased bone formation rates, decreased osteoblast numbers, and decreased serum osteocalcin, a marker of bone formation, all pointing towards osteoblast dysfunction and deficient bone formation as central to the pathogenesis of PBC-related osteoporosis[5,16-18].

Osteoblast dysfunction is a multifactorial process caused both by decreased osteoblast stimulation and increased osteoblast inhibition. Serum levels of insulin-like growth factor-1 (IGF-1), an osteoblast trophic factor, are lower in cirrhotics compared to controls[19]. Supplementation of IGF-1 in cirrhotic rats results in improvement in bone mass and bone density[20]. Vitamin K is also involved in bone metabolism through carboxylation of the non-collagenous bone protein, osteocalcin, and has been shown to stimulate osteoblastogenesis and inhibit osteoclastogenesis[21,22]. Vitamin K levels may be decreased in patients with severe cholestasis and impaired fatty-soluble vitamin absorption resulting in impaired osteoblast function. Indeed a meta-analysis does indicate some reduction in bone loss with vitamin K supplementation, but was not performed exclusively in patients with chronic liver disease[23].

Elevated levels of bilirubin, bile salts, and altered fibronectin production may also play a role in decreased bone formation through osteoblast inhibition in PBC. In one study, plasma mitogenic activity of osteoblasts was significantly lower in patients with cholestatic liver disease compared to healthy controls[24]. In addition, removal of bilirubin by plasma photobleaching resulted in improved plasma mitogenic activity[24]. Similarly, elevated lithocolic acid concentrations have been shown to decrease osteoblast survival through impaired

vitamin D stimulation of osteoblast gene transcription[25]. Patients with chronic liver disease also exhibit altered hepatic fibronectin production, resulting in increased production of a fibronectin isoform containing the oncofetal domain, which inhibits osteoblast-mediated mineralization in humans and mice[26].

Increased bone resorption may also play a role in osteoporosis in PBC in certain populations such as post-menopausal women and men with hypogonadism[5,14,16]. One study showed increased osteoblast numbers and increased eroded surface area indicative of increased resorption, but only in female patients with cholestatic liver disease[16]. Estrogen promotes apoptosis of osteoclasts and its absence results in a sharp decline in bone mineral density (BMD) after menopause[27,28]. Men with cholestatic liver disease show no signs of increased bone resorption despite similar degrees of osteopenia[16].

Theoretically, calcium and vitamin D deficiencies may develop in those with cholestatic liver disease leading to secondary hyperparathyroidism and increased bone resorption. Data is conflicting, however. Some studies have found decreased calcium absorption and serum vitamin D levels in PBC patients compared to controls[5,29], but others have found normal vitamin D, calcium, and PTH levels even among osteoporotic patients with PBC[16,17,26]. In addition, vitamin D supplementation to normal levels has not been shown to improve BMD in PBC[30,31]. While vitamin D absorption may be impaired in those taking cholestyramine, this is overcome by increasing the oral dose of vitamin D[32].

Diagnosis and monitoring

No data exists as to the optimal timing of screening and monitoring for osteoporosis in PBC, however, expert opinion recommends bone densitometry be performed in all PBC patients at diagnosis[11]. The World Health Organization defines osteoporosis as BMD at the spine or proximal femur less than 2.5 standard deviations (SDs) below the mean of a young adult population (expressed as a T score)[33]. Osteopenia refers to those with a T score between -1.0 and -2.5 SDs below the mean. Patients who initially have normal bone densitometry should be reassessed every 2-3 years with repeat bone densitometry, while those with additional risk factors for low bone density and fractures (*i.e.*, severe cholestasis, long-term corticosteroid use, postmenopausal women, BMI < 19, menopause before age 45, alcohol abuse, smoking) should be reassessed annually. Serum calcium, phosphorus, 25-vitamin D, and parathyroid hormone levels should also be checked at diagnosis of PBC and yearly thereafter[14,34,35]. Patients in whom treatment has been initiated should have repeat BMD measured every 1-2 years[36].

MANAGEMENT

Prevention

General measures to prevent bone loss in PBC are extrapolated from osteoporosis risk factors in the general population. Epidemiologic data suggests lifestyle factors such as tobacco and alcohol use, low dietary calcium intake, and low levels of exercise are associated with decreased bone density and fracture risk[33]. Based on this, tobacco and alcohol cessation are recommended in all PBC patients to reduce risk of bone loss. A balanced diet with adequate levels of calcium and vitamin D should also be encouraged. General recommendations for daily dietary calcium and vitamin D intake in at-risk populations (women older than 50) are 1200 mg and 800 IU daily, respectively[33].

Evidence for routine weight-bearing exercise comes from menopausal women. In a 16-year prospective study of early-postmenopausal women with mild osteopenia, those who exercised regularly had significant improvement in BMD compared to controls. This finding was sustained and even greater at 16 years compared to 4[37].

Treatment

Timing of treatment is based on recommendations from the postmenopausal osteoporosis literature[36]. Treatment should be initiated in all PBC patients with osteoporosis (T score < -2.5). The ideal time to start treatment in those with osteopenia (T score < -1 to -2.5) is less well established. The National Osteoporosis Foundation recommends treatment in individuals with a prior hip or vertebral fracture or 10-year hip fracture risk ≥ 3% or osteoporosis-related fracture risk ≥ 20% based on the World Health Organization Fracture Risk Assessment Tool in those with osteopenia[36]. In PBC, patients with a T score < 1.5 appear to be at increased risk of fracture[3] and initiation of specific treatment could be considered at this point (Figure 1). A number of different therapeutic approaches have been evaluated, however, most are limited by small sample size and short follow-up (Tables 1 and 2). The duration of treatment is unclear even in the postmenopausal population, though the National Osteoporosis Foundation emphasizes that treatment should not be indefinite and generally is continued for anywhere from 2 to 5 years based on individual risk assessment[36].

THERAPEUTIC OPTIONS

Vitamin D and calcium supplementation

In PBC patients with osteopenia or osteoporosis, vitamin D and calcium supplementation has not been shown to improve BMD or reduce fracture risk, though given few side effects and potential for deficiency, supplementation is generally recommended[14,34,35]. The only randomized controlled trial (RCT) looking at calcitriol supplementation did not find a significant improvement in BMD from baseline at one year, though BMD did significantly worsen in those who received no treatment[38]. Most studies use some combination of calcium and vitamin D supplementation as standard of care in both the intervention and control groups, though

Table 1 Summary of results in randomized-controlled trials of active agent *vs* placebo or no treatment

Agent	Treatment	No. of patients (*n*)	BMD changes at 1 yr (%)	BMD changes at 2 yr (%)	Fractures (*n*)
Etidronate	400 mg/d (3 mo cycles)	6 (etidronate)	+1.0 (L), +0.2 (F)	N/A	0
Wolfhagen *et al*[43], 1997		6 (no treatment)[3]	-1.7 (L), +0.4 (F)		0
Lindor *et al*[6], 2000	400 mg/d (3 mo cycles)	29 (etidronate)	+0.7 (L), +1.3 (F)	+1.0 (L), +0.5 (F)	4 (V)
		31 (placebo)	-0.6 (L), +0.9 (F)	+2.6 (L), +0.8 (F)	4 (V)
Alendronate	70 mg/wk	15 (alendronate)	+10.4 (L)[1,2], +1.4 (F)[1]	N/A	1 (V), 0 (P)
Zein *et al*[44], 2005		13 (placebo)	-0.1 (L)[1], -2.1 (F)[1]		0 (V), 1 (P)
HRT	50 mcg twice weekly TD	8 (HRT)	+3.1 (L)[2], +1.7 (F)[1,2]	N/A	0
Ormarsdottir *et al*[51], 2004	estradiol + 2.5 mg/d progestin	9 (no treatment)[3]	+1.0 (L), -0.6 (F)[1]		0
Boone *et al*[9], 2006	0.05 mg/d TD estradiol + 0.25 mg/d TD progestin	8 (HRT)	N/A	-0.6 (L), +0.2 (F)	0 (V)
		14 (placebo)		-0.8 (L), -3.7 (F)[2]	2 (V)
Sodium fluoride	50 mg/d sodium fluoride	8 (fluoride)	N/A	+2.9 (L)[1]	0
Guañabens *et al*[7], 1992		8 (placebo)		-6.6 (L)[1,2]	0
Calcitriol	0.5 mcg/d BID calcitriol	17 (calcitriol)	+0.1 (L)[1]	N/A	N/A
Shiomi *et al*[38], 1999		17 (no treatment)[3]	-3.1 (L)[1]		
Vitamin K	45 mg/d vitamin K_2	15 (vitamin K)	+0.3 (L)[1]	-0.8 (L)[1]	N/A
Nishiguchi *et al*[57], 2001		15 (no treatment)[3]	-3.5 (L)[1]	-6.9 (L)[1]	

[1]Statistical significance ($P < 0.05$) between groups; [2]Statistical difference ($P < 0.05$) from baseline; [3]Patients in no treatment groups received vitamin D and calcium supplementation. L: Lumbar; F: Femoral; V: Vertebral; P: Peripheral; BMD: Bone mineral density.

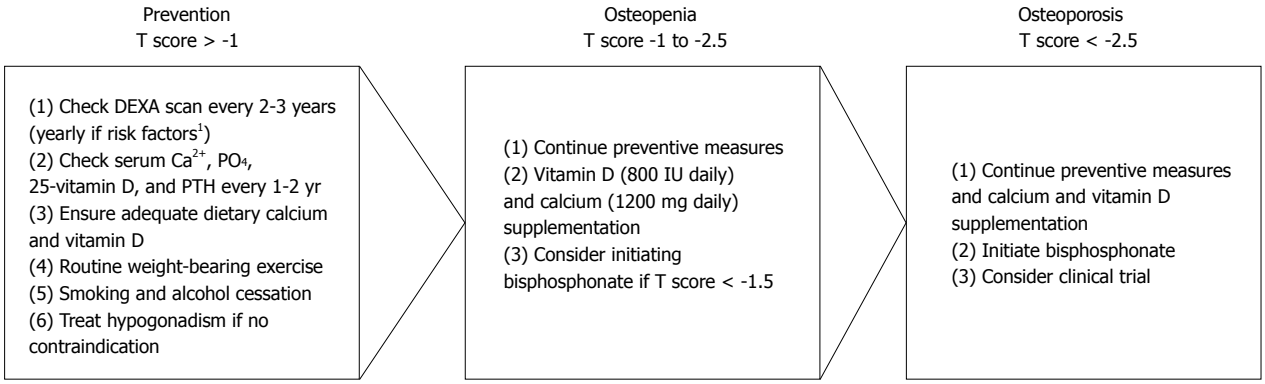


Figure 1 Summary of prevention and treatment strategies for osteoporosis in Primary biliary cholangitis. [1]Prolonged steroid use (> 3 mo), BMI < 19 kg/m^2, heavy alcohol or tobacco use.

notably, BMD continues to worsen in the control group despite supplementation[9,39,40].

Bisphosphonates

Bisphosphonates reduce bone resorption and are effective in increasing BMD while reducing fractures in postmenopausal osteoporosis[41]. Their effectiveness in PBC is not as clear due to the small number of studies with small study size and short follow-up. A 2011 Cochrane review of bisphosphonates in PBC concluded there was insufficient data supporting improved BMD and fracture risk[42]. It appears etidronate is not effective in improving BMD and preventing fractures in PBC. In two RCTs comparing etidronate to calcium alone or placebo, neither demonstrated an improvement in BMD from baseline with etidronate[6,43], though one did show significantly less bone loss compared to calcium alone[43]. Neither showed a difference in fracture risk over 1-2 years of follow-up[6,43].

Alendronate, a nitrogen-containing bisphosphonate as opposed to etidronate, has had more promising results in PBC. Only one RCT has been performed comparing alendronate to placebo in PBC[44]. In this study, 34 patients were randomized to receive oral alendronate or placebo. After 1 year, patients in the alendronate arm experienced a 10.4% increase in lumbar spine BMD compared to -0.12% for placebo[44]. However, there was no difference in fracture incidence over a year and the study included only one male and two post-menopausal females[44].

Several studies have also compared bisphosphonates to one another. Alendronate was found to be superior to etidronate in terms of BMD improvement in a small trial of 32 women with PBC and osteopenia in which patients were randomized to receive cyclical etidronate or weekly alendronate[45]. The alendronate group had significantly higher lumbar BMD (5.8%) at the end of 2 years compared to the etidronate group (1.9%). There was no significant improvement in either lumbar or femoral BMD in the etidronate group from baseline. However, 2 patients in the alendronate group experienced new non-vertebral fractures during the study period compared to 1 in the etidronate group[45].

Ibandronate was also compared to alendronate in

Table 2 Summary of results in comparative randomized controlled trials of different agents for treatment of osteoporosis in primary biliary cholangitis

Agent	Treatment	No. of patients (*n*)	BMD changes at 1 yr (%)	BMD changes at 2 yr (%)	Fractures (*n*)
Guañabens *et al*[39], 1997					
Etidronate	400 mg/d (3 mo cycles)	13 (etidronate)	-0.1 (L), -0.4 (F)	+0.5 (L)[2], -0.2 (F)	0 (V), 3 (P)
Sodium fluoride	50 mg/d sodium fluoride	10 (fluoride)	-1.7 (L), -0.6 (F)	-2.1 (L), -1.5 (F)	2 (V), 2 (P)
Guañabens *et al*[45], 2003					
Alendronate	10 mg/d	13 (alendronate)		+5.8 (L)[1,2], +3.9 (F)[1,2]	0 (V), 2 (P)
Etidronate	400 mg/d (3 mo cycles)	13 (etidronate)		+1.9 (L)[1], +0.4 (F)[1]	0 (V), 1 (P)
Guañabens *et al*[2], 2005					
Alendronate	10 mg/d	16 (alendronate)	+3.3 (L)[2], +1.2 (F)[2]		
Alendronate	70 mg/wk	10 (alendronate)	+1.2 (L), -0.3 (F)		
Guañabens *et al*[46], 2013					
Ibandronate	150 mg/mo	14 (ibandronate)	+3.8 (L), +1.0 (F)	+5.7 (L)[2], +1.1 (F)	0 (V), 0 (P)
Alendronate	70 mg/wk	19 (alendronate)	+4.6 (L), +1.4 (F)	+4.5 (L)[2], +2.5 (F)	1 (V), 0 (P)

[1]Statistical significance ($P < 0.05$) between groups; [2]Statistical difference ($P < 0.05$) from baseline. L: Lumbar; F: Femoral; V: Vertebral; P: Peripheral; BMD: Bone mineral density.

a more recent study of 42 post-menopausal women with PBC and osteoporosis[46]. In this study, women were randomized to receive IV ibandronate monthly or weekly oral alendronate. There was no significant difference in BMD between the 2 groups at the end of 2 years, however, compliance was significantly better in the ibandronate group[46]. One patient in the alendronate group developed a new vertebral fracture. Another advantage of IV administration is the avoidance of the theoretical concern of esophagitis and variceal bleeding with oral bisphosphonates in cirrhotics. However, none of the studies with alendronate and etidronate noted any esophagitis or variceal bleeds[6,40,43-46].

Evidence for the use of bisphosphonates in PBC is limited with only one small trial of alendronate showing improvement in BMD compared to placebo, though this occurred almost exclusively in postmenopausal women, and none demonstrating fracture risk reduction[6,43,44]. Similarly powered trials in postmenopausal osteoporosis have shown improvement benefit in terms of both fracture reduction and BMD improvement[47], and the PBC-specific benefit of bisphosphonates remains unclear.

Hormone replacement

Estrogens also have strong anti-resorptive effect on bones and for a period of time were widely used in postmenopausal osteoporosis[48]. Concerns over worsening of cholestasis initially limited their use in PBC. However, several observational studies did not show any significant worsening in liver disease with hormone replacement therapy (HRT)[49,50] and subsequently two RCTs similarly found no worsening in liver disease[9,51]. In these RCTs, those randomized to transdermal estrogen and progesterone had femoral and vertebral BMD compared to controls after 1-2 years of treatment[9,51], though only one showed an improvement in BMD from baseline[51] and neither showed a reduction in fracture risk[9,51]. In addition, both studies noted an increase in noncholestatic adverse events, such as vaginal bleeding and headaches, in the HRT arm leading to increased dropout[9,51]. Hormone replacement is also associated with increased risk of venous thromboembolism, stroke, ischemic heart disease, and breast cancer and is not recommended in women older than age 60 or greater than 10 years after menopause[52]. Hormone replacement may be effective in improving BMD in PBC, though no improvement in fracture risk has been shown and side effects limit their use.

Calcitonin

Calcitonin has been shown to have some effect in postmenopausal osteoporosis, however, data in PBC is lacking. A crossover study of IV calcitonin for 6 mo compared to oral calcium supplementation did not find a significant difference between the two and BMD ultimately fell in both groups[53]. A 3-year study of vitamin D, calcium, and IM calcitonin found significantly less BMD loss in treatment patients compared to controls who received no treatment[54,55]. However, there was no significant improvement in the calcitonin group and, since the control group received no therapy, the stabilization of BMD may have been from calcium and vitamin D supplementation[55].

Selective estrogen receptor modulators

Raloxifene is a selective estrogen receptor modulator that maintains the antiresorptive effects of estrogen in bone with anti-estrogen effects in the uterus and breast. A single pilot study has examined the efficacy of raloxifene in PBC. In this study, 9 women with PBC treated with raloxifene were compared to 3 age-matched controls[56]. After 1 year, there was a small (0.02 g/cm^2 *vs* 0.00 g/cm^2) but significant improvement in lumbar BMD compared to controls, though there was no difference in femoral BMD and no data on fracture risk[56].

Sodium fluoride

Sodium fluoride increases bone formation and has been shown to be effective in postmenopausal osteoporosis,

but is not as effective as anti-resorptive agents. One randomized, controlled trial examined sodium fluoride in PBC and found a significant increase in BMD (+2.9%) compared to placebo (-6.6%) in 22 women with PBC followed for 2 years[7]. No fractures occurred in either group[7]. A subsequent trial comparing sodium fluoride to etidronate, however, found etidronate to be superior and actually observed a decrease in femoral BMD in the sodium fluoride group after 2 years[39]. In addition, 2 patients in the sodium fluoride group experienced new vertebral fractures compared to none in the etidronate group[39].

Vitamin K

Patients with severe cholestasis may have decreased absorption of fat-soluble vitamins, such as vitamin K. Vitamin K is involved in bone formation through stimulation of osteoblastogenesis[21,22]. One meta-analysis did indicate some reduction in bone loss with vitamin K supplementation, but did not focus on patients with cholestatic liver disease[23]. One small RCT in PBC found significantly less bone loss in vitamin K patients compared to controls, but was ultimately not effective in improving BMD[57].

Parathyroid hormone

Human parathyroid hormone (PTH) improves BMD and reduces fractures in postmenopausal osteoporosis through stimulating bone formation, the major driver of osteoporosis in PBC[58,59]. The recombinant form consisting of the bioactive portion of the hormone (teriparatide) is approved for treatment of postmenopausal osteoporosis, but has not been studied in PBC. Recombinant human PTH 1-34 (rhPTH 1-34) has been studied in rats that have undergone biliary ductal ligation[60]. In this study, rats that underwent biliary ductal ligation had significant worsening in BMD compared to rats that underwent a sham operation. Biliary ductal ligation rats were then administered rhPTH 1-34 at 40 and 80 μg/kg/d. Those rats who received 40 μg/kg/d experienced significant improvement in femoral and tibial BMD compared to untreated rats and those who received 80 μg/kg/d (who did not experience significant improvement compared to untreated rats)[60]. No trials have been undertaken in humans with PBC.

PBC-specific therapies

Therapies directed at PBC itself have not been shown to improve bone disease. Patients enrolled in a randomized, controlled trial of ursodeoxycholic acid (UDCA) were followed over a 3-year period with dual-photon densitometry annually. After 3 years, there was no significant difference in lumbar BMD between UDCA and placebo[61].

In the initial phase 3 trial of obeticholic acid, BMD was measured at baseline and 12 mo[62]. BMD continued to decline in the obeticholic acid groups (both 5-10 mg and 10 mg groups), however, this decline was significantly less at the femoral neck compared to placebo. BMD decreased in both groups at the lumbar spine as well and, while there was a trend toward less decline in the obeticholic acid groups, there was no significant difference[62]. There was no difference in fracture rates[62].

No data currently exists evaluating the effects of fibrates on BMD when used to treat PBC.

Future therapies

Concerns over long-term efficacy and safety of bisphosphonate use in postmenopausal osteoporosis have led to ongoing development of new medications. Abaloparatide, a parathyroid hormone-related peptide analog, was recently approved by the Food and Drug Administration for treatment of postmenopausal osteoporosis and may avoid the pro-resorptive and hypercalcemic effects of teriparatide[63]. Along with teriparatide, these recombinant human PTH agents differ in that they promote bone formation, the primary deficit in PBC, as opposed to decrease resorption, the driving issue in postmenopausal osteoporosis and the current target of most treatments. Based on our understanding of the pathogenesis of osteoporosis in PBC, future studies should include therapies that promote bone formation and ample male and premenopausal female PBC patients to decrease the likelihood that results reflect only treatment of postmenopausal osteoporosis.

CONCLUSION

Osteoporosis is a common complication of PBC resulting in a significantly increased risk of fractures especially in those with a T score < -1.5. Severity of bone disease is related to the severity and duration of the underlying liver disease as well as increasing age. The mechanism of decreased bone density primarily involves decreased bone formation resulting from decreased osteoblast function. Increased bone resorption may also play a role in postmenopausal women.

For prevention of osteoporosis, mitigation of risk factors is recommended through smoking and alcohol cessation as well as a balanced diet and regular weight-bearing exercise. Treatment is recommended in patients who have experienced fractures or have osteoporosis (T score < -2.5), though may also be considered in those with a T score < -1.5 in PBC. No treatment has been adequately shown to reduce fractures in PBC, though the bisphosphonates ibandronate and alendronate may be effective in increasing BMD. HRT may also be effective in improving BMD in PBC, though with more side effects. Treatment of the underlying liver disease with UDCA or OCA does not appear to effectively treat the bone disease. Ultimately further research is required, with special attention to anabolic bone agents, to identify effective treatment in PBC-related osteoporosis.

REFERENCES

1 **Newton J**, Francis R, Prince M, James O, Bassendine M, Rawlings D, Jones D. Osteoporosis in primary biliary cirrhosis revisited. *Gut*

2001; **49**: 282-287 [PMID: 11454807 DOI: 10.1136/gut.49.2.282]
2 **Guañabens N**, Parés A, Ros I, Caballería L, Pons F, Vidal S, Monegal A, Peris P, Rodés J. Severity of cholestasis and advanced histological stage but not menopausal status are the major risk factors for osteoporosis in primary biliary cirrhosis. *J Hepatol* 2005; **42**: 573-577 [PMID: 15763344 DOI: 10.1016/j.jhep.2004.11.035]
3 **Guañabens N**, Cerdá D, Monegal A, Pons F, Caballería L, Peris P, Parés A. Low bone mass and severity of cholestasis affect fracture risk in patients with primary biliary cirrhosis. *Gastroenterology* 2010; **138**: 2348-2356 [PMID: 20178794 DOI: 10.1053/j.gastro.2010.02.016]
4 **Guichelaar MM**, Kendall R, Malinchoc M, Hay JE. Bone mineral density before and after OLT: long-term follow-up and predictive factors. *Liver Transpl* 2006; **12**: 1390-1402 [PMID: 16933236 DOI: 10.1002/lt.20874]
5 **Guañabens N**, Parés A, Mariñoso L, Brancós MA, Piera C, Serrano S, Rivera F, Rodés J. Factors influencing the development of metabolic bone disease in primary biliary cirrhosis. *Am J Gastroenterol* 1990; **85**: 1356-1362 [PMID: 2220729]
6 **Lindor KD**, Jorgensen RA, Tiegs RD, Khosla S, Dickson ER. Etidronate for osteoporosis in primary biliary cirrhosis: a randomized trial. *J Hepatol* 2000; **33**: 878-882 [PMID: 11131448 DOI: 10.1016/S0168-8278(00)80118-X]
7 **Guañabens N**, Parés A, del Rio L, Roca M, Gómez R, Muñoz J, Rodés J. Sodium fluoride prevents bone loss in primary biliary cirrhosis. *J Hepatol* 1992; **15**: 345-349 [PMID: 1447501]
8 **Guañabens N**, Parés A, Navasa M, Martínez de Osaba MJ, Hernández ME, Muñoz J, Rodés J. Cyclosporin A increases the biochemical markers of bone remodeling in primary biliary cirrhosis. *J Hepatol* 1994; **21**: 24-28 [PMID: 7963418]
9 **Boone RH**, Cheung AM, Girlan LM, Heathcote EJ. Osteoporosis in primary biliary cirrhosis: a randomized trial of the efficacy and feasibility of estrogen/progestin. *Dig Dis Sci* 2006; **51**: 1103-1112 [PMID: 16865577 DOI: 10.1007/s10620-006-8015-x]
10 **Cohen SM**, Te HS, Levitsky J. Operative risk of total hip and knee arthroplasty in cirrhotic patients. *J Arthroplasty* 2005; **20**: 460-466 [PMID: 16124961 DOI: 10.1016/j.arth.2004.05.004]
11 **Lindor KD**, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ; American Association for Study of Liver Diseases. Primary biliary cirrhosis. *Hepatology* 2009; **50**: 291-308 [PMID: 19554543 DOI: 10.1002/hep.22906]
12 **Long RG**, Varghese Z, Meinhard EA, Skinner RK, Wills MR, Sherlock S. Parenteral 1,25-dihydroxycholecalciferol in hepatic osteomalacia. *Br Med J* 1978; **1**: 75-77 [PMID: 620204]
13 **Compston JE**, Crowe JP, Horton LW. Treatment of osteomalacia associated with primary biliary cirrhosis with oral 1-alpha-hydroxy vitamin D3. *Br Med J* 1979; **2**: 309 [PMID: 476441]
14 **Bernstein CN**, Leslie WD, Leboff MS. AGA technical review on osteoporosis in gastrointestinal diseases. *Gastroenterology* 2003; **124**: 795-841 [PMID: 12612917 DOI: 10.1016/S0016-5085(03)01062-X]
15 **Hay JE**. Bone disease in cholestatic liver disease. *Gastroenterology* 1995; **108**: 276-283 [PMID: 7806050]
16 **Guichelaar MM**, Malinchoc M, Sibonga J, Clarke BL, Hay JE. Bone metabolism in advanced cholestatic liver disease: analysis by bone histomorphometry. *Hepatology* 2002; **36**: 895-903 [PMID: 12297836 DOI: 10.1053/jhep.2002.36357]
17 **Hodgson SF**, Dickson ER, Wahner HW, Johnson KA, Mann KG, Riggs BL. Bone loss and reduced osteoblast function in primary biliary cirrhosis. *Ann Intern Med* 1985; **103**: 855-860 [PMID: 4062087]
18 **Ackerman Z**, Weinreb M, Amir G, Pollak RD. Bone mineral metabolism and histomorphometry in rats with cholestatic liver disease. *Liver* 2002; **22**: 166-172 [PMID: 12028412]
19 **Gallego-Rojo FJ**, Gonzalez-Calvin JL, Muñoz-Torres M, Mundi JL, Fernandez-Perez R, Rodrigo-Moreno D. Bone mineral density, serum insulin-like growth factor I, and bone turnover markers in viral cirrhosis. *Hepatology* 1998; **28**: 695-699 [PMID: 9731561 DOI: 10.1002/hep.510280315]
20 **Cemborain A**, Castilla-Cortázar I, García M, Quiroga J, Muguerza B, Picardi A, Santidrián S, Prieto J. Osteopenia in rats with liver cirrhosis: beneficial effects of IGF-I treatment. *J Hepatol* 1998; **28**: 122-131 [PMID: 9537849 DOI: 10.1016/S0168-8278(98)80211-0]
21 **Houben RJ**, Rijkers DT, Stanley TB, Acher F, Azerad R, Käkönen SM, Vermeer C, Soute BA. Characteristics and composition of the vitamin K-dependent gamma-glutamyl carboxylase-binding domain on osteocalcin. *Biochem J* 2002; **364**: 323-328 [PMID: 11988107]
22 **Koshihara Y**, Hoshi K, Okawara R, Ishibashi H, Yamamoto S. Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture. *J Endocrinol* 2003; **176**: 339-348 [PMID: 12630919 DOI: 10.1677/joe.0.1760339]
23 **Cockayne S**, Adamson J, Lanham-New S, Shearer MJ, Gilbody S, Torgerson DJ. Vitamin K and the prevention of fractures: systematic review and meta-analysis of randomized controlled trials. *Arch Intern Med* 2006; **166**: 1256-1261 [PMID: 16801507 DOI: 10.1001/archinte.166.12.1256]
24 **Janes CH**, Dickson ER, Okazaki R, Bonde S, McDonagh AF, Riggs BL. Role of hyperbilirubinemia in the impairment of osteoblast proliferation associated with cholestatic jaundice. *J Clin Invest* 1995; **95**: 2581-2586 [PMID: 7769100 DOI: 10.1172/JCI117959]
25 **Ruiz-Gaspà S**, Guañabens N, Enjuanes A, Peris P, Martinez-Ferrer A, de Osaba MJ, Gonzalez B, Alvarez L, Monegal A, Combalia A, Parés A. Lithocholic acid downregulates vitamin D effects in human osteoblasts. *Eur J Clin Invest* 2010; **40**: 25-34 [PMID: 20055894 DOI: 10.1111/j.1365-2362.2009.02230.x]
26 **Kawelke N**, Bentmann A, Hackl N, Hager HD, Feick P, Geursen A, Singer MV, Nakchbandi IA. Isoform of fibronectin mediates bone loss in patients with primary biliary cirrhosis by suppressing bone formation. *J Bone Miner Res* 2008; **23**: 1278-1286 [PMID: 18348696 DOI: 10.1359/jbmr.080313]
27 **Hughes DE**, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nat Med* 1996; **2**: 1132-1136 [PMID: 8837613 DOI: 10.1038/nm1096-1132]
28 **Armas LA**, Recker RR. Pathophysiology of osteoporosis: new mechanistic insights. *Endocrinol Metab Clin North Am* 2012; **41**: 475-486 [PMID: 22877425 DOI: 10.1016/j.ecl.2012.04.006]
29 **Bengoa JM**, Sitrin MD, Meredith S, Kelly SE, Shah N, Baker AL, Rosenberg IH. Intestinal calcium absorption and vitamin D status in chronic cholestatic liver disease. *Hepatology* 1984; **4**: 261-265 [PMID: 6706300]
30 **Herlong HF**, Recker RR, Maddrey WC. Bone disease in primary biliary cirrhosis: histologic features and response to 25-hydroxyvitamin D. *Gastroenterology* 1982; **83**: 103-108 [PMID: 6978827]
31 **Matloff DS**, Kaplan MM, Neer RM, Goldberg MJ, Bitman W, Wolfe HJ. Osteoporosis in primary biliary cirrhosis: effects of 25-hydroxyvitamin D3 treatment. *Gastroenterology* 1982; **83**: 97-102 [PMID: 7075948]
32 **Compston JE**, Thompson RP. Intestinal absorption of 25-hydroxyvitamin D and osteomalacia in primary biliary cirrhosis. *Lancet* 1977; **1**: 721-724 [PMID: 66519]
33 **Office of the Surgeon General (US)**. Bone Health and Osteoporosis: A Report of the Surgeon General. *US Heal Hum Serv* (Internet) 2004; **437** [PMID: 20945569 DOI: 10.2165/00002018-200932030-00004]
34 **Parés A**, Guañabens N. Osteoporosis in primary biliary cirrhosis: pathogenesis and treatment. *Clin Liver Dis* 2008; **12**: 407-24 [PMID: 18456188 DOI: 10.1016/j.cld.2008.02.005]
35 **Glass LM**, Su GL. Metabolic Bone Disease in Primary Biliary Cirrhosis. *Gastroenterol Clin North Am* 2016; **45**: 333-343 [PMID: 27261902 DOI: 10.1016/j.gtc.2016.02.009]
36 **Cosman F**, de Beur SJ, LeBoff MS, Lewiecki EM, Tanner B, Randall S, Lindsay R; National Osteoporosis Foundation. Clinician's Guide to Prevention and Treatment of Osteoporosis. *Osteoporos Int* 2014; **25**: 2359-2381 [PMID: 25182228 DOI: 10.1007/s00198-014-2794-2]
37 **Kemmler W**, Engelke K, von Stengel S. Long-Term Exercise and Bone Mineral Density Changes in Postmenopausal Women--Are There Periods of Reduced Effectiveness? *J Bone Miner Res* 2016;

31: 215-222 [PMID: 26234411 DOI: 10.1002/jbmr.2608]
38 **Shiomi S**, Masaki K, Habu D, Takeda T, Nishiguchi S, Kuroki T, Ochi H. Calcitriol for bone loss in patients with primary biliary cirrhosis. *J Gastroenterol* 1999; **34**: 241-245 [PMID: 10213125]
39 **Guañabens N**, Parés A, Monegal A, Peris P, Pons F, Alvarez L, de Osaba MJ, Roca M, Torra M, Rodés J. Etidronate versus fluoride for treatment of osteopenia in primary biliary cirrhosis: preliminary results after 2 years. *Gastroenterology* 1997; **113**: 219-224 [PMID: 9207281 DOI: 10.1016/S0016-5085(97)70098-2]
40 **Musialik J**, Petelenz M, Gonciarz Z. Effects of alendronate on bone mass in patients with primary biliary cirrhosis and osteoporosis: preliminary results after one year. *Scand J Gastroenterol* 2005; **40**: 873-874 [PMID: 16109666 DOI: 10.1080/00365520510023279]
41 **Sanderson J**, Martyn-St James M, Stevens J, Goka E, Wong R, Campbell F, Selby P, Gittoes N, Davis S. Clinical effectiveness of bisphosphonates for the prevention of fragility fractures: A systematic review and network meta-analysis. *Bone* 2016; **89**: 52-58 [PMID: 27262775 DOI: 10.1016/j.bone.2016.05.013]
42 **Rudic JS**, Giljaca V, Krstic MN, Bjelakovic G, Gluud C. Bisphosphonates for osteoporosis in primary biliary cirrhosis. *Cochrane Database Syst Rev* 2011; CD009144 [PMID: 22161446 DOI: 10.1002/14651858.CD009144.pub2]
43 **Wolfhagen FH**, van Buuren HR, den Ouden JW, Hop WC, van Leeuwen JP, Schalm SW, Pols HA. Cyclical etidronate in the prevention of bone loss in corticosteroid-treated primary biliary cirrhosis. A prospective, controlled pilot study. *J Hepatol* 1997; **26**: 325-330 [PMID: 9059953]
44 **Zein CO**, Jorgensen RA, Clarke B, Wenger DE, Keach JC, Angulo P, Lindor KD. Alendronate improves bone mineral density in primary biliary cirrhosis: a randomized placebo-controlled trial. *Hepatology* 2005; **42**: 762-771 [PMID: 16175618 DOI: 10.1002/hep.20866]
45 **Guañabens N**, Parés A, Ros I, Alvarez L, Pons F, Caballería L, Monegal A, Martínez de Osaba MJ, Roca M, Peris P, Rodés J. Alendronate is more effective than etidronate for increasing bone mass in osteopenic patients with primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 2268-2274 [PMID: 14572578 DOI: 10.1016/S0002-9270(03)00550-1]
46 **Guañabens N**, Monegal A, Cerdá D, Muxí Á, Gifre L, Peris P, Parés A. Randomized trial comparing monthly ibandronate and weekly alendronate for osteoporosis in patients with primary biliary cirrhosis. *Hepatology* 2013; **58**: 2070-2078 [PMID: 23686738 DOI: 10.1002/hep.26466]
47 **El Dareer SM**, Tillery KF, Hill DL. Disposition of 5-methyltetrahydrohomofolate in mice, dogs, and monkeys. *Cancer Treat Rep* 1979; **63**: 201-207 [PMID: 109197 DOI: 10.1056/NEJM199005033221803]
48 **Hillard TC**, Whitcroft SJ, Marsh MS, Ellerington MC, Lees B, Whitehead MI, Stevenson JC. Long-term effects of transdermal and oral hormone replacement therapy on postmenopausal bone loss. *Osteoporos Int* 1994; **4**: 341-348 [PMID: 7696830]
49 **Olsson R**, Mattsson LA, Obrant K, Mellström D. Estrogen-progestogen therapy for low bone mineral density in primary biliary cirrhosis. *Liver* 1999; **19**: 188-192 [PMID: 10395037 DOI: 10.1111/j.1478-3231.1999.tb00034.x]
50 **Menon KV**, Angulo P, Boe GM, Lindor KD. Safety and efficacy of estrogen therapy in preventing bone loss in primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 889-892 [PMID: 12738473 DOI: 10.1111/j.1572-0241.2003.07341.x]
51 **Ormarsdóttir S**, Mallmin H, Naessén T, Petrén-Mallmin M, Broomé U, Hultcrantz R, Lööf L. An open, randomized, controlled study of transdermal hormone replacement therapy on the rate of bone loss in primary biliary cirrhosis. *J Intern Med* 2004; **256**: 63-69 [PMID: 15189367 DOI: 10.1111/j.1365-2796.2004.01342.x]
52 **de Villiers TJ**, Gass ML, Haines CJ, Hall JE, Lobo RA, Pierroz DD, Rees M. Global Consensus Statement on menopausal hormone therapy. *Maturitas* 2013; **74**: 391-392 [PMID: 23497918 DOI: 10.1016/j.maturitas.2013.02.001]
53 **Camisasca M**, Crosignani A, Battezzati PM, Albisetti W, Grandinetti G, Pietrogrande L, Biffi A, Zuin M, Podda M. Parenteral calcitonin for metabolic bone disease associated with primary biliary cirrhosis. *Hepatology* 1994; **20**: 633-637 [PMID: 8076921]
54 **Floreani A**, Chiaramonte M, Giannini S, Malvasi L, Lodetti MG, Castrignano R, Giacomini A, D'Angelo A, Naccarato R. Longitudinal study on osteodystrophy in primary biliary cirrhosis (PBC) and a pilot study on calcitonin treatment. *J Hepatol* 1991; **12**: 217-223 [PMID: 2051000 DOI: 10.1016/0168-8278(91)90941-4]
55 **Floreani A**, Zappala F, Fries W, Naccarato R, Plebani M, D'Angelo A, Chiaramonte M. A 3-year pilot study with 1,25-dihydroxyvitamin D, calcium, and calcitonin for severe osteodystrophy in primary biliary cirrhosis. *J Clin Gastroenterol* 1997; **24**: 239-244 [PMID: 9252849 DOI: 10.1097/00004836-199706000-00012]
56 **Levy C**, Harnois DM, Angulo P, Jorgensen R, Lindor KD. Raloxifene improves bone mass in osteopenic women with primary biliary cirrhosis: results of a pilot study. *Liver Int* 2005; **25**: 117-121 [PMID: 15698408 DOI: 10.1111/j.1478-3231.2005.01026.x]
57 **Nishiguchi S**, Shimoi S, Kurooka H, Tamori A, Habu D, Takeda T, Kubo S. Randomized pilot trial of vitamin K2 for bone loss in patients with primary biliary cirrhosis. *J Hepatol* 2001; **35**: 543-545 [PMID: 11682046 DOI: 10.1016/S0168-8278(01)00133-7]
58 **Neer RM**, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 2001; **344**: 1434-1441 [PMID: 11346808 DOI: 10.1056/NEJM200105103441904]
59 **Miller PD**, Hattersley G, Riis BJ, Williams GC, Lau E, Russo LA, Alexandersen P, Zerbini CA, Hu MY, Harris AG, Fitzpatrick LA, Cosman F, Christiansen C; ACTIVE Study Investigators. Effect of Abaloparatide vs Placebo on New Vertebral Fractures in Postmenopausal Women With Osteoporosis: A Randomized Clinical Trial. *JAMA* 2016; **316**: 722-733 [PMID: 27533157 DOI: 10.1001/jama.2016.11136]
60 **Dresner-Pollak R**, Gabet Y, Steimatzky A, Hamdani G, Bab I, Ackerman Z, Weinreb M. Human parathyroid hormone 1-34 prevents bone loss in experimental biliary cirrhosis in rats. *Gastroenterology* 2008; **134**: 259-267 [PMID: 18061175 DOI: 10.1053/j.gastro.2007.10.025]
61 **Lindor KD**, Janes CH, Crippin JS, Jorgensen RA, Dickson ER. Bone disease in primary biliary cirrhosis: does ursodeoxycholic acid make a difference? *Hepatology* 1995; **21**: 389-392 [PMID: 7843710]
62 **Nevens F**, Andreone P, Mazzella G, Strasser SI, Bowlus C, Invernizzi P, Drenth JP, Pockros PJ, Regula J, Beuers U, Trauner M, Jones DE, Floreani A, Hohenester S, Luketic V, Shiffman M, van Erpecum KJ, Vargas V, Vincent C, Hirschfield GM, Shah H, Hansen B, Lindor KD, Marschall HU, Kowdley KV, Hooshmand-Rad R, Marmon T, Sheeron S, Pencek R, MacConell L, Pruzanski M, Shapiro D; POISE Study Group. A Placebo-Controlled Trial of Obeticholic Acid in Primary Biliary Cholangitis. *N Engl J Med* 2016; **375**: 631-643 [PMID: 27532829 DOI: 10.1056/NEJMoa1509840]
63 **Khosla S**, Hofbauer LC. Osteoporosis treatment: recent developments and ongoing challenges. *Lancet Diabetes Endocrinol* 2017; **5**: 898-907 [PMID: 28689769 DOI: 10.1016/S2213-8587(17)30188-2]

P- Reviewer: Takahashi T **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 August 21; 24(31): 3521-3530

DOI: 10.3748/wjg.v24.i31.3521

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

MINIREVIEWS

Ubiquitin-proteasome system and oxidative stress in liver transplantation

Norma Alva, Arnau Panisello-Roselló, Marta Flores, Joan Roselló-Catafau, Teresa Carbonell

Norma Alva, Marta Flores, Teresa Carbonell, Department of Cell Biology, Physiology and Immunology, University of Barcelona, Barcelona 08028, Spain

Arnau Panisello-Roselló, Joan Roselló-Catafau, Experimental Pathology Department, Institute of Biomedical Research of Barcelona, Barcelona 08036, Spain

ORCID number: Norma Alva (0000-0001-5039-8270); Arnau Panisello-Roselló (0000-0003-2062-6134); Marta Flores (0000-0002-1062-9676); Joan Roselló-Catafau (0000-0001-7127-4883); Teresa Carbonell (0000-0002-7131-3667).

Author contributions: All authors contribute equally to the writing of the manuscript and approved the final version.

Supported by Fondo de Investigaciones Sanitarias, Ministerio de Economia y Competitividad (Madrid, Spain), No. PI15/00110.

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

Manuscript source: Invited manuscript

Correspondence to: Teresa Carbonell, PhD, Associate Professor, Research Scientist, Department of Cell Biology, Physiology and Immunology, University of Barcelona, Avda Diagonal, 643, Barcelona 08028, Catalonia, Spain. tcarbonell@ub.edu
Telephone: +34-93-4035924

Received: May 5, 2018
Peer-review started: May 5, 2018
First decision: May 17, 2018
Revised: June 28, 2018
Accepted: June 30, 2018

Article in press: June 30, 2018
Published online: August 21, 2018

Abstract

A major issue in organ transplantation is the development of a protocol that can preserve organs under optimal conditions. Damage to organs is commonly a consequence of flow deprivation and oxygen starvation following the restoration of blood flow and reoxygenation. This is known as ischemia-reperfusion injury (IRI): a complex multifactorial process that causes cell damage. While the oxygen deprivation due to ischemia depletes cell energy, subsequent tissue oxygenation due to reperfusion induces many cascades, from reactive oxygen species production to apoptosis initiation. Autophagy has also been identified in the pathogenesis of IRI, although such alterations and their subsequent functional significance are controversial. Moreover, proteasome activation may be a relevant pathophysiological mechanism. Different strategies have been adopted to limit IRI damage, including the supplementation of commercial preservation media with pharmacological agents or additives. In this review, we focus on novel strategies related to the ubiquitin proteasome system and oxidative stress inhibition, which have been used to minimize damage in liver transplantation.

Key words: Liver transplant; Ischemia-reperfusion injury; Oxidative stress; Proteasome; Redox regulation; Ubiquitin

Core tip: Ischemia-reperfusion injury is a complex multifactorial process that causes cell damage during liver transplantation. The role of the ubiquitin proteasome system during liver transplantation remains unclear. The

use of proteasome inhibitors is a new strategy aimed at improving organ preservation.

Alva N, Panisello-Roselló A, Flores M, Roselló-Catafau J, Carbonell T. Ubiquitin-proteasome system and oxidative stress in liver transplantation. *World J Gastroenterol* 2018; 24(31): 3521-3530 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3521.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3521

INTRODUCTION

The definitive treatment option for many liver diseases is liver transplantation; and in these cases, one important issue is the optimization of organ preservation.

During liver transplantation, damage is initiated by vascular occlusion during hepatic resections. This is aggravated by cold storage of the liver graft (usually at 4 ℃) in the preservation solution; and finally by warm reperfusion and subsequent implantation into the recipient. During ischemia, a long period of tissue hypoxia can result in tissue injury and organ dysfunction; as the organ is deprived of oxygen, ATP and energy becomes depleted. Paradoxically, the reintroduction of oxygen during reperfusion aggravates the damage. The whole process has a plethora of consequences that are collectively known as ischemia-reperfusion injury (IRI)[1-3]. While oxygen deprivation due to ischemia depletes cell energy, subsequent reoxygenation due to reperfusion induces many cascades, from induction of reactive oxygen species (ROS)[4] to initiation of apoptosis (Figure 1). Such events are, in part, responsible for organ failure. Variables related to the donor (age, steatosis) and surgery (prolonged ischemia times)[5] are the most commonly reported risk factors for graft dysfunction[6]. Other factors such as shear stress and small graft size[7] have been recognized as important factors associated with oxidative stress, lack of primary function, early dysfunction allograft and biliary complications after liver transplantation[8]. Together with the immunological mechanisms of graft rejection, IRI remains as one of the main clinical problems following organ transplantation.

Current strategies to prevent and modulate IRI include: ischemic pre-[9-15] or post-conditioning[16] protocols ; static cold storage[17] or machine perfusion[18-20] and the use of pharmacological agents[21-23]. The most common methods applied in liver surgery have recently been reviewed[5,24]. Conventional hypothermic cold storage continues to be the main method for liver preservation, largely because of its cost-effectiveness, simplicity and logistics. However, hypothermia can have multiple side effects, including the induction of oxidative stress[25].

Because of the wide range of mechanisms that can contribute to cell damage in IRI (involving ROS, oxidized products, inflammatory mediators and cytokines), adoption of a specific therapeutic strategy often results in only limited improvements in organ transplantation. Given these circumstances, it is worthwhile to focus on the assessment of agents that can counteract the damage induced by oxidative stress. As oxidative stress is the result of an imbalance between the rate of ROS generation and the capacity to detoxify these reactive species[26-28], interventions that could result in ROS scavenging or the detoxifying of ROS products could protect against IRI. Three lines of defence against oxidative stress have been reported[29]. Antioxidant molecules (such as glutathione) represent the first line of antioxidant defence; the second line incorporates enzymatic antioxidants; and the third consists of repair system proteins, including the proteolytic pathways. In this third line, the ubiquitin-proteasome system (UPS) is widely recognized as the main system for degradation of cytosolic proteins[30,31]

The purpose of this review is to present an update of effects of oxidative stress while summarizing recent findings on the role of the UPS in organ preservation and liver transplantation. In this review we outline the current data of the literature, previous search in databases - PubMed, Web of Science and Scopus - that support the hypothesis on the potential involvement of UPS and oxidative stress in IRI. We will focus on the new strategies used to minimize damage in liver transplantation.

STATIC COLD STORAGE, MACHINE PERFUSION AND STORAGE SOLUTIONS

The strategy most commonly adopted to reduce ischemic injury is the cooling of organs and the use of a preservation solution to minimize enzymatic activity and depletion of the energy substrate. Cold storage slows down cellular metabolism[32,33], but this can be responsible for further ATP and energy depletion[34]. In addition, cold has adverse side effects due to the induction of cellular inflammation, alterations of the cytoskeleton[35] , and oxidative stress.

Protective strategies to reduce hepatic IRI include the use of the machine perfusion, which represents a new line of research opposed to static cold storage.

Perfusion machine involves a pulsatile perfusion of the liver with a cold (subnormothermic, hypothermic) perfusate[18,36-40]; with normothermic perfusate[41,42]; or with a gradual increase in of the perfusate temperature[43]. These references confirm that machine perfusion protects against IRI damage in animal models. Livers preserved by subnormothermic machine perfusion at 20 ℃ showed significantly less liver damage at the end of reperfusion compared to cold storage. The release of LDH was reduced while the production of bile, ATP levels, glycogen and glutathione content increased in preserved livers by subnormothermic machine perfusion

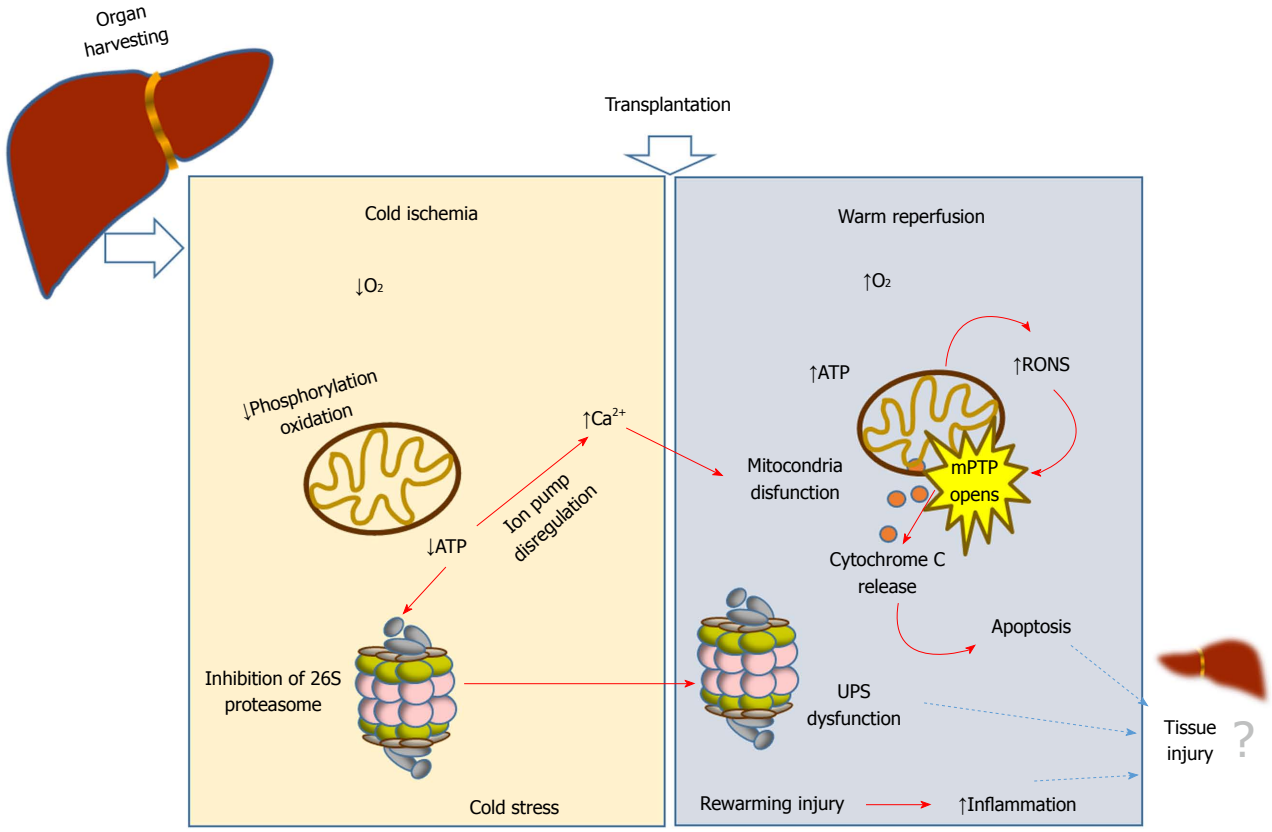


Figure 1 Ischemia-reperfusion injury: A complex multifactorial process that causes cell damage during liver transplantation. While the oxygen deprivation due to ischemia depletes cell energy, subsequent reoxygenation due to reperfusion induces many cascades, from production of reactive oxygen species to initiation of apoptosis. UPS: Ubiquitin-proteasome system; mPTP: mitochondrial permeability transition pore.

than livers submitted to cold storage[44]. Normothermic machine perfusion has also been assessed in discarded human liver grafts[45]. The reported data demonstrate the viability of normothermic perfusion, which results in the continuous production of bile, the fall of lactate levels and the preservation of hepatic morphology. However, the safety and efficacy of machine perfusion is yet to be assessed by randomized controlled clinical trials.

Due to its cost-effectiveness and simplicity, cold storage with conventional hypothermia remains the main method for liver conservation. In fact, major advances in the field of organ preservation have included the development of new improved preservation solutions capable of reducing cold-induced cellular damage[46]. Euro-Collins solution was first developed in the 1970s; while more recently, University of Wisconsin (UW)[47] Celsior[48], Histidine-Ketoglutarate (HTK)[49] and Institute Georges Lopez (IGL-1)[50] solutions tend to be extensively used for liver transplantation. Commercial preservation solutions include oncotic agents, like hydroxy-ethyl starch (HES) in UW and polyethylenglycol-35 (PEG-35) in IGL-1, which confer high viscosity to the media. They also contain metabolic precursors (adenosine and ketoglutarate) and antioxidants (glutathione, allopurinol). Although the use of commercial preservation solutions has improved conditions for liver graft preservation, with the urgent need to expand the donor pool and the subsequent use of suboptimal grafts, new additives have been proposed to combat oxidant and apoptotic damage with the aim of prolonging graft quality during cold storage.

THE ROLE OF OXIDATIVE STRESS IN IRI

ROS are highly reactive and capable of oxidizing lipids, proteins and DNA[51], thereby leading to structural and cellular changes that may cause oxidative stress and cellular apoptosis. Abundant information has been published demonstrating that increased ROS production is involved in IRI pathology[28,50,52,53]. The involvement of ROS was initially observed based on the detection of enhanced production of chemical products generated by the reaction of ROS with cellular components. Lipid peroxidation products, such as malondialdehyde and hydroxynonenal[13,36,54-56] have been widely used as a biomarkers of oxidative stress in IRI. The accelerated ROS production in post-ischemic tissues has been attributed to enzymes capable of reducing molecular oxygen and forming superoxide: xantine oxidase[57-59], NADPH oxidase[60] and nitric oxide synthase[11]. The contribution of each of these enzymes in IRI is assessed in the excellent review by Granger and Kvietys[28]. All this indicates that radicals can be formed from different

sources, and consequently several protective strategies to decrease liver IRI have targeted different sources of ROS: xanthine oxidase (using allopurinol[59,61]) or NADPH oxidase[62], for example. Other strategies have included pharmacological interventions with antioxidants resulting in the neutralization of ROS effects[63-66].

As mentioned above, Jung *et al*[29] describe three main lines of defence against oxidative stress. Thus, to those molecules widely recognized as antioxidants, such as glutathione, and enzymatic antioxidants, they add a third line of defence: repair system proteins. This system includes the proteolytic pathways, such as the UPS[30,31]. The activity of the UPS is necessary so that the cells can cope with oxidative stress, but in turn, the activity of the UPS are also modulated by the redox state[67].

THE UBIQUITIN-PROTEASOME SYSTEM

In order to eliminate damaged proteins, cells have highly regulated mechanisms, such as the autophagy-lysosome pathway and the UPS, which is recognized as the principal system for degrading oxidized cytosolic proteins. Proteasomes are protein complexes that, via proteolysis, degrade unnecessary or damaged proteins. It has always appeared that autophagy within lysosomes is involved in the pathogenesis of hepatic IRI, although the specific alterations it causes and their subsequent functional significance are highly controversial[68]. The use of proteasome inhibitors has been demonstrated to enhance myocardial viability[69,70] and protect liver against IRI[4,71-73], which suggests this may be a promising strategy to reduce the damage inherent to transplantation protocols.

Ubiquitin

Ubiquitin is a highly conserved and small 76-amino-acid protein that acts as a post-translational protein modifier and regulates protein lifespan. Ubiquitin was isolated in the 1970s by Goldstein *et al*[74] and since then has been identified in many cellular processes, including proteasomal proteolysis and also DNA damage repair. Ubiquitin can be attached covalently to a target protein in a process known as ubiquitination. Ubiquitin is conjugated to other proteins through a peptide bond between its C-terminal glycine and a primary amine on the substrate, most typically a lysine residue. Conjugation is dependent on the successive activities of enzymes named E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase)[75]. In the 1980s, biochemical studies elucidated the chemical reactions catalysed by these enzymes[76,77]. E1 activates ubiquitin as a thioester of the active site cysteine residue at expenses of ATP[78]. The activated ubiquitin molecule is then transferred to an E2 enzyme via the formation of a new thioester with the catalytic cysteine residue of the E2 enzyme. E2 transfers the activated ubiquitin moieties to the protein substrate that is specifically bound to a unique ubiquitin ligase: E3. Finally, an isopeptide bond is formed between the C-terminal glycine residue of the ubiquitin (Gly76) and the e-NH2 group of an internal lysine (Lys119) of the protein substrate. Several protein families have been discovered that are evolutionarily related to ubiquitin, as they share the characteristics of the ubiquitin-fold proteins and the capacity to become conjugated to substrates through the action of related E1, E2, and E3 enzymes[79].

Target proteins can be modified by mono- or poly-ubiquitination. The seven lysines in ubiquitin contribute to the assembly of various poly-ubiquitin chains that may be involved in different cellular processes[80]. The K48-linked (Lys 48) poly-ubiquitin chain targets proteins for proteasomal degradation, while the K11-linked poly-ubiquitin chain is involved in endoplasmic reticulum-associated degradation[81] and the K63-linked poly-ubiquitin chain can act in non-proteolytic events, including protein trafficking, DNA repair, and inflammation[82]. Furthermore, ubiquitination can be reversed by the removal of ubiquitin from target proteins by de-ubiquitinating enzymes (DUBs).

Degradation of damaged proteins

The proteasome is a multicatalytic protease involved in the degradation of intracellular proteins. The processing of damaged proteins is mainly mediated by the 20S and 26S proteasomes[76,77].

The 20S proteasome is a large complex, formed of four stacked rings: two alpha and two beta rings, each containing seven different subunits (from α1 to α7 or from β1 to β7). Three of the beta subunits are responsible for the proteolytic activity. β1 exhibits caspase-like activity, while β2 exhibits trypsin-like activity, and β5, chymotrypsin-like activity. Whereas the beta rings are responsible for proteolytic degradation, the alpha rings perform substrate recognition and regulate substrate access to the proteolytic active centre.

The 26S proteasome is formed by the addition of one or two regulatory 19S subunits to an alpha ring in the 20S core proteasome in an ATP-dependent manner. The binding to this regulator increases the proteasomal activity and allows the proteasome to degrade the substrate proteins that have been tagged through the binding of a chain of ubiquitin, as explained above. The ubiquitin chain acts as a labelled sequence to direct proteins to the 26S proteasome where they are degraded. The 19S subunits first remove the poly-ubiquitin "tags" of the targeted proteins and unfold those proteins. Then, the unfolded proteins are degraded by the 20S core of the 26S proteasome. In this way, while the 20S core proteasome can degrade unfolded proteins in an ATP-independent manner, the 26S proteasome is only capable of degrading natively folded and functional proteins in an ATP- and ubiquitin-

dependent manner[83].

ACTIVATION OF THE PROTEASOME UBIQUITIN SYSTEM (UPS) AND PROTEOLYSIS IN IRI

It has been well established that proteolysis is an important regulatory mechanism that helps maintain homeostasis[77]. The proteasome and the UPS are necessary for the control of protein concentrations, to prevent abnormal accumulations and to modulate regulatory proteins involved in cell metabolism. However, proteolysis can have detrimental effects on organs and tissues after transplantation[84]. Free amino acids in the effluent from human livers was used as a marker to predict postoperative graft function. In fact, the prevention of liver graft proteolysis and proteasome activation can be modulated by the use of additives to organ preservation solutions[85,86]. In this way, it has been demonstrated that lactobionate, a component of UW solution, prevents the release of metalloproteinases during cold preservation[85]. Moreover, the UPS is an energy-dependent system and ATP levels affects 26S proteasome assembly, stability, and function[87]. The following processes are dependent on ATP: The first step in ubiquitin conjugation by E1, which is required for the poly-ubiquitination of proteins; the assembly of alpha and beta rings and ATP-dependent proteases; and the degradation of poly-ubiquinated proteins by the 26S proteasome[79,87]. In addition, it is well known that oxygen deprivation leads to a significant decrease in ATP in liver grafts[34], which could affect liver outcomes.

Therefore, during cold storage of organs, and because of the ATP decrease, the formation of 26S proteasomes and poly-ubiquitin-dependent protein degradation are expected to be impaired. However, it has been well established that a subset of 26S proteasomes appears to be activated as ATP levels decline[70]. In that study, Geng *et al*[70] demonstrated that the activation of the 26S proteasome is a pathophysiologically relevant mechanism in cold ischemic myocardial injury. Such observations imply that a subset of 26S is acting as a harmful protease that is activated when the cellular energy supply decreases. The finding that ATP negatively regulates proteasome activity is consistent with previous results concerning increased proteolysis during cold preservation of human liver grafts[84]. Moreover, hypothermia also affects proteasome activity in isolated perfused rat liver, increasing chymotrypsin-like activity[36].

Degradation of protein substrates by 26S proteasomes requires the 19S regulatory cap to recognize ubiquitin protein conjugates and to regulate the entry of the substrate into the proteolytic cavity of the 20S core. Covalent regulation via phosphorylation of a subunit in the 19S regulatory cap of the proteasome allows for a fast increase in the 26S proteasome activity, which becomes an important regulatory mechanism for proteasome control[88-90]. In fact, we have previously reported that phosphorylation of the 19S subunit Rpt6 increases in cold perfused rat livers[36].

The combination of proteasome and oxidative stress in IRI

ROS are capable of oxidatively modifying cell structures. Due to the abundance of proteins, the presence of oxidatively modified proteins and aggregates of oxidized proteins has been reported in the cytosol. There is a certain degree of consensus that proteasomal activity degrades oxidized proteins, although the attribution to 20S or 26S is yet to be elucidated. The 26S proteasome was found to be less active in the degradation of oxidatively damaged proteins[91]. A growing body of evidence suggests that the degradation of oxidatively damaged proteins does not require ATP and polyubiquitination of the substrate[92]. Alternatively, oxidative proteins are removed independently of ubiquitin by the ATP-independent 20S proteasome[93].

The proteasome is responsible for the selective degradation of oxidatively damaged proteins. In this sense it has been shown that certain oxidized proteins degrade faster than their native counterparts[94], and, furthermore, it has been shown that inhibition of the proteasome stabilizes the oxidized proteins[95]. During oxidative stress, the ratio of oxidized to reduced glutathione increases[36]. and this redox status of the cells can also modulate protein ubiquitination by reversible S-thiolation[96,67]. This is concurrent with a decrease in the ubiquitin-activating enzyme, E-1 and ubiquitin conjugates. According to some models, glutathiolation of the E1 or E2 components of a ubiquitinated protein protects it from unnecessary degradation. Thus, S-glutathiolation can be regarded as a general mechanism of a redox signal controlling gene expression. In addition, direct oxidative modifications of the proteasome may also occur, including carbonylation, glycoxidation and modification with lipid peroxidation products. Although it is not clear to what extent these modifications affect the proteasome, they could modulate proteasomal activity. It should be noted that an inefficient proteasomal system would result in an accumulation of protein aggregates in the cytoplasm, as has been reported in brain due to aging or Alzheimer's disease[97]; while excessive protein depletion activity may have deleterious effects by affecting detoxifying systems, membranes, or RNA stability[69,84].

PROTEASOME INHIBITION IN LIVER IRI

The use of proteasome inhibitors has been shown to offer protection and maintain the physiological ubiquitin-protein conjugate pool during cold organ preservation[98]. Table 1 summarizes the protective effects of different proteasome inhibitors against IRI during organ preservation. The potential pharmacological role of proteasome inhibitors was first reported by Campbell *et al*[99]. Those

Table 1 Effect of different proteasome inhibitors on organ preservation

Inhibitor	Organ and condition	Manifestations	Ref.
PS-519	Heart after IRI (rat)	Improved cardiac contractility Improved coronary flow Reduced PMN infiltration	Campbell *et al*[99]
Epoxomicin	Heart after cold ischemia (rat)	Reduced edema formation Preserved ultrastructural integrity	Geng *et al*[70]
MG132	Liver, warm IRI (rat)	Decreased LDH and ALT Increased protein oxidation Decreased antioxidant activities	Alexandrova *et al*[4]
MG132	Liver, cold IRI (rat)	Decreased AST and ALT Reduced inflammation (IL1β and TNFα)	Zaouali *et al*[71]
Bortezomid	Liver, cold IRI (rat)	Decreased AST, ALT and mitochondrial damage Increased bile production Decreased lipid peroxidation Decreased apoptosis (Cyt C and Caspase 3)	Zaouali *et al*[71] Bejaoui *et al*[72]

IRI: Ischemia-reperfusion injury.

authors demonstrated that proteasome inhibition can prevent loss of cardiac contractile function in isolated perfused rat heart. The adherence of polymorphonuclear leukocytes to the endothelium was also reduced. However, controversy regarding whether inhibiting the proteasome is beneficial or detrimental to cardiac function continues[95]. Few studies have examined the role of UPS inhibitors as a strategy to reduce damage in liver IRI. In rat liver subjected to warm IRI, the administration of the proteasome inhibitor MG132 decreased LDH and AST levels during ischemia and reperfusion[4]. In addition, the same authors studied whether MG132 can modulate the prooxidant and antioxidant status of rat liver. The results showed that MG132 did not significantly affect liver lipid peroxidation. However, MG132 increased protein carbonyls and decreased the main antioxidant enzyme activities (catalase and superoxide dismutase).

In contrast to that modulation of oxidative stress, the proteasome inhibitor Bortezomib, used at a non-toxic low dose, up-regulates liver antioxidant enzymes in chronic ethanol-fed rats[86]. Exposure to the proteasome inhibitor increased antioxidant defences by enhancing the levels of mRNA and protein expression transcripts of glutathione reductase, glutathione synthetase and glutathione peroxidase, as well as superoxide dismutase in rat liver. The increase in antioxidant defences was concomitant with enhanced 26S proteasome activity. As mentioned by those authors, the beneficial effects of the proteasome inhibitor Bortezomib could be due to the low dose used and to the reversibility of the drug.

In accordance with the previous research, we have recently demonstrated that the addition of the reversible UPS inhibitors Bortezomib and MG132 to UW solution improved steatotic and non-steatotic rat liver preservation in the face of cold IRI[71]. Both inhibitors prevented liver injury, decreasing AST and ALT, and prevented the release of the inflammatory cytokines IL-1 beta and TNF-alpha. The protective effect of Bortezomib was superior to that of MG132. Bortezomib increased bile production, decreased vascular resistance in fatty rat liver through an increase in nitric oxide generation, prevented lipid peroxidation and mitochondrial damage, and increased AMP-activated protein kinase (AMPK) phosphorylation. It is well known that AMPK phosphorylation is a key process in fatty liver graft preservation, as it can reduce inflammation[9,44].

The supplementation of IGL-1 preservation solution with Bortezomib has also been shown to have protective effects[72], reducing steatotic liver injury and decreasing liver apoptosis (cytochrome c and caspase 3 release) in the face of cold IRI. These effects were partially mediated through the activation of the Akt/mTOR signalling pathway, a key regulator in cell growth and proliferation, and through the phosphorylation of AMPK.

Besides its role in preventing inflammation, AMPK activation also keeps the liver in an energy-conserving state during cold storage. AMPK triggers ATP-producing pathways, balancing the metabolic process towards increasing energy homeostasis in the cell. We have recently found a correlation between AMPK activation, ATP levels, lower proteasome activity and decreased damage in fatty liver grafts preserved in different solutions[73]. As AMPK is regulated and degraded by the UPS, the use of proteasome inhibitors in the preservation solution could avoid AMPK depletion and may contribute to maintaining the beneficial effects of proteasome inhibition after IRI.

CONCLUSION

Remarkable progress has been made over the past few decades regarding the role of the UPS in many cellular processes. However, the function and regulation of the proteasome in organ transplantation still remains unclear. We have proposed that UPS inhibitors reduce IRI in liver grafts *via* up-regulation of AMPK phosphorylation and the consequent preservation of the energy state. As some proteasome inhibitors have been approved for the treatment of different diseases, we propose to explore their use in liver. Well-designed

randomized controlled trials will be needed to evaluate the use of proteasome inhibitors in liver transplantation. The supplementation of low and non-toxic doses of proteasome inhibitors offers a new opportunity for the improvement of organ preservation solutions.

ACKNOWLEDGMENTS

We are grateful to Christopher Evans for language assistance.

REFERENCES

1 **Nagano K**, Gelman S, Bradley EL Jr, Parks D. Hypothermia, hepatic oxygen supply-demand, and ischemia-reperfusion injury in pigs. *Am J Physiol* 1990; **258**: G910-G918 [PMID: 2360637 DOI: 10.1152/ajpgi.1990.258.6.G910]

2 **Iñiguez M**, Dotor J, Feijoo E, Goñi S, Prieto J, Berasain C, Avila MA. Novel pharmacologic strategies to protect the liver from ischemia-reperfusion injury. *Recent Pat Cardiovasc Drug Discov* 2008; **3**: 9-18 [PMID: 18221124]

3 **Duval M**, Plin C, Elimadi A, Vallerand D, Tillement JP, Morin D, Haddad PS. Implication of mitochondrial dysfunction and cell death in cold preservation--warm reperfusion-induced hepatocyte injury. *Can J Physiol Pharmacol* 2006; **84**: 547-554 [PMID: 16902600 DOI: 10.1139/y06-014]

4 **Alexandrova A**, Petrov L, Georgieva A, Kessiova M, Tzvetanova E, Kirkova M, Kukan M. Effect of MG132 on proteasome activity and prooxidant/antioxidant status of rat liver subjected to ischemia/reperfusion injury. *Hepatol Res* 2008; **38**: 393-401 [PMID: 17927769 DOI: 10.1111/j.1872-034X.2007.00270.x]

5 **Gurusamy KS**, Gonzalez HD, Davidson BR. Current protective strategies in liver surgery. *World J Gastroenterol* 2010; **16**: 6098-6103 [PMID: 21182224 DOI: 10.3748/wjg.v16.i48.6098]

6 **Neves DB**, Rusi MB, Diaz LG, Salvalaggio P. Primary graft dysfunction of the liver: definitions, diagnostic criteria and risk factors. *Einstein* (Sao Paulo) 2016; **14**: 567-572 [PMID: 27783749 DOI: 10.1590/s1679-45082016rw3585]

7 **Hori T**, Uemoto S, Walden LB, Chen F, Baine AM, Hata T, Nguyen JH. Pretreatment of Small-for-Size Grafts In Vivo by γ -Aminobutyric Acid Receptor Regulation against Oxidative Stress-Induced Injury in Rat Split Orthotopic Liver Transplantation. *Int J Hepatol* 2013; **2013**: 149123 [PMID: 24223309 DOI: 10.1155/2013/149123]

8 **Verhoeven CJ**, Farid WR, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. *J Hepatol* 2014; **61**: 672-684 [PMID: 24798616 DOI: 10.1016/j.jhep.2014.04.031]

9 **Serafín A**, Roselló-Catafau J, Prats N, Gelpí E, Rodés J, Peralta C. Ischemic preconditioning affects interleukin release in fatty livers of rats undergoing ischemia/reperfusion. *Hepatology* 2004; **39**: 688-698 [PMID: 14999687 DOI: 10.1002/hep.20089]

10 **Bejaoui M**, Pantazi E, Calvo M, Folch-Puy E, Serafín A, Pasut G, Panisello A, Adam R, Roselló-Catafau J. Polyethylene Glycol Preconditioning: An Effective Strategy to Prevent Liver Ischemia Reperfusion Injury. *Oxid Med Cell Longev* 2016; **2016**: 9096549 [PMID: 26981166 DOI: 10.1155/2016/9096549]

11 **Peralta C**, Closa D, Hotter G, Gelpí E, Prats N, Roselló-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. *Biochem Biophys Res Commun* 1996; **229**: 264-270 [PMID: 8954116]

12 **Eipel C**, Glanemann M, Nuessler AK, Menger MD, Neuhaus P, Vollmar B. Ischemic preconditioning impairs liver regeneration in extended reduced-size livers. *Ann Surg* 2005; **241**: 477-484 [PMID: 15729071]

13 **Li JY**, Gu X, Yin HZ, Zhou Y, Zhang WH, Qin YM. Protective effect of ischemic preconditioning on hepatic ischemia-reperfusion injury by advancing the expressive phase of survivin in rats. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 615-620 [PMID: 19073407]

14 **Koti RS**, Seifalian AM, Davidson BR. Protection of the liver by ischemic preconditioning: a review of mechanisms and clinical applications. *Dig Surg* 2003; **20**: 383-396 [PMID: 12840597]

15 **Amador A**, Grande L, Martí J, Deulofeu R, Miquel R, Solá A, Rodriguez-Laiz G, Ferrer J, Fondevila C, Charco R, Fuster J, Hotter G, García-Valdecasas JC. Ischemic pre-conditioning in deceased donor liver transplantation: a prospective randomized clinical trial. *Am J Transplant* 2007; **7**: 2180-2189 [PMID: 17697262 DOI: 10.1111/j.1600-6143.2007.01914.x]

16 **Doeppner TR**, Doehring M, Kaltwasser B, Majid A, Lin F, Bähr M, Kilic E, Hermann DM. Ischemic Post-Conditioning Induces Post-Stroke Neuroprotection via Hsp70-Mediated Proteasome Inhibition and Facilitates Neural Progenitor Cell Transplantation. *Mol Neurobiol* 2017; **54**: 6061-6073 [PMID: 27699598 DOI: 10.1007/s12035-016-0137-3]

17 **Belzer FO**, Southard JH. Principles of solid-organ preservation by cold storage. *Transplantation* 1988; **45**: 673-676 [PMID: 3282347]

18 **Ferrigno A**, Rizzo V, Boncompagni E, Bianchi A, Gringeri E, Neri D, Richelmi P, Freitas I, Cillo U, Vairetti M. Machine perfusion at 20°C reduces preservation damage to livers from non-heart beating donors. *Cryobiology* 2011; **62**: 152-158 [PMID: 21315707 DOI: 10.1016/j.cryobiol.2011.02.004]

19 **Schlegel A**, Dutkowski P. Role of hypothermic machine perfusion in liver transplantation. *Transpl Int* 2015; **28**: 677-689 [PMID: 24852621 DOI: 10.1111/tri.12354]

20 **Hessheimer AJ**, Fondevila C, García-Valdecasas JC. Extracorporeal machine liver perfusion: are we warming up? *Curr Opin Organ Transplant* 2012; **17**: 143-147 [PMID: 22273595 DOI: 10.1097/MOT.0b013e328351082a]

21 **Sola A**, Roselló-Catafau J, Gelpí E, Hotter G. Fructose-1,6-biphosphate in rat intestinal preconditioning: involvement of nitric oxide. *Gut* 2001; **48**: 168-175 [PMID: 11156636]

22 **Padrissa-Altés S**, Franco-Gou R, Boillot O, Serafín A, Rimola A, Arroyo V, Rodés J, Peralta C, Roselló-Catafau J. Effect of angiotensin II and bradykinin inhibition in rat reduced-size liver transplantation. *Liver Transpl* 2009; **15**: 313-320 [PMID: 19242996 DOI: 10.1002/lt.21693]

23 **Zaouali MA**, Ben Abdennebi H, Padrissa-Altés S, Mahfoudh-Boussaid A, Roselló-Catafau J. Pharmacological strategies against cold ischemia reperfusion injury. *Expert Opin Pharmacother* 2010; **11**: 537-555 [PMID: 20163266 DOI: 10.1517/14656560903547836]

24 **Bejaoui M**, Pantazi E, Folch-Puy E, Baptista PM, García-Gil A, Adam R, Roselló-Catafau J. Emerging concepts in liver graft preservation. *World J Gastroenterol* 2015; **21**: 396-407 [PMID: 25593455 DOI: 10.3748/wjg.v21.i2.396]

25 **Carbonell T,** Alva N, Palomeque J. Mechanisms activated by induced hypothermia: positive and adverse effects. In: Delgado JIV, Garza VGF. Hypothermia: Prevention, Recognition and Treatment. New York: Nova Publishers; **2012**: 109-122

26 **Halliwell B**, Gutteridge JM. Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease. *Hum Toxicol* 1988; **7**: 7-13 [PMID: 3278973]

27 **Gutteridge JM**, Halliwell B. Reoxygenation injury and antioxidant protection: a tale of two paradoxes. *Arch Biochem Biophys* 1990; **283**: 223-226 [PMID: 2177320]

28 **Granger DN**, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol* 2015; **6**: 524-551 [PMID: 26484802 DOI: 10.1016/j.redox.2015.08.020]

29 **Jung T**, Catalgol B, Grune T. The proteasomal system. *Mol Aspects Med* 2009; **30**: 191-296 [PMID: 19371762 DOI: 10.1016/j.mam.2009.04.001]

30 **Ciechanover A**. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Biochim Biophys Acta* 2012; **1824**: 3-13 [PMID: 21435401 DOI: 10.1016/

j.bbapap.2011.03.007]
31 **Shang F**, Taylor A. Ubiquitin-proteasome pathway and cellular responses to oxidative stress. *Free Radic Biol Med* 2011; **51**: 5-16 [PMID: 21530648 DOI: 10.1016/j.freeradbiomed.2011.03.031]
32 **Guibert EE**, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. *Transfus Med Hemother* 2011; **38**: 125-142 [PMID: 21566713 DOI: 10.1159/000327033]
33 **Olschewski P**, Gass P, Ariyakhagorn V, Jasse K, Hunold G, Menzel M, Schöning W, Schmitz V, Neuhaus P, Puhl G. The influence of storage temperature during machine perfusion on preservation quality of marginal donor livers. *Cryobiology* 2010; **60**: 337-343 [PMID: 20233587 DOI: 10.1016/j.cryobiol.2010.03.005]
34 **Vajdová K**, Graf R, Clavien PA. ATP-supplies in the cold-preserved liver: A long-neglected factor of organ viability. *Hepatology* 2002; **36**: 1543-1552 [PMID: 12447882 DOI: 10.1053/jhep.2002.37189]
35 **Stefanovich P**, Ezzell RM, Sheehan SJ, Tompkins RG, Yarmush ML, Toner M. Effects of hypothermia on the function, membrane integrity, and cytoskeletal structure of hepatocytes. *Cryobiology* 1995; **32**: 389-403 [PMID: 7656572]
36 **Carbonell T**, Alva N, Sanchez-Nuño S, Dewey S, Gomes AV. Subnormothermic Perfusion in the Isolated Rat Liver Preserves the Antioxidant Glutathione and Enhances the Function of the Ubiquitin Proteasome System. *Oxid Med Cell Longev* 2016; **2016**: 9324692 [PMID: 27800122 DOI: 10.1155/2016/9324692]
37 **Bruinsma BG**, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, Saeidi N, Op den Dries S, Berendsen TA, Smith RN, Markmann JF, Porte RJ, Yarmush ML, Uygun K, Izamis ML. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant* 2014; **14**: 1400-1409 [PMID: 24758155 DOI: 10.1111/ajt.12727]
38 **Op den Dries S**, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, Leuvenink HG, Lisman T, Porte RJ. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. *PLoS One* 2014; **9**: e88521 [PMID: 24551114 DOI: 10.1371/journal.pone.0088521]
39 **Ferrigno A**, Di Pasqua LG, Berardo C, Siciliano V, Rizzo V, Mannucci B, Richelmi P, Croce AC, Vairetti M. Liver Graft Susceptibility during Static Cold Storage and Dynamic Machine Perfusion: DCD versus Fatty Livers. *Int J Mol Sci* 2017; **19**: [PMID: 29301219 DOI: 10.3390/ijms19010109]
40 **Vairetti M**, Ferrigno A, Carlucci F, Tabucchi A, Rizzo V, Boncompagni E, Neri D, Gringeri E, Freitas I, Cillo U. Subnormothermic machine perfusion protects steatotic livers against preservation injury: a potential for donor pool increase? *Liver Transpl* 2009; **15**: 20-29 [PMID: 19109848 DOI: 10.1002/lt.21581]
41 **Brockmann J**, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, Morovat A, Roy D, Winter L, Friend PJ. Normothermic perfusion: a new paradigm for organ preservation. *Ann Surg* 2009; **250**: 1-6 [PMID: 19561463 DOI: 10.1097/SLA.0b013e3181a63c10]
42 **Ravikumar R**, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? *Transpl Int* 2015; **28**: 690-699 [PMID: 25847684 DOI: 10.1111/tri.12576]
43 **Hoyer DP**, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. *Liver Transpl* 2016; **22**: 1223-1230 [PMID: 27398813 DOI: 10.1002/lt.24515]
44 **Vairetti M**, Ferrigno A, Rizzo V, Richelmi P, Boncompagni E, Neri D, Freitas I, Cillo U. Subnormothermic machine perfusion protects against rat liver preservation injury: a comparative evaluation with conventional cold storage. *Transplant Proc* 2007; **39**: 1765-1767 [PMID: 17692606 DOI: 10.1016/j.transproceed.2007.06.002]
45 **op den Dries S**, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, Wiersema-Buist J, Lisman T, Leuvenink HG, Porte RJ. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant* 2013; **13**: 1327-1335 [PMID: 23463950 DOI: 10.1111/ajt.12187]
46 **Duret C**, Moreno D, Balasiddaiah A, Roux S, Briolotti P, Raulet E, Herrero A, Ramet H, Biron-Andreani C, Gerbal-Chaloin S, Ramos J, Navarro F, Hardwigsen J, Maurel P, Aldabe R, Daujat-Chavanieu M. Cold Preservation of Human Adult Hepatocytes for Liver Cell Therapy. *Cell Transplant* 2015; **24**: 2541-2555 [PMID: 25622096 DOI: 10.3727/096368915X687020]
47 **Ben Abdennebi H**, Steghens JP, Margonari J, Ramella-Virieux S, Barbieux A, Boillot O. High-Na+ low-K+ UW cold storage solution reduces reperfusion injuries of the rat liver graft. *Transpl Int* 1998; **11**: 223-230 [PMID: 9638853]
48 **Boudjema K**, Grandadam S, Compagnon P, Salamé E, Wolf P, Ducerf C, Le Treut P, Soubrane O, Cherqui D, Mouchel C, Renault A, Bellissant E. Efficacy and safety of Celsior preservation fluid in liver transplantation: one-year follow up of a prospective, multicenter, non-randomized study. *Clin Transplant* 2012; **26**: 199-207 [PMID: 21517997 DOI: 10.1111/j.1399-0012.2011.01447.x]
49 **Stewart ZA**, Cameron AM, Singer AL, Montgomery RA, Segev DL. Histidine-Tryptophan-Ketoglutarate (HTK) is associated with reduced graft survival in deceased donor livers, especially those donated after cardiac death. *Am J Transplant* 2009; **9**: 286-293 [PMID: 19067658 DOI: 10.1111/j.1600-6143.2008.02478.x]
50 **Zaouali MA**, Ben Abdennebi H, Padrissa-Altés S, Alfany-Fernandez I, Rimola A, Roselló-Catafau J. How Institut Georges Lopez preservation solution protects nonsteatotic and steatotic livers against ischemia-reperfusion injury. *Transplant Proc* 2011; **43**: 77-79 [PMID: 21335159 DOI: 10.1016/j.transproceed.2010.12.026]
51 **Halliwell B,** Gutteridge JMC. Free radicals in biology and medicin. Oxford: Oxford University Press; 1999. ISBN: 0198500459
52 **Bessems M**, 't Hart NA, Tolba R, Doorschodt BM, Leuvenink HG, Ploeg RJ, Minor T, van Gulik TM. The isolated perfused rat liver: standardization of a time-honoured model. *Lab Anim* 2006; **40**: 236-246 [PMID: 16803641 DOI: 10.1258/002367706777611460]
53 **Kron P**, Schlegel A, Mancina L, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion (HOPE) for fatty liver grafts in rats and humans. *J Hepatol* 2017; Epub ahead of print [PMID: 28870676 DOI: 10.1016/j.jhep.2017.08.028]
54 **Polat KY**, Aydinli B, Polat O, Aydin U, Yazici P, Ozturk G, Gundogdu C, Kiziltunc A. The protective effect of aprotinin and alpha-tocopherol on ischemia-reperfusion injury of the rat liver. *Transplant Proc* 2008; **40**: 63-68 [PMID: 18261548 DOI: 10.1016/j.transproceed.2007.11.047]
55 **Gulec B**, Coskun K, Yigitler C, Yigit T, Aydin A, Oner K. Ischemia-reperfusion injury in the liver during renal transplantation: does perfusion solution play any role? *Transplant Proc* 2008; **40**: 59-62 [PMID: 18261547 DOI: 10.1016/j.transproceed.2007.11.023]
56 **Qi Q**, Bie P. Different roles of hepatic hypothermic ischemia and ischemic preconditioning in chemically induced hepatocarcinogenesis in rats. *J Surg Res* 2014; **189**: 213-221 [PMID: 24725680 DOI: 10.1016/j.jss.2014.03.008]
57 **Thompson-Gorman SL**, Zweier JL. Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. *J Biol Chem* 1990; **265**: 6656-6663 [PMID: 2157706]
58 **Peralta C**, Closa D, Xaus C, Gelpí E, Roselló-Catafau J, Hotter G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. *Hepatology* 1998; **28**: 768-773 [PMID: 9731571 DOI: 10.1002/hep.510280325]
59 **Martí R**, Múrio E, Varela E, Bilbao I, Pascual C, Margarit C, Segura RM. Xanthine oxidoreductase and preservation injury in human liver transplantation. *Transplantation* 2004; **77**: 1239-1245 [PMID: 15114092 DOI: 10.1097/01.TP.0000120384.52033.BC]
60 **Jaeschke H**. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G15-G26 [PMID: 12488232 DOI: 10.1152/ajpgi.00342.2002]
61 **Lee WY**, Koh EJ, Lee SM. A combination of ischemic preconditioning and allopurinol protects against ischemic injury through a nitric oxide-dependent mechanism. *Nitric Oxide* 2012; **26**: 1-8 [PMID: 22119149 DOI: 10.1016/j.niox.2011.11.002]
62 **Marden JJ**, Zhang Y, Oakley FD, Zhou W, Luo M, Jia HP, McCray PB Jr, Yaniv M, Weitzman JB, Engelhardt JF. JunD

protects the liver from ischemia/reperfusion injury by dampening AP-1 transcriptional activation. *J Biol Chem* 2008; **283**: 6687-6695 [PMID: 18182393 DOI: 10.1074/jbc.M705606200]
63 **Shi S**, Xue F. Current Antioxidant Treatments in Organ Transplantation. *Oxid Med Cell Longev* 2016; **2016**: 8678510 [PMID: 27403232 DOI: 10.1155/2016/8678510]
64 **Junnarkar SP**, Tapuria N, Dutt N, Fuller B, Seifalian AM, Davidson BR. Bucillamine improves hepatic microcirculation and reduces hepatocellular injury after liver warm ischaemia-reperfusion injury. *HPB* (Oxford) 2009; **11**: 264-273 [PMID: 19590658 DOI: 10.1111/J.1477-2574.2009.00054.X]
65 **de Oca J**, Cuadrado S, Vallet J, Benasco C, Martín E, Ardanuy C, Closa D, Hotter G, Jaurrieta E. Protective effects of lazaroid U74389G on intestinal graft after heterotopic small bowel transplantation in rats. *J Surg Res* 1998; **75**: 18-23 [PMID: 9614851 DOI: 10.1006/jsre.1997.5244]
66 **Yilmaz HR**, Uz E, Yucel N, Altuntas I, Ozcelik N. Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver. *J Biochem Mol Toxicol* 2004; **18**: 234-238 [PMID: 15452882 DOI: 10.1002/jbt.20028]
67 **Demasi M**, Netto LE, Silva GM, Hand A, de Oliveira CL, Bicev RN, Gozzo F, Barros MH, Leme JM, Ohara E. Redox regulation of the proteasome via S-glutathionylation. *Redox Biol* 2013; **2**: 44-51 [PMID: 24396728 DOI: 10.1016/J.REDOX.2013.12.003]
68 **Zhou H**, Zhu J, Yue S, Lu L, Busuttil RW, Kupiec-Weglinski JW, Wang X, Zhai Y. The Dichotomy of Endoplasmic Reticulum Stress Response in Liver Ischemia-Reperfusion Injury. *Transplantation* 2016; **100**: 365-372 [PMID: 26683513 DOI: 10.1097/TP.0000000000001032]
69 **Baker TA**, Geng Q, Romero J, Picken MM, Gamelli RL, Majetschak M. Prolongation of myocardial viability by proteasome inhibition during hypothermic organ preservation. *Biochem Biophys Res Commun* 2010; **401**: 548-553 [PMID: 20875792]
70 **Geng Q**, Romero J, Saini V, Baker TA, Picken MM, Gamelli RL, Majetschak M. A subset of 26S proteasomes is activated at critically low ATP concentrations and contributes to myocardial injury during cold ischemia. *Biochem Biophys Res Commun* 2009; **390**: 1136-1141 [PMID: 19944202 DOI: 10.1016/j.bbrc.2009.10.067]
71 **Zaouali MA**, Bardag-Gorce F, Carbonell T, Oliva J, Pantazi E, Bejaoui M, Ben Abdennebi H, Rimola A, Roselló-Catafau J. Proteasome inhibitors protect the steatotic and non-steatotic liver graft against cold ischemia reperfusion injury. *Exp Mol Pathol* 2013; **94**: 352-359 [PMID: 23305864]
72 **Bejaoui M**, Zaouali MA, Folch-Puy E, Pantazi E, Bardag-Gorce F, Carbonell T, Oliva J, Rimola A, Abdennebi HB, Roselló-Catafau J. Bortezomib enhances fatty liver preservation in Institut George Lopez-1 solution through adenosine monophosphate activated protein kinase and Akt/mTOR pathways. *J Pharm Pharmacol* 2014; **66**: 62-72 [PMID: 24127984 DOI: 10.1111/jphp.12154]
73 **Panisello-Roselló A**, Verde E, Amine Zaouali M, Flores M, Alva N, Lopez A, Folch-Puy E, Carbonell T, Hotter G, Adam R, Roselló-Catafau J. The Relevance of the UPS in Fatty Liver Graft Preservation: A New Approach for IGL-1 and HTK Solutions. *Int J Mol Sci* 2017; **18**: [PMID: 29088097]
74 **Goldstein G**, Scheid M, Hammerling U, Schlesinger DH, Niall HD, Boyse EA. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci USA* 1975; **72**: 11-15 [PMID: 1078892 DOI: 10.1073/pnas.72.1.11]
75 **Ciechanover A**, Elias S, Heller H, Hershko A. "Covalent affinity" purification of ubiquitin-activating enzyme. *J Biol Chem* 1982; **257**: 2537-2542 [PMID: 6277904]
76 **Hershko A**, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998; **67**: 425-479 [PMID: 9759494 DOI: 10.1007/978-1-4899-1922-9_1]
77 **Ciechanover A**. Intracellular protein degradation: from a vague idea through the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Bioorg Med Chem* 2013; **21**: 3400-3410 [PMID: 23485445 DOI: 10.1016/j.bmc.2013.01.056]
78 **Hershko A**, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* 1983; **258**: 8206-8214 [PMID: 6305978]
79 **Hameed DS**, Sapmaz A, Ovaa H. How Chemical Synthesis of Ubiquitin Conjugates Helps To Understand Ubiquitin Signal Transduction. *Bioconjug Chem* 2017; **28**: 805-815 [PMID: 27077728]
80 **Kim W**, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, Harper JW, Gygi SP. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 2011; **44**: 325-340 [PMID: 21906983 DOI: 10.1016/j.molcel.2011.08.025]
81 **Xu P**, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J, Rush J, Hochstrasser M, Finley D, Peng J. Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 2009; **137**: 133-145 [PMID: 19345192]
82 **Chen ZJ**, Sun LJ. Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* 2009; **33**: 275-286 [PMID: 19217402 DOI: 10.1016/j.molcel.2009.01.014]
83 **Jung T**, Grune T. The proteasome and the degradation of oxidized proteins: Part I-structure of proteasomes. *Redox Biol* 2013; **1**: 178-182 [PMID: 24024151 DOI: 10.1016/j.redox.2013.01.004]
84 **Calmus Y**, Cynober L, Dousset B, Lim SK, Soubrane O, Conti F, Houssin D, Giboudeau J. Evidence for the detrimental role of proteolysis during liver preservation in humans. *Gastroenterology* 1995; **108**: 1510-1516 [PMID: 7729644 DOI: 10.1016/0016-5085(95)90701-7]
85 **Adam R**, Delvart V, Karam V, Ducerf C, Navarro F, Letoublon C, Belghiti J, Pezet D, Castaing D, Le Treut YP, Gugenheim J, Bachellier P, Pirenne J, Muiesan P; ELTR contributing centres, the European Liver, Intestine Transplant Association (ELITA). Compared efficacy of preservation solutions in liver transplantation: a long-term graft outcome study from the European Liver Transplant Registry. *Am J Transplant* 2015; **15**: 395-406 [PMID: 25612492 DOI: 10.1111/ajt.13060]
86 **Zaouali MA**, Panisello-Roselló A, Lopez A, Castro Benítez C, Folch-Puy E, García-Gil A, Carbonell T, Adam R, Roselló-Catafau J. Relevance of proteolysis and proteasome activation in fatty liver graft preservation: An Institut Georges Lopez-1 vs University of Wisconsin appraisal. *World J Gastroenterol* 2017; **23**: 4211-4221 [PMID: 28694661 DOI: 10.3748/wjg.v23.i23.4211]
87 **Majetschak M**. Regulation of the proteasome by ATP: implications for ischemic myocardial injury and donor heart preservation. *Am J Physiol Heart Circ Physiol* 2013; **305**: H267-H278 [PMID: 23709597 DOI: 10.1152/ajpheart.00206.2012]
88 **Zhang F**, Hu Y, Huang P, Toleman CA, Paterson AJ, Kudlow JE. Proteasome function is regulated by cyclic AMP-dependent protein kinase through phosphorylation of Rpt6. *J Biol Chem* 2007; **282**: 22460-22471 [PMID: 17565987]
89 **Djakovic SN**, Marquez-Lona EM, Jakawich SK, Wright R, Chu C, Sutton MA, Patrick GN. Phosphorylation of Rpt6 regulates synaptic strength in hippocampal neurons. *J Neurosci* 2012; **32**: 5126-5131 [PMID: 22496558]
90 **Jarome TJ**, Kwapis JL, Ruenzel WL, Helmstetter FJ. CaMKII, but not protein kinase A, regulates Rpt6 phosphorylation and proteasome activity during the formation of long-term memories. *Front Behav Neurosci* 2013; **7**: 115 [PMID: 24009566]
91 **Grune T**, Reinheckel T, Davies KJ. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 1996; **271**: 15504-15509 [PMID: 8663134]
92 **Höhn TJ**, Grune T. The proteasome and the degradation of oxidized proteins: part III-Redox regulation of the proteasomal system. *Redox Biol* 2014; **2**: 388-394 [PMID: 24563857 DOI: 10.1016/j.redox.2013.12.029]
93 **Davies KJ**. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* 2001; **83**: 301-310 [PMID: 11295490]
94 **Shang F**, Nowell TR Jr, Taylor A. Removal of oxidatively damaged proteins from lens cells by the ubiquitin-proteasome

pathway. *Exp Eye Res* 2001; **73**: 229-238 [PMID: 11446773 DOI: 10.1006/exer.2001.1029]

95 **Gomes AV**, Zong C, Ping P. Protein degradation by the 26S proteasome system in the normal and stressed myocardium. *Antioxid Redox Signal* 2006; **8**: 1677-1691 [PMID: 16987021 DOI: 10.1089/ars.2006.8.1677]

96 **Jahngen-Hodge J**, Obin MS, Gong X, Shang F, Nowell TR Jr, Gong J, Abasi H, Blumberg J, Taylor A. Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *J Biol Chem* 1997; **272**: 28218-28226 [PMID: 9353272 DOI: 10.1074/jbc.272.45.28218]

97 **Bonet-Costa V**, Pomatto LC, Davies KJ. The Proteasome and Oxidative Stress in Alzheimer's Disease. *Antioxid Redox Signal* 2016; **25**: 886-901 [PMID: 27392670 DOI: 10.1089/ars.2016.6802]

98 **Majetschak M**, Patel MB, Sorell LT, Liotta C, Li S, Pham SM. Cardiac proteasome dysfunction during cold ischemic storage and reperfusion in a murine heart transplantation model. *Biochem Biophys Res Commun* 2008; **365**: 882-888 [PMID: 18053796]

99 **Campbell B**, Adams J, Shin YK, Lefer AM. Cardioprotective effects of a novel proteasome inhibitor following ischemia and reperfusion in the isolated perfused rat heart. *J Mol Cell Cardiol* 1999; **31**: 467-476 [PMID: 10093058]

P- Reviewer: Chedid MF, Hori T, Rubbini M, Smyrniotis V, Tao R
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 August 21; 24(31): 3531-3537

DOI: 10.3748/wjg.v24.i31.3531

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

ORIGINAL ARTICLE

Basic Study

Stomach wall structure and vessels imaging by acoustic resolution photoacoustic microscopy

Cheng Wang, Yu-Fei Lu, Chun-Miao Cai, Hua-Zhong Xiang, Gang Zheng

Cheng Wang, Yu-Fei Lu, Hua-Zhong Xiang, Gang Zheng, Institute of Biomedical Optics and Optometry, School of Medical Instrument and Food Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China

Chun-Miao Cai, Department of Pathology, Shanghai Tenth People's Hospital Affiliated to Tongji University, Shanghai 200093, China

ORCID number: Cheng Wang (0000-0002-6293-1607); Yu-Fei Lu (0000-0002-1446-0070); Chun-Miao Cai (0000-0003-3692-5120); Hua-Zhong Xiang (0000-0002-2544-4850); Gang Zheng (0000-0002-6818-0509).

Author contributions: Wang C designed and performed the research; Wang C and Lu YF wrote the paper; Cai CM harvested experimental samples and provided the pathology images; Wang C, Lu YF, Xiang HZ, and Gang Z performed the photoacoustic imaging experiment and processed the experiment data.

Supported by the National Nature Science Foundation of China, No. 61378060.

Institutional review board statement: This study was approved by the Laboratory Animal Ethics Committee of Shanghai Tenth People's Hospital. The design is reasonable and in line with the animal requirement. The ethics committee agreed to carry out a formal experiment.

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: Cheng Wang, PhD, Associate Professor, Institute of Biomedical Optics and Optometry, School of Medical Instrument and Food Engineering, University of Shanghai for Science and Technology, No. 516 Jungong Road, Shanghai 200093, China. c.wang@usst.edu.cn
Telephone: +86-21-55271165
Fax: +86-21-55271200

Received: April 12, 2018
Peer-review started: April 13, 2018
First decision: May 17, 2018
Revised: May 26, 2018
Accepted: June 27, 2018
Article in press: June 27, 2018
Published online: August 21, 2018

Abstract

AIM

To image stomach wall blood vessels and tissue, layer-by-layer.

METHODS

We built up the acoustic resolution photoacoustic microscopy (AR-PAM) system for imaging layered tissues, such as the stomach wall. A tunable dye laser system was coupled to a fiber bundle. The fibers of the bundle were placed in nine directions with an incident angle of 45° around a high-frequency ultrasound transducer attached to the acoustic lens. This structure formed a dark field on the tissue surface under the acoustic lens and the nine light beams from the fibers to be combined near the focal point of the acoustic lens. The sample piece was cut from a part of the porcine stomach into a petri dish. In order to realize photoacoustic depth imaging of tumor, we designed a tumor model based on indocyanine green (ICG) dye. The ICG solution (concentration of 129 μM/mL)

was mixed into molten gel, and then a gel mixture of ICG (concentration of 12.9 μM/mL) was injected into the stomach submucosa. The injection quantity was controlled by 0.1 mL to make a small tumor model.

RESULTS

An acoustic resolution photoacoustic microscopy based on fiber illumination was established and an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm has been accomplished. We tuned the laser wavelength to 600 nm. The photoacoustic probe was driven to do B-scan imaging in tissue thickness of 200 μm. The photoacoustic micro-image of mucosa and submucosa of the tissue have been obtained and compared with a pathological photograph of the tissue stained by hematoxylin-eosin staining. We have observed more detailed internal structure of the tissue. We also utilized this photoacoustic microscopy to image blood vessels inside the submucosa. High contrast imaging of the submucosa tumor model was obtained using ICG dye.

CONCLUSION

This AR-PAM is able to image layer-by-layer construction and some blood vessels under mucosa in the stomach wall without any contrast agents.

Key words: Photoacoustic imaging; Stomach; Layered tissue; Acoustic resolution; Fiber

Core tip: In order to image layered tissue and blood vessels, acoustic resolution photoacoustic microscopy based on fiber illumination was established and an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm was accomplished. Layer-by-layer imaging of the stomach tissue and stomach mucosa blood vessels were obtained. High contrast imaging of the submucosa tumor model was obtained using ICG dye.

Wang C, Lu YF, Cai CM, Xiang HZ, Zheng G. Stomach wall structure and vessels imaging by acoustic resolution photoacoustic microscopy. *World J Gastroenterol* 2018; 24(31): 3531-3537 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3531.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3531

INTRODUCTION

Stomach submucosal tumors (SMT) are any intramural growth underneath the mucosa of the gastrointestinal tract[1]. These tumors are very hard to find because they are usually asymptomatic and therefore most often discovered as accidental findings during surgery, autopsy, and so on. When examining submucosal tumors, standard optical stomach endoscopy, capsule optical endoscopy and push-and-pull enteroscopy together with barium contrast X-ray do not alone provide sufficient information. Endoscopic ultrasound (EUS), computed tomography (CT) and magnetic resonance imaging (MRI) are recommended as supplementary tools[2] because CT and MRI often lack either sufficient spatial resolution or satisfactory contrast (or both) to be effective for early-stage tumor imaging[3]. Optimal EUS imaging of an SMT needs submersion of the tumor under water. However, benign SMTs, for example the submucosal inflammation, cannot be distinguished endosonographically[4]. Therefore, early-phase tumor detection or in situ characterization of diseased tissue is challenging for EUS. Because the mechanism of EUS imaging is ultrasound imaging, this is based on tissue bulk mechanical properties. Tumor tissue boundaries and blood vessel structures are clinically relevant and provide necessary information for assessing disease stage and planning treatment therapies.

Recently, optical endoscopic imaging modalities, like narrow band imaging endoscopy[5], endoscopic optical coherent tomography[6] and confocal laser endomicroscopy[7] have been developed. They can detect tissue or tissue changes with high sensitivity and high spatial resolution. Because of the strong optical scattering properties of tissue, these techniques are unable to image targets beyond a 1-2 mm depth. Photoacoustic imaging has a lot of advantages, such as, endogenous optical chromophore contrast enables label-free imaging of the microvasculature with a high resolution[8], and *in vivo* blood vessel photoacoustic imaging can cover the length scale from a superficial capillary[9] to an abdominal aorta[10], demonstrating the potential of photoacoustic imaging to bridge the resolution and penetration gaps between microvascular microscopy, clinical angiography and so on[11,12]. This paper's aim is to demonstrate that photoacoustic imaging is able to image the layered tissue and blood vessels under the surface of the tissue. In the future, we can design and develop an photoacoustic endoscopic imaging system to image deeper tissue with higher resolution for diagnosis and treatment.

MATERIALS AND METHODS

AR-PAM system

The acoustic resolution photoacoustic microscopy (AR-PAM) system for stomach wall imaging was shown in Figure 1A. A tunable dye laser system pumped by a Q-switch Nd:YAG laser (ND6000, continuum) was used to provide laser pulse with a pulse repetition of 10 Hz and a pulse width of 5.5 ns. The tunable range of the laser light was 415 nm to 940 nm. A pair of concave and convex lenses expanded and collimated the light beam to approximately 5 mm in diameter. Then coupling to a fiber bundle that was composed by nine optical fibers (FT400UMT, Thorlabs) which have a 400 μm core

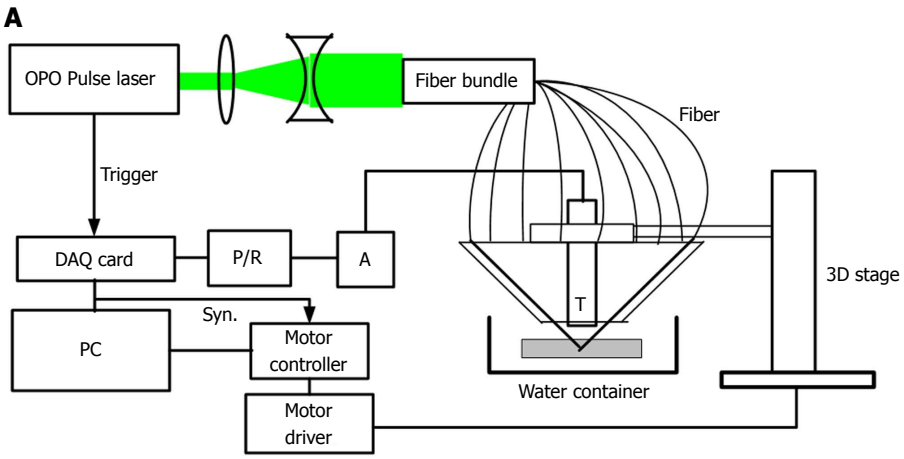


Figure 1 Schematic of the acoustic resolution photoacoustic microscopy system (A) and photograph of the photoacoustic probe (B). P/R: Pulser/receiver; A: Preamplifier; T: Transducer, PC: Personal computer.

diameter and numerical aperture of 0.39 were placed in nine directions (40° intervals in 360°) around a high-frequency (50-MHz, bandwidth of 30 MHz) ultrasound transducer attached with the acoustic lens (the f/# is 1.3 and focal length is 4 mm). The cross angle between the fibers with the transducer axis direction were set to 45°, so that it formed a dark field on the tissue surface under the acoustic lens and the nine light beams from the fibers were combined near the focal point of the acoustic lens, *i.e*. it is then weakly focused into the tissue with the focal region coaxially overlapping the ultrasonic focus inside the tissue. In an optically transparent medium, the optical focus is about 2 mm in diameter, which is much wider than the ultrasonic focus. The focal acoustic transducer and output end of the fibers were fastened with a holder made by a 3D printer. We can refer to it as a photoacoustic probe (Figure 1B). According to the parameters of the ultrasonic transducer, an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm can be accomplished. The photoacoustic probe is translated in a water bath. A window at the bottom of the water container is sealed with an optically and ultrasonically transparent disposable polyethylene membrane (thickness: 0.04 mm). After commercial ultrasound gel is applied to the region of interest on the sample for acoustic coupling, the sample is placed between the sample supporter and the water container for imaging. The photoacoustic wave is recorded at each location of the ultrasonic transducer and subsequently converted into a one-dimensional (1D) depth-resolved image (A-scan) based on the sound velocity in soft tissue (1.54 mm/us). Images were generated by one dimension (B-Scan) in the X or Y direction or two-dimensional (C-Scan) raster scanning of the photoacoustic probe in the X-Y plane with a step size of 30 μm. In addition to, we can also scan in the Z direction with a step of 200 μm for producing an image of the deeper tissue by utilizing deeply penetrable diffused light to excite photoacoustic signals. At each scanning position, signals were recorded by an 8 bits digitizer card (DP1400, Agilent Tech, United States) after it was amplified by a preamplifier (AU-2A-0150-BNC, MTEQ, United States) and then amplified and filtered (high pass 1 MHz) by a pulser/receiver (5073PR, Olympus, Japan). No signal averaging is performed. As shown in Figure 2, photoacoustic imaging of a hair with a diameter of 80 μm were obtained with this photoacoustic system for verifying the imaging ability. Figure 2A was a B-scan image and Figure 2B was a C-scan image of the hair.

Preparing a porcine stomach wall sample

A porcine stomach was received the day after the animal was sacrificed for an unrelated study. The sample piece was cut from a part of the porcine stomach into a petri

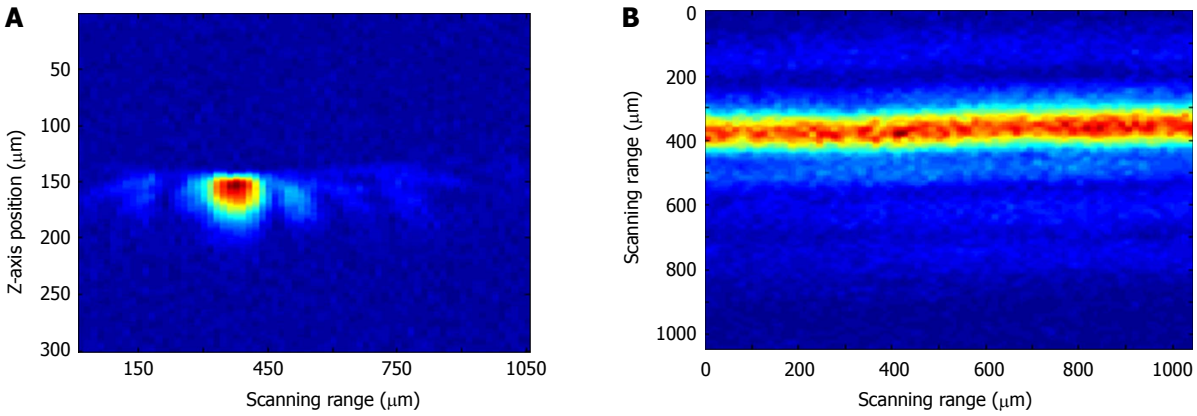


Figure 2 Photoacoustic image of a hair with a diameter of 80 μm. A: B-scan imaging. B: C-scan imaging.

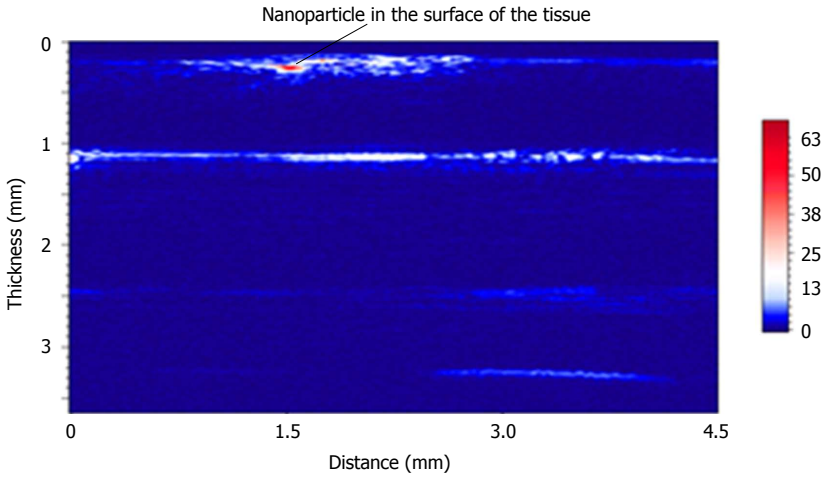


Figure 3 Imaging of layer-by-layer structure of stomach wall.

dish with a diameter of 140 mm and a thickness of 22 mm. In order to obtain a photoacoustic depth image of the tumor, we designed a tumor model based on indocyanine green (ICG) dye, wherein the ICG solution at a concentration of 129 μM/mL was mixed into molten gel, and then the gel mixture of ICG at a concentration of 12.9 μM/mL was injected into the stomach submucosa at a 2-3 mm position. The injection quantity was controlled to 0.1 mL to make a small tumor model.

RESULTS

In order to verify photoacoustic imaging can also be used to implement stratified imaging of the stomach wall, we tuned the laser wavelength to 600 nm. The photoacoustic probe obtained many B-scan images at tissue thickness direction with a lateral scanning step of 200 μm. Then, all of the B-scan images were combined to complete an image of the layered structure of the stomach wall (Figure 3). A particle with the diameter of 300 μm was placed on the surface of stomach tissue, in order to indicate the surface position of the tissue. As shown in Figure 4, the photoacoustic microimage of the mucosa and submucosa of the tissue have been obtained and compared with a pathological photograph of the tissue stained by hematoxylin-eosin staining. We have observed more detailed internal structure of the tissue by the photoacoustic imaging.

In addition, we also utilized this photoacoustic microscopy to image blood vessels inside the submucosa. The dissected stomach wall after imaging by photoacoustic microscopy is shown in Figure 5A. The vessels under submucosa after imaging by photoacoustic microscopy is shown in Figure 5B. We very clearly saw the artery vessels and vein vessels.

In order to realize the photoacoustic depth imaging of the tumor, we designed a labeled tumor model based on ICG dye, wherein 0.1 mL ICG gelatin solution with a concentration of 129 μM/mL was injected into the stomach submucosa at 2-3 mm deep. After the ICG-gel mixture was injected into the tissue and coagulated for 20 minutes, the C-scan imaging at different depths was completed. When doing the scanning, the output wavelength of the laser was tuned to 700 nm because is the absorption peak of the ICG dye with molar extinction coefficient of 1.1×10^5 cm^{-1}/M in water. This will improve the signal: noise ratio of the image.

Some diffusion phenomena of the ICG resolution

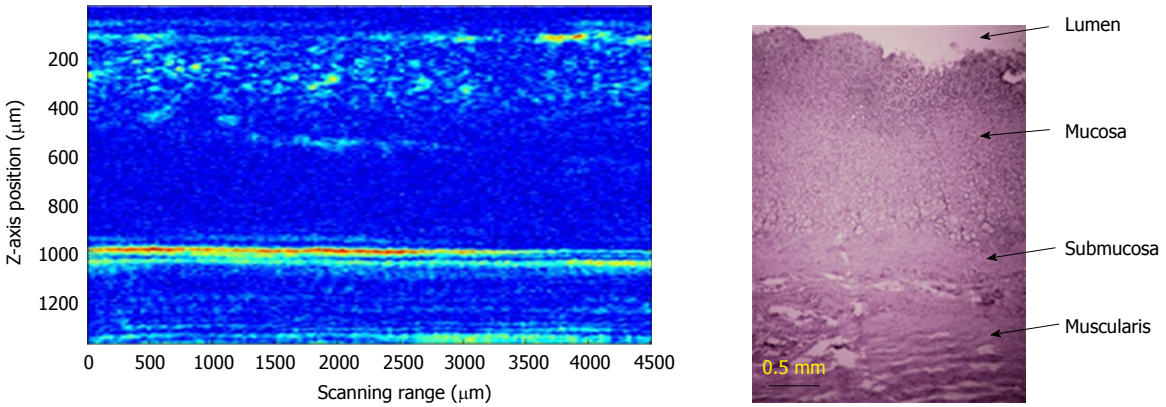


Figure 4 Compared photoacoustic image with pathological photograph stained by hematoxylin-eosin staining for mucosa and submucosa of the tissue.

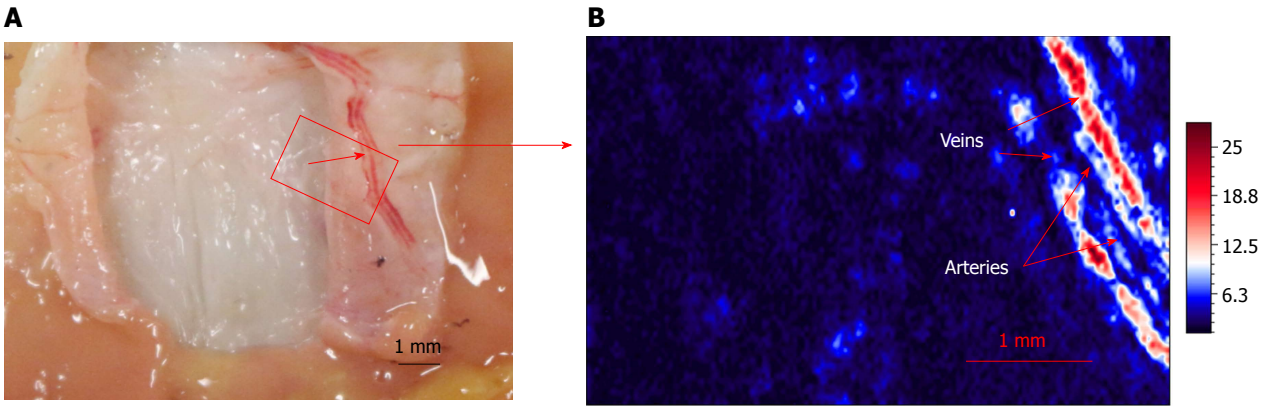


Figure 5 Photograph of the dissected stomach wall after imaging by photoacoustic microscopy (A) and photoacoustic imaging under surface of the tissue of 500 μm (red arrow indicate vessels) (B).

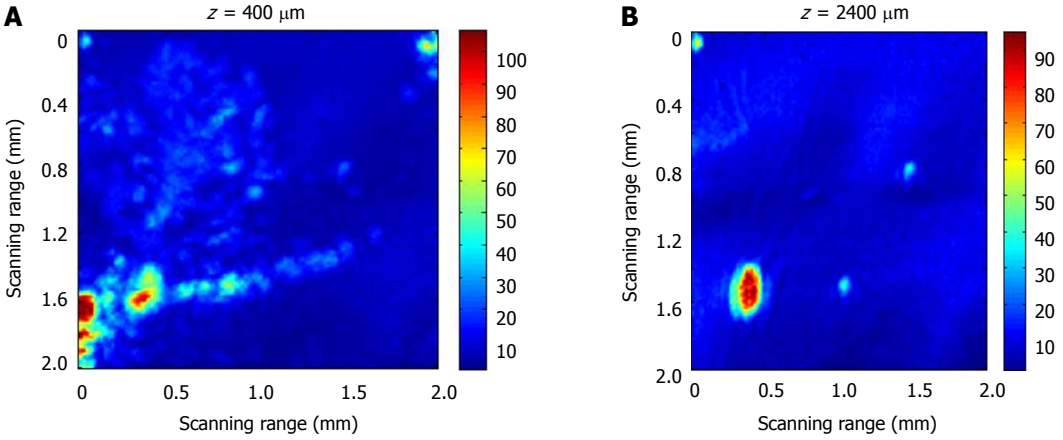


Figure 6 Photoacoustic imaging with laser wavelength of 700 nm for contrast agent with concentration of 12.9 μM/mL in depth of 400 μm (A) and 2400 μm (B).

at the mucosal site of 400 μm was observed (Figure 6A). The ICG dye may have combined with blood in the vessels, so some tubular structures were observed. At a depth of 2400 μm, the ICG labeled tumor model was still visible (Figure 6B). This indicated that we can achieve deeper detection.

DISCUSSION

Ultrasound uses high-frequency sound waves to produce images of the organs and structures inside the body such as ovaries, uterus, liver, gallbladder, pancreas, or aorta. EUS combines endoscopy and ultrasound in order to obtain images and information about the digestive tract and the surrounding tissue and organs. EUS can obtain information about the layers of the intestinal wall as well as adjacent areas such as lymph nodes and the blood vessels. EUS provides doctors with more information than other imaging tests by providing detailed images of the digestive tract. Although contrast-enhanced

ultrasonic techniques[13,14] such as Doppler ultrasound[15] are able to image blood vessels, the resolution of these imaging techniques are much lower than that of PAM, which has recently achieved lateral resolution up to 15 mm[16]. Additionally, photoacoustic imaging provides functional information with endogenous contrast and with the aid of an exogenous contrast agent. Therefore, photoacoustic endoscopy based on photoacoustic tomography and EUS, which has achieved spatially coincident photoacoustic and ultrasonic imaging, provides unprecedented information and promotes morphologic and functional understanding of the gastrointestinal tract imaging[17]. But the research on tissue structure based on photoacoustic imaging technique has not been published yet. This paper reported that utilizing AR-PAM to image layered tissue in the stomach wall and blood vessels under the mucosa in s without any contrasting agents. In the next step, this acoustic resolution photoacoustic microscopy will combine with endoscopy and minimize the photoacoustic probe to make a micro-photoacoustic endoscopy for obtaining morphologic and functional information with higher resolution and depth *in vivo*.

In conclusion, we have presented a fiber illumination based acoustic resolution photoacoustic microscopy method. We have realized an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm. This system was utilized to image layered stomach wall tissue and blood vessels under the mucosa without any contrasting agents. Using ICG dye enhance a tumor model, the tumor model was imaged to depths of 2400 μm with very high contrast. The photoacoustic microscopy has the ability to image layered tissue with high resolution and high contrast.

ARTICLE HIGHLIGHTS

Research background

When checking submucosal tumors, traditional methods (such as standard optical stomach endoscopy, capsule optical endoscopy and push-and-pull enteroscopy together with barium contrast X-ray) can't provide accurate information. Computed tomography and magnetic resonance imaging are often lower in resolution and contrast. Optimal endoscopic ultrasound (EUS) imaging of stomach submucosal tumors (SMT) needs submersion of the tumor under water. However, benign SMTs, for example the submucosal inflammation, cannot be distinguished endosonographically. Therefore, it is still a challenge for EUS to detect early-phase tumor *in situ*. Our team aimed to demonstrate that photoacoustic imaging is able to image layered tissue and blood vessels under the surface of the tissue. In the future, we can design and develop a photoacoustic endoscopic imaging system to image deeper tissues with higher resolution for diagnosis and treatment.

Research motivation

To image layered tissue and blood vessels in layered tissue.

Research objectives

Our aim is to demonstrate that photoacoustic imaging can detect the structure of layered tissue and blood vessels beneath the surface of the tissue.

Research methods

Our team established the acoustic resolution photoacoustic microscopy (AR-PAM) system for stomach wall structure imaging. Photoacoustic microimaging of the stomach wall structure was compared to an HE pathology image to verify the structure, vessels, and vessel direction.

Research results

As a result, we have established a fiber illumination based acoustic resolution photoacoustic microscopy method. The imaging ability has an axial resolution of 25 μm and a lateral resolution of 50 μm in its focal zone range of 500 μm. We have observed more detailed internal structure of the tissue from AR-PAM imaging. We also utilized this photoacoustic microscopy to image blood vessels inside the submucosa. By using ICG dye enhance a tumor model in the submucosa, the images obtained at a depth of 2400 μm depth had very high contrast.

Research conclusions

In this study, we have established a fiber illumination based acoustic resolution photoacoustic microscopy. Layer-by-layer imaging of the stomach tissue and blood vessels under stomach mucosa were obtained. By using ICG dye to enhance the tumor model, high contrast images at a depth of 2400 μm tissue were obtained. This proved that photoacoustic microscopy has the ability to image layered tissue and deep tissue targets with high resolution and high contrast. In the near future, this technique combined with endoscopy will supply a simple tool for physicians to see differences in layered tissues and imaging of tumor angiogenesis in submucosa.

Research perspectives

This technique combined with endoscopy will supply a simple tool to visualize layered tissues and tumor angiogenesis in submucosa. Scientific research should aim at solving practical problems in clinical practice. Innovative scientific research is demonstrated in the ability to effectively solve the difficult problems doctors encounter in clinical practice.

REFERENCES

1 **Chak A**. EUS in submucosal tumors. *Gastrointest Endosc* 2002; **56**: S43-S48 [PMID: 12297748 DOI: 10.1016/S0016-5107(02)70085-0]
2 **Hsu CC**, Chen JJ, Changchien CS. Endoscopic features of metastatic tumors in the upper gastrointestinal tract. *Endoscopy* 1996; **28**: 249-253 [PMID: 8739742 DOI: 10.1055/s-2007-1005437]
3 **Hu S**, Wang LV. Photoacoustic imaging and characterization of the microvasculature. *J Biomed Opt* 2010; **15**: 011101 [PMID: 20210427 DOI: 10.1117/1.3281673]
4 **Shim CS**, Jung IS. Endoscopic removal of submucosal tumors: preprocedure diagnosis, technical options, and results. *Endoscopy* 2005; **37**: 646-654 [PMID: 16010609 DOI: 10.1055/s-2005-861477]
5 **Han S**, Hsu A, Wassef WY. An update in the endoscopic management of gastric cancer. *Curr Opin Gastroenterol* 2016; **32**: 492-500 [PMID: 27662061 DOI: 10.1097/MOG.0000000000000318]
6 **Ding Z**, Qiu J, Shen Y, Chen Z, Bao W. Lens-free all-fiber probe with an optimized output beam for optical coherence tomography. *Opt Lett* 2017; **42**: 2814-2817 [PMID: 28708176 DOI: 10.1364/OL.42.002814]
7 **Karia K**, Kahaleh M. A Review of Probe-Based Confocal Laser Endomicroscopy for Pancreaticobiliary Disease. *Clin Endosc* 2016; **49**: 462-466 [PMID: 27642847 DOI: 10.5946/ce.2016.086]
8 **Zhang HF**, Maslov K, Stoica G, Wang LV. Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging. *Nat Biotechnol* 2006; **24**: 848-851 [PMID: 16823374 DOI: 10.1038/nbt1220]
9 **Maslov K**, Zhang HF, Hu S, Wang LV. Optical-resolution photoacoustic microscopy for in vivo imaging of single capillaries. *Opt Lett* 2008; **33**: 929-931 [PMID: 18451942 DOI: 10.1364/OL.33.000929]
10 **Brecht HP,** Su R, Fronheiser M, Ermilov SA, Conjusteau A, Liopo A, Motamedi M, Oraevsky AA. Optoacoustic 3D whole-body tomography: experiments in nude mice. *Proc SPIE* 2009; **7177**: 71770E [DOI: 10.1117/12.812319]
11 **Wang X**, Xie X, Ku G, Wang LV, Stoica G. Noninvasive imaging

of hemoglobin concentration and oxygenation in the rat brain using high-resolution photoacoustic tomography. *J Biomed Opt* 2006; **11**: 024015 [PMID: 16674205 DOI: 10.1117/1.2192804]

12 **Wang Y**, Hu S, Maslov K, Zhang Y, Xia Y, Wang LV. In vivo integrated photoacoustic and confocal microscopy of hemoglobin oxygen saturation and oxygen partial pressure. *Opt Lett* 2011; **36**: 1029-1031 [PMID: 21478972 DOI: 10.1364/OL.36.001029]

13 **Ignee A**, Jenssen C, Hocke M, Dong Y, Wang WP, Cui XW, Woenckhaus M, Iordache S, Saftoiu A, Schuessler G, Dietrich CF. Contrast-enhanced (endoscopic) ultrasound and endoscopic ultrasound elastography in gastrointestinal stromal tumors. *Endosc Ultrasound* 2017; **6**: 55-60 [PMID: 28218202 DOI: 10.4103/2303-9027.200216]

14 **Dong Y**, Wang WP, Mao F, Ji ZB, Huang BJ. Application of imaging fusion combining contrast-enhanced ultrasound and magnetic resonance imaging in detection of hepatic cellular carcinomas undetectable by conventional ultrasound. *J Gastroenterol Hepatol* 2016; **31**: 822-828 [PMID: 26480287 DOI: 10.1111/jgh.13202]

15 **Xuan JW**, Bygrave M, Jiang H, Valiyeva F, Dunmore-Buyze J, Holdsworth DW, Izawa JI, Bauman G, Moussa M, Winter SF, Greenberg NM, Chin JL, Drangova M, Fenster A, Lacefield JC. Functional neoangiogenesis imaging of genetically engineered mouse prostate cancer using three-dimensional power Doppler ultrasound. *Cancer Res* 2007; **67**: 2830-2839 [PMID: 17363606 DOI: 10.1158/0008-5472.CAN-06-3944]

16 **Xing W**, Wang L, Maslov K, Wang LV. Integrated optical- and acoustic-resolution photoacoustic microscopy based on an optical fiber bundle. *Opt Lett* 2013; **38**: 52-54 [PMID: 23282835 DOI: 10.1364/OL.38.000052]

17 **Yang JM**, Favazza C, Chen R, Yao J, Cai X, Maslov K, Zhou Q, Shung KK, Wang LV. Simultaneous functional photoacoustic and ultrasonic endoscopy of internal organs in vivo. *Nat Med* 2012; **18**: 1297-1302 [PMID: 22797808 DOI: 10.1038/nm.2823]

P- Reviewer: Goral V **S- Editor**: Wang JL **L- Editor**: Filipodia
E- Editor: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2018 August 21; 24(31): 3538-3546

DOI: 10.3748/wjg.v24.i31.3538

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

ORIGINAL ARTICLE

Basic Study

Clinical correlation of B7-H3 and B3GALT4 with the prognosis of colorectal cancer

Ting Zhang, Fang Wang, Jing-Yi Wu, Zhi-Chao Qiu, Yan Wang, Fen Liu, Xiao-Song Ge, Xiao-Wei Qi, Yong Mao, Dong Hua

Ting Zhang, Fen Liu, Dong Hua, Institute of Cancer, Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, China

Fang Wang, Jing-Yi Wu, Zhi-Chao Qiu, Yan Wang, Xiao-Song Ge, Yong Mao, Dong Hua, Department of Oncology, Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, China

Fang Wang, Jing-Yi Wu, Zhi-Chao Qiu, Yan Wang, Wuxi School of Medicine, Jiangnan University, Wuxi 214122, Jiangsu Province, China

Xiao-Wei Qi, Department of Pathology, Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, China

ORCID number: Ting Zhang (0000-0001-6859-6400); Fang Wang (0000-0002-8821-6695); Jing-Yi Wu (0000-0002-9219-2382); Zhi-Chao Qiu (0000-0003-0837-6144); Yan Wang (0000-0002-6310-0987); Fen Liu (0000-0003-1891-6992); Xiao-Song Ge (0000-0002-4180-5900); Xiao-Wei Qi (0000-0001-7761-5095); Yong Mao (0000-0001-5507-8292); Dong Hua (0000-0001-5569-1708).

Author contributions: Zhang T, Ge XS, Qi XW, Mao Y and Hua D designed the research; Zhang T, Wu JY, Qiu ZC, Wang Y and Liu F performed the research; Zhang T and Wang F analyzed the data; Zhang T, Wang F and Wu JY wrote the paper; Ge XS, Qi XW, Mao Y and Hua D revised the paper.

Supported by the Natural Science Foundation of Jiangsu Province, No. BK20171150 and the National Natural Science Foundation of China, No. 81502042.

Institutional review board statement: This study was reviewed and approved by Affiliated Hospital of Jiangnan University Institutional Review Board.

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: Dong Hua, MD, PhD, Chief Doctor, Professor, Department of Oncology, Affiliated Hospital of Jiangnan University, 200 Huihe Road, Wuxi 214062, Jiangsu Province, China. wx89211@163.com
Telephone: +86-510-88682109
Fax: +86-510-85808820

Received: May 11, 2018
Peer-review started: May 11, 2018
First decision: May 16, 2018
Revised: May 25, 2018
Accepted: June 25, 2018
Article in press: June 25, 2018
Published online: August 21, 2018

Abstract

AIM

To investigate the expression and clinical significance of B7 homolog 3 (B7-H3) and β-1,3-galactosyltransferase-4 (B3GALT4) in colorectal cancer (CRC) patients.

METHODS

Using tissue microarray, we identified the expression of B7-H3 and B3GALT4 in 223 CRC patient samples by immunohistochemistry and evaluated the possible correlation between B7-H3 and B3GALT4 and clinical

outcomes. Further, the mRNA and protein expression were identified to establish the regulatory relationship of B7-H3 with B3GALT4 *in vitro*.

RESULTS

A significant positive correlation between B7-H3 and B3GALT4 was observed in CRC specimens (r = 0.219, P = 0.001). High expression of B7-H3 was identified as a significant independent predictor of poor overall survival (OS) [hazard ratio (HR) = 1.781; 95%CI: 1.027-3.089; P = 0.040]. Moreover, high expression of B3GALT4 was also recognized as an independent predictor of inferior OS (HR = 1.597; 95%CI: 1.007-2.533; P = 0.047). Additionally, CRC patients expressing both high B7-H3 and high B3GALT4 contributed to a significant decrease in OS (HR = 2.283; 95%CI: 1.289-4.042; P = 0.005). In CRC cell lines with stable expression of high B7-H3, the mRNA and protein expressions of B3GALT4 were significantly upregulated. Similarly, the expression of B3GALT4 was significantly reduced when expression of B7-H3 was knocked down.

CONCLUSION

The expression of B3GALT4 in CRC is positively correlated with B7-H3 expression *in vitro*. B7-H3/B3GLAT4 may be used as dual prognostic biomarkers for CRC.

Key words: B7 homolog 3; β-1,3-galactosyltransferase-4; Colorectal cancer; Prognosis; Correlation

Core tip: The present study for the first time revealed the expression of β-1,3-galactosyltransferase-4 (B3GALT4) in colorectal cancer (CRC) and its correlation with B7 homolog 3 (B7-H3) *in vitro*. Overall, the findings of the present study suggest that B7-H3 and B3GALT4 are novel prognostic biomarkers for CRC and highlight the significance of both B7-H3 and B3GALT4 as promising therapeutic targets for CRC. Thus, here we present our preliminary work on the relationship of the immune function and glycosylation of tumor-associated protein in CRC.

Zhang T, Wang F, Wu JY, Qiu ZC, Wang Y, Liu F, Ge XS, Qi XW, Mao Y, Hua D. Clinical correlation of B7-H3 and B3GALT4 with the prognosis of colorectal cancer. *World J Gastroenterol* 2018; 24(31): 3538-3546 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3538.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3538

INTRODUCTION

Colorectal cancer (CRC) is the most prevalent gastrointestinal tract malignancy worldwide among both men and women. Overall CRC incidence and mortality rates have been declining over the recent decades, yet CRC ranks third overall among other malignancies in men and women[1]. In China, there were approximately 376300 new cases and 191000 potential deaths for CRC in 2015[2]. Despite several therapeutic advancements including surgical resection, neoadjuvant chemoradiotherapy, and targeted therapy, the prognosis of CRC patients remains relatively poor. With unprecedented survival benefits in selected patients, immunotherapy has become a promising treatment strategy and continuous progress has been made with immunomodulatory agents that target immune system checkpoints such as anti-programmed death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4)[3].

B7 homolog 3 (B7-H3), also known as CD276, is a type I transmembrane glycoprotein that belongs to the B7/CD28 immunoglobulin superfamily. B7-H3 mRNA is widely expressed on many tissues and cell types. However, B7-H3 protein is not constitutively expressed on T-cells, natural killer cells and antigen-presenting cells, and its expression can be induced on immune cells. While limited expression has been noticed on normal tissues, overexpression is observed in a majority of human malignancies including CRC[4] and has been correlated with the poor prognosis of patients[5]. Moreover, B7-H3 has been suggested to promote tumor growth, migration, invasion, and metastasis. Thus, it may be a potential therapeutic target for active immunotherapy.

The β-1,3-galactosyltransferase-4 (B3GALT4) gene belongs to the β-1,3-galactosyltransferase (β3GalT) gene family, which encodes type II membrane-bound glycoproteins and is located in the centromeric segment of the human MHC class II region[6]. This gene is abundantly expressed in human organs and tissues, predominantly in the brain and involved in GM1/GD1 ganglioside synthesis[7]. The β3GalT family plays an essential role in the O-glycosylation process. The surface of cancer cells express glycoproteins, which are rich in O-glycosylation domains[8]. Thus, the family may be closely related to the tumor. Besides, Seko *et al*[9] had confirmed that B3GALT4 could be used as a novel biomarker for the diagnosis of gynecological cancers. However, there are insufficient reports about the correlation between B3GALT4 and CRC.

Therefore, the present study aimed to investigate the clinical correlation of B7-H3 and B3GALT4 with CRC. Further, correlation between the expression of B7-H3 and B3GALT4 was evaluated to determine their prognostic significance in CRC.

MATERIALS AND METHODS

Patients and tissue samples

The medical records of patients who received a histopathological diagnosis and underwent surgery for CRC at Affiliated Hospital of Jiangnan University between June 2008 and December 2011 were retrieved. A total

of 223 formalin-fixed paraffin-embedded CRC tissue samples were included in the study. These patients did not receive radiotherapy or chemotherapy before the surgery. Only histologically confirmed cases were included in the study. However, patients who received chemo- or radiotherapy before surgery and cases with incomplete clinical data were excluded from the study. The study was approved by the Medical Ethics Committee of Affiliated Hospital of Jiangnan University, and written informed consent was obtained from all patients. All the patients were followed up by telephone up to October 31, 2017, to obtain the survival data. The median follow-up was 79 mo (range, 6-114 mo).

Tissue microarray preparation

Two experienced pathologists examined the section stained with hematoxylin and eosin and marked the carcinoma sites in the corresponding paraffin block. Using a manual tissue microarrayer (Quick-Ray, UNITMA, Seoul, Korea), tissue cylinders with a 1.0 mm diameter were punched from representative tissue areas of each donor tissue block and implanted into the hole of the premade recipient paraffin block (UNITMA, Seoul, Korea). The tissue microarray paraffin block was then constructed and cut into 4 μm thick continuous sections, which were attached to anti-dewaxing slides and stored at room temperature.

Immunochemistry

The tissue paraffin blocks were serially sectioned into a 4 μm thickness, dewaxed in xylene, and hydrated in an ethanol gradient. Antigen retrieval was performed by heating the tissue sections at 100 ℃ for 30 min in citrate buffer. Moreover, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. Subsequently, the sections were incubated in 5% bovine serum albumin at room temperature for 30 min. Primary antibodies, mouse anti-human B7-H3 monoclonal antibody (1:200, Santa Cruz, Dallas, TX, United States) and rabbit anti-human B3GALT4 monoclonal antibody (1:50, Abcam, Cambridge, MA, United States) respectively were added drop-wise followed by overnight incubation at 4 ℃. The slides were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min. The immunostaining was carried out by staining with 3,3′-diaminobenzidine tetrahydrochloride solution (GTVisionII Immunohistochemistry Detection Kit for Rabbit/Mouse, Gene Tech, Shanghai, China), counter-stained with hematoxylin, dehydrated, and mounted. Sections were examined under a microscope.

Evaluation of immunochemistry staining

Two independent pathologists performed a blinded manner review of the sections. Both the intensity and extent of immunological staining were analyzed semi-quantitatively. According to the percentage of positively stained cells, the sections were graded by five levels: 0 (≤ 5%), 1 (6%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (> 76%). The staining intensity was scored similarly, with 0 used for negative staining, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive. The scores for the percentage of positive cells and the staining intensity were multiplied to generate an immune-reactive score for each specimen (0-12). Based on the total score, scores of 0-3 were considered as the low expression group, and scores of 4-12 were defined as the high expression group.

Cell lines and culture

The two human CRC cell lines, SW480 and Caco-2, which exhibited different expression levels of B7-H3 were maintained in our lab. We constructed SW480 cells that expressed a high level of B7-H3 (SW480-B7-H3) by transfection with an overexpression plasmid, and Caco-2 cells were stably transfected with a B7-H3 shRNA (Caco-2-shB7-H3). Cells transfected with a mock vector were used as negative controls (SW480-NC and Caco-2-shNC). SW480-NC and SW480-B7-H3 were cultured in L-15 medium (HyClone GE Healthcare Life Sciences, South Logan, UT, United States). Caco-2-NC and Caco-2-shB7-H3 were maintained in RPMI-1640 medium (the same as above). All the media were supplemented with 10% fetal bovine serum (Clark Bioscience, Houston, TX, United States). The cells were incubated at 37 ℃ in a humidified atmosphere with 5% CO_2.

RNA extraction and cDNA synthesis

Total RNA was extracted from 1 × 10^6 cells using TRIzol (Invitrogen, Carlsbad, CA, United States), according to the manufacturer's protocol. A NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, United States) was used to estimate the purity and concentration of total RNA by measuring the optical density. The first strand cDNA was generated from 1 μg RNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) following the manufacturer's instructions.

Real-time quantitative polymerase chain reaction

The expression levels of B7-H3 and B3GALT4 were detected using the QuantiNova™ SYBR Green PCR Kit (QIAGEN, Hilden, Germany) on ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). β-actin was used as an internal control for normalization of the expression data. The first strand cDNA was amplified in a total 20 μL PCR reaction mixture: 10 μL 2 x QuantiNova SYBR Green PCR Master Mix, 0.1 μL QN ROX Reference Dye, 0.4 μL of each specific primer set, 2 μL cDNA and ddH_2O added to 20 μL. The sequences of primers were as follows: β-actin 5′-CATGTACGTTGCTATCCAGGC-3′ (sense), 5′-CTCCTTAATGTCACGCACGAT-3′ (antisense); B7-H3 5′-AGCACTGTGGTTCTGCCTCACA-3′ (sense), 5′-CACCAGCTGTTTGGTATCTGTCAG-3′ (antisense); B3GALT4 5′-ACTCCTACCGCAACCTCA-3′ (sense), 5′-CACATCATCGTCCGTCTT-3′ (antisense). Each sample and internal control gene were run in triplicate, using an amplification condition of denaturation at 95 ℃ for

2 min, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 10 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of the target genes.

Western blotting

Total proteins were extracted from 1×10^6 cells by using ice-cold RIPA lysis buffer containing a cocktail of protease inhibitors (KeyGEN BioTECH, China) for 30 min. The concentrations of whole cell proteins were determined using a BCA Protein Assay Kit (CWBio, Beijing, China) and adjusted to the same concentration. For western blotting assay, the proteins of equal quantity were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane (Merck Millipore, Germany). After blocking with 5% nonfat dry milk for 1 h at room temperature, the membranes were incubated with the primary antibodies at a concentration of 1:1000 at 4 ℃ overnight. The antibodies included rabbit anti-human B7-H3 monoclonal antibody (Abcam, Cambridge, MA, United States), rabbit anti-human B3GALT4 monoclonal antibody (Abcam, Cambridge, MA, United States) and mouse anti-human β-actin monoclonal antibody (Beyotime, Nantong, China). After washing with TBST (TBS with 0.1% Tween), the membranes were incubated with a corresponding goat-anti-mouse or goat-anti-rabbit IgG-horseradish peroxidase secondary antibody (1:1000, Beyotime, Nantong, China) for 1 h at room temperature. The membranes were finally treated with enhanced chemiluminescence (ECL) assay reagents (absin, Shanghai, China) and the immunoreactive bands were visualized using the ChemiDoc™ XRS+ system with Image Lab™ Software (Bio-Rad, Hercules, CA, United States). Quantitative analysis was carried out with Quantity One (Bio-Rad, Hercules, CA, United States).

Statistical analysis

Statistical analysis of clinical data was performed using the software package SPSS 19.0 software (IBM, Chicago, IL, United States). Chi-square test analyzed the association between B7-H3 or B3GALT4 and the clinicopathological data. The non-parametric Spearman test evaluated the correlation of B7-H3 and B3GALT4 expression. Overall survival (OS) was plotted using the Kaplan-Meier method and was compared using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazard model. All the *in vitro* experiments were performed in triplicate. A non-paired *t*-test analyzed differences in mean values between groups. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, United States). A *P*-value less than 0.05 indicated a statistically significant difference.

RESULTS

Expression of B7-H3 and B3GALT4 and their relationship with clinicopathological features of CRC

The expression of B7-H3 and B3GALT4 was analyzed using immunochemistry in CRC tissue. Positive staining of B7-H3 was predominantly detected in the membrane and cytoplasm (Figure 1C), and the location of B3GALT4 expression was identified in the cytoplasm (Figure 1D). The positive rates of the B7-H3 and B3GALT4 expressions were 70.4% (157/223) and 54.7% (122/223), respectively. Further, a highly significant correlation between the positive expression of B7-H3 with the depth of tumor invasion (P = 0.049), distant metastasis (P = 0.034) and differentiation (P = 0.017) was observed. However, the expression of B3GALT4 had no significant correlation with clinicopathological parameters (Table 1).

Correlation between B7-H3 and B3GALT4 expression in CRC

Of the 223 CRC cases, 97 (43.5%) patients exhibited higher expression of both B7-H3 and B3GALT4, and 41 (18.4%) showed lower expression of both. Also, there were 85 (38.1%) cases with either B7-H3 low or B3GALT4 low. Spearmen's Correlation was used to examine the association of B7-H3 expression with B3GALT4 expression in CRC. The result showed that there was a highly significant positive correlation between B7-H3 and B3GALT4 expression (r = 0.219, P = 0.001, Table 2).

High expression of B7-H3 and B3GALT4 in patients with CRC is associated with poor OS

Notably, patients with high expression of B7-H3 had a significantly worse OS as compared to patients with low expression (P = 0.037) (Figure 2A). Similarly, the OS in the high B3GALT4 expression group was significantly inferior to that in the low expression group (P = 0.044) (Figure 2B). Moreover, a subgroup of patients with high expression of B7-H3 and high B3GALT4 exhibited a significantly worse prognosis as compared to patients with a low expression of B3GALT4 (P = 0.016) (Figure 2C). However, in a subgroup of patients with low expression of both B7-H3 and B3GALT4 prognosis of patients with CRC was unaffected (P = 0.061) (Figure 2D).

Furthermore, the univariate Cox proportional hazard model analysis was performed to evaluate the risk factors related to the prognosis of patients with CRC. As shown in Table 3, high expression of B7-H3, high expression of B3GALT4, high expression of both B7-H3 and B3GALT4, depth of tumor invasion (T3/4), lymph node metastasis (N1/2), distant metastasis, TNM stage (III/IV), neural invasion, and vascular invasion were correlated with the OS of patients with CRC. However, there was no correlation of OS with gender, age, tumor location, colon cancer site, mucinous adenocarcinoma, differentiation, and B7-H3 low expression group (Table 3). Besides, the multivariate analysis revealed that distant metastasis, TNM stage (III/IV), and vascular invasion were significant independent prognostic factors for OS of patients with CRC (Table 3).

Table 1 Expression of B7 homolog 3 and β-1,3-galactosyltransferase-4 in colorectal cancer patients and their correlation with clinicopathological parameters

Clinicopathological parameter	Case (*n*)	B7-H3 expression		*P* value	B3GALT4 expression		*P* value
		Low	High		Low	High	
Gender				0.616			0.213
Male	124	35	89		63	61	
Female	99	31	68		42	57	
Age (yr)				0.259			0.791
< 60	87	22	65		40	47	
≥ 60	136	44	92		65	71	
Tumor location				0.361			0.905
Colon	88	23	65		41	47	
Rectum	135	43	92		64	71	
Colon cancer site				0.119			0.352
Right-sided	37	7	30		20	17	
Left-sided	186	59	127		85	101	
Depth of tumor invasion				0.049			0.527
T1/2	64	25	39		28	36	
T3/4	159	41	118		77	82	
Lymph node metastasis				0.433			0.145
N0	116	37	79		60	56	
N1/2	107	29	78		45	62	
Distant metastasis				0.034			0.425
Yes	16	1	15		6	10	
No	207	65	142		99	108	
TNM stage				0.297			0.068
Ⅰ / Ⅱ	113	37	76		60	53	
Ⅲ/Ⅳ	110	29	81		45	65	
Neural invasion				0.146			0.683
Yes	32	6	26		14	18	
No	191	60	131		91	100	
Vascular invasion				0.079			0.585
Yes	35	6	29		15	20	
No	188	60	128		90	98	
Mucinous adenocarcinoma				0.579			0.506
Yes	20	7	13		8	12	
No	203	59	144		97	106	
Differentiation				0.017			0.186
Poor	73	14	59		39	34	
Moderate/well	150	52	98		66	84	

B7-H3: B7 homolog 3; B3GALT4: β-1,3-galactosyltransferase-4.

Table 2 Correlation between B7 homolog 3 and β-1,3-galactosyltransferase-4 expression in colorectal cancer

B3GALT4 expression	B7-H3 expression		*r*	*P* value
	Low	High		
Low	41	60	0.219	0.001
High	25	97		

B7-H3: B7 homolog 3; B3GALT4: β-1,3-galactosyltransferase-4.

Correlation between B7-H3 and B3GALT4 in CRC cell lines

We further investigated the relationship between expression of B7-H3 and B3GALT4 *in vitro*. The mRNA and protein expression was detected in the CRC cell lines with different B7-H3 expression levels, which were maintained in our lab. Both the mRNA and protein expression of B7-H3 and B3GALT4 were significantly upregulated in SW480-B7-H3 cell line expressing a high level of B7-H3 as compared to SW480-NC cell line. Similarly, as compared to Caco-2-shNC cell line, Caco-2-shB7-H3 cell line with low expression of B7-H3 significantly downregulated the expression of B3GALT4. Therefore, B7-H3 and B3GALT4 had a positive correlation both in mRNA and protein expression *in vitro* (Figure 3).

DISCUSSION

CRC continues to be a major cause of cancer-related morbidity and mortality with over 1200000 cases diagnosed every year, and more than 600000 patients

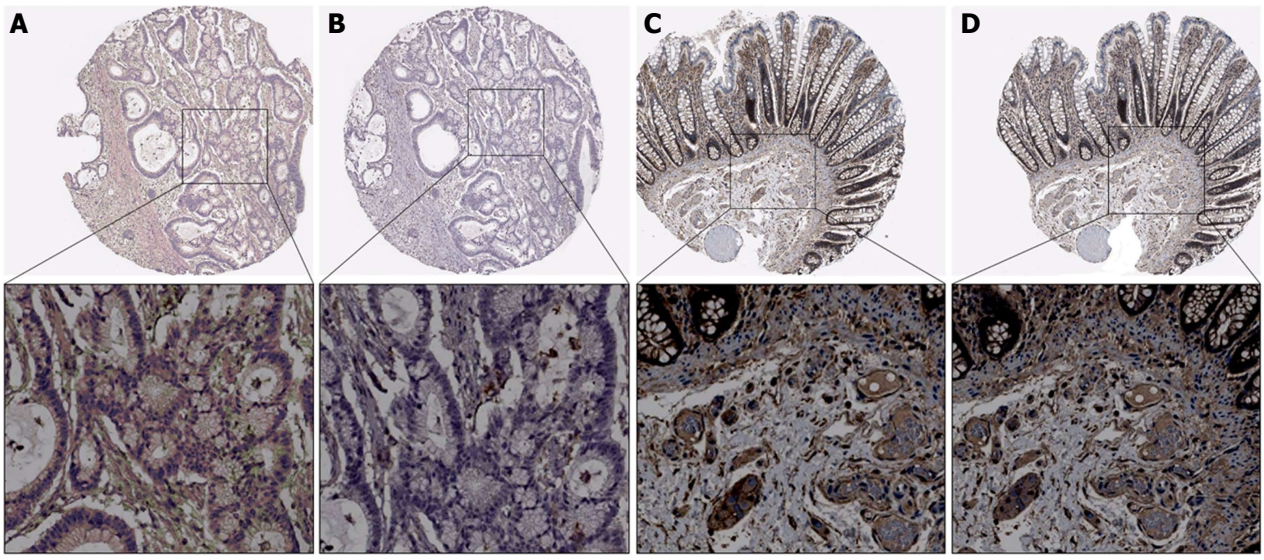


Figure 1 The representative immunohistochemical images of B7 homolog 3 and β-1,3-galactosyltransferase-4 expression in colorectal cancer tissues. A: Low expression of B7 homolog 3 (B7-H3); B: Low expression of β-1,3-galactosyltransferase-4 (B3GALT4); C: High expression of B7-H3; D: High expression of B3GALT4 (100 ×). The images below show the magnification of each zone (400 ×). A and B are from one patient; C and D are from another patient.

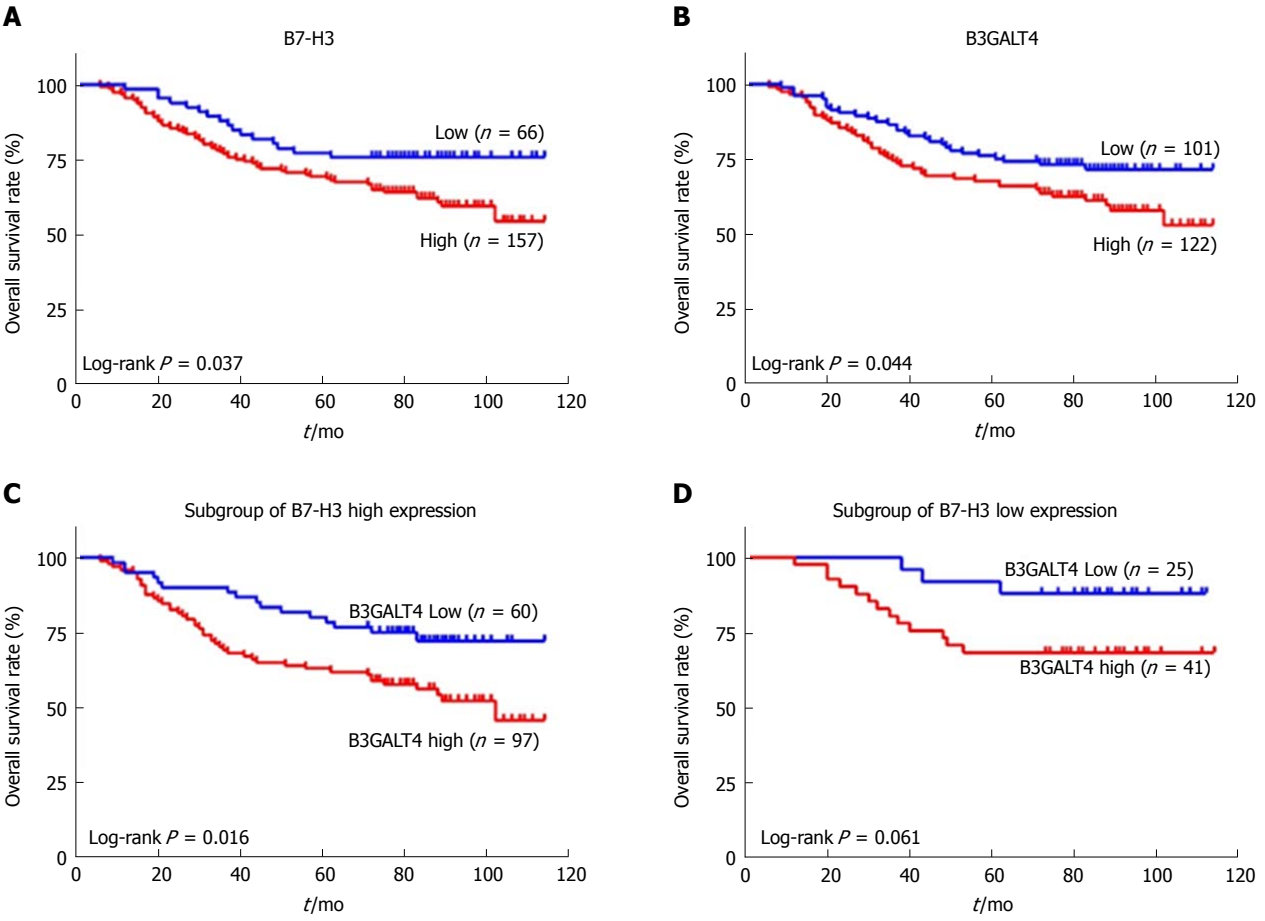


Figure 2 Kaplan-Meier survival curves of the 223 patients with colorectal cancer expressing B7 homolog 3 and β-1,3-galactosyltransferase-4. A: Patients with low expression of B7 homolog 3 (B7-H3) *vs* high expression of B7-H3; B: Patients with low expression of β-1,3-galactosyltransferase-4 (B3GALT4) *vs* high expression of B3GALT4; C: Subgroup of patients with high expression of B7-H3 and low expression of B3GALT4 *vs* high expression of B3GALT4; D: Subgroup of patients with low expression of B7-H3 and high expression of B3GALT4 *vs* low expression of B3GALT4.

succumb to the disease worldwide. Moreover, five-year survival rates for stage I CRC was more than 90% where as for stage IV was 10%[10]. Given the poor prognosis of CRC, the search for novel diagnostic and

Table 3 Univariate and multivariate Cox proportional hazards analyses of overall survival in patients with colorectal cancer

Clinical parameter	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender (male *vs* female)	0.999 (0.637-1.566)	0.995		
Age (≥ 60 *vs* < 60) (yr)	0.951 (0.603-1.500)	0.829		
Tumor location (rectum *vs* colon)	1.351 (0.863-2.117)	0.188		
Colon cancer site (left-sided *vs* right-sided)	0.970 (0.534-1.760)	0.919		
Depth of tumor invasion (T3/4 *vs* T1/2)	2.522 (1.362-4.670)	0.003	1.807 (0.949-3.443)	0.072
Lymph node metastasis (N1/2 *vs* N0)	2.922 (1.801-4.739)	0.000	0.322 (0.077-1.341)	0.119
Distant metastasis (yes *vs* no)	9.353 (5.206-16.804)	0.000	4.635 (2.224-9.660)	0.000
TNM stage (Ⅲ/Ⅳ *vs* Ⅰ/Ⅱ)	3.497 (2.114-5.783)	0.000	7.490 (1.636-34.280)	0.009
Neural invasion (yes *vs* no)	2.240 (1.320-3.802)	0.003	0.800 (0.410-1.561)	0.514
Vascular invasion (yes *vs* no)	2.531 (1.529-4.188)	0.000	1.962 (1.082-3.558)	0.026
Mucinous adenocarcinoma (yes *vs* no)	1.063 (0.488-2.314)	0.877		
Differentiation (poor *vs* moderate/well)	0.799 (0.504-1.267)	0.340		
B7-H3 (high *vs* low)	1.781 (1.027-3.089)	0.040	1.289 (0.721-2.307)	0.392
B3GALT4 (high *vs* low)	1.597 (1.007-2.533)	0.047	1.209 (0.721-2.307)	0.392
B7-H3 and B3GALT4 (high *vs* low)	0.737 (0.552-0.983)	0.038	1.153 (0.845-1.572)	0.369
B7-H3 high (B3GALT4 high *vs* low)	2.283 (1.289-4.042)	0.005		
B7-H3 low (B3GALT4 high *vs* low)	0.321 (0.091-1.127)	0.076		

B7-H3: B7 homolog 3; B3GALT4: β-1,3-galactosyltransferase-4.

prognostic biomarkers is highly desirable to prevent CRC-related deaths.

Immune checkpoint signaling pathways are most frequently modulated in cancer to inhibit the nascent anti-tumor immune response. Checkpoint antibody inhibitors, such as CTLA-4 and PD-1/PD-L1 are the most extensively studied novel class of inhibitors. Currently, drugs inhibiting these pathways are employed for a wide variety of malignancies and have demonstrated durable anti-tumor activities in a subset of cancer patients[11,12]. Furthermore, investigations on new inhibitory pathways are also undergoing, and drugs are being investigated including those targeting B7-H3[13]. As an immune checkpoint protein, B7-H3 has been recognized as an immunoregulatory molecule for T cell activation, proliferation, and cytokine production[14]. Thus far, overexpression of B7-H3 has been reported in several different cancer types and the expression level of B7-H3 correlated with tumor growth, invasion, metastasis, malignant stage, and recurrence rate[15]. Furthermore, several groups have generated anti-B7-H3 antibodies and reported overall suppression of tumor growth *in vitro* and *in vivo*[16]. Therefore, it is expected that B7-H3 plays a crucial role in cancer diagnosis and treatment. The present study revealed that B7-H3 was highly expressed in CRC tissue (70.4%) and significantly associated with the depth of tumor invasion (P = 0.049), distant metastasis (P = 0.034) and differentiation (P = 0.017). These results were consistent with previous findings[17,18].

Furthermore, B7-H3 is a type I transmembrane glycoprotein and plays a role in immunosuppression. Chen *et al*[19] demonstrated that glycoprotein B7-H3 was involved in the pathological process and tumor growth of oral squamous cell carcinoma, and the glycans of B7-H3 from oral cancer cells comprised of terminal α-galactoses and more diverse N-glycan structures with higher fucosylation than that of healthy cells. In addition, the presence of a carbohydrate-lectin receptor interaction between the tumor-associated B7-H3 from oral cancer cells and immune cells was detected[19]. B3GALT4, encoding a UDP-galactose: β-N-acetylgalactosamine β-1,3-galactosyltransferase activity, belongs to the human β-3-galactosyltransferase gene family[7]. Galactosyltransferase (GT) of the glycosyltransferase family, is the most common Golgi "marker" enzyme, catalyzes the transfer of galactose to glycoprotein-bound acetylglucosamine. The enzyme is provided with one N-linked oligosaccharide and palmitate residues and plays a vital role in the process of O-glycosylation. Moreover, in patients suffering from ovarian and breast cancer, increased expression of GT enzyme activity has been reported[20]. Enzymatic activity of B3GALT4 and T5 in ovarian cancer tissues, and significant overexpression of B3GALT4/T5 mainly in stage I uterine corpus cancers, indicated that B3GALT4/T5 is a novel tumor marker for uterine corpus cancer and other gynecological cancers[9]. Consequently, B7-H3 and B3GALT4 were both correlated with glycosylation and tumor progession. However, there was a paucity of literature on the expression and association of B7-H3 and B3GALT4 in CRC. Therefore, the association of B7-H3 and B3GALT4 expression in CRC tissues and cell lines was investigated.

The present study revealed that high expression of B7-H3 or B3GALT4 reduced the OS rate of CRC patients independently (P = 0.037, P = 0.044, respectively). Furthermore, overexpression of both B7-H3 and B3GALT4 in CRC resulted in poorer prognosis (P = 0.016). Besides, there was a significant positive correlation between B7-H3 and B3GALT4 (r = 0.219, P = 0.001). Taken together, B7-H3 and B3GALT4 could serve as a novel, reliable prognostic marker for CRC. Furthermore, we detected that the expression of B3GALT4 was positively altered following B7-H3 expression *in vitro*.

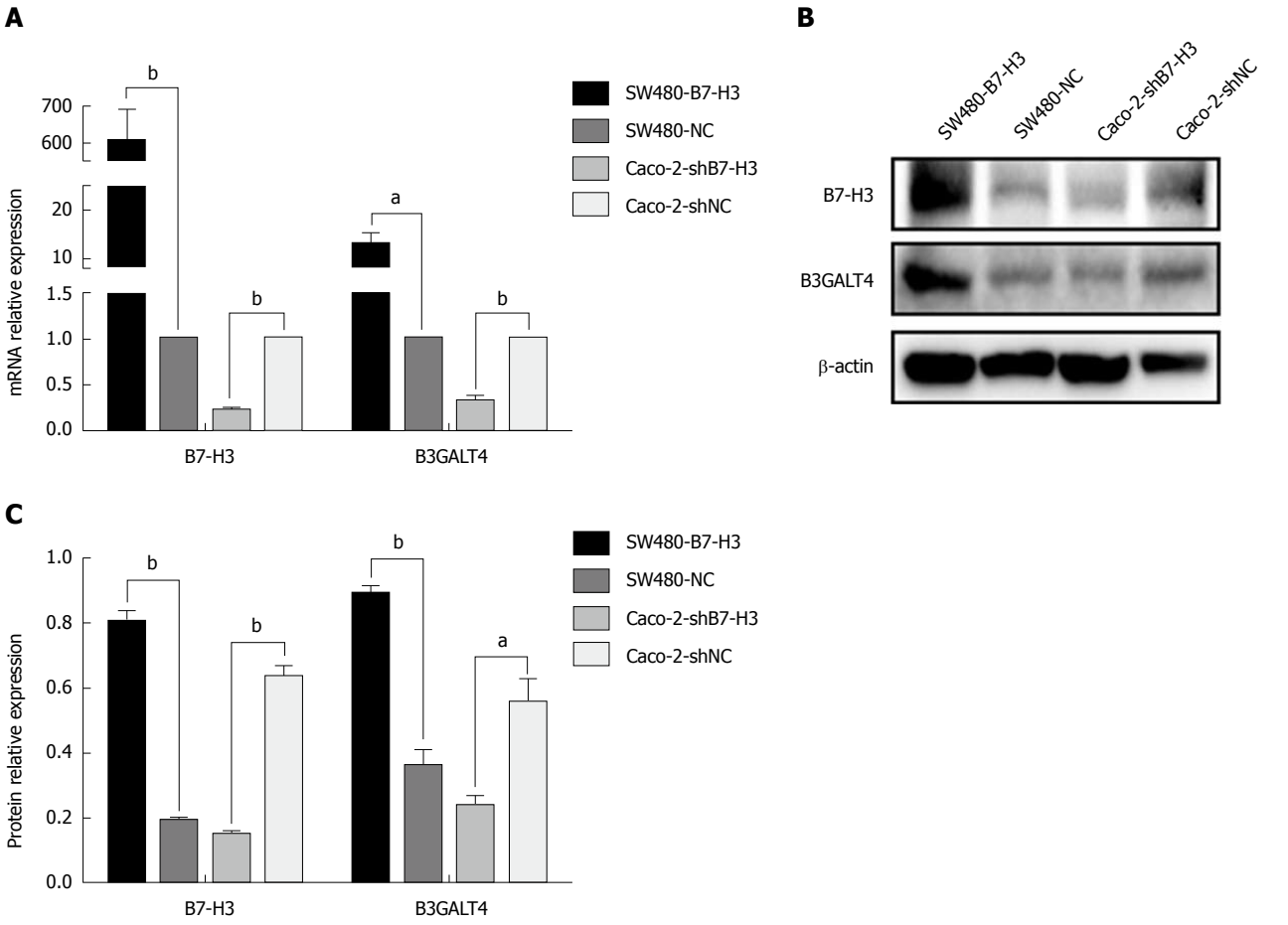


Figure 3 The expression of B7 homolog 3 and β-1,3-galactosyltransferase-4 in colorectal cancer cell lines. A: Real-time quantitative polymerase chain reaction results of relative mRNA expression; B: Western blotting results for protein expression; C: Statistical analysis of protein expression ($^{a}P < 0.05$, $^{b}P < 0.01$).

Combined with the results of clinical samples, this study indicated that B7-H3 could positively regulate B3GALT4 at the mRNA as well as the protein level. However, the underlying mechanism of regulation of B7-H3 and B3GALT4 in CRC warrants further investigation.

In summary, the present study for the first time revealed the expression of B3GALT4 in CRC and its correlation with B7-H3 *in vitro*. Overall, the findings of the present study suggested that B7-H3 and B3GALT4 are novel prognostic biomarkers for CRC and highlight the significance of both B7-H3 and B3GALT4 as promising therapeutic targets for CRC. Thus, here we present our preliminary work on the relationship of the immune function and glycosylation of tumor-associated protein in CRC.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the most prevalent gastrointestinal tract malignancy worldwide. The prognosis of CRC patients remains relatively poor. B7 homolog 3 (B7-H3) mRNA is widely expressed on many tissues and cell types. However, B7-H3 protein is not constitutively expressed on T-cells, natural killer cells and antigen-presenting cells. The β-1,3-galactosyltransferase-4 (B3GALT4), which belongs to β-1,3-galactosyltransferase (β3GalT) gene family is abundantly expressed in human organs and tissues, predominantly in brain and involved in GM1/GD1 ganglioside synthesis. The β3GalT family may be closely related to the tumor.

Research motivation

There are insufficient reports about the correlation between B3GALT4 and CRC.

Research objectives

The aim of the present study is to investigate the clinical correlation of B7-H3 and B3GALT4 with CRC, and the correlation between the expression of B7-H3 and B3GALT4 was evaluated to determine their prognostic significance in CRC.

Research methods

The authors identified the expression of B7-H3 and B3GALT4 in 223 CRC patient samples by immunohistochemistry and evaluated the possible correlation between B7-H3 and B3GALT4 and clinical outcomes. The mRNA and protein expression were also identified to establish the regulatory relationship of B7-H3 with B3GALT4 *in vitro*.

Research results

A significant positive correlation between B7-H3 and B3GALT4 was observed in CRC specimens. High expression of B7-H3 was identified as a significant independent predictor of poor overall survival (OS). High expression of B3GALT4 was also recognized as an independent predictor of inferior OS. In CRC cell lines with the stable expression of high B7-H3, the mRNA and protein expressions of B3GALT4 were significantly upregulated. The expression of B3GALT4 was significantly reduced when expression of B7-H3 was knocked down.

Research conclusions

The expression of B3GALT4 in CRC and its positive correlation with B7-H3 *in vitro* was revealed, as well as B7-H3/B3GLAT4 as dual prognostic biomarkers for CRC.

Research perspectives

The present study suggested that B7-H3 and B3GALT4 are novel prognostic biomarkers for CRC, and the significance of both B7-H3 and B3GALT4 as promising therapeutic targets for CRC are highlighted.

REFERENCES

1 **Siegel RL**, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, Jemal A. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017; **67**: 177-193 [PMID: 28248415 DOI: 10.3322/caac.21395]

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 **Zhang Y**, Chen Z, Li J. The current status of treatment for colorectal cancer in China: A systematic review. *Medicine* (Baltimore) 2017; **96**: e8242 [PMID: 28984783 DOI: 10.1097/MD.0000000000008242]

4 **Castellanos JR**, Purvis IJ, Labak CM, Guda MR, Tsung AJ, Velpula KK, Asuthkar S. B7-H3 role in the immune landscape of cancer. *Am J Clin Exp Immunol* 2017; **6**: 66-75 [PMID: 28695059]

5 **Ye Z**, Zheng Z, Li X, Zhu Y, Zhong Z, Peng L, Wu Y. B7-H3 Overexpression Predicts Poor Survival of Cancer Patients: A Meta-Analysis. *Cell Physiol Biochem* 2016; **39**: 1568-1580 [PMID: 27626927 DOI: 10.1159/000447859]

6 **Shiina T**, Kikkawa E, Iwasaki H, Kaneko M, Narimatsu H, Sasaki K, Bahram S, Inoko H. The beta-1,3-galactosyltransferase-4 (B3GALT4) gene is located in the centromeric segment of the human MHC class II region. *Immunogenetics* 2000; **51**: 75-78 [PMID: 10663566 DOI: 10.1007/s002510050012]

7 **Amado M**, Almeida R, Carneiro F, Levery SB, Holmes EH, Nomoto M, Hollingsworth MA, Hassan H, Schwientek T, Nielsen PA, Bennett EP, Clausen H. A family of human beta3-galactosyltransferases. Characterization of four members of a UDP-galactose:beta-N-acetyl-glucosamine/beta-nacetyl-galactosamine beta-1,3-galactosyltransferase family. *J Biol Chem* 1998; **273**: 12770-12778 [PMID: 9582303 DOI: 10.1074/jbc.273.21.12770]

8 **Wandall HH**, Blixt O, Tarp MA, Pedersen JW, Bennett EP, Mandel U, Ragupathi G, Livingston PO, Hollingsworth MA, Taylor-Papadimitriou J, Burchell J, Clausen H. Cancer biomarkers defined by autoantibody signatures to aberrant O-glycopeptide epitopes. *Cancer Res* 2010; **70**: 1306-1313 [PMID: 20124478 DOI: 10.1158/0008-5472.CAN-09-2893]

9 **Seko A**, Kataoka F, Aoki D, Sakamoto M, Nakamura T, Hatae M, Yonezawa S, Yamashita K. Beta1,3-galactosyltransferases-4/5 are novel tumor markers for gynecological cancers. *Tumour Biol* 2009; **30**: 43-50 [PMID: 19225246 DOI: 10.1159/000203129]

10 **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]

11 **Seidel JA**, Otsuka A, Kabashima K. Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations. *Front Oncol* 2018; **8**: 86 [PMID: 29644214 DOI: 10.3389/fonc.2018.00086]

12 **Abdo J**, Cornell DL, Mittal SK, Agrawal DK. Immunotherapy Plus Cryotherapy: Potential Augmented Abscopal Effect for Advanced Cancers. *Front Oncol* 2018; **8**: 85 [PMID: 29644213 DOI: 10.3389/fonc.2018.00085]

13 **Marin-Acevedo JA**, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next generation of immune checkpoint therapy in cancer: new developments and challenges. *J Hematol Oncol* 2018; **11**: 39 [PMID: 29544515 DOI: 10.1186/s13045-018-0582-8]

14 **Picarda E**, Ohaegbulam KC, Zang X. Molecular Pathways: Targeting B7-H3 (CD276) for Human Cancer Immunotherapy. *Clin Cancer Res* 2016; **22**: 3425-3431 [PMID: 27208063 DOI: 10.1158/1078-0432.CCR-15-2428]

15 **Li G**, Quan Y, Che F, Wang L. B7-H3 in tumors: friend or foe for tumor immunity? *Cancer Chemother Pharmacol* 2018; **81**: 245-253 [PMID: 29299639 DOI: 10.1007/s00280-017-3508-1]

16 **Shi J**, Zhang DL, Cui ZC, Wang HM. Preparation and application of a novel monoclonal antibody specific for human B7-H3. *Mol Med Rep* 2016; **14**: 943-948 [PMID: 27222007 DOI: 10.3892/mmr.2016.5288]

17 **Sun J**, Chen LJ, Zhang GB, Jiang JT, Zhu M, Tan Y, Wang HT, Lu BF, Zhang XG. Clinical significance and regulation of the costimulatory molecule B7-H3 in human colorectal carcinoma. *Cancer Immunol Immunother* 2010; **59**: 1163-1171 [PMID: 20333377 DOI: 10.1007/s00262-010-0841-1]

18 **Mao Y**, Sun J, Wang WP, Zhang XG, Hua D. Clinical significance of costimulatory molecule B7-H3 expression on CD3(+) T cells in colorectal carcinoma. *Chin Med J* (Engl) 2013; **126**: 3035-3038 [PMID: 23981607]

19 **Chen JT**, Chen CH, Ku KL, Hsiao M, Chiang CP, Hsu TL, Chen MH, Wong CH. Glycoprotein B7-H3 overexpression and aberrant glycosylation in oral cancer and immune response. *Proc Natl Acad Sci USA* 2015; **112**: 13057-13062 [PMID: 26438868 DOI: 10.1073/pnas.1516991112]

20 **Strous GJ**. Golgi and secreted galactosyltransferase. *CRC Crit Rev Biochem* 1986; **21**: 119-151 [PMID: 3093147 DOI: 10.3109/10409238609113610]

P- Reviewer: Bordonaro M, Sipos F, Sulkowski S
S- Editor: Wang JL **L- Editor**: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2018 August 21; 24(31): 3547-3555

DOI: 10.3748/wjg.v24.i31.3547

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Favorable clinical outcome of nonalcoholic liver cirrhosis patients with coronary artery disease: A population-based study

Ming-Chang Tsai, Tzu-Wei Yang, Chi-Chih Wang, Yao-Tung Wang, Wen-Wei Sung, Ming-Hseng Tseng, Chun-Che Lin

Ming-Chang Tsai, Tzu-Wei Yang, Chi-Chih Wang, Chun-Che Lin, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Ming-Chang Tsai, Chi-Chih Wang, Yao-Tung Wang, Wen-Wei Sung, Chun-Che Lin, Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

Ming-Chang Tsai, Tzu-Wei Yang, Chi-Chih Wang, Wen-Wei Sung, Ming-Hseng Tseng, Chun-Che Lin, School of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

Tzu-Wei Yang, Institute and Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 300, Taiwan

Yao-Tung Wang, Division of Pulmonary Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Wen-Wei Sung, Department of Urology, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Ming-Hseng Tseng, Department of Medical Informatics, Chung Shan Medical University, Taichung 402, Taiwan

ORCID number: Ming-Chang Tsai (0000-0002-7687-2329); Tzu-Wei Yang (0000-0002-1522-8177); Chi-Chih Wang (0000-0002-8222-0503); Yao-Tung Wang (0000-0002-0300-0896); Wen-Wei Sung (0000-0002-2375-0029); Ming-Hseng Tseng (0000-0001-8868-1610); Chun-Che Lin (0000-0002-2474-6734).

Author contributions: All authors reviewed the manuscript and completed final approval; Tsai MC, Yang TW, Tseng MH and Lin CC contributed to study concept and design; Tsai MC, Yang TW and Tseng MH acquired the data; Tsai MC, Yang TW, Wang YT, Sung WW, Tseng MH and Lin CC analyzed and interpreted the data; Tsai MC and Yang TW wrote the manuscript daft; Tsai MC, Yang TW, Wang CC, Wang YT, Sung WW, Tseng MH and Lin CC made critical revision on the manuscript for important intellectual content; Tsai MC, Yang TW and Tseng MH performed statistical analysis; Lin CC obtained funding; Tseng MH and Lin CC contributed to study supervision.

Supported by Chung Shan Medical University Hospital, Taichung, Taiwan, No. CSH- 2013-C-032.

Institutional review board statement: This study was approved by the Institutional Review Board (IRB) of Chung Shan Medical University Hospital, Taiwan.

Informed consent statement: The IRB waived the need of informed consent for this retrospective study based on an encrypted National Health Insurance Research Database (NHIRD).

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: The data of our study were retrieved from the National Health Insurance Research Database (NHIRD) in Taiwan. The application for the data was reviewed and approved by the National Health Insurance (NHI). NHIRD are available *via* http://nhird.nhri.org.tw/.

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: Chun-Che Lin, MD, PhD, Professor,

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chung Shan Medical University Hospital, No.110, Sec. 1, Jianguo N. Rd., South Dist., Taichung 402, Taiwan. forest65@csmu.edu.tw
Telephone: +886-4-24739595
Fax: +886-4-24739220

Received: April 19, 2018
Peer-review started: April 19, 2018
First decision: June 6, 2018
Revised: July 11, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: August 21, 2018

Abstract

AIM

To elucidate the prevalence and risk of mortality of nonalcoholic liver cirrhosis (LC) patients with coronary artery disease (CAD).

METHODS

The study cohort included newly diagnosed nonalcoholic LC patients age ⩾ 40 years old without a diagnosis of CAD from 2006 until 2011 from a longitudinal health insurance database. The mean follow-up period for the study cohort was 1152 ± 633 d. The control cohort was matched by sex, age, residence, and index date. Hazard ratios (HRs) were calculated using the Cox proportional hazard model and the Kaplan-Meier method.

RESULTS

After exclusion, a total of 3409 newly diagnosed nonalcoholic cirrhotic patients were identified from one million samples from the health insurance database. We found that CAD (5.1% *vs* 17.4%) and hyperlipidemia (20.6% *vs* 24.1%) were less prevalent in nonalcoholic LC patients than in normal subjects (all $P < 0.001$), whereas other comorbidities exhibited an increased prevalence. Among the comorbidities, chronic kidney disease exhibited the highest risk for mortality (adjusted HR (AHR) = 1.76; 95%CI: 1.55-2.00, $P < 0.001$). Ascites or peritonitis exhibited the highest risk of mortality among nonalcoholic cirrhotic patients (AHR = 2.34; 95%CI: 2.06-2.65, $P < 0.001$). Finally, a total of 170 patients developed CAD after a diagnosis of nonalcoholic LC. The AHR of CAD in nonalcoholic LC patients was 0.56 (95%CI: 0.43-0.74, $P < 0.001$). The six-year survival rates for nonalcoholic LC patients with and without CAD were 52% and 50%, respectively ($P = 0.012$).

CONCLUSION

We conclude that CAD was less prevalent and associated with a reduced risk of mortality in nonalcoholic cirrhotic patients.

Key words: Nonalcoholic liver cirrhosis; Coronary artery disease; Population-based study

Core tip: Coronary artery disease (CAD) is less prevalent and associated with a reduced risk of mortality in nonalcoholic liver cirrhosis (LC) patients. Nonalcoholic LC patients with CAD exhibit an increased six-year survival rate compared to cirrhotic patients without CAD. The LC complication rates did not differ between nonalcoholic LC patients with and without CAD. Of note, nonalcoholic LC patients with ascites or peritonitis exhibited the highest risk of mortality among LC complications.

Tsai MC, Yang TW, Wang CC, Wang YT, Sung WW, Tseng MH, Lin CC. Favorable clinical outcome of nonalcoholic liver cirrhosis patients with coronary artery disease: A population-based study. *World J Gastroenterol* 2018; 24(31): 3547-3555 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3547.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3547

INTRODUCTION

Liver cirrhosis (LC) is the 14th leading cause of death in adults and accounted for one million deaths in 2010 worldwide[1]. In Taiwan, LC and chronic liver disease constituted the 8th most common cause of death in 2011[2]. These conditions contribute to numerous lethal complications and comorbidities that result in poor clinical outcomes. Variceal bleeding, systemic infection, hepatic encephalopathy, and hepatocellular carcinoma (HCC) are the primary complications of LC and causes of mortality[3,4]. In addition, LC is associated with systemic comorbidities, such as hemorrhagic stroke and digestive tract malignancies[5-7].

Controversy has emerged regarding the association between LC and coronary artery disease (CAD) in recent decades[7-17]. Several comorbidities of cirrhotic patients have been regarded as cardioprotective factors (*e.g.*, low blood pressure, coagulopathy, thrombocytopenia, malnutrition, and lower serum cholesterol levels)[8,18-20]. However, case-controlled studies have revealed conflicting results and an increased prevalence of CAD in LC patients compared to the normal population[7,9,17,21,22]. A different etiology with an increased proportion of alcoholic and nonalcoholic steatohepatitis (NASH)-related LC in Western populations may have contributed to this result. Furthermore, the application of interferon and nucleot(s)ide analogues for viral hepatitis aided in prolonging survival in recent decades[23,24]. Age-related risks associated with CAD also may have led to an increased prevalence of CAD.

There is a paucity of available data regarding the association between nonalcoholic LC and CAD. Taiwan is an endemic area of viral hepatitis where the majority of LC in patients is caused by hepatitis B (HBV) and hepatitis C (HCV) viral infections[25]. Hence, the nonalcoholic LC population in Taiwan is an ideal cohort in which the confounding effect of alcohol consumption

is minimized.

The aim of our study was to elucidate the association between nonalcoholic LC and CAD using a national population-based database in Taiwan. Additionally, we analyzed the prevalence, hazard ratio (HR), and survival for concomitant comorbidities and complications.

MATERIALS AND METHODS

Study population and design

This study was a nationwide retrospective longitudinal population-based cohort study based on the Taiwanese National Health Insurance research database (NHIRD). The National Health Insurance program has provided compulsory medical insurance for greater than 99% of the Taiwanese population since March 1, 1995. We identified all LC patients with a first-time diagnosis of LC using International Classification of Disease, 9th Revision, Clinical Modification (ICD-9-CM) codes (ICD-9-CM code: 571.5) for the period from 2004 to 2011. The following patients were excluded from the study: patients who had LC or CAD before the end of 2005, had LC after diagnosis of CAD from 2006 to 2011, were younger than 40 years old, had a diagnosis of alcoholic LC (ICD-9-CM code: 571.2) or biliary LC (ICD-9-CM code: 571.6), and were outpatients with less than two follow-up visits after the first visit. The control subjects were matched with the patients in the study cohort at a ratio of five to one (5 control subjects *per* case patient) in terms of sex, age (40-49, 50-59, 60-69, and > 69 years), residence, and entry year.

Other comorbidity data and the ICD-9-CM codes are summarized in Table Appendix 1. The diagnosis of comorbidities was defined as having three outpatient visits or one admission. Hyperlipidemia was defined as having a lipid profile with any one of the following factors: total cholesterol (TC) $\geqslant$ 2000 mg/L, low-density lipoprotein cholesterol (LDL-C) $\geqslant$ 1600 mg/L, or a triglyceride (TG) level $\geqslant$ 1500 mg/L. Complications of LC that occurred after enrollment included esophageal varices (EV) (ICD-9-CM codes: 456-456.21), EV with bleeding (ICD-9-CM codes: 456.0, 456.20) concomitant with ICD-9 procedure codes for ligation of EV or sclerotherapy, ascites (ICD-9-CM codes: 789.5 and 568.82) or peritonitis (ICD-9-CM codes: 567.xx), and hepatic encephalopathy (ICD-9-CM code: 572.2).

End point measurement

We followed up all the subjects enrolled from the index date to the end of 2011. The mean follow-up period for the newly diagnosed nonalcoholic LC cohort (3409 patients) was 1152 $\pm$ 633 d with a median of 1169 d, maximum of 2920 d, and minimum of 7 d. Patients with newly diagnosed CAD (one inpatient or three outpatient codes) were categorized based on the first incidence of CAD. The first-time diagnosis of CAD was identified as the primary end point, and the death of the subject served as the secondary end point. All-cause mortality was analyzed in both the study and control cohorts.

Statistical analysis

Microsoft SQL Server 2008 R2 (Microsoft Corporation, Redmond, WA, United States) was used to perform data processing. Statistical analyses were conducted using SPSS software 19.0 (SPSS, Inc., Chicago, IL, United States). Chi-square tests and analysis of variance (ANOVA) were used to analyze the demographic data, concomitant comorbidities, and complications of cirrhosis. A Cox proportional hazards model was developed to calculate the overall mortality HRs of all comorbidities and complications in cirrhotic patients. The results are expressed in unadjusted and adjusted HRs (AHRs) with 95% confidence intervals (CI). The six-year cumulative survival and survival curve were calculated using the Cox regression method and Kaplan-Meier method. A two-tailed *P* value less than or equal to 0.05 was considered statistically significant.

RESULTS

Primary outcome: CAD was less prevalent in patients with nonalcoholic LC

A total of 10142 cases of LC were retrieved from one million random samples in the NHIRD from 2004 to 2011. To ensure that only newly diagnosed LC cases were included, we excluded patients who met the following criteria: LC diagnosed before the end of 2005, CAD occurred before LC, alcoholic or biliary LC, and age younger than 40 years old. Finally, a total of 3409 newly diagnosed nonalcoholic LC patients between 2006 and 2011 were identified and included in the study (Figure 1). To select age-, sex-, residence-, and entry year-matched controls, we further excluded 173 cirrhotic patients to achieve 100% randomization. A total of 3236 patients with nonalcoholic LC and 16180 matched controls were analyzed for all demographic characteristics (Table 1). Patients with nonalcoholic LC were more likely to have comorbidities including hemorrhagic stroke, hypertension, heart failure (HF), diabetes mellitus (DM), chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD) (all $P < 0.001$), and ischemic stroke ($P = 0.005$). In contrast, hyperlipidemia was less prevalent in nonalcoholic LC patients ($P < 0.001$). During six years of follow-up, 165 (5.1%) patients in the nonalcoholic LC cohort and 2814 (17.4%) subjects in the control cohort developed CAD ($P < 0.001$).

Nonalcoholic LC patients with and without CAD

As shown in Table 2, we stratified nonalcoholic LC patients ($n = 3409$) into two groups according to the presence of CAD. Nonalcoholic LC patients with CAD were older ($\geqslant$ 60 years old), more likely female, and exhibited increased concomitant comorbidities of ischemic stroke, hypertension, HF, DM, CKD, hyperlipidemia, and COPD (all $P < 0.001$). When we compared the complications of liver cirrhosis between the two groups, nonalcoholic LC patients with CAD had fewer LC-related complications, including EV with bleeding, ascites or peritonitis, and hepatic encephalo-

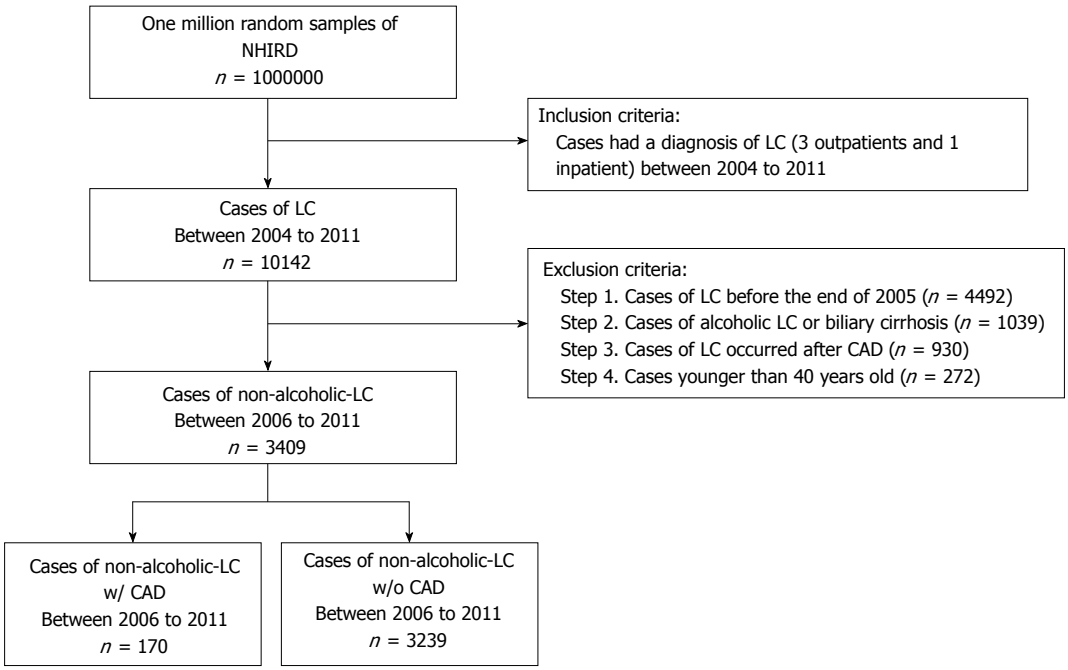


Figure 1 Study design.

Table 1 Characteristics of non-alcoholic liver cirrhosis cohort and the matched control cohort *n* (%)

Variable	Non-alcoholic LC patients, *n* = 3236	Control, *n* = 16180	*P* value
Sex			1.000
Male	2083 (64.4)	10415 (64.4)	
Female	1153 (35.6)	5765 (35.6)	
Age (yr)			1.000
40-49	565 (17.5)	2825 (17.5)	
50-59	885 (27.3)	4425 (27.3)	
60-69	766 (23.7)	3830 (23.7)	
> 69	1020 (31.5)	5100 (31.5)	
Residence			1.000
Metropolis	1834 (56.7)	9170 (56.7)	
General area	1362 (42.1)	6810 (42.1)	
Remote areas	40 (1.2)	200 (1.2)	
Coronary artery disease	165 (5.1)	2814 (17.4)	< 0.001
Cerebrovascular disease	413 (12.8)	1580 (9.8)	< 0.001
Hemorrhage	110 (3.4)	283 (1.7)	< 0.001
Ischemia	333 (10.3)	1413 (8.7)	0.005
Hypertension	1620 (50.1)	6672 (41.2)	< 0.001
Heart failure	252 (7.8)	972 (6.0)	< 0.001
Diabetes mellitus	1075 (33.2)	3141 (19.4)	< 0.001
Chronic kidney disease	616 (19.0)	1216 (7.5)	< 0.001
Hyperlipidemia	668 (20.6)	3903 (24.1)	< 0.001
Chronic obstructive pulmonary disease	534 (16.5)	1976 (12.2)	< 0.001

LC: Liver cirrhosis.

pathy (Supplementary Table 1). However, no significant differences were noted between the two groups.

Secondary outcome: Increased survival rate in the nonalcoholic LC with CAD group

We analyzed 3409 nonalcoholic LC patients and 16180 control subjects with a mean follow-up period of six years, and six-year survival rates were 50% and 85%, respectively ($P < 0.001$) (Figure 2A). When we compared nonalcoholic LC patients with and without CAD, the six-year survival rates were 52% and 50%, respectively ($P = 0.012$) (Figure 2B). The Cox regression proportional hazard model corresponding to each group also demonstrated a better survival rate for the nonalcoholic LC with CAD group than for those without CAD (AHR: 0.56; 95%CI: 0.43-0.74; $P < 0.001$) (Table 3).

HRs of mortality in LC patients with different comorbidities and complications

Table 3 presents the HRs of mortality in LC patients with different comorbidities. Increased HRs were observed for males, older patients, cerebrovascular disease (CVD), HF, DM, CKD and COPD. Among these factors, CKD exhibited the highest AHR of mortality (AHR: 1.76; 95%CI: 1.55-2.00). The AHRs of CAD, hypertension, and hyperlipidemia were reduced. Hyperlipidemia exhibited the lowest AHR of 0.52 (95%CI: 0.44-0.62).

When we analyzed the HRs of LC complications that occurred after enrollment, all complications exhibited increased values for both unadjusted HRs and AHRs. Ascites or peritonitis exhibited the highest AHR at 2.34 (95%CI: 2.06-2.65) (Supplementary Table 2).

Finally, we compared the effect of the number of comorbidities and complications on the survival rate in LC patients. Cirrhotic patients with one, two, or three comorbidities had HRs of 1.35, 1.72, and 1.95 (95%CI: 1.19-1.55, 1.47-2.00, and 1.57-2.43), respectively (Supplementary Table 3). However, the HRs for one, two, or t three LC complications were 3.56, 4.63, and 5.15 (95%CI: 3.14-4.03, 3.94-5.44, and 3.82-6.94), respectively (Supplementary Table 4).

Table 2 Characteristics of non-alcoholic liver cirrhosis patients with and without coronary artery disease *n* (%)

Variable	Non-alcoholic LC with CAD, *n* = 170	Non-alcoholic LC without CAD, *n* = 3239	*P* value
Sex			0.094
Male	99 (58.2)	2091 (64.6)	
Female	71 (41.8)	1148 (35.4)	
Age (yr)			< 0.001
40-49	18 (10.6)	554 (17.1)	
50-59	25 (14.7)	877 (27.1)	
60-69	46 (27.1)	751 (23.2)	
> 69	81 (47.6)	1057 (32.6)	
Residence			0.484
Metropolis	89 (52.4)	1830 (56.5)	
General area	79 (46.5)	1358 (41.9)	
Remote areas	2 (1.2)	51 (1.6)	
Cerebrovascular disease	46 (27.1)	400 (12.3)	< 0.001
Hemorrhage	9 (5.2)	103 (3.2)	0.132
Ischemia	37 (21.8)	327 (10.1)	< 0.001
Hypertension	139 (81.8)	1594 (49.2)	< 0.001
Heart failure	54 (31.8)	215 (6.6)	< 0.001
Diabetes mellitus	84 (49.4)	1051 (32.4)	< 0.001
Chronic renal failure	68 (40.0)	587 (18.1)	< 0.001
Hyperlipidemia	54 (31.8)	656 (20.3)	< 0.001
Chronic obstructive pulmonary disease	49 (28.8)	521 (16.1)	< 0.001

LC: Liver cirrhosis; CAD: Coronary artery disease.

DISCUSSION

In the present study, we observed a reduced prevalence of CAD and an increased survival rate for concomitant CAD in nonalcoholic LC patients. A lower prevalence of atherosclerosis and CAD was first described in an autopsy-based study in 1960[10]. A reduced prevalence of ischemic events was also reported for ischemic stroke[8,26]. However, conflicting data have been reported in some retrospective cohort and case-controlled studies[8,9,13,16,17,21,22]. First, the increased prevalence of CAD found in LC patients might be due to the different etiologies of LC in these Western population-based cohorts. An increased proportion of NASH and alcohol-related LC was noted compared to cohorts in viral hepatitis endemic areas[13,16,21,22]. NASH and alcohol consumption are generally associated with increased CAD risk factors. Nonetheless, the prevalence of CAD was reduced in end-stage liver disease patients assessed for liver transplantation who had alcoholic LC when compared to those with nonalcoholic LC[27]. Although moderate consumption of alcohol (< 30 g/d) has been reported as a protective factor against CAD[12], controversy still exists regarding heavy drinkers. In comparison, most subjects in our study had viral hepatitis-related LC. Few metabolic disorders were associated with steatohepatitis (such as hyperlipidemia), and no alcohol consumption was reported in our study cohort.

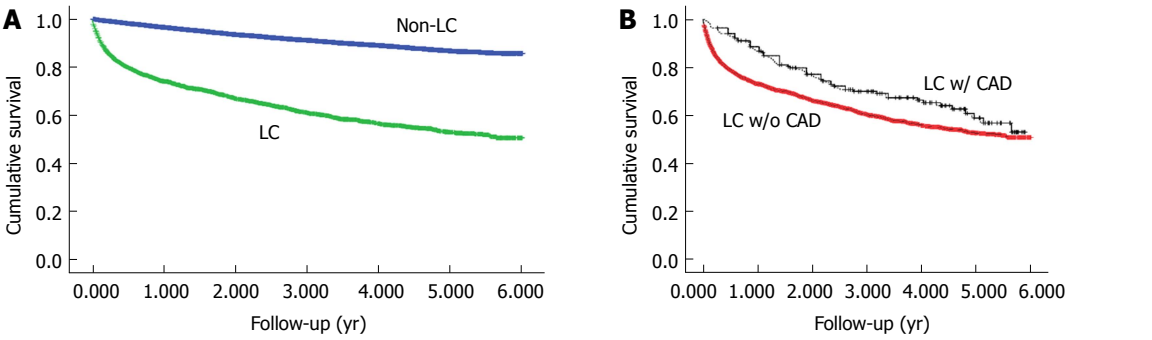
Second, lower blood pressure, better serum lipid profiles (including lower LDL-C, TC, and TG), and co-agulopathy in LC patients were regarded as protective factors against CAD[8,18-20,22]. However, viral hepatitis *per se* can influence vessel function and cause endothelial dysfunction, which is correlated with atherosclerotic disease progression[28]. A retrospective cohort study indicated that hepatitis C virus-infected subjects exhibited an increased risk for CAD, although better blood pressure and lipid profiles were observed in the population[29,30]. Consistent with previous reports, we observed a reduced prevalence of hyperlipidemia in cirrhotic patients. The proportion of comorbidities, including CVD, hypertension, HF, DM, CKD, hyperlipidemia, and COPD, was increased in the nonalcoholic LC with CAD patients[7]. The increase in these age-related comorbidities may be attributed to the older age in this study cohort. Third, CAD is an age-related disease. We hypothesize that better liver function may contribute to increased survival in the cirrhotic patients with CAD cohort. When survival is prolonged, age-related CAD risks also increase[31].

Ischemic stroke is a systemic thromboembolic event. Compared to normal subjects, the prevalence of ischemic stroke was reduced in cirrhotic patients[32]. In contrast, hemorrhagic events increased[7,33]. However, in our study, both ischemic and hemorrhagic stroke were significantly increased in nonalcoholic LC subjects. Of note, the prevalence of hemorrhagic stroke in non-alcoholic LC patients was two-fold higher than that in normal subjects (3.4% *vs* 1.7%, $P < 0.001$), which was considerably increased compared to ischemic stroke (10.3% *vs* 8.7%, $P = 0.005$). Older age and higher proportions of concomitant hypertension, DM, and COPD (as a result of smoking) in our nonalcoholic LC cohort might explain this result. These comorbidities have been reported as significant risk factors for all types of stroke[34].

We analyzed the association among comorbidities, complications, and HRs in LC patients. Consistent with previous studies, cirrhosis concomitant with CVD, HF, CKD, or COPD was regarded as a risk factor for mortality in both the Charlson comorbidity index and the Cirrhosis-specific Comorbidity Scoring System (CirCom)[35-37]. In addition, CKD had the highest HR in our study and exhibited increased risk scores in the two prognostic scoring systems. All the complications in LC patients were risk factors in our study. However, CAD, hypertension, and hyperlipidemia were associated with a lower HR and improved survival in this study. Although acute myocardial infarction was found to be a risk factor of mortality in nonalcoholic LC patients[36], the relationship among chronic stable CAD, hypertension, hyperlipidemia, and LC mortality remains unclear. Among these factors, cirrhotic patients with greater than three comorbidities exhibited the worst prognosis with at least a 1.954-fold increased risk of mortality.

Limitations

There were some limitations in our study. First, this is a retrospective cohort study. Although nonalcoholic LC and CAD subjects were retrieved from the Taiwanese



Survival rate/yr	0	1	2	3	4	5	6
Non-LC	0.97	0.93	0.91	0.89	0.87	0.85	0.85
LC	0.74	0.67	0.61	0.56	0.53	0.50	0.50

Survival rate/yr	0	1	2	3	4	5	6
LC w/ CAD	0.87	0.76	0.70	0.66	0.59	0.52	0.52
LC w/o CAD	0.73	0.66	0.60	0.56	0.52	0.50	0.50

Figure 2 Kaplan-Meier analysis of overall survival. A: Cumulative survival using the Kaplan-Meier method for nonalcoholic LC patients and controls during the follow-up period; B: Cumulative survival using the Kaplan-Meier method for nonalcoholic LC patients with and without CAD during the follow-up period. LC: Liver cirrhosis; CAD: Coronary artery disease.

Table 3 Hazard ratios of mortality in non-alcoholic liver cirrhosis patients with comorbidities using Cox regression model

Variable	Unadjusted hazard ratio			Adjusted hazard ratio[1]		
	Risk ratio	95%CI	*P* value	Risk ratio	95%CI	*P* value
Sex						
Male	1.20	1.07-1.35	0.002	1.34	1.19-1.51	< 0.001
Female (reference)						
Age (yr)						
40-49 (reference)						
50-59	1.04	0.85-1.27	0.688	1.08	0.89-1.32	0.446
60-69	1.29	1.06-1.57	0.013	1.40	1.14-1.72	< 0.001
> 69	2.51	2.10-3.00	< 0.001	2.73	2.25-3.30	< 0.001
Coronary artery disease						
No (reference)						
Yes	0.71	0.55-0.93	0.012	0.56	0.43-0.74	< 0.001
Cerebrovascular disease						
No (reference)						
Yes	1.25	1.08-1.45	0.004	1.08	0.93-1.27	0.323
Hypertension						
No (reference)						
Yes	0.98	0.88-1.09	0.709	0.75	0.66-0.85	< 0.001
Heart failure						
No (reference)						
Yes	1.50	1.26-1.78	< 0.001	1.23	1.02-1.48	0.027
Diabetes mellitus						
No (reference)						
Yes	1.03	0.92-1.16	0.609	1.12	0.99-1.26	0.082
Chronic kidney disease						
No (reference)						
Yes	1.89	1.67-2.13	< 0.001	1.76	1.55-2.00	< 0.001
Hyperlipidemia						
No (reference)						
Yes	0.51	0.44-0.60	< 0.001	0.52	0.44-0.62	< 0.001
Chronic obstructive pulmonary disease						
No (reference)						
Yes	1.30	1.13-1.48	< 0.001	0.99	0.858-1.141	0.888

[1]Adjustments were made for sex, age, coronary heart disease, cerebrovascular disease, hypertension, heart failure, diabetes mellitus, chronic kidney disease, hyperlipidemia, and chronic obstructive pulmonary disease.

NHIRD database according to ICD-9 codes, data were lacking regarding the severity of cirrhosis (*e.g.*, Child-Turcotte-Pugh scores). In this study, we excluded patients who had less than three outpatient visits with the same diagnosis. Furthermore, we analyzed the complications of cirrhosis to evaluate the severity. Second, the severity of concomitant comorbidities, such as hypertension, DM, and hyperlipidemia, cannot be evaluated. In addition, other known risk factors, such as smoking status and family history of CAD, were not recorded in the database. Third, the severity of CAD was not recorded in the database. A prospective case-

controlled study revealed an increased prevalence of nonobstructive CAD (*i.e.,* narrowing < 50%) in cirrhotic patients compared to normal controls using computerized coronary angiography. This feature may contribute to the increased survival rates for LC patients with CAD. The severity of CAD should be further evaluated in future studies[17]. Finally, the different follow-up periods for each patient may impact the HRs of mortality in nonalcoholic LC patients.

In conclusion, CAD was less prevalent in nonalcoholic LC patients. Among cirrhotic patients, an increased number of concomitant comorbidities was found in cirrhotic patients with CAD. However, improved survival was found in nonalcoholic LC patients with CAD when compared to those without CAD.

ARTICLE HIGHLIGHTS

Research background

The risk of mortality in nonalcoholic liver cirrhosis (LC) patients with coronary artery disease (CAD) is unclear. Previous case-control studies demonstrated conflicting results potentially due to the different etiologies of LC. LC patients with alcoholic and nonalcoholic fatty liver disease-related metabolic disorders exhibit an increased risk of CAD. In contrast, hemostatic defects that occur in LC, such as thrombocytopenia, coagulopathy, and low blood pressure, are not considered as potential protective factors of cirrhosis against atherosclerotic events.

Research motivation

The results of CAD risk in LC patients are controversial. In contrast to the present study, previous works using alcoholic LC-based cohorts may have been confounded by the increased risk of metabolic syndrome in heavy drinkers. To the best of our knowledge, the risk of CAD in nonalcoholic cirrhotic patients is not well established.

Research objectives

The aim of the study was to elucidate the prevalence and risk of mortality in nonalcoholic cirrhotic patients. The comorbidities and LC-related complications were also important prognostic factors among cirrhotic patients. The result of this study can provide a better understanding of CAD risk in LC patients.

Research methods

We collected 10142 LC patients diagnosed from 2004 to 2011 using the Taiwanese National Health Insurance research database. After exclusion of subjects who were treated before the end of 2005, had alcoholic or biliary cirrhosis, had LC occurring after CAD, and were younger than 40 years old, a total of 3409 LC patients were enrolled in the study. The comorbidities and complications of LC were collected. The first-time diagnosis of CAD was identified as the primary end point, and the death of the subject served as the secondary end point. All-cause mortality was analyzed in both the study and control cohorts. A Cox proportional hazards model was developed to calculate the overall mortality hazard ratios of all comorbidities and complications in cirrhotic patients. The six-year cumulative survival and survival curve were calculated using the Cox regression method and the Kaplan-Meier method.

Research results

CAD was less prevalent in nonalcoholic LC patients than in controls. Nonalcoholic LC patients with CAD were associated with a reduced risk of mortality. The six-year survival rates were increased in patients with CAD compared to patients without CAD in the nonalcoholic LC cohort. As this is a retrospective cohort study, further prospective studies are needed to confirm this finding.

Research conclusions

In this study, we demonstrate that CAD is less prevalent and associated with a reduced risk for mortality in nonalcoholic LC patients. This result confirms previous studies regarding the lower risk for atherosclerotic events (*i.e.* ischemic stroke and coronary artery disease) in alcohol-related and nonalcohol-related LC cohorts. Although viral hepatitis can cause endothelial dysfunction, which correlates with atherosclerotic disease progression, we propose that LC has a more powerful protective effect against atherosclerotic events based on the favorable cardiovascular risk profiles, such as thrombocytopenia, coagulopathy, and low blood pressure. The strengths of the study include the large sample size cohort and risk adjustments for comorbidities and complications. Finally, we conclude that nonalcoholic LC patients with CAD exhibit a favorable outcome.

Research perspectives

Future prospective research should focus on the advantages and disadvantages of antiplatelet therapy in the prevention of CAD in nonalcoholic LC patients. Additionally, it is advised that future studies should compare alcoholic and nonalcoholic LC cohorts and evaluate the effect of viral treatment.

REFERENCES

1 **Lozano R**, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De León FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2095-2128 [PMID: 23245604 DOI: 10.1016/S0140-6736(12)61728-0]

2 **Department of Health EY**, ROC. Statistics of Causes of Death. 2011

3 **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

4 **Amin J**, Law MG, Bartlett M, Kaldor JM, Dore GJ. Causes of death after diagnosis of hepatitis B or hepatitis C infection: a large community-based linkage study. *Lancet* 2006; **368**: 938-945 [PMID: 16962883 DOI: 10.1016/S0140-6736(06)69374-4]

5 **Sun LM**, Lin MC, Lin CL, Liang JA, Jeng LB, Kao CH, Lu

CY. Nonalcoholic Cirrhosis Increased Risk of Digestive Tract Malignancies: A Population-Based Cohort Study. *Medicine* (Baltimore) 2015; **94**: e2080 [PMID: 26656334 DOI: 10.1097/MD.0000000000002080]
6 **Jepsen P**, Lash TL, Vilstrup H. The clinical course of alcoholic cirrhosis: development of comorbid diseases. A Danish nationwide cohort study. *Liver Int* 2016; **36**: 1696-1703 [PMID: 27124269 DOI: 10.1111/liv.13151]
7 **Parikh NS**, Navi BB, Kumar S, Kamel H. Association between Liver Disease and Intracranial Hemorrhage. *J Stroke Cerebrovasc Dis* 2016; **25**: 543-548 [PMID: 26679070 DOI: 10.1016/j.jstrokecerebrovasdis.2015.11.005]
8 **Berzigotti A**, Bonfiglioli A, Muscari A, Bianchi G, Libassi S, Bernardi M, Zoli M. Reduced prevalence of ischemic events and abnormal supraortic flow patterns in patients with liver cirrhosis. *Liver Int* 2005; **25**: 331-336 [PMID: 15780058 DOI: 10.1111/j.1478-3231.2005.01002.x]
9 **Tiukinhoy-Laing SD**, Rossi JS, Bayram M, De Luca L, Gafoor S, Blei A, Flamm S, Davidson CJ, Gheorghiade M. Cardiac hemodynamic and coronary angiographic characteristics of patients being evaluated for liver transplantation. *Am J Cardiol* 2006; **98**: 178-181 [PMID: 16828588 DOI: 10.1016/j.amjcard.2006.01.089]
10 **HOWELL WL**, MANION WC. The low incidence of myocardial infarction in patients with portal cirrhosis of the liver: A review of 639 cases of cirrhosis of the liver from 17,731 autopsies. *Am Heart J* 1960; **60**: 341-344 [PMID: 14403495 DOI: 10.1016/0002-8703(60)90192-7]
11 **Carey WD**, Dumot JA, Pimentel RR, Barnes DS, Hobbs RE, Henderson JM, Vogt DP, Mayes JT, Westveer MK, Easley KA. The prevalence of coronary artery disease in liver transplant candidates over age 50. *Transplantation* 1995; **59**: 859-864 [PMID: 7701580 DOI: 10.1097/00007890-199503270-00010]
12 **Mukamal KJ**, Rimm EB. Alcohol's effects on the risk for coronary heart disease. *Alcohol Res Health* 2001; **25**: 255-261 [PMID: 11910702]
13 **Kalaitzakis E**, Björnsson E. Coronary artery disease in liver cirrhosis: does the aetiology of liver disease matter? *J Hepatol* 2009; **51**: 962-963; author reply 963-964 [PMID: 19692136 DOI: 10.1016/j.jhep.2009.06.012]
14 **Marui A**, Kimura T, Tanaka S, Miwa S, Yamazaki K, Minakata K, Nakata T, Ikeda T, Furukawa Y, Kita T, Sakata R; CREDO-Kyoto Investigators. Coronary revascularization in patients with liver cirrhosis. *Ann Thorac Surg* 2011; **91**: 1393-1399 [PMID: 21396626 DOI: 10.1016/j.athoracsur.2011.01.022]
15 **Raval Z**, Harinstein ME, Skaro AI, Erdogan A, DeWolf AM, Shah SJ, Fix OK, Kay N, Abecassis MI, Gheorghiade M, Flaherty JD. Cardiovascular risk assessment of the liver transplant candidate. *J Am Coll Cardiol* 2011; **58**: 223-231 [PMID: 21737011 DOI: 10.1016/j.jacc.2011.03.026]
16 **Gologorsky E**, Pretto EA Jr, Fukazawa K. Coronary artery disease and its risk factors in patients presenting for liver transplantation. *J Clin Anesth* 2013; **25**: 618-623 [PMID: 23994032 DOI: 10.1016/j.jclinane.2013.06.001]
17 **An J**, Shim JH, Kim SO, Lee D, Kim KM, Lim YS, Lee HC, Chung YH, Lee YS. Prevalence and prediction of coronary artery disease in patients with liver cirrhosis: a registry-based matched case-control study. *Circulation* 2014; **130**: 1353-1362 [PMID: 25095888 DOI: 10.1161/CIRCULATIONAHA.114.009278]
18 **Tripodi A**, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011; **365**: 147-156 [PMID: 21751907 DOI: 10.1056/NEJMra1011170]
19 **D'Arienzo A**, Manguso F, Scaglione G, Vicinanza G, Bennato R, Mazzacca G. Prognostic value of progressive decrease in serum cholesterol in predicting survival in Child-Pugh C viral cirrhosis. *Scand J Gastroenterol* 1998; **33**: 1213-1218 [PMID: 9867102 DOI: 10.1080/00365529850172593]
20 **Lisman T**, Caldwell SH, Burroughs AK, Northup PG, Senzolo M, Stravitz RT, Tripodi A, Trotter JF, Valla DC, Porte RJ; Coagulation in Liver Disease Study Group. Hemostasis and thrombosis in patients with liver disease: the ups and downs. *J Hepatol* 2010; **53**: 362-371 [PMID: 20546962 DOI: 10.1016/j.jhep.2010.01.042]
21 **Kadayifci A**, Tan V, Ursell PC, Merriman RB, Bass NM. Clinical and pathologic risk factors for atherosclerosis in cirrhosis: a comparison between NASH-related cirrhosis and cirrhosis due to other aetiologies. *J Hepatol* 2008; **49**: 595-599 [PMID: 18662837 DOI: 10.1016/j.jhep.2008.05.024]
22 **Kalaitzakis E**, Rosengren A, Skommevik T, Björnsson E. Coronary artery disease in patients with liver cirrhosis. *Dig Dis Sci* 2010; **55**: 467-475 [PMID: 19242795 DOI: 10.1007/s10620-009-0738-z]
23 **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]
24 **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]
25 **Mokdad AA**, Lopez AD, Shahraz S, Lozano R, Mokdad AH, Stanaway J, Murray CJ, Naghavi M. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. *BMC Med* 2014; **12**: 145 [PMID: 25242656 DOI: 10.1186/s12916-014-0145-y]
26 **Kane WC**, Aronson SM. Cerebrovascular disease in an autopsy population. 4. Reduced frequency of stroke in patients with liver cirrhosis. *Trans Am Neurol Assoc* 1971; **96**: 259-260 [PMID: 5159099]
27 **Patel S**, Kiefer TL, Ahmed A, Ali ZA, Tremmel JA, Lee DP, Yeung AC, Fearon WF. Comparison of the frequency of coronary artery disease in alcohol-related versus non-alcohol-related endstage liver disease. *Am J Cardiol* 2011; **108**: 1552-1555 [PMID: 21890080 DOI: 10.1016/j.amjcard.2011.07.013]
28 **Ciccone MM**, Principi M, Ierardi E, Di Leo A, Ricci G, Carbonara S, Gesualdo M, Devito F, Zito A, Cortese F, Scicchitano P. Inflammatory bowel disease, liver diseases and endothelial function: is there a linkage? *J Cardiovasc Med* (Hagerstown) 2015; **16**: 11-21 [PMID: 25427048 DOI: 10.2459/JCM.0000000000000149]
29 **Butt AA**, Xiaoqiang W, Budoff M, Leaf D, Kuller LH, Justice AC. Hepatitis C virus infection and the risk of coronary disease. *Clin Infect Dis* 2009; **49**: 225-232 [PMID: 19508169 DOI: 10.1086/599371]
30 **Roed T**, Lebech AM, Kjaer A, Weis N. Hepatitis C virus infection and risk of coronary artery disease: a systematic review of the literature. *Clin Physiol Funct Imaging* 2012; **32**: 421-430 [PMID: 23031062 DOI: 10.1111/j.1475-097X.2012.01152.x]
31 **Savji N**, Rockman CB, Skolnick AH, Guo Y, Adelman MA, Riles T, Berger JS. Association between advanced age and vascular disease in different arterial territories: a population database of over 3.6 million subjects. *J Am Coll Cardiol* 2013; **61**: 1736-1743 [PMID: 23500290 DOI: 10.1016/j.jacc.2013.01.054]
32 **Chen YH**, Chen KY, Lin HC. Non-alcoholic cirrhosis and the risk of stroke: a 5-year follow-up study. *Liver Int* 2011; **31**: 354-360 [PMID: 20860634 DOI: 10.1111/j.1478-3231.2010.02350.x]
33 **Lai CH**, Cheng PY, Chen YY. Liver cirrhosis and risk of intracerebral hemorrhage: a 9-year follow-up study. *Stroke* 2011; **42**: 2615-2617 [PMID: 21757678 DOI: 10.1161/STROKEAHA.111.617076]
34 **O'Donnell MJ**, Xavier D, Liu L, Zhang H, Chin SL, Rao-Melacini P, Rangarajan S, Islam S, Pais P, McQueen MJ, Mondo C, Damasceno A, Lopez-Jaramillo P, Hankey GJ, Dans AL, Yusoff K, Truelsen T, Diener HC, Sacco RL, Ryglewicz D, Czlonkowska A, Weimar C, Wang X, Yusuf S; INTERSTROKE investigators. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. *Lancet* 2010; **376**: 112-123 [PMID: 20561675 DOI: 10.1016/S0140-6736(10)60834-3]
35 **Charlson ME**, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373-383 [PMID: 3558716 DOI: 10.1016/0021-9681(87)90171-8]

36 **Jepsen P**, Vilstrup H, Andersen PK, Lash TL, Sørensen HT. Comorbidity and survival of Danish cirrhosis patients: a nationwide population-based cohort study. *Hepatology* 2008; **48**: 214-220 [PMID: 18537190 DOI: 10.1002/hep.22341]

37 **Jepsen P**, Vilstrup H, Lash TL. Development and validation of a comorbidity scoring system for patients with cirrhosis. *Gastroenterology* 2014; **146**: 147-156; quiz e15-e16 [PMID: 24055278 DOI: 10.1053/j.gastro.2013.09.019]

P- Reviewer: Chang ST, Ciccone MM, Posadas-Sánchez R
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 August 21; 24(31): 3556-3566

DOI: 10.3748/wjg.v24.i31.3556

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

ORIGINAL ARTICLE

Prospective Study

PillCamColon2 after incomplete colonoscopy - A prospective multicenter study

Peter Baltes, Marc Bota, Jörg Albert, Michael Philipper, Hans-Georg Hörster, Friedrich Hagenmüller, Ingo Steinbrück, Ralf Jakobs, Matthias Bechtler, Dirk Hartmann, Horst Neuhaus, Jean-Pierre Charton, Rupert Mayershofer, Horst Hohn, Thomas Rösch, Stefan Groth, Tanja Nowak, Peter Wohlmuth, Martin Keuchel

Peter Baltes, Marc Bota, Martin Keuchel, Clinic for Internal Medicine, Bethesda Krankenhaus Bergedorf, Hamburg 21029, Germany

Jörg Albert, Department of Internal Medicine I, Klinikum der JW. Goethe Universität, Frankfurt 60590, Germany

Michael Philipper, Gastroenterologische Schwerpunktpraxis, Düsseldorf 40227, Germany

Hans-Georg Hörster, Gastropraxis Rheydt, Mönchengladbach 41236, Germany

Friedrich Hagenmüller, Ingo Steinbrück, 1st Medical Department, Asklepios Klinikum Altona, Hamburg 22763, Germany

Ralf Jakobs, Matthias Bechtler, Medical Clinic C, Klinikum der Stadt Ludwigshafen, Ludwigshafen 67063, Germany

Dirk Hartmann, Clinic for Internal Medicine, Sana Klinikum Lichtenberg, Berlin 10365, Germany

Horst Neuhaus, Jean-Pierre Charton, Clinic for Internal Medicine, Evangelisches Krankenhaus, Düsseldorf 40217, Germany

Rupert Mayershofer, Gastroenterologie am Burgweiher, Bonn 53123, Germany

Horst Hohn, Schwerpunktpraxis Gastroenterologie, Koblenz 56068, Germany

Thomas Rösch, Stefan Groth, Clinic for Interdisciplinary Endoscopy, University Hospital Eppendorf, Hamburg 20251, Germany

Tanja Nowak, CorporateHealth International, Hamburg 20149, Germany

Peter Wohlmuth, Biometry and Data Management, Asklepios Proresearch, Hamburg 20099, Germany

ORCID number: Peter Baltes (0000-0001-6318-6015); Marc Bota (0000-0003-0265-8642); Jörg Albert (0000-0002-3420-7371); Michael Philipper (0000-0002-9557-9662); Hans-Georg Hörster (0000-0003-0563-6596); Friedrich Hagenmüller (0000-0001-7436-8527); Ingo Steinbrück (0000-0002-8861-2903); Ralf Jakobs (0000-0002-1902-5692); Matthias Bechtler (0000-0002-2449-5373); Dirk Hartmann (0000-0002-9783-6431); Horst Neuhaus (0000-0001-6200-0688); Jean-Pierre Charton (0000-0003-4654-3062); Rupert Mayershofer (0000-0001-8310-1381); Horst Hohn (0000-0001-7931-6910); Thomas Rösch (0000-0003-2270-2495); Stefan Groth (0000-0003-2022-8806); Tanja Nowak (0000-0002-2648-087X); Peter Wohlmuth (0000-0002-1636-7865); Martin Keuchel (0000-0001-5288-4993).

Author contributions: Albert J, Hagenmüller F, Jakobs R, Hartmann D, Neuhaus H, Wohlmuth P and Keuchel M designed the study; Baltes P, Bota M, Albert J, Philipper M, Hörster HG, Steinbrück I, Bechtler M, Hartmann D, Charton JP, Mayershofer R, Hohn H and Keuchel M collected, analysed and reviewed the data; Baltes P, Bota M and Keuchel M managed the study; Baltes P, Bota M, Nowak T and Keuchel M wrote the manuscript; Rösch T and Groth S revised the manuscript; all authors read and approved the final version of the manuscript.

Institutional review board statement: The research was approved by the Ethics Committee of Hamburg Chamber of Physicians.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Peter Baltes, Michael Philliper, Ingo Steinbrück, and Horst Hohn received lecture fees from Given Imaging, Jörg Albert, Martin Keuchel received study support and lecture fees from Given Imaging, Tanja Nowak was an employee of Given Imaging, the other authors have nothing to declare. The study was supported by Given Imaging, Hamburg, Germany by providing the capsules and an unrestricted grant.

CONSORT 2010 statement: The authors have read the

CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

Manuscript source: Unsolicited manuscript

Correspondence to: Peter Baltes, MD, Doctor, Senior Consultant, Clinic for Internal Medicine, Bethesda Krankenhaus Bergedorf, Akademisches Lehrkrankenhaus der Universität Hamburg, Glindersweg 80, Hamburg 21029, Germany. baltes@bkb.info
Telephone: +49-40-725541201
Fax: +49-40-725541202

Received: June 9, 2018
Peer-review started: June 9, 2018
First decision: July 4, 2018
Revised: July 18, 2018
Accepted: July 21, 2018
Article in press: July 21, 2018
Published online: August 21, 2018

Abstract

AIM

To evaluate the ability of PillCamColon2 to visualize colonic segments missed by incomplete optical colonoscopy (OC) and to assess the diagnostic yield.

METHODS

This prospective multicentre study included 81 patients from nine centres who underwent second-generation colon capsule endoscopy (CCE) following incomplete OC performed by an experienced gastroenterologist (> 1000 colonoscopies). Patients with stenosis were excluded. According to patient preferences, CCE was performed the following day (protocol A) after staying on clear liquids and 0.75 L Moviprep in the morning or within 30 d after new split-dose Moviprep (protocol B). Boosts consisted of 0.75 L and 0.25 L Moviprep, and phospho-soda was given as a rescue if the capsule was not excreted after seven hours.

RESULTS

Seventy-four patients were analysed (51% of them in group A; 49% in group B). Bowel cleansing was adequate in 67% of cases, and CCE could visualize colonic segments missed by incomplete colonoscopy in 90% of patients under protocol A and 97% of patients under protocol B (P = 0.35, n.s.). Significant polyps including adenocarcinoma were detected in 24% of cases. Detection rates for all polyps and significant polyps per patient were similar in both protocols. Polyps were found predominantly in the right colon (86%) in segments that were not reached by OC. Extracolonic findings - such as reflux esophagitis, suspected Barrett esophagus, upper GI-bleeding, gastric polyps, gastric erosions and angiectasia - were detected in eight patients. PillCamColon2 capsule was retained in the ileum of one patient (1.4%) without symptoms and removed during an uneventful resection for unknown Crohn's disease that was diagnosed as the cause of anemia, which was the indication for colonoscopy. CCE was well tolerated. One patient suffered from self-limiting vomiting after consuming the phospho-soda.

CONCLUSION

Second-generation CCE using a low-volume preparation is useful after incomplete OC, and it allows for the detection of additional relevant findings, but cleansing efficiency could be improved.

Key words: Colon capsule endoscopy; PillCamColon2; Incomplete colonoscopy; Low volume prep; Moviprep; Phospho-soda; Cleanliness level; Complementation rate; Polyps

Core tip: Colonoscopy is the gold standard for visualization of the colon, but it may be incomplete, not reaching the cecum. Second-generation colon capsule endoscopy (CCE) with low-volume preparations could complement incomplete colonoscopies in 90% of cases, and it could help to detect additional relevant colonic and extracolonic findings. Protocols with either CCE the day following an incomplete colonoscopy or within 30 d after a new low-volume preparation were both feasible and well tolerated; however, the protocols could be improved with respect to bowel cleanliness and complete colon visualization.

Baltes P, Bota M, Albert J, Philipper M, Hörster HG, Hagenmüller F, Steinbrück I, Jakobs R, Bechtler M, Hartmann D, Neuhaus H, Charton JP, Mayershofer R, Hohn H, Rösch T, Groth S, Nowak T, Wohlmuth P, Keuchel M. PillCamColon2 after incomplete colonoscopy - A prospective multicenter study. *World J Gastroenterol* 2018; 24(31): 3556-3566 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i31/3556.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i31.3556

INTRODUCTION

Flexible optical colonoscopy (OC) is the gold standard to detect and treat colorectal diseases and proved to be safe and effective in colorectal cancer screening (CRC)[1]. However, OC may be incomplete for various reasons, with completion rates between 90% and 98%[2-5] for screening colonoscopy and 81%-94% in mixed series[6-9]. Risk factors for incomplete colonoscopy

are female gender, elder patients, previous pelvic or abdominal surgery, diverticulosis, redundant colon, stenosing tumors, poor preparation, inflammatory bowel disease; in contrast sedation, male gender and higher body mass index were reported as protective factors[4,6,7,10,11]. Of the patients with incomplete OC, 4.3% had advanced neoplasia in the right colon[5]. A five-year follow-up study showed a higher risk for colorectal cancer in patients with failed colonoscopy[12], demonstrating the need for additional investigations in these patients. PillCamColon has been cleared by the US Food and Drug Administration (FDA) for detection of colon polyps in patients after an incomplete optical colonoscopy with adequate preparation. Later the indication had been expanded to the detection of colon polyps in patients with evidence of lower gastrointestinal origin and major risks for colonoscopy or moderate sedation.

First-generation PillCamColon1 with a constant rate of two frames per second (fps) had limited sensitivity compared to a colonoscopy[13-15]. Technical improvements of the second-generation PillCamColon2 that was used in the present study included adaptive frame rates (4 fps and 35 fps) and an increased viewing angle. It also included automatic small bowel detection with consecutive timing of boosters to shorten transit times and to improve cleansing levels[16], which was shown to have a significantly higher diagnostic yield for polyps[17]. A decisive factor for CCE accuracy is bowel cleansing; recent data have shown that low-volume Polyethylene glycol (PEG)-based protocols may be feasible instead of high-volume preparations[18,19].

Our study aimed to assess the impact of second-generation CCE after incomplete colonoscopies by analyzing the complementation rates and incremental diagnostic yields with two different low-volume cleansing protocols. Patients were offered to either stay on clear liquids with a CCE the next day or to start a new bowel preparation procedure within 30 d.

MATERIALS AND METHODS

Study design and population

This prospective, multicentre study including nine centres (tertiary care hospitals or private endoscopy offices) in Germany. It was approved by the ethics committee of Hamburg Chamber of Physicians (PV3467, 20.05.2010). The www.clinicaltrials.gov identifier is NCT01480635.

All patients included in the study were 18 years or older who had incomplete colonoscopies that were performed by an experienced endoscopist (> 1000 colonoscopies performed). Incomplete OC was defined as a failure to reach the cecum or ileo-cecal anastomosis due to looping, bowel angulation, adhesions, and intolerance of sedation or inflammation. The presumed area of the colon reached by OC was documented, as were reasons for termination, detection of polyps and tumours, other findings and adverse events. Patients with stenosis, inadequate preparation, or exchange of endoscope were excluded. Other exclusion criteria for CCE have been described previously[18]. Written informed consent was obtained from all patients.

Study protocol

After an incomplete colonoscopy, patients were rescheduled for the following day (protocol A) or they were given a separate appointment within 30 d (protocol B), according to their preference. CCE was performed with PillCamColon2 (Given Imaging, Yoqneam, Israel) after a low-volume cleansing regimen with PEG and ascorbic acid (Moviprep, Norgine, Marburg/Lahn, Germany). Following capsule ingestion, the boosts consisted of 0.75 L after small bowel detection and 0.25 L Moviprep if the capsule had not been excreted five hours after ingestion. 30 mL sodium picosulfate (NaP; Fleet, Recordati, Ulm, Germany) was used as an additional boost if the capsule was not excreted seven hours after ingestion. In protocol A, patients stayed on clear liquids after the colonoscopy, and they received an additional cleansing of 0.75 L Moviprep the next morning (at the latest, one hour before CCE). In protocol B, patients were allowed to eat after the OC. A new bowel cleansing procedure was performed within 30 d: split-dose 1 L of Moviprep was consumed in the evening and 1 L was consumed in the morning, each followed by 1 L of water. Boosts with Moviprep or NaP were identical in both protocols (Table 1).

CCE studies were read with Rapid7 or Rapid8 software in each centre by an experienced endoscopist with additional experience in capsule endoscopy (> 1000 colonoscopies, > 100 small bowel capsule endoscopies (SBCE) and > 25 CCEs performed). All readers had completed a dedicated two-day CCE evaluation course. Polyp size was estimated with the integrated software tool.

For both examination modalities, cleansing levels were documented, as described previously[15], using four grades: excellent, good (adequate), fair and poor (inadequate). Each colon segment [that is, cecum, ascending colon (AC), transverse colon (TC), left colon (LC) and rectum, see Figure 1] and overall cleansing status were evaluated.

For CCE, visualization of colonic segments, complementation of previous colonoscopy, completeness of CCE, and adverse events were recorded. Complete CCE was defined as excretion of capsule during recording time or by identification of the haemorrhoidal plexus. Detection of polyps, significant polyps, tumours or other relevant findings were documented for segments reached and not reached by the previous standard colonoscopy. According to previous studies, significant polyps were defined by size (⩾ 6 mm) or number (⩾ 3)[19-21]. Other findings were considered important if they explained the indication for the colonoscopy or if they had further diagnostic or therapeutic implications.

Table 1 Study protocols A and B for colon capsule endoscopy after incomplete optical colonoscopy

Time	Procedure	
Evening and morning before colonoscopy	Standard bowel prep for colonoscopy	
	Incomplete colonoscopy (OC)	
After incomplete colonoscopy	Patient´s choice of protocol A or B for colon capsule endoscopy (CCE)	
	Protocol A (CCE next day)	**Protocol B** (CCE within 30 d)
After incomplete colonoscopy	Patient stays on clear liquids	Patient can eat
2 d before CCE		Low residue diet
Day before CCE	NA	Clear liquids only
Evening before CCE		1 L Moviprep + 1 L water
Morning of CCE	0.75 L Moviprep + water	1 l Moviprep + 1 L water
	Colon capsule ingestion	
Small bowel detection	0.75 L Moviprep + water (1st boost)	
5 h after ingestion	0.5 L Moviprep + water (2nd boost)[1]	
7 h after ingestion	30 mL NaP + water (´Rescue boost)[1]	
	Bisacodyl supp[1]	
11 h after ingestion	Removal of equipment[1]	

[1]If capsule is not excreted earlier. OC: Optical colonoscopy.

Follow-up took place via a telephone call one week after the procedure. During the call, adverse events were documented, and capsule excretion was confirmed. Any adverse events were recorded, as well as the time of capsule excretion.

Study endpoints

The primary outcome parameter was complementation rate of CCE in patients with incomplete OC, which was defined by visualization of colonic segments not reached by OC. The secondary outcome parameters were as follows: additional (incremental) diagnostic yield of CCE compared to incomplete OC (all polyps, significant polyps defined by size or number and other significant findings) in segments not reached by OC; CCE findings in the upper gastrointestinal (GI) tract and small bowel; rate of complete CCE, as defined above; cleansing level of the colon (overall and segments) following the low-volume protocol for CCE; visualization of the Z-Line; and adverse events (number, type and severity).

Statistical analysis

Continuous variables were reported as mean and standard deviation, and categorical variables were reported as percentage. The null hypothesis (H0) for the primary endpoint (complementation of incomplete colonoscopy) was constructed using data from a former study that used PillCamColon1 and that showed a complementation rate of 50%[22]. Accordingly, H0 was set to $\mu = 0.5$ with an expectation for complementation of OC by PillCamColon2 of 0.67. Using the Fisher exact test with a power of 80% (which is equal to 1-β), 74 patients had to be recruited. Categorical values were compared using a chi-squared test (χ^2), while continuous values with a normal distribution were compared by a Student´s *t*-test. *P* values < 0.05 were considered significant. An intention-to-treat (ITT) analysis was performed for complementation rates, cleansing levels, detection of significant polyps and safety. Statistical review was performed by one of the authors (Peter Wohlmuth).

RESULTS

Demographics

Eighty-one consecutive patients were enrolled from nine participating centres between 2010 and 2013. Seven patients (four in the protocol A group and three in the protocol B group) had to be excluded due to technical failure (*n* = 1), protocol noncompliance as a result of incorrect timing of CCE (*n* = 4), exchange of colonoscope (*n* = 1) or early removal of recorder by the patient (*n* = 1) (Figure 2). In total, data of 74 patients were analysed per protocol. Demographics are shown in Table 2.

Reasons for referral to colonoscopy were CRC screening (22%), anemia (15%), hematochezia (15%), irregular stool (12%), abdominal pain (12%), B symptoms (7%), colitis (5%) and other reasons (12%). Thirty-six patients (48.6%) had prior abdominal surgery, while 14 patients (19%) had more than one surgical intervention. Most common surgeries were appendectomies (23%) and hysterectomies (19%). Only three patients had colonic surgery (4%), one patient had an ileocecal and one patient had a Billroth Ⅱ anastomosis.

Standard OC

An experienced endoscopist (> 1000 colonoscopies performed) performed OC with a standard colonoscope. The mean duration of the procedure was 45 ± 17 min (range: 15-101 min). Unfavourable anatomy was the reason for termination in 92% of procedures. OC reached the sigmoid colon in 27% of cases, the descending colon (DC) in 4% of cases, the splenic flexure in 12% of cases, the TC in 14% of cases, the hepatic flexure in 35% of cases and the AC in 8% of cases. Adequate cleansing was achieved in 76% of procedures. In 12 of 74 patients (16%), polyps were detected, with a mean size of 6 ± 4.2 mm. Six

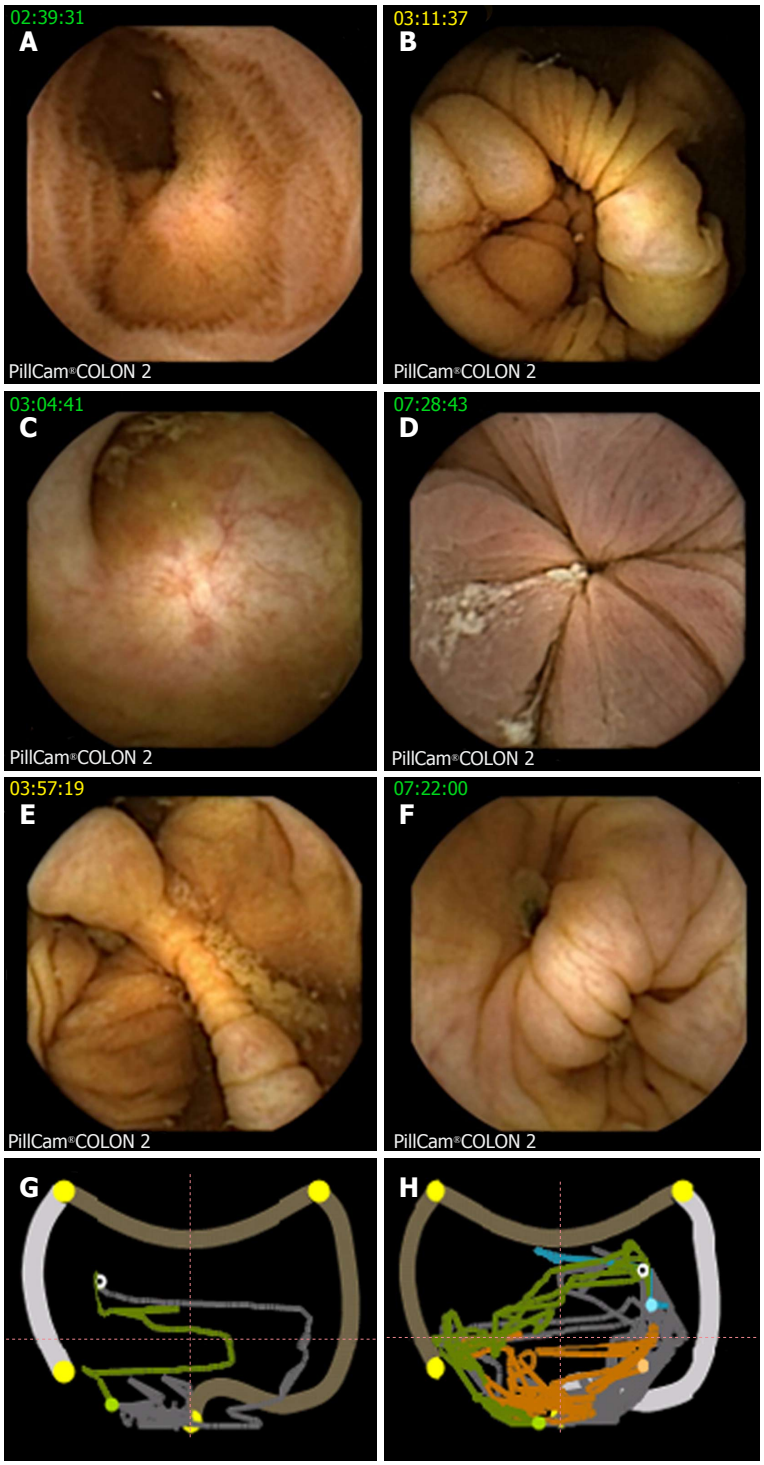


Figure 1 Landmarks at colon capsule endoscopy. A: Terminal ileum; B: IC valve; C: Appendix; D: Hemorrhoidal plexus, hepatic flexure - CCE image (E) with corresponding localization trace (G), white circle showing actual capsule position, green colon already displayed, grey colon yet to be analyzed, orange small bowel, blue stomach, outer pictogram the position to the colonic segment as manually defined by setting the landmarks; F and H: CCE image and localization trace of splenic flexure in another patient. CCE: Colon capsule endoscopy.

patients had more than one polyp (range: 2-5 polyps). Diverticula were found in 35 patients. Other findings were diverticulitis, erosions, angiectasias and erythema.

Colon capsule endoscopy

Primary endpoint: complementation of incomplete colonoscopy: Incomplete colonoscopy could be complemented by CCE in 69 of 74 patients (93%; see Table 2). Complete CCE was achieved in 48 of 74 patients (65%). In four additional patients, the capsule reached the rectum but did not visualize the haemorrhoidal plexus (5%).

Complementation of OC could be achieved by CCE with protocol A in 89.5% of procedures and with protocol

Table 2 Demographics, reasons for termination of optical colonoscopy, and results of colon capsule endoscopy for protocol A and B *n* (%)

	Protocol A (CCE next day)	Protocol B (CCE within 30 d)	Significance
Demographics			
Patients	38 (51.4)	36 (48.6)	
Female	20/38 (52.6)	24/36 (66.7)	
Age, mean ± SD	68.0 ± 12.8 yr	63.9 ± 13.0 yr	
Body mass index	26.0 ± 3.9	26.5 ± 4.9	
Reasons for termination of colonoscopy			
Looping of colon	23/38 (60.5)	23/36 (63.9)	
Angulation of colon	6/38 (15.8)	5/36 (13.9)	
Susp. adhesions	5/38 (13.2)	6/36 (16.7)	
Risk of perforation	2/38 (5.3)	0	$P = 0.710$ (NS); χ^2 test
Sedation problems	2/38 (5.3)	2/36 (5.6)	
Results of CCE			
Complete CCE	24/38 (63.3)	24/36 (66.7)	$P = 0.560$ (NS); χ^2 test
Complementation of colonoscopy	34/38 (89.5)	35/36 (97.2)	$P = 0.350$ (NS); χ^2 test
Adequate cleansing	25/36 (69.4)	23/36 (63.9)	$P = 0.820$ (NS); χ^2 test
Patients with significant colon polyps	10/38 (26.3)	11/36 (30.6)	$P = 0.500$ (NS); χ^2 test
Patients with other colon findings	0	Angiectasia ($n = 3$) Diverticulitis ($n = 1$)	$P = 0.045$; χ^2 test
Patients with small bowel findings	Angiectasia ($n = 1$) Crohn's disease ($n = 1$)	0	$P = 0.174$ (NS); χ^2 test
Patients with upper GI findings	Reflux esophagitis ($n = 1$) Upper GI-bleeding ($n = 1$) Gastric polyps ($n = 1$)	Reflux-esophagitis ($n = 1$) Susp. Barrett esophagus ($n = 1$) Gastric erosions ($n = 1$)	$P = 0.949$ (NS); χ^2 test

CCE: Colon capsule endoscopy.

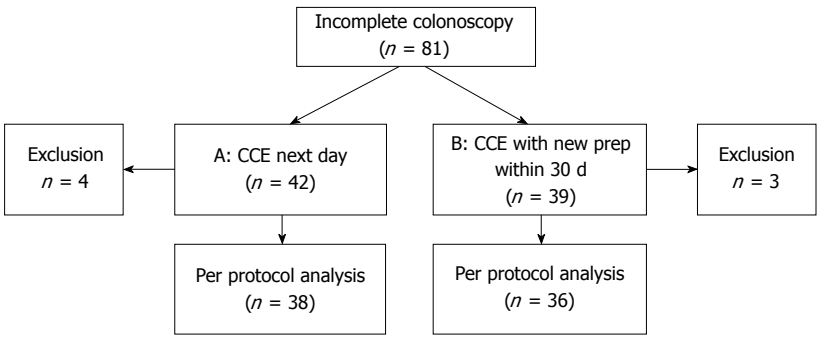


Figure 2 Flow chart protocol A (colon capsule endoscopy the day after colonoscopy) and protocol B (colon capsule endoscopy within 30 d after incomplete colonoscopy).

B in 97.2% of procedures ($P = 0.35$, not significant; Table 2). There were no differences between the rates of complementation using CCE for protocol A or B and the diagnostic yield. In the patients with incomplete visualization of the missing segments ($n = 5$), the capsule was able to display the hepatic flexure in two patients. One patient had a capsule retention in the small bowel because of unknown stenosing Crohn's disease. In one patient, the capsule did not reach the colon during recording time, and in one patient, visualization of the colon was incomplete due to recording gaps. The capsule was excreted within seven hours of ingestion and before the need for an additional NaP booster in 17 of 38 patients (44.7%) following protocol A, and in 12 of 36 patients (33.3%; $P = 0.25$, not significant) in protocol B.

Secondary endpoints: CCE was performed on the day after colonoscopy in 38 patients (protocol A; 51%) or within 30 d in 36 patients (protocol B; 49%). Overall, cleansing was adequate in 48 of 72 (67%), and cleansing was adequate in the cecum in 58% of cases, in the AC in 65% of cases, in the TC in 77% of cases, in the LC in 70% of cases and in the rectum in 63% of cases (Figure 3); there were no differences between the protocols. Two capsules did not allow for visualization of the colon. Poor cleansing was rare (4 of 72 patients; 5.6%).

CCE detected 76 polyps in 35 of 74 patients (47%; Figure 4). Twenty-one patients (28%) had significant polyps (Table 3), and 14 patients had an insignificant number of polyps. In 9 of 21 patients, polyp size was ⩾ 6 mm; in 3 patients, the number of polyps was ⩾ 3; and in 9 patients, both parameters were positive. A total of 59 polyps (mean size 8 ± 4.5 mm) were detected in the 21

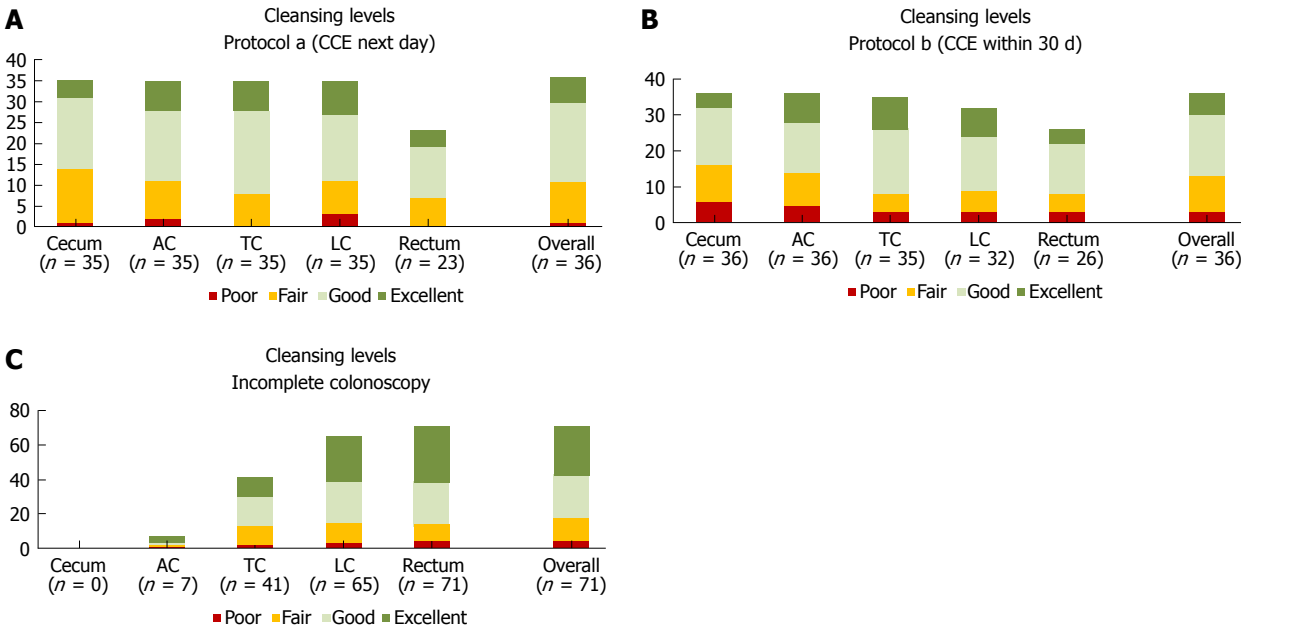


Figure 3 Cleansing levels for the colon segments: cecum, ascending colon, transverse colon, left colon, rectum and overall classification for all segments. Absolute number of patients for each level (poor, fair, good, excellent) are shown A for CCE with protocol a (next day), B for CCE with protocol b (within 30 d), and C for incomplete colonoscopy. AC: Ascending colon; CCE: Colon capsule endoscopy; LC: Left colon; TC: Transverse colon.

patients with significant polyps. Significant polyps were predominantly found in the ascending colon in segments that had not been reached by OC (86%). Incremental diagnostic yield of CCE compared to previous incomplete OC per patient for detecting significant polyps was 24%, and there were no differences between the two protocols. A cecal polyp of 26 mm turned out to be an adenocarcinoma, and hemicolectomy was performed in this patient.

Z-line was visualized in 45 of 74 patients (60.8%). CCE findings in the upper GI tract were reflux esophagitis (n = 2), suspected Barrett´s esophagus (n = 1), hemorrhagic gastropathy (n = 1), gastric polyps (n = 1; consecutive gastroscopy revealed foveolar hyperplasia in previously undiagnosed atrophic gastritis with vitamin B12 deficiency) and upper GI bleeding (n = 1).

In the small bowel, angiectasia and previously unknown Crohn's disease was detected in one patient each. In colon segments missed by incomplete colonoscopy, angiectasias were detected in three patients and diverticulitis was detected in one patient (all patients were part of protocol B).

ITT analysis of all 81 patients found a complementation rate of 89%, adequate cleansing in 65%, significant polyps in 26% of patients, and no additional adverse events. The low complementation rate that was observed in this cohort is consistent with the exclusion criteria [technical problems, n = 1, and noncompliance of study protocol, n = 6 (for example, early removal of recorder and incorrect CCE timing)].

In one patient (protocol A), the capsule was retained in the small bowel without symptoms. Surgery was indicated following a new diagnosis of stenosing and fistulating Crohn's disease with iron deficiency anemia (indication for colonoscopy). During an uneventful surgery, the capsule was retrieved. Another patient (protocol B) complained of self-limiting nausea and vomiting after the NaP boost.

DISCUSSION

In this prospective, multicentre study, second-generation CCE allowed for the visualization of colon segments that were not reached by a previously incomplete colonoscopy in 93% of 74 patients. Fifty-one percent of patients opted for protocol A, and 49% for protocol B. The complementation rate following incomplete OC was higher in group B (97%) compared with group A (89%), but the results did not reach statistical significance. There were no differences between the rate of complete CCE, the cleansing level, the diagnostic yield or the number of adverse events.

The complementation rate identified in the present study corresponded well with results from recent trials using a first-generation colon capsule. A Spanish study found a complementation rate of incomplete colonoscopies of 85.3% and a diagnostic yield of 45%[23]. A French trial including patients as well with contraindicated as incomplete OC, complete CCE was achieved in 83% of patients. An Italian trial reported a complete PillcamColon2 CCE after incomplete OC in 98% of patients using a separate preparation with Senna tablets, 4 L of PEG and two boosters with NaP and gastrografin[24]. This rate was higher than in our low-volume protocol, but it was accompanied more often by preparation-related complaints (28.0% *vs* 1.4%). Nevertheless, our primary aim to complement colon visualization was achieved in 93% of patients, which was similar to a Greek study that achieved results

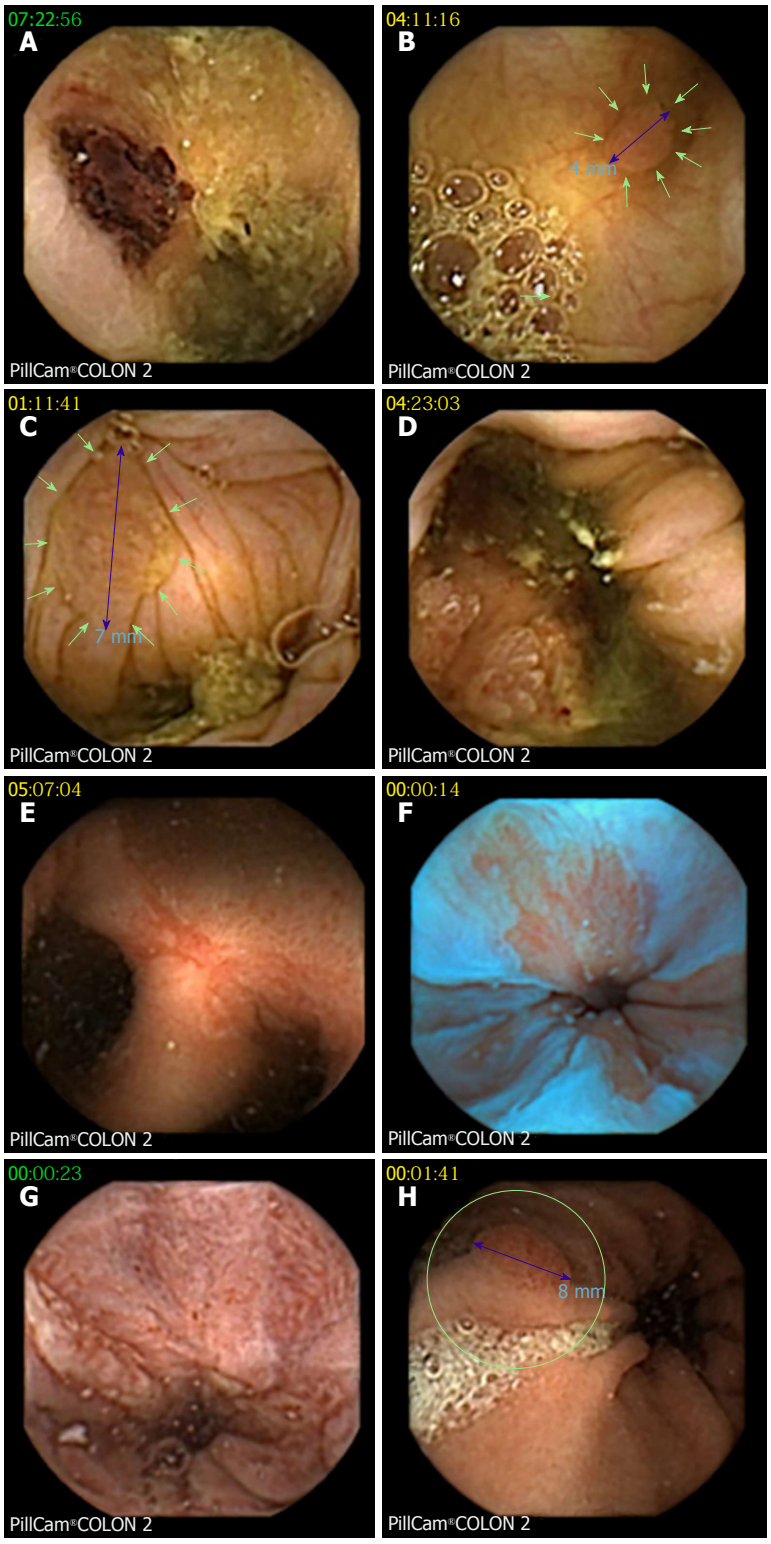


Figure 4 Examples of findings at colon capsule endoscopy. A: Biopsy tattoo after optical colonoscopy; B: One of three 4 mm polyps in the ascending colon, confirmed at consecutive balloon enteroscopy as tubular adenoma; C: Significant (7 mm) polyp; D: Cecal adenocarcinoma (confirmed by surgery); E: Fistulating and stenosing Crohn´s disease of the ileum (confirmed by enteroclysis, CT scan, and surgery); F: Irregular Z-line suggestive of Barrett´s esophagus (blue mode image); G: Reflux esophagitis; H: Gastric polyp (consecutive gastroscopy found foveolar hyperplasia and autoimmune gastritis with vitamin B12 deficiency).

using PillCamColon1 in 91% of patients[14]. In the present study, unfavourable anatomy was the reason for terminating the colonoscopy, which was usually performed under propofol sedation, in 92% of patients. In contrast, pain was reported by 45% of patients as the reason for incomplete colonoscopy in the Italian trial. This selection bias toward unfavourable anatomy might have also influenced the rate of complete consecutive CCEs (65%) in the present study. It may also be relevant when comparing our results to series

Table 3 Details of the 21/74 patients in whom significant polyps/tumors were detected during colon capsule endoscopy

No.	Significance based on	Size of largest polyp (mm)	Number of polyps	Localization of polyps	Farest point reached in OC	All segments with polyps in CCE reached by colonoscopy
1	Size/number	6	5	Rectum	HF	Yes
2	Size	8	2	Sigma	SF	Yes
3	Size/number	8	3	Sigma	AC	Yes
4	Size/number	8	3	2 × Cecum, 1 × AC	TC	No
5	Size	14	1	AC	HF	No
6	Size	26	1	AC	SF	No
7	Size	6	2	AC	Sigma	No
8	Size/number	10	3	2 × AC, 1 × TC	SF	No
9	Size/number	10	3	2 × LC, 1 × AC	Sigma	No
10	Size	10	1	AC	SF	No
11	Size/number	9	3	2 ×AC, 1 × LC	HF	No
12	Size	6	1	AC	Sigma	No
13	Size	8	2	1 × AC/1 × LC	HF	No
14	Size	7	1	HF	Sigma	No
15	Number	5	4	AC, TC, 2 × sigma	Sigma	No
16	Size/number	12	4	1 × Cecum, 1 × AC, 1 × LC, 1 × rectum	Sigma	No
17	Number	4	3	1 × Cecum, 2 × rectum	TC	No
18	Number	5	5	2 × AC, 1 × TC, 2 × LC	HF	No
20	Size/number	10	7	4 × AC, 3 × LC	TC	No
20	Size	9	2	2 × Cecum	LC	No
21	Size/number	12	3	AC	LC	No

AC: Ascending colon; HF: Hepatic flexure; TC: Transverse colon; SF: Splenic flexure; LC: Left colon; OC: Optical colonoscopy.

with unselected patients without previously incomplete colonoscopies. In these trials, complete CCE was achieved in 76% of patients using a similar low-volume preparation[18], in 88% of patients using PEG[25], in 76% of patients using NaP[14] and in 98% of patients using a PEG-, NaP- or gastrografin-based regimen[24].

In the present study, colon cleansing using a low-volume preparation was adequate in 67% of patients, and the results were similar in both protocols. These results are comparable to other studies that used PillCamColon1 for incomplete colonoscopies with PEG preparation and NaP boosts (65%)[23], and 60% *vs* 63% in the right and left colon, respectively[14]. Adequate cleansing following use of the PillcamColon1 was observed in 76% of patients following consumption of 1-2 L Moviprep and two NaP boosts in a mixed cohort, including 28% of patients with contraindicated colonoscopy without anatomical problems[19]. A recent Spanish multicentre trial found adequate cleansing levels in 75% of patients using a standard 4 L PEG preparation with a NaP booster for PillCamColon2 after an incomplete colonoscopy[26]. Even in a large trial in a screening population without negative selection towards unfavorable anatomy, CCE was technically insufficient in 9% of patients due to inadequate cleansing or rapid transit of the capsule[27].

Twenty-four percent of our patients (ITT 22%) had additional relevant finding following the CCE, which led to a recommendation for further diagnostics or treatment. Similarly, PillCamColon1 was useful in guiding management after failed OC in a Spanish trial[23].

Most of the significant polyps (86%), including a carcinoma, were found in the AC. Our data confirmed previous observations[5] that CCE can identify relevant lesions in segments not reached by incomplete OC (Table 3).

Device-assisted colonoscopy with either spiral endoscopy and double or single balloons have identical rates, similar to CCE, of complementing incomplete OC of about 90%[28-31]. These flexible endoscopic techniques additionally provide the possibility to directly treat detected lesions, but they require equipment and expertise, and they are restricted to specialized centres. A cap-assisted colonoscopy was also successful in 93% of patients, and one perforation occurred with this technique after failed OC[32].

Computed tomographic colonography (CTC) after incomplete OC has a good diagnostic yield and may be advantageous if extra-colonic findings are considered[33], for example, in the case of tumour. However, CTC detected fewer small polyps than CCE[24]. Furthermore, radiation-free CCE could additionally be used to visualize the Z-line in 60.8% of patients, as previously reported for PillCamColon1 (60%)[34]. In the present trial, eight patients had relevant extra-colonic findings, seven of which were presumably not detectable with CTC.

Capsule retention occurred in one patient, corresponding to a retention rate of 1.4%, which was similar to the rate reported for SBCE[35]. This capsule retention resulted in a new diagnosis of Crohn´s disease and was considered to be diagnostic rather than a complication. However, possible retention must also be considered when applying CCE to complement incomplete OC and should be included as part of the informed consent discussion. Although adhesions were supposed to be the cause of incomplete OC in some patients, no related clinical problems or capsule retention following CCE

were observed.

In conclusion, second-generation CCE using low-volume bowel preparations is useful, well tolerated and is able to detect additional relevant lesions. Risk of retention is as low as in SBCE, but must be considered. Similar results were found in the present study between the two protocols for the complementation rate and presence of significant polyps.

Limitations

Patients could choose between preparation protocols for CCE without randomization. The area reached by the colonoscopy was described, but tattooing was only optional. Long-term follow-up was not part of the present study.

ARTICLE HIGHLIGHTS

Research background

Optical colonoscopy (OC) is the gold standard for visualization of the colon. However, it may be incomplete e.g. due to unfavorable anatomy. Colon capsule endoscopy (CCE) is cleared by the US Food and Drug Administration for patients with previously incomplete OC. Second generation CCE has been shown to have a higher sensitivity for detection of colon polyps than first generation. Low volume bowel prep with Moviprep has been shown to be feasible for CCE.

Research motivation

Bowel preparation for CCE is more extensive than for OC. Thus, we aimed to evaluate, if second generation CCE is feasible using either repeated low volume bowel prep or staying on clear liquids following an incomplete OC.

Research objectives

Main research objective was the ability of CCE to visualize those colon segments not reached by incomplete OC. Secondary objectives were additional diagnostic yield of CCE, rate of complete colon visualization by CCE, cleansing levels, and safety.

Research methods

In this prospective multicenter study 81 patients underwent second generation colon capsule endoscopy with PillCamColon2 after incomplete OC. CCE was performed either the following day (protocol A) after staying on clear liquids and 0.75 L Moviprep in the morning or within 30 d after new split-dose Moviprep (protocol B). Boosts consisted of 0.75 L and 0.25 L Moviprep, and phospho-soda as rescue if the capsule was not excreted after 7 hours.

Research results

Seventy-four patients were finally analyzed per protocol. Of those, cleansing was adequate in 67% of cases and CCE could visualize the colonic segments missed by incomplete colonoscopy in 90% (protocol A) and 97% (protocol B, $P = 0.35$, n.s.) of the patients. Detection rates were similar with both protocols: Significant polyps and one adenocarcinoma were detected in 24% of cases. Polyps were found predominantly in the right colon (86%) in segments not reached by OC. Extra-colonic findings as reflux esophagitis, suspected Barrett esophagus, upper GI-bleeding, gastric polyps, gastric erosions, and angiectasia were detected in 8 patients. One capsule (1.4%) was retained in the ileum without symptoms and removed during uneventful resection for unknown Crohn`s disease diagnosed as cause of unclear anemia. CCE was well tolerated. One patient suffered from self-limiting vomiting after phospho-soda.

Research conclusions

Second generation CCE using low volume prep is useful to complement incomplete OC, detects additional relevant findings including extra-colonic lesions, and is well tolerated. CCE is feasible the following day after staying on clear liquids or after new prep within 30 d. Potential risk of capsule retention must be considered.

Research perspectives

Future studies should address improvement of colon cleansing levels and completeness of CCE after incomplete OC. Cost-effectiveness of CCE after incomplete OC should be addressed by future research in comparison with other methods as CT colonoscopy, MR colonoscopy, and device assisted colonoscopy.

REFERENCES

1 **Pox CP**, Altenhofen L, Brenner H, Theilmeier A, Von Stillfried D, Schmiegel W. Efficacy of a nationwide screening colonoscopy program for colorectal cancer. *Gastroenterology* 2012; **142**: 1460-1467.e2 [PMID: 22446606 DOI: 10.1053/j.gastro.2012.03.022]

2 **Brenner H**, Altenhofen L, Katalinic A, Lansdorp-Vogelaar I, Hoffmeister M. Sojourn time of preclinical colorectal cancer by sex and age: estimates from the German national screening colonoscopy database. *Am J Epidemiol* 2011; **174**: 1140-1146 [PMID: 21984657 DOI: 10.1093/aje/kwr188]

3 **Sarwar S**, Anwar MM, Ryan B, O'Morain C. Colonoscopy completion rates - are we prepared for a national screening programme? *Ir Med J* 2007; **100**: 585-587 [PMID: 18196881]

4 **Koido S**, Ohkusa T, Nakae K, Yokoyama T, Shibuya T, Sakamoto N, Uchiyama K, Arakawa H, Osada T, Nagahara A, Watanabe S, Tajiri H. Factors associated with incomplete colonoscopy at a Japanese academic hospital. *World J Gastroenterol* 2014; **20**: 6961-6967 [PMID: 24944489 DOI: 10.3748/wjg.v20.i22.6961]

5 **Neerincx M**, Terhaar sive Droste JS, Mulder CJ, Räkers M, Bartelsman JF, Loffeld RJ, Tuynman HA, Brohet RM, van der Hulst RW. Colonic work-up after incomplete colonoscopy: significant new findings during follow-up. *Endoscopy* 2010; **42**: 730-735 [PMID: 20669092 DOI: 10.1055/s-0030-1255523]

6 **Dafnis G**, Granath F, Påhlman L, Ekbom A, Blomqvist P. Patient factors influencing the completion rate in colonoscopy. *Dig Liver Dis* 2005; **37**: 113-118 [PMID: 15733524 DOI: 10.1016/j.dld.2004.09.015]

7 **Shah HA**, Paszat LF, Saskin R, Stukel TA, Rabeneck L. Factors associated with incomplete colonoscopy: a population-based study. *Gastroenterology* 2007; **132**: 2297-2303 [PMID: 17570204 DOI: 10.1053/j.gastro.2007.03.032]

8 **Loffeld RJ**, van der Putten AB. The completion rate of colonoscopy in normal daily practice: factors associated with failure. *Digestion* 2009; **80**: 267-270 [PMID: 19923819 DOI: 10.1159/000236030]

9 **Hansel SL**, Prechel JA, Horn B, Crowell MD, DiBaise JK. Observational study of the frequency of use and perceived usefulness of ancillary manoeuvres to facilitate colonoscopy completion. *Dig Liver Dis* 2009; **41**: 812-816 [PMID: 19467939 DOI: 10.1016/j.dld.2009.03.010]

10 **Tsai MS**, Su YH, Liang JT, Lai HS, Lee PH. Patient factors predicting the completion of sedation-free colonoscopy. *Hepatogastroenterology* 2008; **55**: 1606-1608 [PMID: 19102351]

11 **Crispin A**, Birkner B, Munte A, Nusko G, Mansmann U. Process quality and incidence of acute complications in a series of more than 230,000 outpatient colonoscopies. *Endoscopy* 2009; **41**: 1018-1025 [PMID: 19856246 DOI: 10.1055/s-0029-1215214]

12 **Britton EJ**, Sidhu S, Geraghty J, Psarelli E, Sarkar S. The 5-year outcome of patients having incomplete colonoscopy. *Colorectal Dis* 2015; **17**: 298-303 [PMID: 25605376 DOI: 10.1111/codi.12901]

13 **Spada C**, Hassan C, Galmiche JP, Neuhaus H, Dumonceau JM, Adler S, Epstein O, Gay G, Pennazio M, Rex DK, Benamouzig R, de Franchis R, Delvaux M, Devière J, Eliakim R, Fraser C, Hagenmuller F, Herrerias JM, Keuchel M, Macrae F, Munoz-Navas M, Ponchon T, Quintero E, Riccioni ME, Rondonotti E, Marmo R, Sung JJ, Tajiri H, Toth E, Triantafyllou K, Van Gossum A, Costamagna G; European Society of Gastrointestinal Endoscopy.

Colon capsule endoscopy: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. *Endoscopy* 2012; **44**: 527-536 [PMID: 22389230 DOI: 10.1055/s-0031-1291717]

14 **Triantafyllou K**, Viazis N, Tsibouris P, Zacharakis G, Kalantzis C, Karamanolis DG, Ladas SD. Colon capsule endoscopy is feasible to perform after incomplete colonoscopy and guides further workup in clinical practice. *Gastrointest Endosc* 2014; **79**: 307-316 [PMID: 24060522 DOI: 10.1016/j.gie.2013.07.061]

15 **Van Gossum A**, Munoz-Navas M, Fernandez-Urien I, Carretero C, Gay G, Delvaux M, Lapalus MG, Ponchon T, Neuhaus H, Philipper M, Costamagna G, Riccioni ME, Spada C, Petruzziello L, Fraser C, Postgate A, Fitzpatrick A, Hagenmüller F, Keuchel M, Schoofs N, Devière J. Capsule endoscopy versus colonoscopy for the detection of polyps and cancer. *N Engl J Med* 2009; **361**: 264-270 [PMID: 19605831 DOI: 10.1056/NEJMoa0806347]

16 **Adler S**, Hassan C, Metzger Y, Sompolinsky Y, Spada C. Accuracy of automatic detection of small-bowel mucosa by second-generation colon capsule endoscopy. *Gastrointest Endosc* 2012; **76**: 1170-1174 [PMID: 23025975 DOI: 10.1016/j.gie.2012.07.034]

17 **Spada C**, Pasha SF, Gross SA, Leighton JA, Schnoll-Sussman F, Correale L, González Suárez B, Costamagna G, Hassan C. Accuracy of First- and Second-Generation Colon Capsules in Endoscopic Detection of Colorectal Polyps: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2016; **14**: 1533-1543.e8 [PMID: 27165469 DOI: 10.1016/j.cgh.2016.04.038]

18 **Hartmann D**, Keuchel M, Philipper M, Gralnek IM, Jakobs R, Hagenmüller F, Neuhaus H, Riemann JF. A pilot study evaluating a new low-volume colon cleansing procedure for capsule colonoscopy. *Endoscopy* 2012; **44**: 482-486 [PMID: 22275051 DOI: 10.1055/s-0031-1291611]

19 **Pioche M**, de Leusse A, Filoche B, Dalbiès PA, Adenis Lamarre P, Jacob P, Gaudin JL, Coulom P, Letard JC, Borotto E, Duriez A, Chabaud JM, Crampon D, Gincul R, Levy P, ben-Soussan E, Garret M, Lapuelle J, Saurin JC. Prospective multicenter evaluation of colon capsule examination indicated by colonoscopy failure or anesthesia contraindication. *Endoscopy* 2012; **44**: 911-916 [PMID: 22893133 DOI: 10.1055/s-0032-1310008]

20 **Eliakim R**, Yassin K, Niv Y, Metzger Y, Lachter J, Gal E, Sapoznikov B, Konikoff F, Leichtmann G, Fireman Z, Kopelman Y, Adler SN. Prospective multicenter performance evaluation of the second-generation colon capsule compared with colonoscopy. *Endoscopy* 2009; **41**: 1026-1031 [PMID: 19967618 DOI: 10.1055/s-0029-1215360]

21 **Spada C**, Hassan C, Marmo R, Petruzziello L, Riccioni ME, Zullo A, Cesaro P, Pilz J, Costamagna G. Meta-analysis shows colon capsule endoscopy is effective in detecting colorectal polyps. *Clin Gastroenterol Hepatol* 2010; **8**: 516-522 [PMID: 20215066 DOI: 10.1016/j.cgh.2010.02.018]

22 **Triantafyllou K**, Tsibouris P, Kalantzis C, Papaxoinis K, Kalli T, Kalantzis N, Ladas SD. PillCam Colon capsule endoscopy does not always complement incomplete colonoscopy. *Gastrointest Endosc* 2009; **69**: 572-576 [PMID: 19231502 DOI: 10.1016/j.gie.2008.10.047]

23 **Alarcón-Fernández O**, Ramos L, Adrián-de-Ganzo Z, Gimeno-García AZ, Nicolás-Pérez D, Jiménez A, Quintero E. Effects of colon capsule endoscopy on medical decision making in patients with incomplete colonoscopies. *Clin Gastroenterol Hepatol* 2013; **11**: 534-540.e1 [PMID: 23078891 DOI: 10.1016/j.cgh.2012.10.016]

24 **Spada C**, Hassan C, Barbaro B, Iafrate F, Cesaro P, Petruzziello L, Minelli Grazioli L, Senore C, Brizi G, Costamagna I, Alvaro G, Iannitti M, Salsano M, Ciolina M, Laghi A, Bonomo L, Costamagna G. Colon capsule versus CT colonography in patients with incomplete colonoscopy: a prospective, comparative trial. *Gut* 2015; **64**: 272-281 [PMID: 24964317 DOI: 10.1136/gutjnl-2013-306550]

25 **Spada C**, Hassan C, Munoz-Navas M, Neuhaus H, Deviere J, Fockens P, Coron E, Gay G, Toth E, Riccioni ME, Carretero C, Charton JP, Van Gossum A, Wientjes CA, Sacher-Huvelin S, Delvaux M, Nemeth A, Petruzziello L, de Frias CP, Mayershofer R, Amininejad L, Dekker E, Galmiche JP, Frederic M, Johansson GW, Cesaro P, Costamagna G. Second-generation colon capsule endoscopy compared with colonoscopy. *Gastrointest Endosc* 2011; **74**: 581-589.e1 [PMID: 21601200 DOI: 10.1016/j.gie.2011.03.1125]

26 **Nogales Ó**, García-Lledó J, Luján M, Nicolás D, Juanmartiñena JF, González-Suárez B, Sánchez Ceballos F, Couto I, Olmedo J, Garfia C, Carretero C, Fernández Urién I, Rodríguez S, Asteinza M, Olivencia P, Masedo Á, Muñoz-Navas M, Merino B, González Asanza C. Therapeutic impact of colon capsule endoscopy with PillCam™ COLON 2 after incomplete standard colonoscopy: a Spanish multicenter study. *Rev Esp Enferm Dig* 2017; **109**: 322-327 [PMID: 28229607 DOI: 10.17235/reed.2017.4369/2016]

27 **Rex DK**, Adler SN, Aisenberg J, Burch WC Jr, Carretero C, Chowers Y, Fein SA, Fern SE, Fernandez-Urien Sainz I, Fich A, Gal E, Horlander JC Sr, Isaacs KL, Kariv R, Lahat A, Leung WK, Malik PR, Morgan D, Papageorgiou N, Romeo DP, Shah SS, Waterman M. Accuracy of capsule colonoscopy in detecting colorectal polyps in a screening population. *Gastroenterology* 2015; **148**: 948-957.e2 [PMID: 25620668 DOI: 10.1053/j.gastro.2015.01.025]

28 **Becx MC**, Al-Toma A. Double-balloon endoscopy: an effective rescue procedure after incomplete conventional colonoscopy. *Eur J Gastroenterol Hepatol* 2014; **26**: 519-522 [PMID: 24584269 DOI: 10.1097/MEG.0000000000000067]

29 **Dzeletovic I**, Harrison ME, Pasha SF, Crowell MD, Decker GA, Gurudu SR, Leighton JA. Comparison of single- versus double-balloon assisted-colonoscopy for colon examination after previous incomplete standard colonoscopy. *Dig Dis Sci* 2012; **57**: 2680-2686 [PMID: 22615017 DOI: 10.1007/s10620-012-2227-z]

30 **Schembre DB**, Ross AS, Gluck MN, Brandabur JJ, McCormick SE, Lin OS. Spiral overtube-assisted colonoscopy after incomplete colonoscopy in the redundant colon. *Gastrointest Endosc* 2011; **73**: 515-519 [PMID: 21353848 DOI: 10.1016/j.gie.2010.11.047]

31 **Sulz MC**, Frei R, Semadeni GM, Sawatzki M, Borovicka J, Meyenberger C. The role of single-balloon colonoscopy for patients with previous incomplete standard colonoscopy: Is it worth doing it? *Surg Endosc* 2016; **30**: 1876-1882 [PMID: 26183958 DOI: 10.1007/s00464-015-4405-5]

32 **Lee YT**, Hui AJ, Wong VW, Hung LC, Sung JJ. Improved colonoscopy success rate with a distally attached mucosectomy cap. *Endoscopy* 2006; **38**: 739-742 [PMID: 16673307 DOI: 10.1055/s-2006-925238]

33 **Pullens HJ**, van Leeuwen MS, Laheij RJ, Vleggaar FP, Siersema PD. CT-colonography after incomplete colonoscopy: what is the diagnostic yield? *Dis Colon Rectum* 2013; **56**: 593-599 [PMID: 23575398 DOI: 10.1097/DCR.0b013e3182781668]

34 **Schoofs N**, Devière J, Van Gossum A. PillCam colon capsule endoscopy compared with colonoscopy for colorectal tumor diagnosis: a prospective pilot study. *Endoscopy* 2006; **38**: 971-977 [PMID: 17058159 DOI: 10.1055/s-2006-944835]

35 **Liao Z**, Gao R, Xu C, Li ZS. Indications and detection, completion, and retention rates of small-bowel capsule endoscopy: a systematic review. *Gastrointest Endosc* 2010; **71**: 280-286 [PMID: 20152309 DOI: 10.1016/j.gie.2009.09.031]

P- Reviewer: Dutta AK, Paduani G, Teramoto-Matsubara OT
S- Editor: Gong ZM **L- Editor**: A **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