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

World Journal of *Gastroenterology*

World J Gastroenterol 2019 July 7; 25(25): 3108-3282

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 25 Number 25 July 7, 2019

OPINION REVIEW

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

Contents

World Journal of Gastroenterology

Volume 25 Number 25 July 7, 2019

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Tatsuo Kanda, MD, PhD, Associate Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, Tokyo 173-8610, Japan

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. The *WJG* Editorial Board consists of 701 experts in gastroenterology and hepatology from 58 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, *etc*. The *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

The *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 Scopus. The 2019 edition of Journal Citation Report® cites the 2018 impact factor for *WJG* as 3.411 (5-year impact factor: 3.579), ranking *WJG* as 35th among 84 journals in gastroenterology and hepatology (quartile in category Q2). CiteScore (2018): 3.43.

RESPONSIBLE EDITORS FOR THIS ISSUE

Responsible Electronic Editor: *Yu-Jie Ma*

Proofing Production Department Director: *Yun-Xiaojian Wu*

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Subrata Ghosh, Andrzej S. Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

EDITORIAL OFFICE
Ze-Mao Gong, Director

PUBLICATION DATE
July 7, 2019

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3108-3115

DOI: 10.3748/wjg.v25.i25.3108

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

OPINION REVIEW

Advanced imaging in surveillance of Barrett's esophagus: Is the juice worth the squeeze?

Sara A Cerrone, Arvind J Trindade

ORCID number: Sara A Cerrone (0000-0002-4442-7466); Arvind J Trindade (0000-0002-4132-0014).

Author contributions: All the authors contributed to conception and design; analysis and interpretation of the data; drafting of the article; critical revision of the article for important intellectual content; and final approval of the article.

Conflict-of-interest statement: The authors have declared no conflicts of interest.

Manuscript source: Invited manuscript

Received: February 18, 2019
Peer-review started: February 18, 2019
First decision: April 30, 2019
Revised: May 8, 2019
Accepted: May 18, 2019
Article in press: May 18, 2019
Published online: July 7, 2019

Sara A Cerrone, Arvind J Trindade, Division of Gastroenterology, Long Island Jewish Medical Center, Zucker School of Medicine at Hofstra/Northwell, Northwell Health System, New Hyde Park, NY 11040, United States

Corresponding author: Arvind J Trindade, MD, Director of Endoscopy, Division of Gastroenterology, Long Island Jewish Medical Center, Associate Professor of Medicine, Zucker School of Medicine at Hofstra/Northwell, Northwell Health System, 270-05 76th Avenue, New Hyde Park, NY 11040, United States. arvind.trindade@gmail.com
Telephone: +1-718-4707281
Fax: +1-718-4705509

Abstract

Esophageal cancer is on the rise. The known precursor lesion is Barrett's esophagus (BE). Patients with dysplasia are at higher risk of developing esophageal cancer. Currently the gold standard for surveillance endoscopy involves taking targeted biopsies of abnormal areas as well as random biopsies every 1-2 cm of the length of the Barrett's. Unfortunately studies have shown that this surveillance can miss dysplasia and cancer. Advanced imaging technologies have been developed that may help detect dysplasia in BE. This opinion review discusses advanced imaging in BE surveillance endoscopy and its utility in clinical practice.

Key words: Barrett's esophagus; Advanced imaging; Chromoendoscopy; Endomicroscopy

Core tip: Barrett's esophagus (BE) is a precursor of esophageal cancer, the incidence of which is on the rise worldwide. Advanced imaging in BE includes dye chromoendoscopy, electronic chromoendoscopy narrow band imaging (NBI), confocal laser endomicroscopy and volumetric laser endomicroscopy (VLE). The decision to perform these procedures ultimately depends on if the benefit outweighs the cost and any added time performing the procedure. In our practice the added benefits of NBI and VLE outweighs the costs and added time and thus we have incorporated this into our Barrett's surveillance routine.

Citation: Cerrone SA, Trindade AJ. Advanced imaging in surveillance of Barrett's esophagus: Is the juice worth the squeeze? *World J Gastroenterol* 2019; 25(25): 3108-3115
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3108.htm

P-Reviewer: Hashimoto N
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3108

INTRODUCTION

Barrett's esophagus (BE) is the development of specialized intestinal metaplasia in the esophagus. The exact incidence of BE is not known, but it is estimated to be from 0.2%-2% per year[1]. It is a premalignant lesion for adenocarcinoma of the esophagus. Though the rates of esophageal squamous cell carcinoma and distal gastric cancers are declining, the incidence of esophageal adenocarcinoma is rising more than any other malignancy[2]. Reports have quoted an average annual increase of up to 17%[2]. This increase may be due to environmental and population factors, but also due to insufficient detection of Barrett's and insufficient surveillance protocols for patients with known BE[3,4]. The Seattle protocol guides the surveillance procedure of many endoscopists, which consists of 4-quadrant biopsies at intervals of every 1-2 cm and separate samples of areas of mucosal irregularity, may miss a significant number of areas of low-or high-grade dysplasia[5]. In a large multicenter study, 53% of patients who underwent consecutive surveillance endoscopies documenting non-dysplastic tissue or intestinal metaplasia without dysplasia, developed high grade dysplasia and/or cancer within a mean of 3 years[1].

Unfortunately, the mortality for esophageal adenocarcinomas is very high, with a mean 5-year survival rate of less than 20% for advanced disease. Many patients are diagnosed at presentation with advanced disease, and there is a need to find better means to identify patients at earlier stages. BE confers a 30-40 fold increased risk for esophageal adenocarcinoma, but it is unclear if such focus on surveillance in BE patients has improved outcomes[2]. Patients with surveillance-detected BE have higher rates of survival at two years compared to patients that are diagnosed outside of a surveillance program (73.3% *vs* 12.5%, $P = 0.02$), yet few patients (3.9%) are diagnosed with BE before their cancer diagnosis[2]. In other studies, there was no association between surveillance in BE and decreased risk of death from esophageal adenocarcinoma (OR = 0.99; 95%CI: 0.36-2.75) and the detection of advanced disease was equivalent in surveillance and non-surveillance groups[4]. Therefore, current surveillance strategies may be ineffective in improving patient outcomes.

Endoscopic surveillance of known BE may be improved through advanced imaging. Advanced imaging technologies allow visualization of abnormalities that may not be seen on routine endoscopic evaluation. These are also termed as red flag technologies as they point attention to abnormal areas that can be consistent with dysplasia or early cancer.

Preservation and Incorporation of Valuable Endoscopic Innovations (PIVI) was developed in 2011 by the American Society of Gastrointestinal Endoscopy (ASGE) to recognize important clinical questions and develop diagnostic and/or therapeutic thresholds for endoscopic technologies related to these clinical questions[6]. In 2016, performance thresholds were established to evaluate real-time imaging-assisted modalities used for endoscopic targeted biopsies in the endoscopic surveillance of non-dysplastic BE which included chromoendoscopy (using acetic acid and methylene blue), electronic chromoendoscopy [using narrow-band imaging (NBI)], and both probe and endoscopic based confocal laser endomicroscopy (CLE). Volumetric laser endomicroscopy (VLE) was not evaluated in the PIVI initiative given the recent release on the market at the time and thus lack of studies. These per-formance thresholds included: (1) Sensitivity of ≥ 90% and a negative predictive value of ≥ 98% for detecting high grade dysplasia (HGD) or early adenocarcinoma (EAC) compared to standard protocol, and (2) Imaging technology with high (80%) specificity to allow reduction in the number of biopsies compared to random biopsies. Acetic acid chromoendoscopy, narrow band imaging and endoscopic CLE met the thresholds set by the ASGE PIVI, and thus the ASGE Technology Committee endorsed using these modalities to guide surveillance in patients with previously non-dysplastic BE.

Given the number of tools on the market, the main question to ask is, is it worth performing any of these technologies? Ultimately it comes down to how cumbersome is it to perform the technology compared to the yield of additional cases of dysplasia being detected that changes management. Cost to the patient and health care system is also a factor that can contribute to usage of a technology. We describe the technologies from these viewpoints in this mini-review.

DYE CHROMOENDOSCOPY

Dye chromoendoscopy uses chemical agents to highlight mucosal changes of dysplasia to allow for improved detection of abnormalities[3]. Such dye agents include methylene blue, indigo carmine and acetic acid. Methylene blue is absorbed by non-dysplastic intestinal-type epithelium and can also be used to detect Barrett's mucosa. In several studies comparing rates of detection of intestinal metaplasia and dysplasia between methylene blue and 4-quadrant biopsies, the rates for methylene blue were similar than 4-quadrant biopsies, but the number of biopsies used to detect those changes were lower with methylene blue[7]. Other studies have found four-quadrant biopsies detect significantly more dysplasia than methylene blue[8]. In addition, there is concern that methylene blue may damage DNA in Barrett's epithelium potentially leading to errors in diagnosis[9]. These solutions are difficult to handle and use endoscopically and therefore their use is not standard. Indigo carmine has also been shown to be effective in detecting mucosal patterns, but when compared to high-resolution white light endoscopy, indigo carmine showed no significant difference in detecting early neoplasia[10,11]. Given its cumbersome use and lack of incremental yield *vs* high definition white light endoscopy, its use is also not standard.

Acetic acid can be used to enhance different mucosal pit patterns in columnar epithelium. Certain pit patterns have shown high sensitivity and specificity for intestinal metaplasia. In a meta-analyses, acetic acid chromoendoscopy has shown a sensitivity of 96.6%, negative predictive value of 98.3% and a specificity of 84.5% in detecting dysplasia or esophageal adenocarcinoma[6].

Acetic acid is a safe, rapid, and inexpensive. It can highlight ridged and villous patterns that are associated with mucosal abnormalities associated with dysplasia. Therefore, its use is high yield in surveillance in BE[12].

ELECTRONIC CHROMOENDOSCOPY

Electronic chromoendoscopy techniques involve the use of NBI (Olympus America, Center Valley, PA, United States) and I-Scan (Pentax Medical, Montvale, NJ, United States). Blue light imaging and linked color imaging (Fujifilm, Tokyo, Japan) are newer electronic chromoendosocpy platforms. They will not be discussed here due to limited data in Barrett's esophagus. NBI highlights vascular patterns on the mucosal surface by using spectral narrow-band optical fibers[13]. Figure 1 shows a patient with Barrett's and high-grade dysplasia with NBI imaging. I-Scan uses a post-processing technology to highlight contrast between squamous and columnar epithelia[3]. Patterns detected by NBI have been shown to predict histology. In one study, a ridge/villous pattern predicted the presence of intestinal metaplasia with a sensitivity of 93.5% and a specificity of 86.7%, and an irregular/distorted pattern predicted high-grade dysplasia with a sensitivity of 100% and sensitivity of 98.7%[14]. In a prospective, blinded, tandem endoscopy study of 65 patients comparing NBI-targeted biopsies to random biopsies *via* the Seattle protocol with high definition white light, NBI was able to identify more patients with dysplasia (57% *vs* 43%, $P < 0.001$) and also found high grades of dysplasia (18% higher grade *vs* 0%, $P < 0.001$)[15]. In another study comparing results in patient who were first screened for Barrett's with high definition white light and then NBI-targeted biopsies at a 6-wk interval, there was no significant difference in detection of intestinal metaplasia and dysplasia ($P = 0.15$), but NBI required a significantly fewer number of biopsies to make a diagnosis (3.6 *vs* 7.6, $P < 0.001$)[16]. A study comparing computed virtual chromoendoscopy to conventional chromoendoscopy with acetic acid, a sensitivity of 83% and 92% for high grade intraepithelial neoplasia was found, respectively, with no significant difference between the two methods ($P = 0.617$)[17].

Overall, electronic chromoendoscopy shows high sensitivity for the detection of high-grade dysplasia in patients with BE and provides a means to more efficiently biopsy patients. Its ability to detect low grade dysplasia is comparable to that of high-resolution white light endoscopy. We recommend using electronic chromoendoscopy given its ease of use (turning on a switch on the endoscope processor), low cost (already incorporated into the scope technology), and that it meets PIVI thresholds.

CONFOCAL LASER ENDOMICROSCOPY (CLE)

A CLE examination of the gut mucosa is performed using endoscopically delivered laser light. This light is reflected through a pinhole onto sensors that relay the signals to a computer, which provides a cross-sectional microscopic image of the mucosa[18].

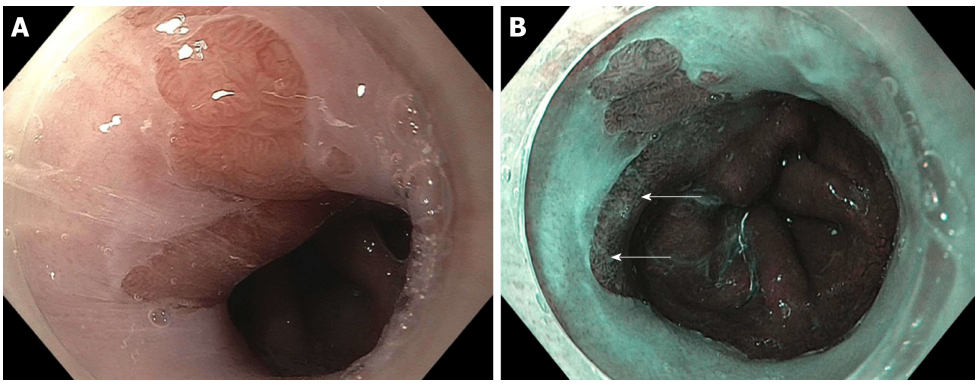


Figure 1 A patient with Barrett's and high-grade dyspalsia with narrow band imaging. A: A segment of Barrett's esophagus on high definition white light endoscopy (HDWLE); and B: narrow band imaging (NBI) from a patient with prior long segment disease post two sessions of endoscopic resection, 4 sessions of radiofrequency ablation, and one session of cryotherapy. The HDWLE did not show any features concerning for dysplasia. The NBI shows an area of disrupted vessels (upper yellow arrow, lower white arrow) concerning for dysplasia.

CLE is almost analogous to looking real time at a microscope during the endoscopy exam. There is limited data on the learning curve for use of CLE in Barrett's, but it appears favorable[19]. This allows for detailed analysis of the intestinal mucosa and *in vivo* histology during ongoing endoscopy[20]. CLE has shown high accuracy rates (85%-94%) for the detection of high-grade dysplasia and a sensitivity of 80% in the identification of advanced neoplasia with good interobserver agreement[20,21]. In an *ex vivo* study, CLE was shown to have positive and negative predictive values for high-grade dysplasia/early cancer of 44% and 83%, respectively[22]. When combined with high-definition white light endoscopy, probe-based CLE was able to detect all cases of high-grade dysplasia/early cancer in this study, but this was not statistically significant compared to these imaging methods alone[23].

Currently endoscope-based CLE (e-CLE) is not available for use in the United States, but the probe-based CLE (pCLE) version (Cellvizio, Mauna Kea Technologies, MA, United States) is. The pCLE version images a small area at a time and thus has a narrow field of view. Thus, it is cumbersome to use in long segments of Barrett's where advanced imaging is more of a need *vs* short segments. In our practice we find CLE to be helpful when wanting to examine a specific area if considering a biopsy *vs* endoscopic resection, however its routine use for surveillance is limited given its narrow field of view; especially since it does not meet PIVI thresholds. It should be noted that CLE also requires intravenous fluorescein which has been reported to be safe in GI procedures[23].

VOLUMETRIC LASER ENDOMICROSCOPY (VLE)

VLE (NvisionVLE, Ninepoint Medical, Bedford, MA, USA) is the latest advanced imaging technology in Barrett's. It is second-generation optical coherence tomography using infrared light to produce real-time, high-resolution, cross-sectional microstructure imaging of tissue[24]. VLE can scan a 6-cm length of the esophagus in approximately 90 s, providing surface and subsurface wide-field cross-sectional imaging with an axial resolution of 7 μm, and to a depth of 3 mm[25]. *Ex-vivo* studies comparing VLE features to endoscopic resection specimens have demonstrated sensitivities of 86%-90% and specificities of 88%-93% for the detection of dysplasia in BE[26]. The benefits to VLE is that an entire segment of Barrett's can be imaged in a short period of time, abnormalities can be laser marked for targeting, the interobserver variability among experts is limited[27], and the learning curve for image interpretation appears favorable[28]. In a large retrospective study comparing the dysplasia yield in BE's patients undergoing Seattle protocol biopsies, VLE without laser markings, and VLE with laser markings (VLEL), both VLE with and without laser marking had statistically significant differences in dysplasia yield compared to Seattle protocol, (14% *vs* 1%, $P = 0.001$) and (11% *vs* 1%, $P = 0.003$), respectively[25]. VLE appears to be a safe form of advanced imaging. In a case series on 52 patients, the safety and feasibility of the NVision VLE system was assessed. Of the 52 patients undergoing VLE, only 2 minor adverse events were reported which includes mucosal lacerations that did not require therapy or intervention[29]. VLE does not appear to significantly increase endoscopic risk to patient but can lead longer procedure times, estimated 22 ± 6 min standard deviation, which can be of anesthetic concern[30].

The downside to VLE is that a large amount of information is presented that may be overwhelming or time consuming to interpret. Artificial intelligence (AI) technology has been developed that has recently been released and is under study in a prospective fashion[31]. This may help physicians process the large amount of information and images that are presented at one time. The AI technology is termed intelligent real-time image segmentation (IRIS) and highlights three VLE features associated with dysplasia . The three features of dysplasia include hyper-reflective surface, hypo-reflective structures, and a lack of layering. A hyper-reflective surface indicates a high surface signal (appears darker) relative to the subsurface. The image feature is represented as a pink color bar at the tissue surface. The lack of layering image feature is represented by an orange color bar at the exterior edge of the VLE image space. The hypo-reflective structure (usually glandular structures) is represented by a blue image overlay on top of the structure. IRIS displays an *en face* image of the scanned esophagus. There is also a luminal *en face* view that reconstructs the Barrett's segment in regard to the three features. These allow for easier identification of overlap between the three colors and is high yield for areas that could be dysplastic. Figures 2-4 show VLE images with IRIS in patients with Barrett's esophagus and high-grade dysplasia. Figure 5 shows the endoscopy view of the targeted laser marks (upper yellow arrow, lower yellow arrow) placed using volumetric laser endomicroscopy.

Prospective *in vivo* studies are needed looking at the sensitivity and specificity of VLE in dysplasia detection for BE. A prospective multi-center study examining this has been completed and we are awaiting results[32]. We suspect VLE is the most sensitive tool for the detection of dysplasia in BE. For reference, acetic acid chromoendoscopy, narrow band imaging and endoscopic CLE have been shown to have high sensitivity of close to 90% in various studies. The VLE scoring system is evolving as quickly as the technology is being developed. The traditional current VLE scoring system (OCT-SI) generates a dysplasia score after the combination of 2 independent criteria (surface to subsurface signal intensity and glandular archi-tecture). The novel VLE diagnostic algorithm (VLE-DA) where a segment of BE is first characterized as having complete or partial effacement, then further categorized by subsurface intensity and number of atypical glands respectively. In a head to head comparison for the detection of dysplasia, pCLE, OCT-SI and VLE-DA were evaluated. The sensitivity for pCLE was 76% (95%CI: 59-88), for OCT-SI was 70% (95%CI: 52-84) and for VLE-DA was 86% (95%CI: 69-96)[33]. Finally cost utility studies are needed comparing the benefit of finding dysplasia and preventing cancer and its associated costs.

CONCLUSION

Advanced imaging in BE can be useful in management of these patients if it helps increase yield of dysplasia detection or help change management in a procedure. The decision to perform these procedures ultimately depends on if the benefit outweighs the cost and added time performing the procedure. In our practice the added benefits of narrow band imaging and volumetric laser endomicroscopy outweighs the costs and added time and thus we have incorporated this into our Barrett's surveillance routine.

Future research may dictate which advanced imaging techniques become incorporated in the gastrointestinal society guidelines, but for now if the sensitivity, specificity, and cost of an exam is acceptable locally for a center/endoscopic imaging expert[34], then the advanced imaging tool is generally acceptable and thus worth the squeeze!

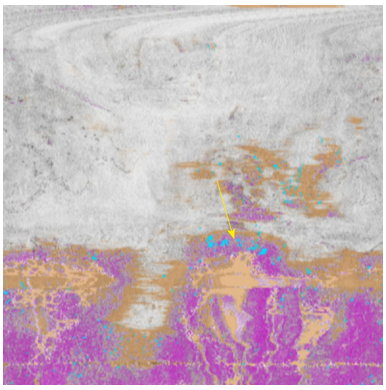


Figure 2 Volumetric laser endomicroscopy with artifical intelligence from the same patient as in Figure 1 with an *en-face* view showing an area of overlap (yellow arrow) between three features of dysplasia (orange is lack of layering, blue is glandular structures, and pink is a hyper-reflective surface).

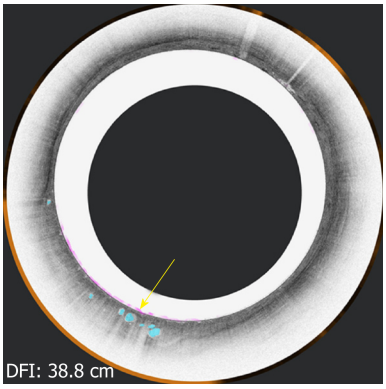


Figure 3 Volumetric laser endomicroscopy from the same patient showing cross-sectional view of the area of overlap (yellow arrow 5.73" 5.41") between three features of dysplasia (orange is lack of layering, blue is glandular structures, and pink is a hyper-reflective surface).

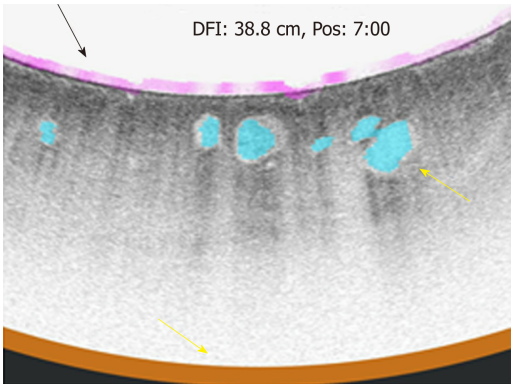


Figure 4 Volumetric laser endomicroscopy with artificial intelligence showing an up close snap shot of the abnormal area of overlap between three features of dysplasia (orange is lack of layering, blue is glandular structures, and pink is a hyper-reflective surface).

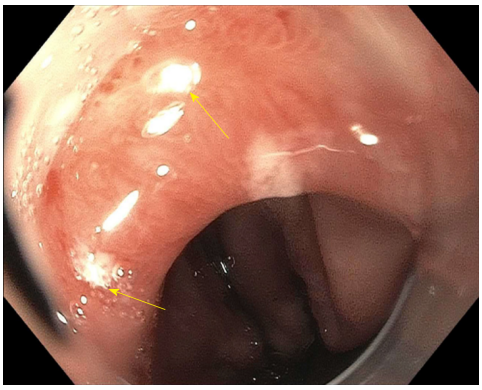


Figure 5 Endoscopy view of the targeted laser marks (upper yellow arrow, lower yellow arrow) placed using volumetric laser endomicroscopy. This corresponds to the same area highlighted by narrow band imaging. The pathology showed high-grade dysplasia.

REFERENCES

1 **Sharma P**, Falk GW, Weston AP, Reker D, Johnston M, Sampliner RE. Dysplasia and cancer in a large multicenter cohort of patients with Barrett's esophagus. *Clin Gastroenterol Hepatol* 2006; **4**: 566-572 [PMID: 16630761 DOI: 10.1016/j.cgh.2006.03.001]

2 **Corley DA**, Levin TR, Habel LA, Weiss NS, Buffler PA. Surveillance and survival in Barrett's adenocarcinomas: a population-based study. *Gastroenterology* 2002; **122**: 633-640 [PMID: 11874995]

3 **Bhat SK**, McManus DT, Coleman HG, Johnston BT, Cardwell CR, McMenamin U, Bannon F, Hicks B, Kennedy G, Gavin AT, Murray LJ. Oesophageal adenocarcinoma and prior diagnosis of Barrett's oesophagus: a population-based study. *Gut* 2015; **64**: 20-25 [PMID: 24700439 DOI: 10.1136/gutjnl-2013-305506]

4 **Corley DA**, Mehtani K, Quesenberry C, Zhao W, de Boer J, Weiss NS. Impact of endoscopic surveillance on mortality from Barrett's esophagus-associated esophageal adenocarcinomas. *Gastroenterology* 2013; **145**: 312-319.e1 [PMID: 23673354 DOI: 10.1053/j.gastro.2013.05.004]

5 **Spechler SJ**, Sharma P, Souza RF, Inadomi JM, Shaheen NJ; American Gastroenterological Association. American Gastroenterological Association technical review on the management of Barrett's esophagus. *Gastroenterology* 2011; **140**: e18-52; quiz e13 [PMID: 21376939 DOI: 10.1053/j.gastro.2011.01.031]

6 **ASGE Technology Committee**. Thosani N, Abu Dayyeh BK, Sharma P, Aslanian HR, Enestvedt BK, Komanduri S, Manfredi M, Navaneethan U, Maple JT, Pannala R, Parsi MA, Smith ZL, Sullivan SA, Banerjee S. ASGE Technology Committee systematic review and meta-analysis assessing the ASGE Preservation and Incorporation of Valuable Endoscopic Innovations thresholds for adopting real-time imaging-assisted endoscopic targeted biopsy during endoscopic surveillance of Barrett's esophagus. *Gastrointest Endosc* 2016; **83**: 684-98.e7 [PMID: 26874597 DOI: 10.1016/j.gie.2016.01.007]

7 **Horwhat JD**, Maydonovitch CL, Ramos F, Colina R, Gaertner E, Lee H, Wong RK. A randomized comparison of methylene blue-directed biopsy versus conventional four-quadrant biopsy for the detection of intestinal metaplasia and dysplasia in patients with long-segment Barrett's esophagus. *Am J Gastroenterol* 2008; **103**: 546-554 [PMID: 17970838 DOI: 10.1111/j.1572-0241.2007.01601.x]

8 **Lim CH**, Rotimi O, Dexter SP, Axon ATR. Randomized crossover study that used methylene blue or random 4-quadrant biopsy for the diagnosis of dysplasia in Barrett's esophagus. *Gastrointest Endosc* 2006; **64**: 195-199 [DOI: 10.1016/j.gie.2005.07.025]

9 **Olliver JR**, Wild CP, Sahay P, Dexter S, Hardie LJ. Chromoendoscopy with methylene blue and associated DNA damage in Barrett's oesophagus. *Lancet* 2003; **362**: 373-374 [PMID: 12907012]

10 **Sharma P**, Weston AP, Topalovski M, Cherian R, Bhattacharyya A, Sampliner RE. Magnification chromoendoscopy for the detection of intestinal metaplasia and dysplasia in Barrett's oesophagus. *Gut* 2003; **52**: 24-27 [PMID: 12477754 DOI: 10.1136/gut.52.1.24]

11 **Kara MA**, Peters FP, Rosmolen WD, Krishnadath KK, ten Kate FJ, Fockens P, Bergman JJ. High-resolution endoscopy plus chromoendoscopy or narrow-band imaging in Barrett's esophagus: a prospective randomized crossover study. *Endoscopy* 2005; **37**: 929-936 [PMID: 16189764 DOI: 10.1055/s-2005-870433]

12 **Guelrud M**, Herrera I, Essenfeld H, Castro J. Enhanced magnification endoscopy: a new technique to identify specialized intestinal metaplasia in Barrett's esophagus. *Gastrointest Endosc* 2001; **53**: 559-565 [PMID: 11323579]

13 **Gono K**, Obi T, Yamaguchi M, Ohyama N, Machida H, Sano Y, Yoshida S, Hamamoto Y, Endo T. Appearance of enhanced tissue features in narrow-band endoscopic imaging. *J Biomed Opt* 2004; **9**: 568-577 [PMID: 15189095 DOI: 10.1117/1.1695563]

14 **Sharma P**, Bansal A, Mathur S, Wani S, Cherian R, McGregor D, Higbee A, Hall S, Weston A. The utility of a novel narrow band imaging endoscopy system in patients with Barrett's esophagus. *Gastrointest Endosc* 2006; **64**: 167-175 [PMID: 16860063 DOI: 10.1016/j.gie.2005.10.044]

15 **Wolfsen HC**, Crook JE, Krishna M, Achem SR, Devault KR, Bouras EP, Loeb DS, Stark ME, Woodward TA, Hemminger LL, Cayer FK, Wallace MB. Prospective, controlled tandem endoscopy study of narrow band imaging for dysplasia detection in Barrett's Esophagus. *Gastroenterology* 2008; **135**: 24-31 [PMID: 18442484 DOI: 10.1053/j.gastro.2008.03.019]

16 **Sharma P**, Bansal A, Hawes R, Rastogi A, Singh M, Curvers WL, Hall M, Mathur SC, Wani SB, Singh V, Verma A, Higbee AD, Fockens P, Bergman J. Detection of metaplasia and neoplasia in patients with Barrett's esophagus using high-definition white light endoscopy vs narrow band imaging: a prospective, multi-center, randomized, crossover trial. *Gastrointest Endosc* 2009; **69**: AB135 [DOI: 10.1016/j.gie.2009.03.181]

17 **Pohl J**, May A, Rabenstein T, Pech O, Nguyen-Tat M, Fissler-Eckhoff A, Ell C. Comparison of computed virtual chromoendoscopy and conventional chromoendoscopy with acetic acid for detection of neoplasia in Barrett's esophagus. *Endoscopy* 2007; **39**: 594-598 [PMID: 17611913 DOI: 10.1055/s-2007-966649]

18 **Kiesslich R**, Gossner L, Goetz M, Dahlmann A, Vieth M, Stolte M, Hoffman A, Jung M, Nafe B, Galle PR, Neurath MF. In vivo histology of Barrett's esophagus and associated neoplasia by confocal laser endomicroscopy. *Clin Gastroenterol Hepatol* 2006; **4**: 979-987 [PMID: 16843068 DOI: 10.1016/j.cgh.2006.05.010]

19 **Liu J**, Li M, Li Z, Zuo XL, Li CQ, Dong YY, Zhou CJ, Li YQ. Learning curve and interobserver agreement of confocal laser endomicroscopy for detecting precancerous or early-stage esophageal squamous cancer. *PLoS One* 2014; **9**: e99089 [PMID: 24897112 DOI: 10.1371/journal.pone.0099089]

20 **Kiesslich R**, Burg J, Vieth M, Gnaendiger J, Enders M, Delaney P, Polglase A, McLaren W, Janell D, Thomas S, Nafe B, Galle PR, Neurath MF. Confocal laser endoscopy for diagnosing intraepithelial neoplasias and colorectal cancer in vivo. *Gastroenterology* 2004; **127**: 706-713 [PMID: 15362025]

21 **Pohl H**, Rösch T, Vieth M, Koch M, Becker V, Anders M, Khalifa AC, Meining A. Miniprobe confocal laser microscopy for the detection of invisible neoplasia in patients with Barrett's oesophagus. *Gut* 2008; **57**: 1648-1653 [PMID: 18755886 DOI: 10.1136/gut.2008.157461]

22 **Sharma P**, Meining AR, Coron E, Lightdale CJ, Wolfsen HC, Bansal A, Bajbouj M, Galmiche JP, Abrams JA, Rastogi A, Gupta N, Michalek JE, Lauwers GY, Wallace MB. Real-time increased detection of neoplastic tissue in Barrett's esophagus with probe-based confocal laser endomicroscopy: final results of an international multicenter, prospective, randomized, controlled trial. *Gastrointest Endosc* 2011; **74**: 465-472 [PMID: 21741642 DOI: 10.1016/j.gie.2011.04.004]

23 **Wallace MB**, Meining A, Canto MI, Fockens P, Miehlke S, Roesch T, Lightdale CJ, Pohl H, Carr-Locke D, Löhr M, Coron E, Filoche B, Giovannini M, Moreau J, Schmidt C, Kiesslich R. The safety of intravenous fluorescein for confocal laser endomicroscopy in the gastrointestinal tract. *Aliment Pharmacol Ther* 2010; **31**: 548-552 [PMID: 20002025 DOI: 10.1111/j.1365-2036.2009.04207.x]

24 **Trindade AJ**, Leggett CL, Chang KJ. Volumetric laser endomicroscopy in the management of Barrett's esophagus. *Curr Opin Gastroenterol* 2017; **33**: 254-260 [PMID: 28402993 DOI: 10.1097/MOG.0000000000000366]

25 **Alshelleh M**, Inamdar S, McKinley M, Stewart M, Novak JS, Greenberg RE, Sultan K, Devito B, Cheung M, Cerulli MA, Miller LS, Sejpal DV, Vegesna AK, Trindade AJ. Incremental yield of dysplasia detection in Barrett's esophagus using volumetric laser endomicroscopy with and without laser marking compared with a standardized random biopsy protocol. *Gastrointest Endosc* 2018; **88**: 35-42 [PMID: 29410080 DOI: 10.1016/j.gie.2018.01.032]

26 **Wolfsen HC**. Volumetric Laser Endomicroscopy in Patients With Barrett Esophagus. *Gastroenterol Hepatol (NY)* 2016; **12**: 719-722 [PMID: 28035200]

27 **Trindade AJ**, Inamdar S, Smith MS, Chang KJ, Leggett CL, Lightdale CJ, Pleskow DK, Sejpal DV, Tearney GJ, Thomas RM, Wallace MB. Volumetric laser endomicroscopy in Barrett's esophagus: interobserver agreement for interpretation of Barrett's esophagus and associated neoplasia among high-frequency users. *Gastrointest Endosc* 2017; **86**: 133-139 [PMID: 27899321 DOI: 10.1016/j.gie.2016.11.026]

28 **Trindade AJ**, Inamdar S, Smith MS, Rosen L, Han D, Chang KJ, Leggett CL, Lightdale CJ, Pleskow DK, Sejpal DV, Tearney GJ, Thomas RM, Wallace MB. Learning curve and competence for volumetric laser endomicroscopy in Barrett's esophagus using cumulative sum analysis. *Endoscopy* 2018; **50**: 471-478 [PMID: 29179229 DOI: 10.1055/s-0043-121569]

29 **Wolfen H**. Sharma P, Wallace M, Leffett C, Tearney G, Wang K, Safety and feasibility of volumetric laser endomicroscopy in patients with Barrett's esophagus (with videos). *Gastrointest Endo* 2015; 82 [DOI: 10.1016/j.gie.2015.03.1968]

30 **Swager AF**, de Groof AJ, Meijer SL, Weusten BL, Curvers WL, Bergman JJ. Feasibility of laser marking in Barrett's esophagus with volumetric laser endomicroscopy: first-in-man pilot study. *Gastrointest Endosc* 2017; **86**: 464-472 [PMID: 28161451 DOI: 10.1016/j.gie.2017.01.030]

31 Volumetric Laser Endomicrosopy with Intelligent Real-time Image Segmentation. [accessed 2019 Feb 2]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/record/NCT03814824 ClinicalTrials.gov Identifier: NCT03814824

32 Barrett's Dysplasia Detection Pilot Trial Using the NvisionVLE® Imaging System (DDP). [accessed 2019 Jan 29]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02864043 ClinicalTrials.gov Identifier: NCT02864043

33 **Leggett CL**, Gorospe EC, Chan DK, Muppa P, Owens V, Smyrk TC, Anderson M, Lutzke LS, Tearney G, Wang KK. Comparative diagnostic performance of volumetric laser endomicroscopy and confocal laser endomicroscopy in the detection of dysplasia associated with Barrett's esophagus. *Gastrointest Endosc* 2016; **83**: 880-888.e2 [PMID: 26344884 DOI: 10.1016/j.gie.2015.08.050]

34 **Sharma P**, Brill J, Canto M, DeMarco D, Fennerty B, Gupta N, Laine L, Lieberman D, Lightdale C, Montgomery E, Odze R, Tokar J, Kochman M. White Paper AGA: Advanced Imaging in Barrett's Esophagus. *Clin Gastroenterol Hepatol* 2015; **13**: 2209-2218 [PMID: 26462567 DOI: 10.1016/j.cgh.2015.09.017]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3116-3122

DOI: 10.3748/wjg.v25.i25.3116

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

OPINION REVIEW

Fate plasticity in the intestine: The devil is in the detail

Simon Buczacki

ORCID number: Simon Buczacki (0000-0002-2975-416X).

Author contributions: Buczacki S conceived, researched and wrote this opinion review.

Supported by a Fellowship grant from Cancer Research UK C14094/A27178; and core funding from Wellcome and MRC to the Wellcome-MRC Cambridge Stem Cell Institute.

Conflict-of-interest statement: The author has declared no conflict of interest.

Manuscript source: Invited manuscript

Received: February 21, 2019
Peer-review started: February 22, 2019
First decision: April 30, 2019
Revised: May 14, 2019
Accepted: May 31, 2019
Article in press: June 1, 2019
Published online: July 7, 2019

P-Reviewer: Vagholkar KR
S-Editor: Ma RY

Simon Buczacki, Cambridge Stem Cell Institute, Jeffrey Cheah Biomedical Centre, Addenbrooke's Biomedical Campus, Cambridge CB2 0AF, United Kingdom

Corresponding author: Simon Buczacki, FRCS, MBChB, PhD, Senior Scientist, Surgical Oncologist, Cambridge Stem Cell Institute, Jeffrey Cheah Biomedical Centre, Addenbrooke's Biomedical Campus, Robinson Way, Cambridge CB2 0AF, United Kingdom.
sjab2@cam.ac.uk
Telephone: +44-1223-256879
Fax: +44-1223-256879

Abstract

The intestinal epithelium possesses a remarkable ability for both proliferation and regeneration. The last two decades have generated major advances in our understanding of the stem cell populations responsible for its maintenance during homeostasis and more recently the events that occur during injury induced regeneration. These fundamental discoveries have capitalised on the use of transgenic mouse models and *in vivo* lineage tracing to make their conclusions. It is evident that maintenance is driven by rapidly proliferating crypt base stem cells, but complexities associated with the technicality of mouse modelling have led to several overlapping populations being held responsible for the same behaviour. Similarly, it has been shown that essentially any population in the intestinal crypt can revert to a stem cell state given the correct stimulus during epithelial regeneration. Whilst these observations are profound it is uncertain how relevant they are to human intestinal homeostasis and pathology. Here, these recent studies are presented, in context with technical considerations of the models used, to argue that their conclusions may indeed not be applicable in understanding "homeostatic regeneration" and experimental suggestions presented for validating their results in human tissue.

Key words: Intestinal stem cell; Plasticity; Lgr5; Regeneration

Core tip: Recent advances, using transgenic mice, in understanding cellular hierarchies in the intestinal epithelium have identified numerous cell populations which retain the ability to change their fate in response to injury. Here, these new studies are presented in the context of a discussion about what represents a relevant epithelial injury to understand 'homeostatic regeneration'. Experimental suggestions are proposed for validating animal findings to translate our current knowledge to better understand human intestinal epithelial maintenance.

L-Editor: A
E-Editor: Ma YJ

Citation: Buczacki S. Fate plasticity in the intestine: The devil is in the detail. *World J Gastroenterol* 2019; 25(25): 3116-3122
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3116.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3116

INTRODUCTION

The intestinal lining is one of the most rapidly proliferating epithelia in humans. In the small intestine this single-cell thick structure is thrown into folds consisting of villi that protrude into the lumen and crypts that are embedded in the intestinal wall. The colonic epithelium is similar although lacks villi. At a histological level the small intestine and colon are also alike. In the small intestine the most prevalent cell type is the absorptive enterocyte and, in the colon, the colonocyte. Both organs also possess secretory goblet, enteroendocrine and tuft cells (Figure 1). The main cellular difference between the small intestine and colon is the presence of secretory Paneth cells. These long-lived secretory and niche cells are only found in the bottom of small intestinal crypts and rarely found in the normal colon. A functionally similar cell type, termed the deep crypt secretory cell, has however recently been shown to exist in colonic crypts[1,2]. Both epithelia are highly proliferative and retain a remarkable ability for regeneration following injury. Homeostatic proliferation throughout the intestine takes place in the bottom of the crypts, being most active in the so-called transit amplifying zone – an area directly above the crypt base. Differentiation occurs as cells migrate up the crypts onto either villi or the colonic mucosal plateau.

Over the last two decades the intestine has become an area of great interest in stem cell biology and is arguably the prototypical organ for the study of epithelial homeostasis and regeneration partly due to the unique structure of the crypt that facilitates ready quantification of stem cell clonogenicity. Following seminal findings using lineage tracing, of the clonogenic function of undifferentiated crypt cells during homeostasis, the concept of cellular plasticity has more recently been explored using contemporary *in vivo* techniques. Plasticity is defined as a change in cell fate in response to a stimulus. The results of these new studies have however led the field into a complex and confusing period where, on face value, it appears that almost any cell type in the intestinal epithelium can revert to a stem cell state during regeneration. In this opinion review I discuss both the important original and more recent studies and propose that whilst the findings are striking they may not be entirely relevant for our understanding of "homeostatic regeneration". Here, I define homeostatic regeneration as the cellular changes that occur during the response to injury classically occurring during mammalian life and commonly encountered pathologies.

Evidence for the existence of intestinal stem cells was first demonstrated in the 1970s by Cheng and Leblond who showed that after treating mice with tritiated thymidine, crypt base columnar cells (CBCs) developed labelled phagosomes following phagocytosis of nearby non-viable cells[3]. Subsequent tracing of these labelled phagosomes over time found they were inherited by all the differentiated cell types of the epithelium. These experiments proposed that stem cells present in the base of intestinal crypts could generate all the differentiated cell types of the intestinal epithelium. Following this, attention focussed on cells in the so-called +4 position that appeared both quiescent and undifferentiated – a feature commonly found in stem cells in other organs[4]. The field however underwent a sea change in 2007 following the publication from Hans Clevers' laboratory demonstrating that *Lgr5* expression marked rapidly proliferating CBCs in the small intestine and colon which were capable of profound clonal capacity as shown using a lineage tracing technique in mice[5]. This highly elegant study provided the first direct proof that rapidly cycling Lgr5+ CBCs were the bona fide homeostatic stem cells of throughout the intestine. There then followed a period of intense debate about the nature of the +4 cell with several groups showing marker overlay of genes of interest with cells in this position also possessing stem cell capacity including *Bmi1*, *Hopx* and *mTert*[6-8]. Interestingly, like the original Barker *et al*[5] study, all three of these studies primarily used the location of promoter driven reporter expression to define the anatomical location of cells expressing the respective gene of interest. It is however unknown what degree of gene activity is required to drive reporter expression and this can be compounded by the introduction of *Cre* recombinase as a conditional activator of reporter expression as is often used in lineage tracing studies. Highlighting these issues, two separate

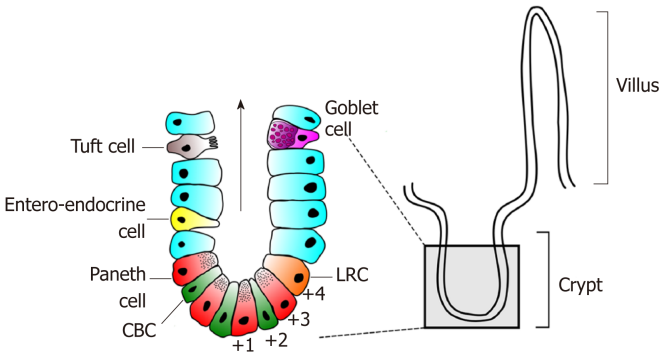


Figure 1 Schematic of the arrangement of cells in the small intestine. CBC: Crypt base columnar cell; LRC: Label retaining cell.

studies of *Bmi1* expressing cells using different models; *Bmi1-CreER* and *Bmi1-GFP* (both knocked in at the endogenous locus) show different results with one study finding the cells to be stem cells and the other mature enteroendocrine cells[6,9]. The Cre enzyme is also seen to possess apparent regional differences in expression when under the control of reportedly pan intestinal promoters; Cre is often found to have greater activity the more proximal in the intestinal tract making it hard to compare with stem cell behaviour in the distal small intestine and colon[10,11]. Whether this is due to promoter, enzyme intrinsic or reporter differences is incompletely understood. These concerns can be compounded when a CreER system is used to drive conditional recombination. In this situation off-target effects of both tamoxifen and impaired stem cell function following activation of Cre have been reported by two separate studies[12,13]. These important studies indicate that quantification of stem cell behaviour following tamoxifen driven Cre activation may not be accurate or representative of the true *in vivo* situation.

Tissue specific gene promoters can also have problems with both sensitivity and specificity for all cells on the crypt-villus axis. A comparison between two intestinal, reportedly pan-epithelial Cre models, *Villin-Cre* and *Ah-Cre* showed unexpected variation in the *Ah-Cre* driven recombination that failed to target a cell population that was capable of driving regeneration[14]. Previously the *Ah-Cre* model had been reported to induce recombination in all IECs other than terminally differentiated Paneth cells[10]. However, in the study by Parry *et al*[14] the authors found that *Ah-Cre* also failed to induce recombination in a non-Paneth cell putative reserve stem cell population. The implication of this work is that quantifying global clonal output using *Ah-Cre* driven reporters may not include all potentially clonogenic populations and erroneous conclusions could be drawn.

It is also unknown how long reporter proteins persist and are visible for, following cessation of their production. It is likely that different reporters have varying stability that could confound analysis if induced conditionally and thus temporally where expression may still be visualised in daughter cells that aren't expressing the mRNA of the gene of interest. This may lead to an assumption that reporter expression is directly correlated with gene expression which has not definitively be proven. Cells may also express low levels of the gene of interest and still possess the same functionality as those with high levels of expression (as the marker itself is unlikely to directly drive function) but low expressors may fail to be identified by reporter expression. Indeed, precisely these concerns were found valid when bulk gene expression comparisons were used to compare the transcriptome of CBC Lgr5+ cells with +4 located Bmi1+ cells suggesting that these cells are in reality one and the same and the +4 cell may well be *Lgr5* expressing[15]. Cumulatively, all these early studies certainly provide direct evidence that homeostatic stem cells exist at the base of the intestinal crypts but also demonstrate the clear difficulties in using transgenic mouse models to dissect sub-populations and functionality at a high level of detail.

Given the striking ability of the intestinal epithelium to regenerate, a natural progression of recent research focus has been to understand which cell populations are responsible for this behaviour. Theoretically there are two possible cell types responsible for regeneration; a distinct quiescent sub-population waiting to become activated when required or a population that has one role during homeostasis but that can revert to a stem-like state during injury *i.e.*, plastic. The first named population found to have a role in regeneration were *Dll1* expressing crypt cells[16]. Using a combination of lineage tracing in combination with irradiation the Clevers group found that during homeostasis these cells were proliferating secretory progenitors but

following irradiation and cell death they acquired stem cell capacity and were thus capable of regenerating the injured epithelium. The question of what the apparently quiescent +4 cell represented was further addressed by Doug Winton's group using a novel split-Cre mouse to conditionally genetically mark label-retaining cells (LRCs)[17]. Supporting the findings of the Clevers group this study found LRCs to be a slowly-cycling Paneth and enteroendocrine cell progenitor that similarly reverted to a stem cell-like fate during injury induced regeneration albeit at very low frequency - partly due to the complexities of the transgenic model employed.

Following the finding of plasticity in *Dll1* cells and LRCs, attention has focussed on whether other populations can perform similar functions. Indeed, it has now been shown that goblet cells, enteroendocrine cells, enterocytes and Paneth cells are all capable of contextually acquiring stem cell capacity[9,18-28]. Analysis of these reports however shows wide variation in the types of injury models employed varying from relatively mild oral dextran sulfate sodium (DSS) that induces mucosal inflammation, to lethal whole-body irradiation (12Gy), making it difficult to compare results between studies (Table 1). More recently it has also been shown that some secretory progenitor populations even during homeostasis may stochastically acquire stemness[22,26,28]. This bi-fated character of some secretory progenitors was originally demonstrated in 2004 where a small number of *Ngn3* enteroendocrine cell progenitors were also found to have clonal/stem cell capacity during homeostasis[29]. Whilst it is entirely plausible that there are a wide range of cell types capable of plasticity there are evidently those that cannot, as clone formation has never been found arising on villi even during classical injury induced regeneration. Schwitalla *et al*[30] have however demonstrated that aberrant elevated NF-kB signalling in apparently terminally differentiated enterocytes on the villi can cause de-differentiation to a tumour-initiating *Lgr5*+ status. This finding proposes that at the very least, if given a strong enough stimulus, even terminally differentiated villus-based enterocytes may acquire some stem cell characteristics.

Cumulatively, these studies show widespread plasticity amongst almost every cell type described to-date in the murine intestinal epithelium however there are many inconsistencies between studies driven primarily from the technical issues related to ascribing identity and plasticity (Table 2). It could also be argued that the apparent broadly found plasticity may not be relevant to advancing our understanding of what cell types are *actually* at play in humans during routine epithelial insult. It has been known for decades that cell types can be reprogrammed to different identities and this forms the fundamental basis of induced pluripotent stem cells (iPS) technology. In Waddington's classical model of the epigenetic landscape of differentiation it is therefore clear that cells can traverse between several deep valleys given the appropriate stimulus to ascend the elevations between[31]. Evidently, any cell type if pushed hard enough can de-differentiate or trans-differentiate but the question remains what represents a *physiological injury* and what cell types are involved in the subsequent "homeostatic regeneration"? It may well be the case that during different forms of injury such as that seen between Crohn's disease and ulcerative colitis varying cell types are mobilised *via* plasticity to the stem cell state to try and repair the damaged epithelium.

How then can the field move forward to make meaningful in-roads into translating these previous animal findings to the benefit of patients? There are two areas that would seem ripe for development – tools for lineage tracing in humans and better validated injury models in mice. Lineage tracing in mice is primarily performed through generating transgenic mouse strains which is clearly impossible in humans. There are however several new tools that could permit lineage tracing analysis in human tissue albeit by quantifying the clonal output of all potentially clonogenic cells. Two important recent reports have made use of next generation sequencing technology to identify clones through the quantification of somatically acquired mosaic mutations found in human and murine tissue[32,33]. The advances now made in sequencing technologies enabling combined single cell DNA (scDNAseq) and RNA sequencing (scRNAseq) allow for similar sequencing approaches with higher coverage to uncover cellular hierarchies from human tissue in both disease and homeostasis.

Clonal marking in humans can also be performed by quantifying mitochondrial DNA (mtDNA) mutations through dual immunohistochemical (IHC) staining for the mitochondrially encoded enzyme cytochrome-c oxidase (CCO)[34]. Cells acquire mtDNA mutations infrequently and can be identified through this IHC technique as cumulatively more are acquired in the many mtDNA copies of the CCO gene *via* stochastic genetic drift. As the mutation is genetic and therefore heritable, the clonal output of cells acquiring these mutations can be quantified. More recently several new similarly working but genomic DNA (gDNA) encoded neutral IHC clonal marks have been described by the Winton laboratory[35]. Importantly, both CCO staining and the

Table 1 Mouse injury models used for plasticity studies

Study	Plastic cell identified	Injury model used
Tian *et al*[18]	Bmi1+ cell	DTR Lgr5+ ablation
Roth *et al*[19]	Paneth cell	12Gy radiation
van Es *et al*[16]	Dll1+ cell	6Gy radiation
Buczacki *et al*[17]	Label-retaining cell	6Gy radiation, doxorubicin or hydroxyurea
Asfaha *et al*[20]	Upper crypt progenitor	12Gy radiation +/- 5-Fluorouracil
Tetteh *et al*[27]	Alpi1+ enterocyte	DTR Lgr5+ ablation
Jadhav *et al*[9]	Goblet cell progenitors	DTR Lgr5+ ablation
Yan *et al*[21]	Enteroendocrine cell	12Gy radiation
Ishibashi *et al*[22]	Atoh1+ cell	DSS (1.75%) for 5 d
Nusse *et al*[23]	Lgr5- crypt cell	Parasite infection
Schmitt *et al*[24]	Paneth cell	DSS (3%) for 1 wk
Tomic *et al*[26]	Atoh1+ cell	6Gy radiation, AOM or 2% DSS
Yu *et al*[25]	Lyz1+ Paneth cell	12Gy radiation
Castillo *et al*[28]	Atoh1+ cell	DSS (2.5%-3%) for 5 d

newer clonal marks allow the quantification of clonal behaviour in situ from human tissue. Using these techniques on matched sections of diseased and normal tissue allows an understanding, at the numerical level, of changes in behaviour such as seen in plasticity. Further, combining these lineage tracing approaches with scRNAseq could provide profound insights, like those described in the mouse, to understanding intestinal homeostasis and plasticity in humans.

CONCLUSION

Finally, there is an urgent need to better define the cellular effects of common human intestinal epithelial injuries to identify appropriate murine model equivalents. The current use of multiple different forms of injury models which only bear a passing relationship to human disease in that there is some degree of cell death is far from ideal. The interplay between epithelial loss, stromal tissue, immune cells, vasculature and resident microbiota is highly complex and very likely inadequately modelled in our current simplistic models of injury performed on mice housed in clean animal facilities. Here, the opposite approach to that presented earlier could be used: Human studies into the precise cellular events occurring during various injuries could inform the development of better murine injury models. It would appear that the field is reaching the limitations of what can be achieved with current tools and models and in order to advance as rapidly as previously, new approaches are required that maximise on novel technologies and translationally relevant models.

Table 2 Concepts leading to difficulties in ascribing behaviour to cell types in the intestine

CreER and Tamoxifen	Toxicity
	Off-target effects
Incongruity between reporter expression and protein expression	Regional differences
	Chronicity of reporter stability
	Reporter and mRNA expression differences
Inconsistent injury models	Intestinal specific effects including incomplete cell type eradication *e.g.*, diphtheria toxin mediated cell death
	Off-target whole body effects *e.g.*, irradiation
	Representative of "homeostatic regeneration"
	Different cell-type responses to different injuries
Laboratory differences	Microbiota
	Diet
	Area of intestine examined
	Strain differences between laboratories due to inbreeding

REFERENCES

1 **Rothenberg ME**, Nusse Y, Kalisky T, Lee JJ, Dalerba P, Scheeren F, Lobo N, Kulkarni S, Sim S, Qian D, Beachy PA, Pasricha PJ, Quake SR, Clarke MF. Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice. *Gastroenterology* 2012; **142**: 1195-1205.e6 [PMID: 22333952 DOI: 10.1053/j.gastro.2012.02.006]

2 **Sasaki N**, Sachs N, Wiebrands K, Ellenbroek SI, Fumagalli A, Lyubimova A, Begthel H, van den Born M, van Es JH, Karthaus WR, Li VS, López-Iglesias C, Peters PJ, van Rheenen J, van Oudenaarden A, Clevers H. Reg4+ deep crypt secretory cells function as epithelial niche for Lgr5+ stem cells in colon. *Proc Natl Acad Sci USA* 2016; **113**: E5399-E5407 [PMID: 27573849 DOI: 10.1073/pnas.1607327113]

3 **Cheng H**, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* 1974; **141**: 537-561 [PMID: 4440635 DOI: 10.1002/aja.1001410407]

4 **Potten CS**. Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. *Nature* 1977; **269**: 518-521 [PMID: 909602]

5 **Barker N**, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003-1007 [PMID: 17934449 DOI: 10.1038/nature06196]

6 **Sangiorgi E**, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 2008; **40**: 915-920 [PMID: 18536716 DOI: 10.1038/ng.165]

7 **Montgomery RK**, Carlone DL, Richmond CA, Farilla L, Kranendonk ME, Henderson DE, Baffour-Awuah NY, Ambruzs DM, Fogli LK, Algra S, Breault DT. Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. *Proc Natl Acad Sci USA* 2011; **108**: 179-184 [PMID: 21173232 DOI: 10.1073/pnas.1013004108]

8 **Takeda N**, Jain R, LeBoeuf MR, Wang Q, Lu MM, Epstein JA. Interconversion between intestinal stem cell populations in distinct niches. *Science* 2011; **334**: 1420-1424 [PMID: 22075725 DOI: 10.1126/science.1213214]

9 **Jadhav U**, Saxena M, O'Neill NK, Saadatpour A, Yuan GC, Herbert Z, Murata K, Shivdasani RA. Dynamic Reorganization of Chromatin Accessibility Signatures during Dedifferentiation of Secretory Precursors into Lgr5+ Intestinal Stem Cells. *Cell Stem Cell* 2017; **21**: 65-77.e5 [PMID: 28648363 DOI: 10.1016/j.stem.2017.05.001]

10 **Ireland H**, Kemp R, Houghton C, Howard L, Clarke AR, Sansom OJ, Winton DJ. Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. *Gastroenterology* 2004; **126**: 1236-1246 [PMID: 15131783]

11 **el Marjou F**, Janssen KP, Chang BH, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D, Robine S. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* 2004; **39**: 186-193 [PMID: 15282745 DOI: 10.1002/gene.20042]

12 **Zhu Y**, Huang YF, Kek C, Bulavin DV. Apoptosis differently affects lineage tracing of Lgr5 and Bmi1 intestinal stem cell populations. *Cell Stem Cell* 2013; **12**: 298-303 [PMID: 23415913 DOI: 10.1016/j.stem.2013.01.003]

13 **Bohin N**, Carlson EA, Samuelson LC. Genome Toxicity and Impaired Stem Cell Function after Conditional Activation of CreER^{T2} in the Intestine. *Stem Cell Reports* 2018; **11**: 1337-1346 [PMID: 30449703 DOI: 10.1016/j.stemcr.2018.10.014]

14 **Parry L**, Young M, El Marjou F, Clarke AR. Evidence for a crucial role of paneth cells in mediating the intestinal response to injury. *Stem Cells* 2013; **31**: 776-785 [PMID: 23335179 DOI: 10.1002/stem.1326]

15 **Muñoz J**, Stange DE, Schepers AG, van de Wetering M, Koo BK, Itzkovitz S, Volckmann R, Kung KS, Koster J, Radulescu S, Myant K, Versteeg R, Sansom OJ, van Es JH, Barker N, van Oudenaarden A, Mohammed S, Heck AJ, Clevers H. The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. *EMBO J* 2012; **31**: 3079-3091 [PMID: 22692129 DOI: 10.1038/emboj.2012.166]

16 **van Es JH**, Sato T, van de Wetering M, Lyubimova A, Yee Nee AN, Gregorieff A, Sasaki N, Zeinstra L, van den Born M, Korving J, Martens ACM, Barker N, van Oudenaarden A, Clevers H. Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. *Nat Cell Biol* 2012; **14**: 1099-1104 [PMID: 23000963 DOI: 10.1038/ncb2581]

17 **Buczacki SJ**, Zecchini HI, Nicholson AM, Russell R, Vermeulen L, Kemp R, Winton DJ. Intestinal label-retaining cells are secretory precursors expressing Lgr5. *Nature* 2013; **495**: 65-69 [PMID: 23446353 DOI: 10.1038/nature11965]

18 **Tian H**, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, de Sauvage FJ. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 2011; **478**: 255-259 [PMID: 21927002 DOI: 10.1038/nature10408]

19 **Roth S**, Franken P, Sacchetti A, Kremer A, Anderson K, Sansom O, Fodde R. Paneth cells in intestinal homeostasis and tissue injury. *PLoS One* 2012; **7**: e38965 [PMID: 22745693 DOI: 10.1371/journal.pone.0038965]

20 **Asfaha S**, Hayakawa Y, Muley A, Stokes S, Graham TA, Ericksen RE, Westphalen CB, von Burstin J, Mastracci TL, Worthley DL, Guha C, Quante M, Rustgi AK, Wang TC. Krt19(+)/Lgr5(-) Cells Are Radioresistant Cancer-Initiating Stem Cells in the Colon and Intestine. *Cell Stem Cell* 2015; **16**: 627-638 [PMID: 26046762 DOI: 10.1016/j.stem.2015.04.013]

21 **Yan KS**, Gevaert O, Zheng GXY, Anchang B, Probert CS, Larkin KA, Davies PS, Cheng ZF, Kaddis JS, Han A, Roelf K, Calderon RI, Cynn E, Hu X, Mandleywala K, Wilhelmy J, Grimes SM, Corney DC, Boutet SC, Terry JM, Belgrader P, Ziraldo SB, Mikkelsen TS, Wang F, von Furstenberg RJ, Smith NR, Chandrakesan P, May R, Chrissy MAS, Jain R, Cartwright CA, Niland JC, Hong YK, Carrington J, Breault DT, Epstein J, Houchen CW, Lynch JP, Martin MG, Plevritis SK, Curtis C, Ji HP, Li L, Henning SJ, Wong MH, Kuo CJ. Intestinal Enteroendocrine Lineage Cells Possess Homeostatic and Injury-Inducible Stem Cell Activity. *Cell Stem Cell* 2017; **21**: 78-90.e6 [PMID: 28686870 DOI: 10.1016/j.stem.2017.06.014]

22 **Ishibashi F**, Shimizu H, Nakata T, Fujii S, Suzuki K, Kawamoto A, Anzai S, Kuno R, Nagata S, Ito G, Murano T, Mizutani T, Oshima S, Tsuchiya K, Nakamura T, Watanabe M, Okamoto R. Contribution of ATOH1^{+} Cells to the Homeostasis, Repair, and Tumorigenesis of the Colonic Epithelium. *Stem Cell Reports* 2018; **10**: 27-42 [PMID: 29233556 DOI: 10.1016/j.stemcr.2017.11.006]

23 **Nusse YM**, Savage AK, Marangoni P, Rosendahl-Huber AKM, Landman TA, de Sauvage FJ, Locksley RM, Klein OD. Parasitic helminths induce fetal-like reversion in the intestinal stem cell niche. *Nature* 2018; **559**: 109-113 [PMID: 29950724 DOI: 10.1038/s41586-018-0257-1]

24 **Schmitt M**, Schewe M, Sacchetti A, Feijtel D, van de Geer WS, Teeuwssen M, Sleddens HF, Joosten R, van Royen ME, van de Werken HJG, van Es J, Clevers H, Fodde R. Paneth Cells Respond to Inflammation and Contribute to Tissue Regeneration by Acquiring Stem-like Features through SCF/c-Kit Signaling. *Cell Rep* 2018; **24**: 2312-2328.e7 [PMID: 30157426 DOI: 10.1016/j.celrep.2018.07.085]

25 **Yu S**, Tong K, Zhao Y, Balasubramanian I, Yap GS, Ferraris RP, Bonder EM, Verzi MP, Gao N. Paneth Cell Multipotency Induced by Notch Activation following Injury. *Cell Stem Cell* 2018; **23**: 46-59.e5 [PMID: 29887318 DOI: 10.1016/j.stem.2018.05.002]

26 **Tomic G**, Morrissey E, Kozar S, Ben-Moshe S, Hoyle A, Azzarelli R, Kemp R, Chilamakuri CSR, Itzkovitz S, Philpott A, Winton DJ. Phospho-regulation of ATOH1 Is Required for Plasticity of Secretory Progenitors and Tissue Regeneration. *Cell Stem Cell* 2018; **23**: 436-443.e7 [PMID: 30100168 DOI: 10.1016/j.stem.2018.07.002]

27 **Tetteh PW**, Basak O, Farin HF, Wiebrands K, Kretzschmar K, Begthel H, van den Born M, Korving J, de Sauvage F, van Es JH, van Oudenaarden A, Clevers H. Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters. *Cell Stem Cell* 2016; **18**: 203-213 [PMID: 26831517 DOI: 10.1016/j.stem.2016.01.001]

28 **Castillo-Azofeifa D**, Fazio EN, Nattiv R, Good HJ, Wald T, Pest MA, de Sauvage FJ, Klein OD, Asfaha S. Atoh1^{+} secretory progenitors possess renewal capacity independent of Lgr5^{+} cells during colonic regeneration. *EMBO J* 2019; **38** [PMID: 30635334 DOI: 10.15252/embj.201899984]

29 **Schonhoff SE**, Giel-Moloney M, Leiter AB. Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol* 2004; **270**: 443-454 [PMID: 15183725 DOI: 10.1016/j.ydbio.2004.03.013]

30 **Schwitalla S**, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, Canli O, Heijmans J, Huels DJ, Moreaux G, Rupec RA, Gerhard M, Schmid R, Barker N, Clevers H, Lang R, Neumann J, Kirchner T, Taketo MM, van den Brink GR, Sansom OJ, Arkan MC, Greten FR. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* 2013; **152**: 25-38 [PMID: 23273993 DOI: 10.1016/j.cell.2012.12.012]

31 **Waddington CH**. *Organisers and Genes*. Cambridge: Cambridge Univ. Press 1940;

32 **Behjati S**, Huch M, van Boxtel R, Karthaus W, Wedge DC, Tamuri AU, Martincorena I, Petljak M, Alexandrov LB, Gundem G, Tarpey PS, Roerink S, Blokker J, Maddison M, Mudie L, Robinson B, Nik-Zainal S, Campbell P, Goldman N, van de Wetering M, Cuppen E, Clevers H, Stratton MR. Genome sequencing of normal cells reveals developmental lineages and mutational processes. *Nature* 2014; **513**: 422-425 [PMID: 25043003 DOI: 10.1038/nature13448]

33 **Blokzijl F**, de Ligt J, Jager M, Sasselli V, Roerink S, Sasaki N, Huch M, Boymans S, Kuijk E, Prins P, Nijman IJ, Martincorena I, Mokry M, Wiegerinck CL, Middendorp S, Sato T, Schwank G, Nieuwenhuis EE, Verstegen MM, van der Laan LJ, de Jonge J, IJzermans JN, Vries RG, van de Wetering M, Stratton MR, Clevers H, Cuppen E, van Boxtel R. Tissue-specific mutation accumulation in human adult stem cells during life. *Nature* 2016; **538**: 260-264 [PMID: 27698416 DOI: 10.1038/nature19768]

34 **Walther V**, Alison MR. Cell lineage tracing in human epithelial tissues using mitochondrial DNA mutations as clonal markers. *Wiley Interdiscip Rev Dev Biol* 2016; **5**: 103-117 [PMID: 26302049 DOI: 10.1002/wdev.203]

35 **Nicholson AM**, Olpe C, Hoyle A, Thorsen AS, Rus T, Colombé M, Brunton-Sim R, Kemp R, Marks K, Quirke P, Malhotra S, Ten Hoopen R, Ibrahim A, Lindskog C, Myers MB, Parsons B, Tavaré S, Wilkinson M, Morrissey E, Winton DJ. Fixation and Spread of Somatic Mutations in Adult Human Colonic Epithelium. *Cell Stem Cell* 2018; **22**: 909-918.e8 [PMID: 29779891 DOI: 10.1016/j.stem.2018.04.020]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3123-3135

DOI: 10.3748/wjg.v25.i25.3123

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

REVIEW

Revisiting the liver's role in transplant alloimmunity

Nitin Abrol, Caroline C Jadlowiec, Timucin Taner

ORCID number: Nitin Abrol (0000-0002-9948-314X); Caroline C Jadlowiec (0000-0001-7860-9519); Timucin Taner (0000-0003-0641-2930).

Author contributions: Abrol N, Jadlowiec CC and Taner T participated in the literature search and drafting of the manuscript.

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

Manuscript source: Invited manuscript

Received: March 15, 2019
Peer-review started: March 15, 2019
First decision: April 11, 2019
Revised: April 25, 2019
Accepted: May 18, 2019
Article in press: May 18, 2019
Published online: July 7, 2019

P-Reviewer: El-Karaksy H, Guo JS, Ramos E
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

Nitin Abrol, Timucin Taner, William J. von Liebig Center for Transplantation and Clinical Regeneration, Massyo Clinic, Rochester, MN 55905, United States

Caroline C Jadlowiec, Transplant Center, Mayo Clinic, Phoenix, AZ 85259, United States

Corresponding author: Timucin Taner, FACS, MD, PhD, Associate Professor, Surgeon, William J. von Liebig Center for Transplantation and Clinical Regeneration, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905, United States. taner.timucin@mayo.edu
Telephone: +1-507-266-3841
Fax: +1-507-266-9806

Abstract

The transplanted liver can modulate the recipient immune system to induce tolerance after transplantation. This phenomenon was observed nearly five decades ago. Subsequently, the liver's role in multivisceral transplantation was recognized, as it has a protective role in preventing rejection of simultaneously transplanted solid organs such as kidney and heart. The liver has a unique architecture and is home to many cells involved in immunity and inflammation. After transplantation, these cells migrate from the liver into the recipient. Early studies identified chimerism as an important mechanism by which the liver modulates the human immune system. Recent studies on human T-cell subtypes, cytokine expression, and gene expression in the allograft have expanded our knowledge on the potential mechanisms underlying immunomodulation. In this article, we discuss the privileged state of liver transplantation compared to other solid organ transplantation, the liver allograft's role in multivisceral transplantation, various cells in the liver involved in immune responses, and the potential mechanisms underlying immunomodulation of host alloresponses.

Key words: Liver transplantation; Alloimmunity; Liver-kidney transplant; Tolerance; Rejection

Core tip: The liver not only protects itself from host alloimmune responses, but also modulates alloimmune responses to other simultaneously transplanted solid organs like heart or kidney. The titer of donor specific alloantibodies decreases after liver transplantation, making transplantation of other solid organs possible even in highly sensitized high-risk patients. The immune cells from the liver allograft cross-talk with recipient immune cells and modulate the immune system towards tolerance. The cross-talk between these cells suppress the genes involved in alloimmunity and upregulate the genes involved in tissue repair and metabolism.

Citation: Abrol N, Jadlowiec CC, Taner T. Revisiting the liver's role in transplant alloimmunity. *World J Gastroenterol* 2019; 25(25): 3123-3135
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3123.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3123

INTRODUCTION

The liver has baffled researchers for decades because of its complex set of functions and unique architecture. From a metabolic and anatomic standpoint, it has a dual blood supply with the portal vein carrying blood from the gastrointestinal tract and the hepatic artery carrying systemic blood. From an immunological standpoint, the liver is home to many cells of the lymphoid system. Together, the liver's unique architecture and resident immune cells, allow it to play a key role in transplant alloimmunity. It is well recognized that the liver is an immunologically privileged organ, compared to other organs that are commonly transplanted. The liver allograft not only protects itself from the host immune system, but this protection also extends to other simultaneously transplanted solid organs from the same donor. Many researchers have investigated potential mechanisms of this donor-specific hyporesponsiveness. Recent studies on the host T-cell subtypes and gene expression in the allograft after multi-visceral transplants that include the liver, have expanded our knowledge on the liver's role in transplant immunity[1-3]. In this article, we revisit the liver allograft's role in modulating host alloimmunity with special emphasis on combined organ transplants.

LITERATURE SEARCH

For purpose of this review, the Embase and Ovid MEDLINE databases were searched from January 2000 through January 2019 using keywords "Liver transplant*" and "Alloimmunity". The search included Epub ahead of print, in process, and other non-indexed citations. After removing duplicate publications, 242 studies were finally reviewed by title and abstract for selecting full text articles for current review. The studies describing liver-based modulation of cells of the immune system in solid organ solitary liver or multivisceral transplantation were selected for review.

THE LIVER IS AN IMMUNOLOGICALLY PRIVILEGED ORGAN

The liver's immune-privileged status was first recognized in the porcine liver transplantation model[4]. As early as 1965, it was observed that pigs undergoing liver transplantation survived for prolonged periods with limited immunosuppression, whereas other organs, including skin, heart, and kidneys were quickly rejected[4]. This phenomenon has since been observed in other animal models[5].

The first reports of tolerance in human liver transplantation came from Dr. Thomas Starzl and the Pittsburgh group[6,7]. Their early experience showed that 27% of liver transplant recipients could be weaned from all immunosuppression[8]. Subsequently, many other groups tried immunosuppression weaning in patients with stable liver function[6,9-11]. In a pilot study, 60% of carefully selected pediatric liver transplant recipients could be successfully weaned off immunosuppression[10]. This approach, however, was associated with increased rejection [9-11]. Nevertheless, most liver transplant patients require less maintenance immunosuppression than recipients of other solid organs. Likewise, induction immunosuppression, other than steroids, is rarely needed in liver transplantation.

Antibody-mediated rejection

Compared to other solid organ transplants, liver transplant recipients have fewer episodes of antibody-mediated rejection (AMR). While donor specific alloantibodies can cause antibody-mediated hyperacute or acute rejection in other solid organs, their role in liver transplantation remains unclear[12-17]. Liver transplantation is often performed without a prospective cross-match and outcomes do not appear to be related to pre-transplant positive cross-matches[18,19]. The majority of recipients with preformed donor specific antibodies (DSA) show decline in their DSA levels after

liver transplantation (Figure 1). InMoreover, persitent post-transplant DSA, do not appear to be negatively impact allograft survival within the first year following liver transplantation[20]. The observed decline in DSA post-transplant appears to be linked to the overall health of the liver allograft, as persistence of DSA or development of *de novo* DSA are observed more commonly in patients with allograft fibrosis or recurrent disease. However, this protection is not absolute and there is evidence of complement fixation in recipients with persistent DSA in protocol liver biopsies[20]. In patients with *de novo* DSA against class II human leukocyte antigens (HLA), overall survival is inferior when compared to those with no DSA[21]. Importantly, *de novo* DSA are not uncommon in patients in whom immunosuppression withdrawal is attempted, suggesting that most liver transplant patients require some, albeit minimal, immunosuppression to counter the host alloimmune responses[22].

Several factors are felt to play a role in the liver's resistance to antibody-mediated hyperacute rejection. These factors include the liver's dual blood supply, its fenestrated sinusoidal complex, secretion of soluble major histocompatibility complex antigens, and its ability to absorb antibody (Figure 2). In contrast to other solid organs, the microvascular network of the liver is sinusoidal and lined by fenestrated endothelium with a scant underlying basement membrane (Figure 2, g)[23]. This sinusoidal network is in contrast to other organs that not only have a single afferent blood supply, but also have standard capillary microvasculature that results in ischemia when occluded by complement activated immune complexes. In the liver, only the biliary system is truly dependent on capillary microvasculature. This histological variation may result in a more limited, biliary-specific, form of injury in liver transplantation compared to other solid organs[24].

T cell-mediated rejection (TCMR)

Unlike other solid organs, cellular (T cell-mediated) rejection (TCMR) in liver transplantation follows a bimodal pattern of distribution with the majority of cellular rejections occurring very early (< 6 weeks) post-transplant[25]. When early cellular rejection episodes occur in liver transplant patients, these episodes require much less immunosuppression compared to TCMR in heart, pancreas, lungs, or kidney. Similarly, unlike other solid organs, these early episodes of TCMR do not appear to have a long-term impact of patient or allograft survival[25]. In liver transplantation, TCMR can largely be treated by increasing the dose of immunosuppression or by pulse steroids without requiring lymphocyte depleting antibody-based treatment.

THE LIVER'S ROLE IN MULTIVISCERAL TRANSPLANTATION

Liver-induced immunological tolerance to other allografts was first recognized in pigs, when liver allografts were noted to prevent rapid rejection of skin, kidney, and heart from the same donor[4]. This phenomenon was observed to be true for both orthotopic and auxiliary liver transplants[4]. Since these initial animal models, the same observation has been made in human multivisceral transplants[1,2,26-32]. Patients who undergo a combined liver-kidney transplantation (LKT) experience a lower number of kidney TCMR episodes compared to matched solitary kidney transplant recipients (4.2% *vs* 32.6%)[33]. The protective effect of the liver allograft on simultaneously transplanted kidneys persists long term[2,34]. In a study comparing kidney transplantation after liver or heart/lung transplantation, recipients who previously had a liver transplant had fewer episodes of TCMR in their kidneys (20% *vs* 36%)[34]. In addition, the observed rejection episodes were less severe (all rejection episodes were grade IA/IB), and grade II or grade III rejections were seen only after heart/lung transplantation (0% *vs* 16%)[34]. Similar protective effects against AMR have been reported by several groups. In Sweden, auxiliary liver transplantation was performed in a group of highly sensitized kidney patients who were otherwise deemed too risky to transplant, so as to facilitate kidney transplantation, with partial success[35]. We have also demonstrated protection of the heart allograft from AMR in highly sensitized patients by initial liver transplantation from the same donor[27]. All patients in this cohort had pre-existing DSA and positive cross-matches. In this group, there was an immediate decrease in DSA and stable cardiac and liver allograft function at mean follow up of nearly 2 years[27]. Furthermore, there was less cardiac allograft vasculopathy (assessed with 3D volumetric intravascular ultrasound), lower plaque volume, and slower plaque progression in the cardiac allografts of patients who underwent combined liver-heart transplantation[36]. In a series of 13 combined liver-lung transplants, only 3 patients experienced early rejection that was successfully treated with methylprednisolone[28]; this rate is much lower than that seen after

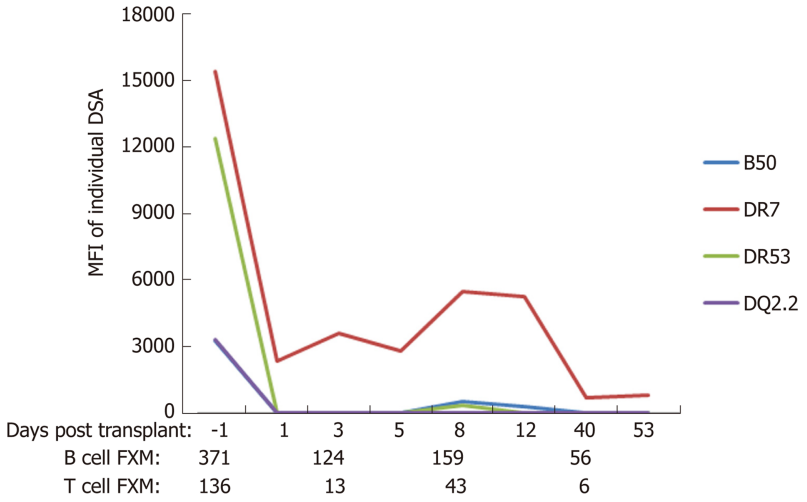


Figure 1 Typical course of donor-specific antibodies and flow cytometric cross match after liver transplant in a patient with fully functional liver allograft who is maintained on triple regimen immunosuppression (tacrolimus, mycophenolate, and prednisone). DSA: Donor specific antibodies; FXM: Flow cytometric cross match.

solitary lung transplantation[28]. Similar protective effects have been observed in combined liver-intestine transplantation[26,37].

OVERVIEW OF ALLOIMMUNITY

Detailed discussion of alloimmunity and downstream pathways after antigen presentation is beyond the scope of this article. Briefly, alloantigens from the transplanted organ are recognized by the host lymphocytes in the secondary lymphoid organs. Dendritic cells, macrophages, B cells, and endothelial cells can play the role of antigen-presenting cells (APC) under various circumstances. Allo-recognition occurs *via* three main pathways: (1) The direct pathway where T-cell receptors on host T cells directly interact with the HLA molecules on the surface of donor APC; (2) The indirect pathway where host APC process donor peptides (mostly derived from donor HLA) and present to host T cells; and (3) The semidirect pathway that involves membrane exchange between donor and host cells or extra-cellular vesicles[38,39]. T cell activation after antigen presentation (Signal 1) requires two additional signals. T-cell receptor interaction occurs through binding of costimulatory molecules on T cells (CD40, CD28) with corresponding ligands on the APCs (CD40L, CD80, CD86) (Signal 2) The presence of T cell stimulatory cytokines in the microenvironment (Signal 3) then results in T cell proliferation (Figure 3).

THE LIVER AS A LYMPHOID AND IMMUNE-REGULATORY ORGAN

Liver architecture is uniquely adapted to provide immunomodulation after exposure to foreign antigens from the gastrointestinal tract. The liver receives a dual blood supply from the high-pressure systemic and the low-pressure portal circulation. These two circulations meet in the hepatic sinusoids resulting in low oxygen saturation, low pressure, and irregular flow facilitating interaction between antigens, T cells and other resident immune cells[40]. Although cell migration occurs in all types of solid organ transplants, the large population of migratory cells in liver allografts may explain the privileged tolerogenicity of the liver compared to other organs (Figure 2)[41]. The hepatocytes are arranged as sheets around the sinusoids. The liver is constantly exposed to microbial antigens carried through the portal circulation. In order to avoid immune activation in response to microbial antigens, liver has developed many molecular modifications. This is evident from high levels of lipopolysaccharide in the portal blood when none is detected in the systemic circulation under normal conditions[42]. Therefore, there is evolutionary advantage to the immunomodulatory role of liver parenchyma. In fact, the liver has been described as a "lymphoid", "immunoregulatory" and "immunomodulatory" organ with various cells playing active role in supporting this function[13,42,40].

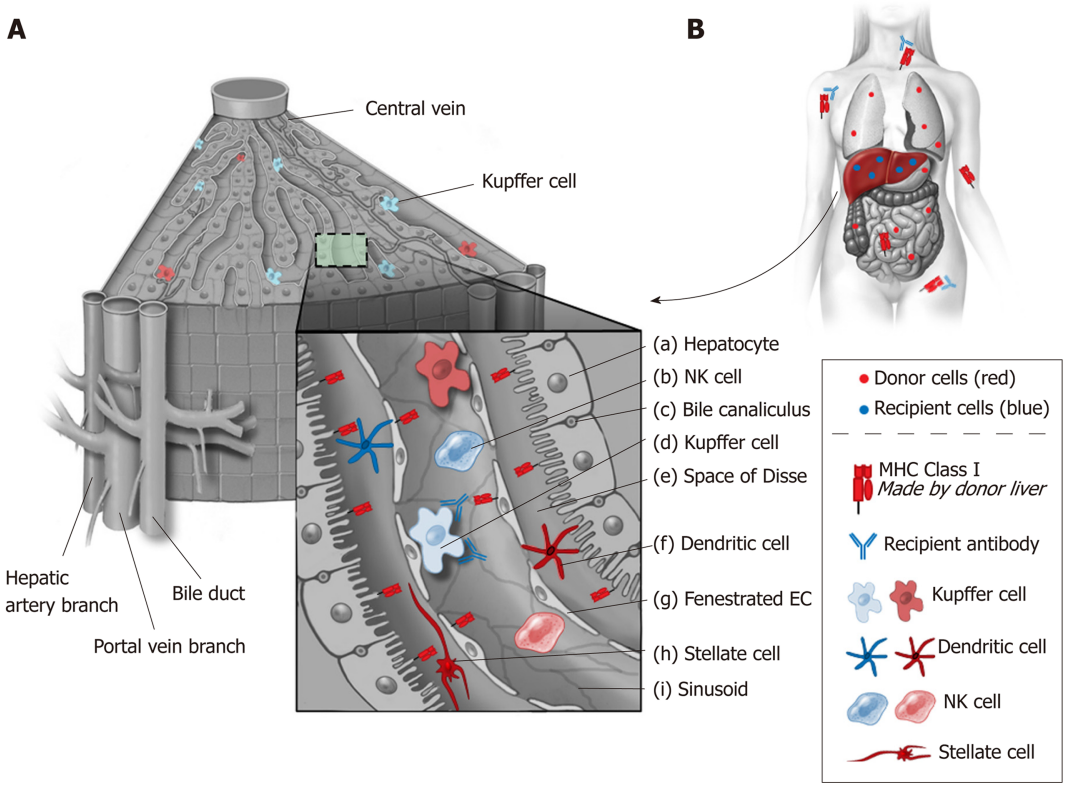


Figure 2 Liver architecture and resident immune cells. A: The liver's unique architecture and the large number of passenger immune cells that accompany it during transplant likely play a role in its immunologic activity. Class I major histocompatibility (MHC) antigens are strongly expressed on bile ducts (c) and to a lesser extent on sinusoidal and endothelial cells (g). By contrast, Class II MHC antigens are primarily expressed on capillary endothelium, sinusoidal cells and dendritic cells (f). It is also recognized that cell surface MHC antigens are not static and can change in response to host and allograft dynamics such as infection and rejection; B: Liver transplants secrete soluble class I MHC antigens that bind and neutralize systemically circulating antibodies. Kupffer cells (d) also are involved in neutralization of antibodies. As such, liver allografts are thought to function as sinks for circulating immune complexes. EC: Endothelial cell; NK: Natural killer; MHC: Major histocompatibility complex.

Immune cells of both lymphoid and myeloid lineage line the thin walled sinusoids, mostly in the space of Disse (Figure 2, e)[42]. These cells include Kupffer cells, dendritic cells, T cells, B cells, natural killer cells, natural killer T cells, hepatic stellate cells (HSC), and hematopoietic stem cells[42,43]. The phenotype of hepatic T cells also differs con-siderably from that observed in the periphery as reflected by a higher ratio (3.5:1 *vs* 1:2) of CD8+ *vs* CD4+ cells[44]. The unique architecture of liver sinusoids (low pressure, fenestrated system, expression of adhesion molecules) allows direct contact of circulating T cells with these cells. These alloantigen recognizing T cells are exposed to IL-10, PD-L1, and lack of co-stimulation leads to their destruction in the liver[42].

Endothelial cells

The sinusoidal endothelial cells (EC) comprise of 50% of non-parenchymal liver cells (Figure 2, g)[40,43]. The sinusoidal EC uniquely lack a basement membrane, are fenestrated, and express scavenger receptors that remove circulatory antigens[42]. ECs also express class I and II HLA and costimulatory molecules, making them potent APCs. However, their main role seems to be induction of tolerance because they respond to antigen stimulation by IL-10 secretion[42]. ECs increase their expression of FasL upon exposure to antigen and induce apoptosis of activated CD4+ T cells[45]. ECs also induce apoptosis of reactive CD8+ T cell *via* a pro-apoptotic Bcl-2 family member Bim[46].

Dendritic cells

The dendritic cells (DC) are professional APCs derived from bone marrow (Figure 2, f). The liver contains two types of DC: Plasmacytoid (pDC) and myeloid (mDC)[42]. The observed frequency of pDC in the liver is more than that in the lymph nodes[42]. The liver contains these cells in immature form. Under normal circumstances, these cells have low expression of the costimulatory molecule CD80[47]. Liver pDC play an important role in innate immunity, as they can produce and secrete IFN-γ[42]. On the other hand, pDC can express PD-L1 on their cell surface, and increased expression of

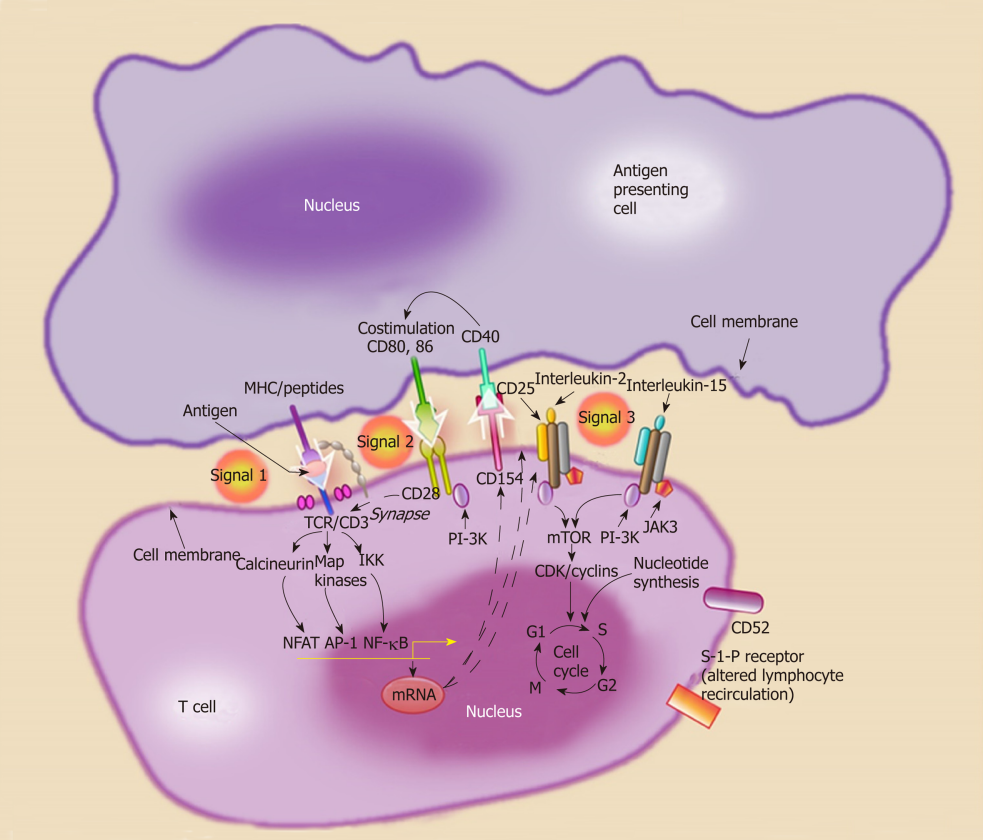


Figure 3 Activation of naïve helper T cells is thought to occur through a three signal pathway. Signal 1, antigen recognition by the T cell receptor complex. Antigens are presented by major histocompatibility complex II cells [antigen presenting cells (APC) such dendritic cells]. Signal 2, co-stimulation, the interaction between the APC (CD80 and CD86) and the T cell (CD28). Signal 3, cellular proliferation and T cell differentiation into effector phenotypes (Th1, Th2), through cytokine stimulation. MHC: Major histocompatibility complex; APC: Antigen presenting cells.

PD-L1 on pDC in tolerant liver transplant patients has been correlated with elevated Tregs[48]. Liver mDC, unlike their counterparts isolated from other organs, appear to have a more inherent tolerant phenotype. For example, under normal circumstances, liver derived mDCs secrete IL-10 and mediate differentiation of T cells into Tregs[49]. Myeloid DC interactions with hepatic stellate cells may play a role in downregulating the immune response[50]. Hepatic stellate cells regulate mDC function by inducing signal transduction, activating transcription, and upregulating indolamine 2,3-dioxygenase (IDO)[50]. Therefore mDC, primed by hepatic stellate cells, have impaired ability to induce allogenic T cell responses[50].

Kupffer cells

The Kupffer cells (KC) are macrophages present in the intra-sinusoidal space and comprise of 15% of all liver cells and 20% of non-parenchymal liver cells (Figure 2, d)[40,43]. Their main role is phagocytosis and cytokine secretion[40]. They also express HLA and costimulatory molecules, therefore they can present antigens to T cells[42]. However, compared to DC, their expression of HLA and costimulatory molecules is low[40]. KC secrete IL-10 and downregulate secretion of proinflammatory cytokines IL-6 and TNF-γ after exposure to lipopolysaccharide[51]. KC have also been found to secrete prostaglandin E2 (PGE2) and 15-deoxy-delta 12,14-PGJ2 (15d-PGJ2)[52]. PGE2 and 15d-PGJ2 inhibit activation of CD4+ T cells[52]. KC can also stimulate Tregs to secrete IL-10[53].

Natural killer cells

The liver contains a high percentage of natural killer (NK) cells (50% of liver lymphocytes) compared to peripheral blood (Figure 2, b)[40,42]. Two type of NK cells exist in the liver: $CD3^{-}CD56^{dim}CD16^{+}CD27^{-}$ (cytotoxic phenotype and $CD3^{-}CD56^{bright}CD16^{-}CD27^{+}$ (cytokine secreting phenotype)[40]. Their role in alloimmunity and rejection appears to be influenced by their origin, such that the NK cells derived from the recipient are involved in rejection while donor-derived NK cells induce tolerance[54]. NK cells have been found to overexpress certain genes in tolerant liver transplant recipients signifying their important role in tolerance induction[55].

This is consistent with the upregulation of NK cell transcripts in tolerant liver transplant patients[56]. In addition, natural killer T cells (NKT), which express markers of NK cells along with the T-cell receptor Vα chain, appear to have a role in liver-induced tolerance, as tolerance is reversed in mice deficient in Vα14 NKT cells [57].

Hepatic stellate cells

Hepatic stellate cells (HSC) (Figure 2, h) are located in the subendothelial space and comprise 10% of the liver cells[43]. Known also as Ito cells, HSC store vitamin A and are involved in various fibrotic processes[43,58]. They express HLA class I, HLA class II and can activate T cells[58]. However, they also express PD-L1 that can lead to tolerance by inactivating activated T cells[42,59,60]. There is evidence that both parenchymal and non-parenchymal cells in the liver cause activation followed by apoptosis of the T cells in the liver allograft as well as *in vitro*[42,61]. Allogenic HSC can migrate to lymph nodes and induce expression of Tregs[62].

Mesenchymal stromal cells

The mesenchymal stromal cells (MSC) have also been localized in the liver[63]. MSC were first described by Friedenstein *et al*[64] as fibroblast like colonies in the bone marrow cultures[64]. Subsequently, MSC have been identified in various organs such as adipose tissue and the liver. These cells are characterized by their ability of trilineage differentiation, plastic adherence, and expression of certain markers on their surface[65]. Though liver derived MSC have not been well characterized yet, extensive research on the bone marrow and adipose tissue-derived MSC shows that MSC have the ability to modulate every cell of the immune system including macrophages, DC, NK cells, B cells, and T cells[66]. Interaction of APC with MSC program the former towards a tolerant phenotype as evident from increased IL-10 secretion[66]. MSC may modulate these responses by IDO[67]. Liver MSC appear to be more potent than the bone marrow- and adipose-derived MSC in their capacity to modulate alloimmune T-cell responses, at least *in vitro*[68].

THE LIVER'S ROLE IN TOLERANCE DEVELOPMENT AND THE UNDERLYING MECHANISMS

"True tolerance" is long-term acceptance of the allograft in the absence of any immunosuppression and without evidence of any DSA or signs of lymphocyte activation on biopsy[38]. True tolerance in human beings is a rare phenomenon. A more common scenario in clinical transplantation is stable graft function for at least 1 year in the absence of immunosuppression ("operational tolerance") or with minimal immunosuppression ("prope tolerance")[38,69-71]. Nearly 25% of adult and 60% of pediatric liver transplantation recipients can achieve operational tolerance[55,72]. While different tolerance mechanisms have been demonstrated in animal models and limited clinical studies, how exactly the liver allograft dampens the host alloimmune responses remains unknown.

Chimerism

The liver contains a population of hematopoietic stem cells[73]. In fact, liver transplants can behave like bone marrow transplants and rare cases of graft versus host disease have been described after liver transplantation[74-76]. In the earliest era of clinical liver transplantation, presence of donor cells in the recipient circulation was observed (chimerism), and chimerism was thought to lead to tolerance. Chimerism was first demonstrated in 1968, with karyotyping studies of male donor livers that had been transplanted into female recipients[77]. It was observed that the majority of the allograft retained its donor specificity, but the bone marrow derived passenger leukocytes, including KCs, were largely replaced with recipient female cells within 100 days. There was also evidence of adoptive immunity, with demonstration of newly acquired immunoglobulin types of donor specificity and donor derived anti-erythrocyte isoagglutinin-associated hemolysis. Despite these subtle clues, it would not be until almost two decades later, that the conviction that donor cells were wholly eliminated by the immune system would be challenged[7]. In 1992, decisive steps were taken to search for donor leukocytes in the blood and tissue of thirty human recipients of successful liver transplants performed up to 29 years prior. Female recipients from male donor were found to have microchimerism in their allografts and extrahepatic tissues 10 to 19 years post-transplant[7].

The early alloresponse after liver transplantation is characterized by recruitment of CD4+ T cells to the allograft and by their proliferation and IFN-γ production[78]. However, later there is selective reduction of T cells in the recipient[78]. At the same

time the donor hematopoietic and T cells migrate from the allograft into the recipient. These donor-derived cells may survive in the recipient for a prolonged period of time and lead to chimerism observed after liver transplantation[79]. If donor hematopoietic cells constitute more than 1% of the recipient tissue, this is termed macrochimerism, and if they are < 1%, microchimerism[79]. In one study, all patients showed chimerism initially after liver transplantation, however chimerism decreased to variable degrees in the first year[79]. The rejection episodes in this study correlated with the lower degree of chimerism[79]. The patients with high degrees of chimerism had measurable *in vitro* alloreactive response after one year suggesting that chimerism did not lead to complete depletion of cytotoxic T cells[79]. Passenger cells in the liver may play a role in tolerance induction[6,7,79,80], as strategies to reduce the number of these passenger cells before transplantation prevents tolerance induction in experimental models[81].

T cell deletion

The unique architecture of the liver and the cross-talk between alloreactive T cells and liver inhabitant cells may play a significant role in tolerance induction by destroying host T cells[42,45,61]. The fenestrated endothelium of hepatic sinusoids facilitates direct contact between T cells and parenchymal cells leading to T cell deletion[74]. There is distinct expression of genes for T cell recruiting cytokines after LKT in tolerant patients[82]. This study found large number of CD3+ T cells and macrophages in the liver allograft but only a few in the simultaneously implanted kidney allograft[82]. It seems that increased expression of chemokines in the liver attracts alloreactive T cells that are subsequently destroyed by coming in contact with various liver cells inherently programmed towards tolerance induction. Another study found donor specific hypo-responsiveness, down regulation of T helper type I cytokine (IFN-γ) and no change in T helper type 2 cytokine (IL10) in the *in vitro* mixed lymphocyte reaction in recipients who achieved operational tolerance[83]. A similar cytokine pattern was found in the allograft on real time reverse transcriptase polymerase reaction (RT-PCR)[83]. Animal experiments have shown that T cells activated in the lymph nodes are capable of mediating immune response but T cells activated in the liver are short lived, defective, and are not able to mount immune responses[84].

Peripheral Tregs

An alternative model is the development of regulatory T cells (Treg) that actively regulate alloreactive T cells[62]. The liver cells secrete cytokines after antigen presentation that differentiates host T cells into a regulatory phenotype[48,49,53,62].

DSA neutralization

Under normal circumstances, liver has strong expression of class I HLA, secretes class I HLA antigens, and has weak class II expression[13,85]. Soluble class I HLA may absorb anti-HLA type I DSA leading to lower risk of antibody mediated rejection. We studied DSA levels in the serum of liver transplant recipients who did not receive any antibody-targeting induction[20]. Nearly 20% of recipients had preformed DSA that markedly decreased in all but three recipients 7 days after transplantation[20]. In rare instances, when DSA persisted, there was compliment activation and C4d deposition in the liver[20]. One year follow-up showed stable function despite antibody-mediated complement activation in patients with persistent DSA[20]. The unique architecture of hepatic sinusoids (fenestrated endothelium, lack of basement membrane, wider lumen) may confer resistance to complement activity. When endothelial injury does occur, it is seen in the microvasculature but not in the sinusoids[86]. This may be the reason for the increased susceptibility of peribiliary plexus to immunological or ischemic damage as its blood supply is derived from the hepatic artery[43,86]. DSA levels in the recipient seem to be the net result of two opposing factors: Host memory cells mounting immune attack and liver mediated neutralization of alloantibodies. Though protection against *de novo* class II DSA is less, incidence of *de novo* class II DSA is lower in liver transplantation compared to kidney transplantation[13].

At Mayo Clinic, we perform nearly 400 solid organ transplants in a year and many are combined liver-kidney transplants. Our group has investigated the liver's role in modulating host alloimmune responses in these combined transplant recipients. Our program also employs protocol kidney biopsies to investigate the extent of subclinical and chronic alloreactivity. In our work, we have found that liver allografts from LKT protect the kidney from hyperacute/acute antibody mediated rejection [odds ratio 0.11, 95% confidence interval (CI) 0.03-0.32] and acute cellular rejection (odds ratio 0.13; 95%CI 0.06-0.27). Moreover, in assessing variables, the presence of a functioning liver allograft was the most predictive factor for protecting kidney allografts from the chronic injury (odds ratio 0.22, 95%CI 0.06-0.59)[1]. Solitary kidney transplant patients with positive DSA had 44% decline in GFR by 5 years while LKT patients with positive DSA had stable GFR[1]. The LKT recipients had a lower frequency of

circulating CD8+, activated CD4+, and effector memory T cells, compared to kidney transplant alone (KTA) recipients[2]. Moreover, surviving T cells in LKT patients had a lower proliferative response to the donor cells (11.9% *vs* 42.9) [2,87]. This donor-specific hypo-responsiveness persisted after the first year of transplant[2]. We further compared molecular changes in the kidney allograft after LKT and KTA by doing RT-PCR on the protocol kidney biopsies[3]. We found that mechanisms underlying the liver's protective role do not only operate inside the liver but extend to the kidney as there were distinct gene expressions seen on the RT-PCR[3]. The kidneys in LKT showed markedly increased expression of genes associated with tissue integrity/metabolism, even in cross-match positive transplants[3]. We hypothesize that liver inhabitant cells migrate into the circulation after liver transplant transplantation to home at the site of inflammation in the second co-transplanted solid organ and modulate host immune cells. While the key cell type is not yet known, this hypothesis is supported by our work and previously published studies[2,3,7,80,88].

Tolerance can be conceptualized as a state of fine balance between two opposing forces: Host immune system and liver mediated immune-regulation. This balance can be tilted towards rejection by stimulation of the immune system by tissue damage[89]. After exposure to endotoxins from infectious agents or Toll Like Receptors resulting from ischemia-reperfusion injury, there is upregulation of class II HLA on hepatic EC[13]. While operational tolerance has been demonstrated in a small number of patients, the majority of liver transplant patients require small amounts of immunosuppression to counter the effect of host alloimmune response.

CONCLUSION

The liver microenvironment is inherently programmed towards induction of tolerance as a result of evolution to avoid immune activation on exposure to the gut delivered antigens. This has important implications for alloimmunity in the context of liver transplantation alone or in multivisceral transplants. Liver-induced protection against the host immune system is likely the result of a multitude of effects including microchimerism, deficient antigen presentation due to lack of costimulation, expression of inhibitory molecules, deletion of activated recipient T cells in the liver, a large antigen load in liver, active secretion of HLA molecules neutralizing alloantibody, and generation of Tregs in peripheral lymph nodes. Liver parenchymal as well as non-parenchymal cells, including MSC, may play a crucial role in some or all of the effects of the liver on the host immune system.

REFERENCES

1 **Taner T**, Heimbach JK, Rosen CB, Nyberg SL, Park WD, Stegall MD. Decreased chronic cellular and antibody-mediated injury in the kidney following simultaneous liver-kidney transplantation. *Kidney Int* 2016; **89**: 909-917 [PMID: 26924059 DOI: 10.1016/j.kint.2015.10.016]

2 **Taner T**, Gustafson MP, Hansen MJ, Park WD, Bornschlegl S, Dietz AB, Stegall MD. Donor-specific hypo-responsiveness occurs in simultaneous liver-kidney transplant recipients after the first year. *Kidney Int* 2018; **93**: 1465-1474 [PMID: 29656904 DOI: 10.1016/j.kint.2018.01.022]

3 **Taner T**, Park WD, Stegall MD. Unique molecular changes in kidney allografts after simultaneous liver-kidney compared with solitary kidney transplantation. *Kidney Int* 2017; **91**: 1193-1202 [PMID: 28233612 DOI: 10.1016/j.kint.2016.12.016]

4 **Calne RY**, Sells RA, Pena JR, Davis DR, Millard PR, Herbertson BM, Binns RM, Davies DA. Induction of immunological tolerance by porcine liver allografts. *Nature* 1969; **223**: 472-476 [PMID: 4894426 DOI: 10.1038/223472a0]

5 **Qian S**, Demetris AJ, Murase N, Rao AS, Fung JJ, Starzl TE. Murine liver allograft transplantation: Tolerance and donor cell chimerism. *Hepatology* 1994; **19**: 916-924 [PMID: 8138266 DOI: 10.1002/hep.1840190418]

6 **Reyes J**, Zeevi A, Ramos H, Tzakis A, Todo S, Demetris AJ, Nour B, Nalesnik M, Trucco M, Abu-Elmagd K. Frequent achievement of a drug-free state after orthotopic liver transplantation. *Transplant Proc* 1993; **25**: 3315-3319 [PMID: 8266556 DOI: 10.1097/00007890-199312000-00051]

7 **Starzl TE**, Demetris AJ, Trucco M, Ramos H, Zeevi A, Rudert WA, Kocova M, Ricordi C, Ildstad S, Murase N. Systemic chimerism in human female recipients of male livers. *Lancet* 1992; **340**: 876-877 [PMID: 1357298 DOI: 10.1016/0140-6736(92)93286-V]

8 **Ramos HC**, Reyes J, Abu-Elmagd K, Zeevi A, Reinsmoen N, Tzakis A, Demetris AJ, Fung JJ, Flynn B, McMichael J. Weaning of immunosuppression in long-term liver transplant recipients. *Transplantation* 1995; **59**: 212-217 [PMID: 7839442 DOI: 10.1097/00007890-199501270-00010]

9 **Eason JD**, Cohen AJ, Nair S, Alcantera T, Loss GE. Tolerance: Is it worth the risk? *Transplantation* 2005; **79**: 1157-1159 [PMID: 15880061 DOI: 10.1097/01.TP.0000162084.46555.10]

10 **Feng S**, Ekong UD, Lobritto SJ, Demetris AJ, Roberts JP, Rosenthal P, Alonso EM, Philogene MC, Ikle D, Poole KM, Bridges ND, Turka LA, Tchao NK. Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants. *JAMA* 2012; **307**: 283-293 [PMID: 22253395 DOI: 10.1001/jama.2011.2014]

11 **Takatsuki M**, Uemoto S, Inomata Y, Egawa H, Kiuchi T, Fujita S, Hayashi M, Kanematsu T, Tanaka K.

Weaning of immunosuppression in living donor liver transplant recipients. *Transplantation* 2001; **72**: 449-454 [PMID: 11502975 DOI: 10.1097/00007890-200108150-00016]
12 **Gloor J**, Cosio F, Lager DJ, Stegall MD. The spectrum of antibody-mediated renal allograft injury: Implications for treatment. *Am J Transplant* 2008; **8**: 1367-1373 [PMID: 18510643 DOI: 10.1111/j.1600-6143.2008.02262.x]
13 **Cheng EY**. The Role of Humoral Alloreactivity in Liver Transplantation: Lessons Learned and New Perspectives. *J Immunol Res* 2017; **2017**: 3234906 [PMID: 28164136 DOI: 10.1155/2017/3234906]
14 **Terasaki PI**. Humoral theory of transplantation. *Am J Transplant* 2003; **3**: 665-673 [PMID: 12780557 DOI: 10.1034/j.1600-6143.2003.00135.x]
15 **Jakab SS**, Navarro VJ, Colombe BW, Daskalakis C, Herrine SK, Rossi S. Human leukocyte antigen and adult living-donor liver transplantation outcomes: An analysis of the organ procurement and transplantation network database. *Liver Transpl* 2007; **13**: 1405-1413 [PMID: 17902126 DOI: 10.1002/lt.21264]
16 **Opelz G**, Döhler B. Effect of human leukocyte antigen compatibility on kidney graft survival: Comparative analysis of two decades. *Transplantation* 2007; **84**: 137-143 [PMID: 17667803 DOI: 10.1097/01.tp.0000269725.74189.b9]
17 **Watson R**, Kozlowski T, Nickeleit V, Woosley JT, Schmitz JL, Zacks SL, Fair JH, Gerber DA, Andreoni KA. Isolated donor specific alloantibody-mediated rejection after ABO compatible liver transplantation. *Am J Transplant* 2006; **6**: 3022-3029 [PMID: 17061997 DOI: 10.1111/j.1600-6143.2006.01554.x]
18 **Doyle HR**, Marino IR, Morelli F, Doria C, Aldrighetti L, McMichael J, Martell J, Gayowski T, Starzl TE. Assessing risk in liver transplantation. Special reference to the significance of a positive cytotoxic crossmatch. *Ann Surg* 1996; **224**: 168-177 [PMID: 8757380 DOI: 10.1097/00000658-199608000-00009]
19 **Gordon RD**, Fung JJ, Markus B, Fox I, Iwatsuki S, Esquivel CO, Tzakis A, Todo S, Starzl TE. The antibody crossmatch in liver transplantation. *Surgery* 1986; **100**: 705-715 [PMID: 3532391]
20 **Taner T**, Gandhi MJ, Sanderson SO, Poterucha CR, De Goey SR, Stegall MD, Heimbach JK. Prevalence, course and impact of HLA donor-specific antibodies in liver transplantation in the first year. *Am J Transplant* 2012; **12**: 1504-1510 [PMID: 22420671 DOI: 10.1111/j.1600-6143.2012.03995.x]
21 **O'Leary JG**, Demetris AJ, Friedman LS, Gebel HM, Halloran PF, Kirk AD, Knechtle SJ, McDiarmid SV, Shaked A, Terasaki PI, Tinckam KJ, Tomlanovich SJ, Wood KJ, Woodle ES, Zachary AA, Klintmalm GB. The role of donor-specific HLA alloantibodies in liver transplantation. *Am J Transplant* 2014; **14**: 779-787 [PMID: 24580828 DOI: 10.1111/ajt.12667]
22 **Feng S**, Demetris AJ, Spain KM, Kanaparthi S, Burrell BE, Ekong UD, Alonso EM, Rosenthal P, Turka LA, Ikle D, Tchao NK. Five-year histological and serological follow-up of operationally tolerant pediatric liver transplant recipients enrolled in WISP-R. *Hepatology* 2017; **65**: 647-660 [PMID: 27302659 DOI: 10.1002/hep.28681]
23 **Wanless IR**, Schiff ER, Meddrey WC, Reddy KR. Physioanatomical Considerations. Schiff ER, Meddrey WC, Reddy KR. *Schiff's Diseases of the Liver*. Chichester (West Sussex): Wiley-Blackwell 2018; 86
24 **Batts KP**, Moore SB, Perkins JD, Wiesner RH, Grambsch PM, Krom RA. Influence of positive lymphocyte crossmatch and HLA mismatching on vanishing bile duct syndrome in human liver allografts. *Transplantation* 1988; **45**: 376-379 [PMID: 3278430 DOI: 10.1097/00007890-198802000-00026]
25 **Jadlowiec CC**, Morgan PE, Nehra AK, Hathcock MA, Kremers WK, Heimbach JK, Wiesner RH, Taner T. Not All Cellular Rejections Are the Same: Differences in Early and Late Hepatic Allograft Rejection. *Liver Transpl* 2019; **25**: 425-435 [PMID: 30615251 DOI: 10.1002/lt.25411]
26 **Abu-Elmagd K**, Reyes J, Todo S, Rao A, Lee R, Irish W, Furukawa H, Bueno J, McMichael J, Fawzy AT, Murase N, Demetris J, Rakela J, Fung JJ, Starzl TE. Clinical intestinal transplantation: New perspectives and immunologic considerations. *J Am Coll Surg* 1998; **186**: 512-25; discussion 525-7 [PMID: 9583691 DOI: 10.1016/S1072-7515(98)00083-0]
27 **Daly RC**, Pereira NL, Taner T, Gandhi MJ, Heimbach JK, Dearani JA, Edwards BS, Kushwaha SS. Combined Heart and Liver Transplantation in Highly Sensitized Patients: Protection of the Cardiac Allograft from Antibody Mediated Rejection by Initial Liver Implantation. *J Heart Lung Transplant* 2017; **36**: S200 [DOI: 10.1016/j.healun.2017.01.525]
28 **Grannas G**, Neipp M, Hoeper MM, Gottlieb J, Lück R, Becker T, Simon A, Strassburg CP, Manns MP, Welte T, Haverich A, Klempnauer J, Nashan B, Strueber M. Indications for and outcomes after combined lung and liver transplantation: A single-center experience on 13 consecutive cases. *Transplantation* 2008; **85**: 524-531 [PMID: 18347530 DOI: 10.1097/TP.0b013e3181636f3f]
29 **Gonwa TA**, Nery JR, Husberg BS, Klintmalm GB. Simultaneous liver and renal transplantation in man. *Transplantation* 1988; **46**: 690-693 [PMID: 3057691 DOI: 10.1097/00007890-198811000-00013]
30 **Olausson M**, Mjörnstedt L, Nordén G, Rydberg L, Mölne J, Bäckman L, Friman S. Successful combined partial auxiliary liver and kidney transplantation in highly sensitized cross-match positive recipients. *Am J Transplant* 2007; **7**: 130-136 [PMID: 17227562 DOI: 10.1111/j.1600-6143.2006.01592.x]
31 **Fung J**, Makowka L, Tzakis A, Klintmalm G, Duquesnoy R, Gordon R, Todo S, Griffin M, Starzl T. Combined liver-kidney transplantation: Analysis of patients with preformed lymphocytotoxic antibodies. *Transplant Proc* 1988; **20**: 88-91 [PMID: 3279673 DOI: 10.1097/00007890-198802000-00057]
32 **Fong TL**, Bunnapradist S, Jordan SC, Selby RR, Cho YW. Analysis of the United Network for Organ Sharing database comparing renal allografts and patient survival in combined liver-kidney transplantation with the contralateral allografts in kidney alone or kidney-pancreas transplantation. *Transplantation* 2003; **76**: 348-353 [PMID: 12883191 DOI: 10.1097/01.TP.0000071204.03720.BB]
33 **Creput C**, Durrbach A, Samuel D, Eschwege P, Amor M, Kriaa F, Kreis H, Benoit G, Bismuth H, Charpentier B. Incidence of renal and liver rejection and patient survival rate following combined liver and kidney transplantation. *Am J Transplant* 2003; **3**: 348-356 [PMID: 12614293 DOI: 10.1034/j.1600-6143.2003.00050.x]
34 **Schachtner T**, Stein M, Reinke P. Kidney transplant recipients after nonrenal solid organ transplantation show low alloreactivity but an increased risk of infection. *Transpl Int* 2016; **29**: 1296-1306 [PMID: 27638250 DOI: 10.1111/tri.12856]
35 **Fung J**, Griffin M, Duquesnoy R, Shaw B, Starzl T. Successful sequential liver-kidney transplantation in a patient with performed lymphocytotoxic antibodies. *Transplant Proc* 1987; **19**: 767-768 [PMID: 3274867 DOI: 10.1105/tpc.115.00038]
36 **Topilsky Y**, Raichlin E, Hasin T, Boilson BA, Schirger JA, Pereira NL, Edwards BS, Clavell AL, Rodeheffer RJ, Frantz RP, Gandhi MJ, Maltais S, Park SJ, Daly RC, Lerman A, Kushwaha SS. Combined heart and liver transplant attenuates cardiac allograft vasculopathy compared with isolated heart transplantation. *Transplantation* 2013; **95**: 859-865 [PMID: 23364484 DOI:

10.1097/TP.0b013e31827eef7e]
37 **Grant D**, Abu-Elmagd K, Mazariegos G, Vianna R, Langnas A, Mangus R, Farmer DG, Lacaille F, Iyer K, Fishbein T; Intestinal Transplant Association. Intestinal transplant registry report: Global activity and trends. *Am J Transplant* 2015; **15**: 210-219 [PMID: 25438622 DOI: 10.1111/ajt.12979]
38 **Beaudreuil S**, Samuel D, Rouas-Freiss N, Durrbach A. New aspect of immunosuppressive treatment in liver transplantation. How could you induce tolerance in liver transplantation? *Transpl Immunol* 2007; **17**: 98-107 [PMID: 17306740 DOI: 10.1016/j.trim.2006.11.007]
39 **Joly E**, Hudrisier D. What is trogocytosis and what is its purpose? *Nat Immunol* 2003; **4**: 815 [PMID: 12942076 DOI: 10.1038/ni0903-815]
40 **Huang H**, Lu Y, Zhou T, Gu G, Xia Q. Innate Immune Cells in Immune Tolerance After Liver Transplantation. *Front Immunol* 2018; **9**: 2401 [PMID: 30473690 DOI: 10.3389/fimmu.2018.02401]
41 **Starzl TE**, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. *Lancet* 1992; **339**: 1579-1582 [PMID: 1351558 DOI: 10.1016/0140-6736(92)91840-5]
42 **Crispe IN**. The liver as a lymphoid organ. *Annu Rev Immunol* 2009; **27**: 147-163 [PMID: 19302037 DOI: 10.1146/annurev.immunol.021908.132629]
43 **Demetris AJ**, Bellamy CO, Gandhi CR, Prost S, Nakanuma Y, Stolz DB. Functional Immune Anatomy of the Liver-As an Allograft. *Am J Transplant* 2016; **16**: 1653-1680 [PMID: 26848550 DOI: 10.1111/ajt.13749]
44 **Taylor AL**, Gibbs P, Bradley JA. Acute graft versus host disease following liver transplantation: The enemy within. *Am J Transplant* 2004; **4**: 466-474 [PMID: 15023138 DOI: 10.1111/j.1600-6143.2004.00406.x]
45 **Tokita D**, Shishida M, Ohdan H, Onoe T, Hara H, Tanaka Y, Ishiyama K, Mitsuta H, Ide K, Arihiro K, Asahara T. Liver sinusoidal endothelial cells that endocytose allogeneic cells suppress T cells with indirect allospecificity. *J Immunol* 2006; **177**: 3615-3624 [PMID: 16951321 DOI: 10.4049/jimmunol.177.6.3615]
46 **Davey GM**, Kurts C, Miller JF, Bouillet P, Strasser A, Brooks AG, Carbone FR, Heath WR. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J Exp Med* 2002; **196**: 947-955 [PMID: 12370256 DOI: 10.1084/jem.20020827]
47 **Bosma BM**, Metselaar HJ, Mancham S, Boor PP, Kusters JG, Kazemier G, Tilanus HW, Kuipers EJ, Kwekkeboom J. Characterization of human liver dendritic cells in liver grafts and perfusates. *Liver Transpl* 2006; **12**: 384-393 [PMID: 16498646 DOI: 10.1002/lt.20659]
48 **Tokita D**, Mazariegos GV, Zahorchak AF, Chien N, Abe M, Raimondi G, Thomson AW. High PD-L1/CD86 ratio on plasmacytoid dendritic cells correlates with elevated T-regulatory cells in liver transplant tolerance. *Transplantation* 2008; **85**: 369-377 [PMID: 18301333 DOI: 10.1097/TP.0b013e3181612ded]
49 **Kushwah R**, Wu J, Oliver JR, Jiang G, Zhang J, Siminovitch KA, Hu J. Uptake of apoptotic DC converts immature DC into tolerogenic DC that induce differentiation of Foxp3+ Treg. *Eur J Immunol* 2010; **40**: 1022-1035 [PMID: 20101618 DOI: 10.1002/eji.200939782]
50 **Sumpter TL**, Dangi A, Matta BM, Huang C, Stolz DB, Vodovotz Y, Thomson AW, Gandhi CR. Hepatic stellate cells undermine the allostimulatory function of liver myeloid dendritic cells *via* STAT3-dependent induction of IDO. *J Immunol* 2012; **189**: 3848-3858 [PMID: 22962681 DOI: 10.4049/jimmunol.1200819]
51 **Knolle P**, Schlaak J, Uhrig A, Kempf P, Meyer zum Büschenfelde KH, Gerken G. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J Hepatol* 1995; **22**: 226-229 [PMID: 7790711 DOI: 10.1016/0168-8278(95)80433-1]
52 **You Q**, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology* 2008; **48**: 978-990 [PMID: 18712788 DOI: 10.1002/hep.22395]
53 **Breous E**, Somanathan S, Vandenberghe LH, Wilson JM. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology* 2009; **50**: 612-621 [PMID: 19575456 DOI: 10.1002/hep.23043]
54 **Harmon C**, Sanchez-Fueyo A, O'Farrelly C, Houlihan DD. Natural Killer Cells and Liver Transplantation: Orchestrators of Rejection or Tolerance? *Am J Transplant* 2016; **16**: 751-757 [PMID: 26690302 DOI: 10.1111/ajt.13565]
55 **Li L**, Wozniak LJ, Rodder S, Heish S, Talisetti A, Wang Q, Esquivel C, Cox K, Chen R, McDiarmid SV, Sarwal MM. A common peripheral blood gene set for diagnosis of operational tolerance in pediatric and adult liver transplantation. *Am J Transplant* 2012; **12**: 1218-1228 [PMID: 22300520 DOI: 10.1111/j.1600-6143.2011.03928.x]
56 **Sarwal MM**. Fingerprints of transplant tolerance suggest opportunities for immunosuppression minimization. *Clin Biochem* 2016; **49**: 404-410 [PMID: 26794635 DOI: 10.1016/j.clinbiochem.2016.01.007]
57 **Ikehara Y**, Yasunami Y, Kodama S, Maki T, Nakano M, Nakayama T, Taniguchi M, Ikeda S. CD4(+) Valpha14 natural killer T cells are essential for acceptance of rat islet xenografts in mice. *J Clin Invest* 2000; **105**: 1761-1767 [PMID: 10862791 DOI: 10.1172/JCI8922]
58 **Winau F**, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, Modlin RL, Liblau RS, Gressner AM, Kaufmann SH. Ito cells are liver-resident antigen-presenting cells for activating T cell responses. *Immunity* 2007; **26**: 117-129 [PMID: 17239632 DOI: 10.1016/j.immuni.2006.11.011]
59 **Limmer A**, Ohl J, Kurts C, Ljunggren HG, Reiss Y, Groettrup M, Momburg F, Arnold B, Knolle PA. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med* 2000; **6**: 1348-1354 [PMID: 11100119 DOI: 10.1038/82161]
60 **Yu MC**, Chen CH, Liang X, Wang L, Gandhi CR, Fung JJ, Lu L, Qian S. Inhibition of T-cell responses by hepatic stellate cells *via* B7-H1-mediated T-cell apoptosis in mice. *Hepatology* 2004; **40**: 1312-1321 [PMID: 15565659 DOI: 10.1002/hep.20488]
61 **Qian S**, Lu L, Fu F, Li Y, Li W, Starzl TE, Fung JJ, Thomson AW. Apoptosis within spontaneously accepted mouse liver allografts: Evidence for deletion of cytotoxic T cells and implications for tolerance induction. *J Immunol* 1997; **158**: 4654-4661 [PMID: 9144477]
62 **Dangi A**, Sumpter TL, Kimura S, Stolz DB, Murase N, Raimondi G, Vodovotz Y, Huang C, Thomson AW, Gandhi CR. Selective expansion of allogeneic regulatory T cells by hepatic stellate cells: Role of endotoxin and implications for allograft tolerance. *J Immunol* 2012; **188**: 3667-3677 [PMID: 22427640 DOI: 10.4049/jimmunol.1102460]
63 **Najimi M**, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, Sokal EM. Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? *Cell Transplant* 2007; **16**: 717-728 [PMID: 18019361 DOI: 10.3727/000000007783465154]

64 **Friedenstein AJ**, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; **3**: 393-403 [PMID: 5523063 DOI: 10.1111/j.1365-2184.1970.tb00347.x]

65 **Dominici M**. Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

66 **Najar M**, Raicevic G, Fayyad-Kazan H, Bron D, Toungouz M, Lagneaux L. Mesenchymal stromal cells and immunomodulation: A gathering of regulatory immune cells. *Cytotherapy* 2016; **18**: 160-171 [PMID: 26794710 DOI: 10.1016/j.jcyt.2015.10.011]

67 **Wang Y**, Chen X, Cao W, Shi Y. Plasticity of mesenchymal stem cells in immunomodulation: Pathological and therapeutic implications. *Nat Immunol* 2014; **15**: 1009-1016 [PMID: 25329189 DOI: 10.1038/ni.3002]

68 **Taner T**, Gustafson M, Hansen M, Dietz A, Stegall M. Liver Mesenchymal Stem Cells Inhibit T Cell Alloresponses. *Am J Transplant* 2017; 17

69 **Calne RY**. Prope tolerance: The future of organ transplantation--from the laboratory to the clinic. *Transplantation* 2004; **77**: 930-932 [PMID: 15077040 DOI: 10.1097/01.TP.0000117776.14277.03]

70 **Cortesini R**, Renna-Molajoni E, Cinti P, Pretagostini R, Ho E, Rossi P, Suciu-Foca Cortesini N. Tailoring of immunosuppression in renal and liver allograft recipients displaying donor specific T-suppressor cells. *Hum Immunol* 2002; **63**: 1010-1018 [PMID: 12392853 DOI: 10.1016/S0198-8859(02)00442-1]

71 **Cortesini R**, Suciu-Foca N. The concept of "partial" clinical tolerance. *Transpl Immunol* 2004; **13**: 101-104 [PMID: 15380540 DOI: 10.1016/j.trim.2004.05.002]

72 **Martínez-Llordella M**, Puig-Pey I, Orlando G, Ramoni M, Tisone G, Rimola A, Lerut J, Latinne D, Margarit C, Bilbao I, Brouard S, Hernández-Fuentes M, Soulillou JP, Sánchez-Fueyo A. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007; **7**: 309-319 [PMID: 17241111 DOI: 10.1111/j.1600-6143.2006.01621.x]

73 **Taniguchi H**, Toyoshima T, Fukao K, Nakauchi H. Presence of hematopoietic stem cells in the adult liver. *Nat Med* 1996; **2**: 198-203 [PMID: 8574965 DOI: 10.1038/nm0296-198]

74 **Bishop GA**, Ierino FL, Sharland AF, Hall BM, Alexander SI, Sandrin MS, Coates PT, McCaughan GW. Approaching the promise of operational tolerance in clinical transplantation. *Transplantation* 2011; **91**: 1065-1074 [PMID: 21544029 DOI: 10.1097/TP.0b013e318215e742]

75 **Chaib E**, Silva FD, Figueira ER, Lima FR, Andraus W, D'Albuquerque LA. Graft-versus-host disease after liver transplantation. *Clinics (Sao Paulo)* 2011; **66**: 1115-1118 [PMID: 21808887 DOI: 10.1590/S1807-59322011000600035]

76 **Kanaan Z**, Tse W. A novel strategy in managing post-liver transplant acute graft-versus-host-disease: The new era (T)rojan horse. *Biol Blood Marrow Transplant* 2016; **22**: S401-S2 [DOI: 10.1016/j.bbmt.2015.11.929]

77 **Kashiwagi N**, Porter KA, Penn I, Brettschneider L, Starzl TE. Studies of homograft sex and of gamma globulin phenotypes after orthotopic homotransplantation of the human liver. *Surg Forum* 1969; **20**: 374-376 [PMID: 4192216]

78 **Ahearn AJ**, Klein I, Hayden T, Lui F, Lee K, Tang Q, Kang S. Liver transplant tolerance: Differences in the persistence of various alloreactive t cell subsets: 2486. *Transplantation* 2010; 90 [DOI: 10.1097/00007890-201007272-00964]

79 **Bettens F**, Tiercy JM, Campanile N, Giostra E, Majno P, Rubbia L, Roosnek E, Mentha G, Villard J. Microchimerism after liver transplantation: Absence of rejection without abrogation of anti-donor cytotoxic T-lymphocyte-mediated alloreactivity. *Liver Transpl* 2005; **11**: 290-297 [PMID: 15719407 DOI: 10.1002/lt.20360]

80 **Starzl TE**, Demetris AJ, Trucco M, Murase N, Ricordi C, Ildstad S, Ramos H, Todo S, Tzakis A, Fung JJ. Cell migration and chimerism after whole-organ transplantation: The basis of graft acceptance. *Hepatology* 1993; **17**: 1127-1152 [PMID: 8514264 DOI: 10.1002/hep.1840170629]

81 **Sun J**, McCaughan GW, Gallagher ND, Sheil AG, Bishop GA. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation* 1995; **60**: 233-236 [PMID: 7645034 DOI: 10.1097/00007890-199508000-00004]

82 **Ingelsten M**, Karlsson-Parra A, Granqvist AB, Mölne J, Olausson M, Haraldsson B, Nyström J. Postischemic inflammatory response in an auxiliary liver graft predicts renal graft outcome in sensitized patients. *Transplantation* 2011; **91**: 888-894 [PMID: 21494202 DOI: 10.1097/TP.0b013e3182100f19]

83 **Takatsuki M**, Uemoto S, Inomata Y, Sakamoto S, Hayashi M, Ueda M, Kanematsu T, Tanaka K. Analysis of alloreactivity and intragraft cytokine profiles in living donor liver transplant recipients with graft acceptance. *Transpl Immunol* 2001; **8**: 279-286 [PMID: 11316071 DOI: 10.1016/S0966-3274(01)00027-2]

84 **Bowen DG**, Zen M, Holz L, Davis T, McCaughan GW, Bertolino P. The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J Clin Invest* 2004; **114**: 701-712 [PMID: 15343389 DOI: 10.1172/JCI21593]

85 **Davies HS**, Pollard SG, Calne RY. Soluble HLA antigens in the circulation of liver graft recipients. *Transplantation* 1989; **47**: 524-527 [PMID: 2646783 DOI: 10.1097/00007890-198903000-00025]

86 **Demetris AJ**. Bellamy C, Hübscher SG, O'Leary J, Randhawa PS, Feng S, Neil D, Colvin RB, McCaughan G, Fung JJ, Del Bello A, Reinholt FP, Haga H, Adeyi O, Czaja AJ, Schiano T, Fiel MI, Smith ML, Sebagh M, Tanigawa RY, Yilmaz F, Alexander G, Baiocchi L, Balasubramanian M, Batal I, Bhan AK, Bucuvalas J, Cerski CTS, Charlotte F, de Vera ME, ElMonayeri M, Fontes P, Furth EE, Gouw ASH, Hafezi-Bakhtiari S, Hart J, Honsova E, Ismail W, Itoh T, Jhala NC, Khettry U, Klintmalm GB, Knechtle S, Koshiba T, Kozlowski T, Lassman CR, Lerut J, Levitsky J, Licini L, Liotta R, Mazariegos G, Minervini MI, Misdraji J, Mohanakumar T, Mölne J, Nasser I, Neuberger J, O'Neil M, Pappo O, Petrovic L, Ruiz P, Sağol Ö, Sanchez Fueyo A, Sasatomi E, Shaked A, Shiller M, Shimizu T, Sis B, Sonzogni A, Stevenson HL, Thung SN, Tisone G, Tsamandas AC, Wernerson A, Wu T, Zeevi A, Zen Y. 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection. *Am J Transplant* 2016; **16**: 2816-2835 [PMID: 27273869 DOI: 10.1111/ajt.13909]

87 **Taner T**, Hansen M, Park W, Stegall M. Phenotypic and functional assessment of t cell alloimmunity after liver transplantation. *Am J Transplant* 2017; 17

88 **Pan Q**, Fouraschen SM, Kaya FS, Verstegen MM, Pescatori M, Stubbs AP, van Ijcken W, van der Sloot A, Smits R, Kwekkeboom J, Metselaar HJ, Kazemier G, de Jonge J, Tilanus HW, Wagemaker G, Janssen HL, van der Laan LJ. Mobilization of hepatic mesenchymal stem cells from human liver grafts. *Liver*

Transpl 2011; **17**: 596-609 [PMID: 21506248 DOI: 10.1002/lt.22260]
89 **Testro AG**, Visvanathan K, Skinner N, Markovska V, Crowley P, Angus PW, Gow PJ. Acute allograft rejection in human liver transplant recipients is associated with signaling through toll-like receptor 4. *J Gastroenterol Hepatol* 2011; **26**: 155-163 [PMID: 21175809 DOI: 10.1111/j.1440-1746.2010.06324.x]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3136-3150

DOI: 10.3748/wjg.v25.i25.3136

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

REVIEW

Hepatocellular carcinoma: Therapeutic advances in signaling, epigenetic and immune targets

Daniel Neureiter, Sebastian Stintzing, Tobias Kiesslich, Matthias Ocker

ORCID number: Daniel Neureiter (0000-0001-9155-5762); Sebastian Stintzing (0000-0002-3297-5801); Tobias Kiesslich (0000-0001-5403-9478); Matthias Ocker (0000-0001-8263-6288).

Author contributions: All authors equally contributed to literature review, writing this paper, critical revision final approval of the final version.

Conflict-of-interest statement: Stintzing S received honoraria for talks and/or for advisory function from: Amgen, Bayer, Merck KGaA, Lilly, Sanofi, Roche, Takeda, Taiho, SIRTEX and Samsung. Ocker M is employee and shareholder of Bayer AG.

Manuscript source: Invited manuscript

Received: April 6, 2019
Peer-review started: April 8, 2019
First decision: April 16, 2019
Revised: May 2, 2019
Accepted: May 18, 2019

Daniel Neureiter, Institute of Pathology, Cancer Cluster Salzburg, Paracelsus Medical University/Salzburger Landeskliniken (SALK), Salzburg 5020, Austria

Sebastian Stintzing, Medical Department, Division of Oncology and Hematology, Campus Charité Mitte, Charité University Medicine Berlin, Berlin 10117, Germany

Tobias Kiesslich, Department of Internal Medicine I, Paracelsus Medical University/Salzburger Landeskliniken (SALK) and Institute of Physiology and Pathophysiology, Paracelsus Medical University, Salzburg 5020, Austria

Matthias Ocker, Translational Medicine Oncology, Bayer AG, Berlin 13353, Germany

Matthias Ocker, Charité University Medicine Berlin, Berlin 10117, Germany

Corresponding author: Matthias Ocker, MD, Professor, Translational Medicine Oncology, Bayer AG, Muellerstrasse 178, Berlin 13353, Germany. matthias.ocker@bayer.com
Telephone: +49-30-468194799
Fax: +49-30-468994799

Abstract

Hepatocellular carcinoma (HCC) remains a global medical burden with rising incidence due to chronic viral hepatitis and non-alcoholic fatty liver diseases. Treatment of advanced disease stages is still unsatisfying. Besides first and second generation tyrosine kinase inhibitors, immune checkpoint inhibitors have become central for the treatment of HCC. New modalities like epigenetic therapy using histone deacetylase inhibitors (HDACi) and cell therapy approaches with chimeric antigen receptor T cells (CAR-T cells) are currently under investigation in clinical trials. Development of such novel drugs is closely linked to the availability and improvement of novel preclinical and animal models and the identification of predictive biomarkers. The current status of treatment options for advanced HCC, emerging novel therapeutic approaches and different preclinical models for HCC drug discovery and development are reviewed here.

Key words: Liver cancer; Immunotherapy; Checkpoint inhibitors; Targeted therapy; Mouse model; Biomarker; Next-generation sequencing; Non-alcoholic steatohepatitis; Fibrosis; Clinical trial

Core tip: Treatment of advanced hepatocellular carcinoma still represents an unmet

Article in press: May 18, 2019
Published online: July 7, 2019

P-Reviewer: Borzio M, Cerwenka H, Hashimoto N, Qi XS, Roohvand F, Sandhu DS, Tajiri K
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

medical need. Novel therapeutic options comprise new tyrosine kinase inhibitors, epigenetic modifiers and increasingly also cell therapy and immune checkpoint inhibitors and combinations of those modalities. Development of better drugs is closely linked to improved preclinical and animal models and has to be accompanied by the implementation of predictive biomarkers, which is still lacking for hepatocellular carcinoma. The current status of these aspects is reviewed in this manuscript.

Citation: Neureiter D, Stintzing S, Kiesslich T, Ocker M. Hepatocellular carcinoma: Therapeutic advances in signaling, epigenetic and immune targets. *World J Gastroenterol* 2019; 25(25): 3136-3150
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3136.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3136

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver, accounting for approximately 85% of all cases. It is considered to be the 6th most commonly diagnosed cancer and the 4th most common cause of cancer related death worldwide, with 2 to 3 times higher rates for men[1]. Major risk factors are chronic viral hepatitis [hepatitis B virus (HBV) and hepatitis C virus (HCV)], aflatoxin exposure, alcohol intake, and the globally increasing high rates of obesity and type 2 diabetes[2]. The 5-year survival rate of advanced HCC remains devastating at 1% and is the poorest of all solid cancers[3]. Treatment of advanced stages of HCC is often limited by the underlying liver disease which is commonly accompanied by cirrhosis and end-stage liver disease with significantly impaired liver function. Due to those different etiologies and pathogenetic mechanisms, the identification of common oncogenic drivers is challenging although heterogeneous sets of mutations could be detected in HCC (Table 1), of which especially telomerase, p53, β-catenin and others were linked to distinct and prognostic HCC subtypes (Table 2)[4,5].

Curative therapy is currently only possible in early stages by complete surgical resection or orthotopic liver transplantation, the latter being limited by availability of donor organs. Locoregional therapies [*e.g.*, transarterial chemoembolization (TACE), different ablation strategies, selective internal radiotherapy (SIRT)] are available for intermediate stages or HCCs not amenable to surgical therapy and can be applied repeatedly also for downstaging in preparation of transplantation or in an adjuvant setting prior to surgery[6,7]. In addition, external beam radiation (EBRT) is a valuable adjuvant therapy option for small HCCs, in combination with surgery or other locoregional therapies or as a bridging option to orthotopic liver transplantation. It can also help to reduce pain in extrahepatic metastases and prolong survival after surgical resection[8]. For further details on locoregional and non-systemic treatment options, we refer the reader to a recent meta-analysis on the management of HCC[9].

Since the introduction of the multi-kinase inhibitor sorafenib about a decade ago, only little progress has been made in treatment of advanced HCC. In this article, we will review the current status of novel drugs for the treatment of advanced HCC including the emerging immune checkpoint inhibitor therapies. We will also highlight recent trends in identifying predictive biomarkers and establishing animal models that closely resemble the complex and diverse human pathophysiology of HCC.

CONVENTIONAL CHEMOTHERAPY AND MOLECULAR TARGETED THERAPIES

Prior to the approval of sorafenib, no standard chemotherapy regimen had been established for treatment of advanced HCC. Randomized trials and meta-analyses showed a poor response rate of different agents like 5-fluorouracil (5-FU), cisplatin, doxorubicin or hormonal therapy (*e.g.*, tamoxifen or somatostatin-analogues). The high intrinsic resistance of HCC is considered due to the high expression of efflux pumps (linked to the physiologic metabolic capacity of the liver parenchyma), altered blood flow and fibrosis as well as high expression and mutations in drug resistance genes like p53. Most tested drugs showed only modest activity with minimal improvement in overall survival but with significant toxicities in combination[6,10].

Table 1 Known dysregulated pathways and genes in hepatocellular carcinoma with mode of action and frequency (modified from[4,5,92])

Pathways / genes	Alteration	Frequency in HCC
AKT-mTOR-MAPK signaling		
RPS6KA3	Mutation	2%%-9%
TSC1 and TSC2	Mutation or deletion	3%-8%
PTEN	Mutation or deletion	1%-3%
FGF3, FGF4 and FGF19	Amplification	4%-6%
PI3KCA	Mutation	0%-2%
Angiogenesis		
VEGFA	Amplification	3%-7%
Antioxidation		
NFE2L2 KEAP1	Mutation Mutation	3%-6% 2%-8%
Cell cycle control/tumor suppressors		
TP53*	Mutation or deletion	12%-45%
RB1	Mutation or deletion	3%-8%
CCND1*	Amplification	5%-14%
Epigenetic and chromatin remodeling		
ARID1A*	Mutation or deletion	4%-17%
ARID2*	Mutation	3%-18%
BAP1	Mutation	5%[117]
Immortalization/telomere maintenance		
ERT*	Promotor mutation amplification	54%-60% 5%-6%
JAK/STAT		
JAK1	Mutation	5%
Metabolic pathways		
Afamin apoptogenic protein 1, mitochondrial	Mutation	Up to 10%[117]
Oncogenes		
MET*	Amplification	30%-50%
MYC	Amplification	4%
TGFβ pathway		
Osteopontin	Mutation	Up to 40%[118]
G2/mitotic-specific cyclin-B2 Cyclin-dependent kinase 1 lymphoid enhancer-binding factor 1		
Integrin α2		
Wnt pathway		
Catenin β1*	Mutation	11%-37%
AXIN1*	Mutation or deletion	5%-15%

HCC: Hepatocellular carcinoma.

The small-molecule multi-kinase inhibitor sorafenib was the first drug to show an overall survival benefit in first-line therapy of advanced HCC (10.7 mo *vs* 7.9 mo; 6.5 mo *vs* 4.2 mo in Asian patients) in randomized controlled trials[11,12]. Since then, only lenvatinib was able to achieve increased overall survival (in a phase 2 study) and was proven to be non-inferior to sorafenib in a recent phase 3 study, reaching a median survival time of 13.6 mo compared to 12.3 mo for sorafenib[13,14].

Similarly, results for new drugs in a second-line setting after failure of sorafenib were mostly disappointing. Regorafenib, a derivative of sorafenib, and the novel multi-kinase inhibitor cabozantinib achieved a significant increase in overall survival in placebo-controlled trials. Regorafenib increased overall survival to 10.6 mo *vs* 7.8 mo[15], while cabozantinib achieved overall survival of 10.2 mo *vs* 8.0 mo with toxicity similar to regorafenib[16]. Lately the vascular endothelial growth factor receptor 2 (VEGFR-2) antibody ramucirumab proved efficacy in sorafenib pretreated patients with an alpha-fetoprotein level of ≥ 400 mg/mL[17]. The randomized phase-III placebo controlled trial REACH-2 showed median overall survival times of 8.5 mo for ramucirumab treated patients *vs* 7.3 mo for the placebo arm [hazard ratio (HR) = 0.7; P < 0.0001]. This is the first study to show a significant survival benefit for a bio-marker (alpha-fetoprotein) selected subgroup of HCC.

Table 2 Summary of classification schemes of hepatocellular carcinoma (modified from[119])

First author	Lee *et al*[120]	Boyault *et al*[121]	Chiang *et al*[122]	Hoshida *et al*[123]	Désert *et al*[124]	TCGA network[117]
Year	2004	2006	2008	2009	2017	2017
HCC cases	91	56	91	232	1133	559
Number of subgroups	2	6	5	3	4	3
Names of classes	Cluster A/B	G1-G6	CTNNB1-proliferation	S1-S3	PP, PV, ECM, STEM	iCluster1-iCluster3
Major applied technology for molecular profiling						
Transcriptomics	X	X	X	X	X	X
Genetic Mutations		X				X
Copy number alterations			X			X
Metabolomics					X	
Epigenomics		X (CDH1 and CDKN2A)				X
Proteomics						X
Major HCC Classes with clinic-pathological features and high mutation rates						
Proliferative phenotype						
Poor outcome	A	G1, G2, G3	Proliferation	S1 + S2	ECM + STEM	iCluster 1 + 3
High AFP						
Moderate to poor differentiation						
P53						
Non-proliferative phenotype						
Good to moderate outcome	B	G5, G6	CTNNB1	S3	PP + PV	iCluster 2
Low AFP						
CTNNB1						

ECM: Extracellular matrix; PP: Periportal; PV: Perivenous; STEM: Stem/progenitor cells; HCC: Hepatocellular carcinoma.

Several other targets for inhibition of receptor tyrosine kinase function in HCC were investigated. Hepatocyte growth factor (HGF) and its receptor c-Met are commonly overexpressed in HCC and have been linked to poor prognosis and re-sistance to *e.g.*, sorafenib treatment[18-20]. c-Met is targeted by several multi-kinase inhibitors like gefitinib or cobazitinib and more recently also selective inhibitors like capmatinib or tepotinib entered clinical trials but results for studies in HCC are still pending[21-23]. Other less selective compounds with c-Met inhibition properties like crizotinib, brivanib or foretinib did not lead to significant prolongation of overall survival (OS) in phase III studies or were not investigated in HCC patients yet[21,22,24,25].

All of these compounds are recommended for patients with preserved liver function, *i.e.*, Child-Pugh score 5, 6 and 7[26]. Treatment options for patients with more advanced liver impairment or cirrhosis are still lacking and represent an urgent medical need. Esp. the use of sorafenib in patients with portal vein tumor thrombosis (PVTT) remains controversial[27]. In a study with 30 patients with advanced HCC and PVTT treated with sorafenib monotherapy, a disease control rate of 33.3% was achieved, including thrombus revascularization in a small number of patients. Yet, OS and progression-free survival (PFS) still remained disappointing with only 3.1 and 2.0 mo, respectively[28]. In combination with TACE, sorafenib was able to induce a significant survival benefit compared to TACE only in patients with type B (13 mo *vs* 6 mo) or type C (15 mo *vs* 10 mo) in a stud enrolling 99 patients[29]. Similar results were obtained in combination with radiofrequency ablation (RFA)[30]. Still, prospective randomized controlled trials on sorafenib or regorafenib monotherapy in this setting are missing and the effect of the combination approach is probably overruling the currently available results[27].

Different combination studies of sorafenib and other agents have been performed. Interestingly, vitamin K was shown to enhance the antitumor effects of sorafenib via reduction of expression of des-γ-carboxy prothrombin (DCP), a proangiogenic growth factor that can also trigger signaling *via* c-Met and which is commonly upregulated after sorafenib treatment[31,32].

Combination studies with other targeted agents showed only a modest increase in survival but had significant increase in drug related adverse events or even showed a worse outcome than single agents like the sorafenib/erlotinib combination[33]. These agents have also been investigated in earlier disease stages but could not demonstrate a survival benefit in combination with locoregional approaches like TACE or in an adjuvant setting[7].

In summary, sorafenib and lenvatinib are options for first-line therapy of advanced HCC, while regorafenib, cabozantinib and ramucirumab can be used as second-line options afterwards.

IMMUNE CHECKPOINT INHIBITORS AND NOVEL IMMUNOTHERAPY TARGETS IN HCC

The development of immune checkpoint inhibitors like anti-CTLA4 (ipilimumab, tremelimumab) or anti-PD-1/PD-L1 (nivolumab, pembrolizumab, tislelizumab, camrelizumab, atezolizumab, durvalumab, avelumab) antibodies has dramatically changed clinical oncology nowadays and achieved sustained treatment responses in an unprecedented manner across different cancer types. Chronic inflammation due to the various underlying etiologies is a mainstay of HCC development. Different immune cell subtypes (T cells, macrophages, Kupffer cells) are currently intensively investigated to understand their role in HCC pathogenesis and to exploit them as novel and specific therapeutic approaches for this disease. Kupffer cells and CD8+ T cells in HCC have been shown to express high levels of PD-1 and PD-L1, thus playing a key role in the immune evasive phenotype of HCC. High expression of PD-L1 on tumor cells was associated with poorer outcome in HCC patients[34-37].

Several immune checkpoint inhibitors are currently investigated in clinical trials as single agents or in combination with neo-epitope releasing locoregional therapies, epigenetic drugs or conventional targeted agents[38].

In a phase 1 study, tremelimumab reached a clinical disease control rate of 76.4% with 17.6% of patients achieving partial response[39]. In combination with subtotal radiofrequency ablation or chemoablation, 26.3% of patients reached partial response and a median overall survival of 12.3 mo. Responders also showed increased infiltration of CD8+ T cells and HCV positive patients had a significant reduction in viral load[40].

Based on positive results of the phase 1/2 CheckMate-040 study, nivolumab received FDA approval as a 2nd line therapy option in HCC[41]. An objective response rate of 20% was reached overall, and patients expressing PD-L1 on tumor cells even reached 26%. A subgroup analysis revealed disease control rates of up to 75% and a median duration of response of 9.9 mo. Results of the phase 3 CheckMate-459 study that compares nivolumab *vs* sorafenib are still pending.

Similar results were obtained for pembrolizumab in the KEYNOTE-224 study. Here, 17% of patients had partial response, 44% had stable disease and overall 77% of responding patients had durable responses of 9 mo or more. Pembrolizumab also received FDA approval as a 2nd line therapy after sorafenib therapy.

Several further studies are currently ongoing to evaluate these checkpoint inhibitors in first or second line therapy of HCC, including the HIMALAYA study that explores durvalumab alone or in combination with tremelimumab *vs* sorafenib or the IMbrave50 study that combines atezolizumab with bevacizumab *vs* sorafenib[42].

Due to its physiologic role in clearing portal vein blood flow from potentially harmful gut content, the liver is a key immunologic organ and contains a high proportion of macrophages (Kupffer cells) and other cells of lymphoid lineage, including B and T cells as well as natural killer (NK) and NKT cells[43]. As outlined above, HCC commonly develops on the basis of chronic inflammatory liver injury and exploiting the immunologic repertoire of the liver. The first promising results of immune checkpoint inhibitors in HCC further support this approach. Different technologies have been developed to apply an adaptive cell transfer as a therapeutic option also in HCC, including application of tumor infiltrating lymphocytes, cytokine induced killer cells or fostering neoepitope release by locoregional ablation techniques[44-46].

Recently, chimeric antigen receptor-engineered T (CAR-T) cells yielded outstanding responses in hematologic malignancies and received FDA approval for the treatment of acute lymphoblastic leukemia[47,48] and diffuse large B-cell lymphoma[49,50]. In brief, T cells of patients are harvested, genetically modified, expanded and reinfused into the patient. CARs consist of an extracellular antigen recognition domain, a hinge/spacer domain, a transmembrane domain and a T cell activation domain (CD3ζ). CARs of the 2nd and 3rd generation have additional costimulatory molecules like CD27 or CD134 in between the transmembrane and the CD3ζ domain to

achieve prolonged T cell expansion and antitumor effects[51]. Success of CAR-T cells in solid tumors is limited by their broader mutational load compared to hematologic malignancies and suitable tumor antigens are thus more difficult to identify. An additional hurdle is the localization of modified T cells to the tumor, which is influenced by tumor angiogenesis, low levels of chemokines to attract T cells into the tumor and, esp. in HCC, a tumor microenvironment (stroma, fibrosis) that does not permit sufficient tissue penetration of large molecules[52]. The latter can be overcome by selective local administration like hepatic artery infusion which makes HCC an interesting option for this therapy approach and several studies using CAR-T cells were therefore initiated in HCC[44,53] (Table 3). Interestingly, most studies use the cell surface glycoprotein glypican-3 (GPC3) as an antigen, which is highly expressed in HCC but not in other adult tissues and has been linked to poor prognosis of HCC[54,55]. Preclinically, CAR-T cells targeting GPC3 were able to eliminate HCC cells and prolong survival of tumor-bearing mice[56,57]. Ongoing studies also evaluate different application routes like hepatic artery infusion, systemic infusion or combination with transarterial (chemo-)embolization and the effect of lymphodepleting conditioning. Preliminary results from study NCT02395250 indicate that GPC3-CAR-T cells are safe in relapsed or refractory Chinese HCC patients. In this study, 1 partial response and 2 stable diseases were observed as best response (from 6 evaluable patients), all durable for more than one year[58].

EPIGENETIC THERAPY WITH HDAC INHIBITORS IN HCC

Epigenetic dysregulation of gene activity is essentially involved in HCC tumorigenesis as evidenced by dysregulation of histone deacetylases *in vitro*, *in vivo* and *in situ*[59,60]. Treatment with HDAC inhibitors (HDACi) therefore represents an attractive therapeutic option in liver cancer that addresses different molecular mechanisms compared to chemotherapy or targeted therapies to inhibit tumor cell growth and promote cell death. HDACi inhibitors commonly inhibit cell cycle progression by re-expression of $p21^{cip1/waf1}$ in a p53-dependent manner but can also mediate alternative cell death pathways like unfolded protein response and ER stress pathways due to their non-specific acetylation of proteins also outside the nu-cleus[61-65]. Although some case reports showed a positive effect of HDACi in com-bination with sorafenib in HCC[66], most studies are disappointing so far. A phase 2 study using belinostat (inhibitor of all zinc dependent HDAC isoforms) in unresectable HCC showed a PFS and OS of 2.6 and 6.6 mo, respectively[67]. The SHELTER study investigated resminostat (oral pan-HDACi with predominant activity against HDAC1, 2 and 3) in combination with sorafenib in a 2nd line setting of adv-anced HCC and demonstrated an OS of 8.0 mo, while monotherapy resminostat reached only 4.1 mo[68]. Interestingly, this combination was also used in a 1st line setting in Asian patients but did not provide evidence for an OS benefit over sorafenib[69].

These studies, like others investigating tyrosine kinase inhibitors, usually enroll patients with progressive, unresectable, locally advanced or metastatic HCC with an overall poor prognosis that limits the chance of achieving PFS or OS advantages[70]. Yet, epigenetic therapies may be able to overcome such hurdles and even enhance the results of immune checkpoint inhibitors in HCC. Epigenetic targeting with the enhancer of zeste homolog 2 (EZH2), a histone-lysine-N-methyltransferase, inhibitor 3-Deazaneplanocin A (DZNep) and the DNA methyltransferase 1 (DNMT1) inhibitor 5-azacytidine reactivated transcriptionally repressed chemokines genes and augmented T cell trafficking to the tumor[71]. Consequently, epigenetic pretreatment may lead to priming of so-called immune cold tumors also in HCC[71,72].

NOVEL BIOMARKERS TO IMPROVE PATIENT OUTCOME

HCC patients are commonly stratified based on their liver function capacity as assessed by Child-Pugh score, which overall seems to be a better predictor for treatment outcome than the underlying etiology of HCC[70,73]. Biomarkers that could predict treatment response are therefore urgently needed, esp. for targeted therapies and immunotherapy approaches[74].

In a recent biomarker analysis of the sorafenib phase 3 STORM trial (BIOSTORM), none of the biomarkers related to angiogenesis or cell proliferation or other molecular markers like gene signatures or mutations could predict a treatment benefit or recurrence-free survival (RFS). Only p-ERK and microvascular invasion were associated with poor RFS. This study proposed a new 146-gene signature that identified about 30% of patients which benefit from sorafenib treatment. Interestingly, those patients

Table 3 Clinical trials with chimeric antigen receptor T cells cells in hepatocellular carcinoma

NCT	Antigen	Phase	Patients	Sponsor	Status	Comments
NCT02715362	GPC3	I/II	30	Company	Recruiting	HAI
NCT03672305	c-Met/PD-L1	I	50	Academic	Not yet recruiting	IV
NCT02723942	GPC3	I/II	60	Academic	Completed	
NCT03198546	GPC3	I	30	Academic	Recruiting	
NCT02395250	GPC3	I	13	Academic	Completed	[58]
NCT03349255	AFP	I	18	Company	Recruiting	IV *vs* HAI
NCT03130712	GPC3	I/II	10	Company	Recruiting	IT
NCT03084380	GPC3	I/II	20	Academic	Not yet recruiting	Combination with TACE
NCT02905188[1]	GPC3	I	14	Academic	Not yet recruiting	
NCT03302403	GPC3	I	48	Academic	Not yet recruiting	
NCT03146234	GPC3	I	20	Academic	Recruiting	
NCT01935843	Her2	I/II	10	Academic	Unknown	
NCT02959151	GPC3	I/II	20	Company	Unknown	
NCT02587689	MUC1	I/II	20	Company	Unknown	
NCT03013712	EpCAM	I/II	60	Academic	Recruiting	

[1]Location of study is United States, all other trials are conducted in China. HAI: Hepatic artery infusion; IT: Intratumoral injection; IV: Intravenous injection; TACE: Transarterial chemoembolization.

were also enriched in CD4+ T and B cells, NK cells and were associated with signature of poor response to immune checkpoint inhibitors[75].

Analysis of the regorafenib phase 3 study in HCC (RESORCE), also recently identified a plasma protein expression profile [angiopoietin 1, cystatin B, oxidized low density lipoprotein (LDL) receptor 1, latency associated peptide of transforming growth factor β (TGF-β) and macrophage inflammatory protein 1α (MIP1α)] and 9 plasma miRNAs that were associated with increased overall survival and time to progression. The proposed soluble plasma protein biomarkers are also known to play a role in inflammation and HCC pathogenesis. Interestingly, none of these predictive biomarkers was so far shown to have prognostic relevance[76].

Overall, the identification and validation of biomarkers in HCC was previously limited by the availability of tissue specimens as international guidelines did not mandate a biopsy sample for diagnostic purposes. Recently, guidelines from EASL recommend taking tissue and liquid biopsies from HCC patients participating in clinical studies which could improve this situation[77]. Biomarker analyses in HCC are often limited by small sample size in respective subgroup analyses due to the diverse etiologic backgrounds like viral hepatitis, NASH/NAFLD, cirrhosis status etc. which all significantly impact on the underlying chronic inflammation or have direct influence on oncogenic pathways.

Numerous new diagnostic and prognostic biomarkers like GPC-3 or c-Met have been proposed recently and were also translated into CAR-T cell based therapy approaches (see above) but no clear predictive biomarker for either targeted therapies or for immune checkpoint inhibitors is available so far[78,79].

Liquid biopsies are capable of detecting genetic and epigenetic alterations as well as expression patterns of DNA, RNA and miRNA from circulating tumor cells, cell-free nucleic acids or from exosomes. Success rate of these technologies is still variable depending on tumor size and stage but with further technological advances, *e.g.*, on next generation sequencing from cell-free DNA, it is rapidly maturing to clinical applicability[80-82].

Assessment of metabolic pathways including proteomics and glycomics could further contribute to biomarker development although current approaches, *e.g.*, detection of CD44v9[83] or Hippocalcin-like 1 (HPCAL1)[84], are used for diagnostic or prognostic settings or to predict disease recurrence and have not been linked to treatment responses.

NEW PRECLINICAL MODELS TO IMPROVE HCC THERAPY

The successful development of novel drugs is largely dependent on the availability of suitable and predictive preclinical models. Besides the specific biochemical (cell-free)

inhibition of a distinct target, novel compounds need to prove their potency in various *in vitro* and *in vivo* model systems before entering human clinical trials.

In the past, high throughput screening was performed on 2D cultures using immortalized cell lines. Although this approach allowed screening large numbers of cell lines, it lacks the complex interaction of different cell types and matrix structures within real tissue. Consequently, more complex 3D culture systems were established, also from primary human cancer samples, that also contain components of extracellular matrix and additional cell types as fibroblasts[85,86]. Recently, spheroids and organoids that mimic organ structure and aggressiveness of human HCC have been established as tools for drug sensitivity screening[87,88]. To reflect the genetic heterogeneity of human cancers, mixtures of barcoded tumor cell lines can be subjected to high throughput screening. This technology secures homogeneous drug exposure to genetically different cell types at the same time and was shown to identify responders and non-responders to specific treatments as well as to create new biomarker hypotheses[89].

Precision-cut liver slices represent an interesting *ex vivo* model system for drug development. Complex tissue architecture is preserved and the model allows to investigate different pathophysiologic conditions and drug testing on primary human tissue samples[90,91].

Animal models, however, still represent standard models for early drug development approaches. As HCC usually develops on the background of chronic underlying liver diseases associated with chronic inflammation, viral infection or fibrotic remodeling, and clear oncogenic driver mutations have not been identified yet, finding suitable and appropriate models still remains an urgent task. An ideal model would therefore in parallel describe the underlying liver disease and tumor development. The increasing role of immunotherapies in HCC also urgently warrants the development of respective immunocompetent models (Table 4)[92].

The subcutaneous implantation of HCC cell lines was extensively used in the past and still has value when using primary cell lines or tissue explants (patient-derived xenograft models). These models are relatively easy to handle and provide an easy readout of tumor growth by caliper measurement. Orthotopic implantation reflects the primary site of tumor formation and crossplay with liver matrix and cells but requires surgical expertise and more advanced imaging technologies to monitor tumor growth. Both systems need immunodeficient mice unless syngeneic murine tumors are used. This limits the application of those models for studying immunotherapy approaches.

As human HCC can also develop upon chronic exposure to toxic agents, chemical induction in mice using diethylnitrosamine (DEN) has been established as a standard method but is limited by high variability of tumor formation and time to develop tumors[93]. Yet, chemical models can easily be combined with other approaches (*e.g.*, fibrosis induction, NASH models, alcohol) and thus provide an option for rapid evaluation of novel drugs under distinct pathophysiologic conditions[94-97].

Genetically engineered mice (GEM) are useful tools to study the contribution and effects of individual genes on HCC pathogenesis. They are technically more demanding and may have a longer latency period than other models. With the option of targeted knock-in and knock-out systems and combinations of those approaches, distinct molecular backgrounds can be analyzed. As today no clear single oncogenic driver for HCC development has been identified, several GEM models have been established as useful tools, *e.g.*, p53-deficient[98] mice or mice overexpressing MYC[99] or WNT pathway components[100,101]. The option to study HBV and HCV transgenic mice is of special interest. We have shown previously that dependent on the host genetic background, ER stress pathways can be activated that are known to lead to cellular stress and chronic inflammation and that are involved in fibrogenesis and ultimately also HCC pathogenesis[102,103].

As outlined above, HCC commonly develops on the basis of an underlying chronic liver disease. Therefore, specific liver disease models are of very high relevance to study HCC pathogenesis and explore new therapeutic options[92]. Several small animal models for the development of liver fibrosis and cirrhosis are currently established. Application of carbon tetrachloride (CCl_4) or thioacetamide (in combination with ethanol)[104] leads to rapid fibrosis development and acute inflammation[105]. While easy to handle, these models can be combined with GEM or orthotopic transplantation models and provide a good tool to study tumor cells with a fibrotic microenvironment.

Although less clear in its pathogenesis for HCC, the increasing prevalence of metabolic liver diseases associated with diabetes mellitus, adipositas, hyperlipidemia, hypertriglyceridemia and metabolic syndrome puts models for NAFLD/NASH in the focus of today's research models[106-108]. NAFLD and NASH can easily be induced by dietary models, *e.g.*, using a high-fat (HFD), high-cholesterol (HCD), high-fructose or

Table 4 Available techniques for induction of hepatocellular carcinoma in relation to temporal and technical aspects as well as major advantages and disadvantages (summarized from[92])

Method and specification	Time to HCCshort (+) to long (+++)	Technical effortslow (+) to high (+++)	Major "Pros" (+) *vs* "Contras" (-)
Chemotoxic agents linked models			
Diethylnitrosamine	++	+	(+) good combination options with other methods
9,10-dimethyl-1,2-benzanthracene			(-) time to HCC not easily predictable
Direct implantation of tumor cells or tissue			
Heterotopic/orthotopic	+	+/++	(+) heterotopic xenografts are often and easily done
			(+) syngeneic orthotopic models better reflect the natural liver microenvironment
Syngeneic/xenografts			(-) xenografts need immunocompromised mice
			(-) orthotopic tumor implants need surgical and imaging experience
Genetically engineered mouse models			
Mouse embryo manipulation	++/+++	+++	(+) hepatocarcinogenesis can be analyzed stepwise
Cre-Lox recombination			(-) effects of manipulated gene(s) could have heterogeneous latency and genetic penetrance
Hydrodynamic injection			
CRISPR-Cas9			
Humanized mouse models			
Immunologically humanized mice	+++	+++	(+) immunotherapeutical issues can be studied based on human cell lines in mice
Genetically humanized mice			(-) establishment difficult due to engraftment failure and development of stable stem cell-derived hepatocytes

HCC: Hepatocellular carcinoma.

methionine and choline-deficient (MCD) diets or combinations of those classical inducers[109]. Yet, dietary models are often limited by not completely following the human course of disease, *e.g.*, lacking HCC formation for high-fat diet or lacking obesity for the MCD diet. Therefore, models have been further refined by combining the dietary stimulus with distinct genetic models like PTEN-deficient mice, MC4R (melanocortin 4 receptor) or ALR (augmenter of liver regeneration) knockouts that reliably lead to HCC formation in 60% to 100% after approximately one year[110]. Recently, it was shown that HFD and HCD triggers liver cancer formation in an ApoE/LDL-receptor double knockout mouse, linking metabolic stress and atherosclerosis to HCC formation[111].

CONCLUSION

Integrative and comprehensive molecular and genomic analyses could classify hepatocellular carcinoma on the basis of landscapes of genetic and molecular signatures (Tables 1 and 2) which could then lead to the identification of predictive biomarkers for novel treatment options and then impact HCC trial design and patient outcome. Consequently, all HCCs should be biopsied and specimen should be intensively investigated applying "omics"-technologies for real precision medicine approaches. Furthermore, the biological roles of the identified driver genes in HCC must be analyzed in the deeper integration of inter- and intratumoral, interpatient, and inter-ethnic tumor heterogeneity in more detail. Deeper knowledge about those drivers is urgently needed, as the underlying pathogenesis of HCC is complex and currently shifting from chronic viral infections to more metabolically driven tumorigenesis as seen in NASH. As inflammation seems to be a common ground for HCC development, it is not surprising that immune checkpoint inhibitors are moving into first line therapy setting and it is expected that this compound class will also

significantly shift the therapeutic landscape in HCC soon. Therefore, tumor models with complex genetically engineering are an essential drug development and technology transfer tool closing the gap between *in vitro* experiments and intensive clinical trials in future (Table 4). Finally, artificial intelligence and machine learning could essentially help to analyze, to classify and to interpret the dramatically increasing and high-dimensional amount of transcriptomic, genomic, epigenomic, metabolic, proteomic and imaging data in HCC[112-114]. The desirable aims of such approaches will be to (1) identify cancer drug targets, (2) predict anticancer sensitivity, toxicity and cancer resistance, and (3) give robust recommendations for therapeutic strategies in the future[115,116].

Overall, the better understanding of the molecular pathogenesis of HCC allows for more stringent patient selection criteria in biomarker-driven studies that can improve patient outcome.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Marengo A**, Rosso C, Bugianesi E. Liver Cancer: Connections with Obesity, Fatty Liver, and Cirrhosis. *Annu Rev Med* 2016; **67**: 103-117 [PMID: 26473416 DOI: 10.1146/annurev-med-090514-013832]

3 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019; **69**: 7-34 [PMID: 30620402 DOI: 10.3322/caac.21551]

4 **Khemlina G**, Ikeda S, Kurzrock R. The biology of Hepatocellular carcinoma: implications for genomic and immune therapies. *Mol Cancer* 2017; **16**: 149 [PMID: 28854942 DOI: 10.1186/s12943-017-0712-x]

5 **Zucman-Rossi J**, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology* 2015; **149**: 1226-1239.e4 [PMID: 26099527 DOI: 10.1053/j.gastro.2015.05.061]

6 **Gosalia AJ**, Martin P, Jones PD. Advances and Future Directions in the Treatment of Hepatocellular Carcinoma. *Gastroenterol Hepatol (NY)* 2017; **13**: 398-410 [PMID: 28867968]

7 **Ikeda K**. Recent advances in medical management of hepatocellular carcinoma. *Hepatol Res* 2019; **49**: 14-32 [PMID: 30308081 DOI: 10.1111/hepr.13259]

8 **Han B**, Li C, Meng H, Gomes Romeiro F, Mancuso A, Zhou Z, Levi Sandri GB, Xu Y, Han T, Han L, Shao L, Qi X. Efficacy and safety of external-beam radiation therapy for hepatocellular carcinoma: An overview of current evidence according to the different target population. *Biosci Trends* 2019; **13**: 10-22 [PMID: 30799321 DOI: 10.5582/bst.2018.01261]

9 **Qi X**, Zhao Y, Li H, Guo X, Han G. Management of hepatocellular carcinoma: an overview of major findings from meta-analyses. *Oncotarget* 2016; **7**: 34703-34751 [PMID: 27167195 DOI: 10.18632/oncotarget.9157]

10 **Eatrides J**, Wang E, Kothari N, Kim R. Role of Systemic Therapy and Future Directions for Hepatocellular Carcinoma. *Cancer Control* 2017; **24**: 1073274817729243 [PMID: 28975834 DOI: 10.1177/1073274817729243]

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

12 **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

13 **Ikeda K**, Kudo M, Kawazoe S, Osaki Y, Ikeda M, Okusaka T, Tamai T, Suzuki T, Hisai T, Hayato S, Okita K, Kumada H. Phase 2 study of lenvatinib in patients with advanced hepatocellular carcinoma. *J Gastroenterol* 2017; **52**: 512-519 [PMID: 27704266 DOI: 10.1007/s00535-016-1263-4]

14 **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

15 **Bruix J**, Qin S, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Breder V, Gerolami R, Masi G, Ross PJ, Song T, Bronowicki JP, Ollivier-Hourmand I, Kudo M, Cheng AL, Llovet JM, Finn RS, LeBerre MA, Baumhauer A, Meinhardt G, Han G; RESORCE Investigators. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **389**: 56-66 [PMID: 27932229 DOI: 10.1016/S0140-6736(16)32453-9]

16 **Abou-Alfa GK**, Meyer T, Cheng AL, El-Khoueiry AB, Rimassa L, Ryoo BY, Cicin I, Merle P, Chen Y, Park JW, Blanc JF, Bolondi L, Klümpen HJ, Chan SL, Zagonel V, Pressiani T, Ryu MH, Venook AP, Hessel C, Borgman-Hagey AE, Schwab G, Kelley RK. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. *N Engl J Med* 2018; **379**: 54-63 [PMID: 29972759 DOI: 10.1056/NEJMoa1717002]

17 **Zhu AX**, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, Assenat E, Brandi G, Pracht M, Lim HY, Rau KM, Motomura K, Ohno I, Merle P, Daniele B, Shin DB, Gerken G, Borg C, Hiriart JB, Okusaka T, Morimoto M, Hsu Y, Abada PB, Kudo M; REACH-2 study investigators. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2019; **20**: 282-296 [PMID:

30665869 DOI: 10.1016/S1470-2045(18)30937-9]
18 **Han P**, Li H, Jiang X, Zhai B, Tan G, Zhao D, Qiao H, Liu B, Jiang H, Sun X. Dual inhibition of Akt and c-Met as a second-line therapy following acquired resistance to sorafenib in hepatocellular carcinoma cells. *Mol Oncol* 2017; **11**: 320-334 [PMID: 28164434 DOI: 10.1002/1878-0261.12039]
19 **Ueki T**, Fujimoto J, Suzuki T, Yamamoto H, Okamoto E. Expression of hepatocyte growth factor and its receptor, the c-met proto-oncogene, in hepatocellular carcinoma. *Hepatology* 1997; **25**: 619-623 [PMID: 9049208 DOI: 10.1002/hep.510250321]
20 **Kaposi-Novak P**, Lee JS, Gòmez-Quiroz L, Coulouarn C, Factor VM, Thorgeirsson SS. Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype. *J Clin Invest* 2006; **116**: 1582-1595 [PMID: 16710476 DOI: 10.1172/JCI27236]
21 **García-Vilas JA**. Medina MÁ Updates on the hepatocyte growth factor/c-Met axis in hepatocellular carcinoma and its therapeutic implications. *World J Gastroenterol* 2018; **24**: 3695-3708 [PMID: 30197476 DOI: 10.3748/wjg.v24.i33.3695]
22 **Bouattour M**, Raymond E, Qin S, Cheng AL, Stammberger U, Locatelli G, Faivre S. Recent developments of c-Met as a therapeutic target in hepatocellular carcinoma. *Hepatology* 2018; **67**: 1132-1149 [PMID: 28862760 DOI: 10.1002/hep.29496]
23 **Qi XS**, Guo XZ, Han GH, Li HY, Chen J. MET inhibitors for treatment of advanced hepatocellular carcinoma: A review. *World J Gastroenterol* 2015; **21**: 5445-5453 [PMID: 25987766 DOI: 10.3748/wjg.v21.i18.5445]
24 **Llovet JM**, Decaens T, Raoul JL, Boucher E, Kudo M, Chang C, Kang YK, Assenat E, Lim HY, Boige V, Mathurin P, Fartoux L, Lin DY, Bruix J, Poon RT, Sherman M, Blanc JF, Finn RS, Tak WY, Chao Y, Ezzeddine R, Liu D, Walters I, Park JW. Brivanib in patients with advanced hepatocellular carcinoma who were intolerant to sorafenib or for whom sorafenib failed: results from the randomized phase III BRISK-PS study. *J Clin Oncol* 2013; **31**: 3509-3516 [PMID: 23980090 DOI: 10.1200/JCO.2012.47.3009]
25 **Johnson PJ**, Qin S, Park JW, Poon RT, Raoul JL, Philip PA, Hsu CH, Hu TH, Heo J, Xu J, Lu L, Chao Y, Boucher E, Han KH, Paik SW, Robles-Aviña J, Kudo M, Yan L, Sobhonslidsuk A, Komov D, Decaens T, Tak WY, Jeng LB, Liu D, Ezzeddine R, Walters I, Cheng AL. Brivanib versus sorafenib as first-line therapy in patients with unresectable, advanced hepatocellular carcinoma: results from the randomized phase III BRISK-FL study. *J Clin Oncol* 2013; **31**: 3517-3524 [PMID: 23980084 DOI: 10.1200/JCO.2012.48.4410]
26 **Ogasawara S**, Chiba T, Ooka Y, Kanogawa N, Saito T, Motoyama T, Suzuki E, Tawada A, Kanai F, Yokosuka O. Sorafenib treatment in Child-Pugh A and B patients with advanced hepatocellular carcinoma: safety, efficacy and prognostic factors. *Invest New Drugs* 2015; **33**: 729-739 [PMID: 25861764 DOI: 10.1007/s10637-015-0237-3]
27 **Qi X**, Guo X. Sorafenib for the treatment of hepatocellular carcinoma with portal vein tumour thrombosis: a systematic review of comparative studies. *Prz Gastroenterol* 2015; **10**: 142-147 [PMID: 26516379 DOI: 10.5114/pg.2015.52470]
28 **Jeong SW**, Jang JY, Shim KY, Lee SH, Kim SG, Cha SW, Kim YS, Cho YD, Kim HS, Kim BS, Kim KH, Kim JH. Practical effect of sorafenib monotherapy on advanced hepatocellular carcinoma and portal vein tumor thrombosis. *Gut Liver* 2013; **7**: 696-703 [PMID: 24312711 DOI: 10.5009/gnl.2013.7.6.696]
29 **Zhu K**, Chen J, Lai L, Meng X, Zhou B, Huang W, Cai M, Shan H. Hepatocellular carcinoma with portal vein tumor thrombus: treatment with transarterial chemoembolization combined with sorafenib--a retrospective controlled study. *Radiology* 2014; **272**: 284-293 [PMID: 24708192 DOI: 10.1148/radiol.14131946]
30 **Giorgio A**, Merola MG, Montesarchio L, Merola F, Santoro B, Coppola C, Gatti P, Amendola F, DI Sarno A, Calvanese A, Matteucci P, Giorgio V. Sorafenib Combined with Radio-frequency Ablation Compared with Sorafenib Alone in Treatment of Hepatocellular Carcinoma Invading Portal Vein: A Western Randomized Controlled Trial. *Anticancer Res* 2016; **36**: 6179-6183 [PMID: 27793949 DOI: 10.21873/anticanres.11211]
31 **Jung DH**, Hwang S, Song GW, Ryoo BY, Kim N, Tak E, Hong HN. An interim safety analysis of hepatocellular carcinoma patients administrating oral vitamin K with or without sorafenib. *Korean J Hepatobiliary Pancreat Surg* 2015; **19**: 1-5 [PMID: 26155269 DOI: 10.14701/kjhbps.2015.19.1.1]
32 **Haruna Y**, Hasegawa N, Imanaka K, Kawamoto S, Inoue A. Clinical Impact of Vitamin K Dosing on Sorafenib Treatment for Hepatocellular Carcinoma. *J Cancer* 2017; **8**: 1988-1994 [PMID: 28819398 DOI: 10.7150/jca.18900]
33 **Zhu AX**, Rosmorduc O, Evans TR, Ross PJ, Santoro A, Carrilho FJ, Bruix J, Qin S, Thuluvath PJ, Llovet JM, Leberre MA, Jensen M, Meinhardt G, Kang YK. SEARCH: a phase III, randomized, double-blind, placebo-controlled trial of sorafenib plus erlotinib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2015; **33**: 559-566 [PMID: 25547503 DOI: 10.1200/JCO.2013.53.7746]
34 **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]
35 **Calderaro J**, Rousseau B, Amaddeo G, Mercey M, Charpy C, Costentin C, Luciani A, Zafrani ES, Laurent A, Azoulay D, Lafdil F, Pawlotsky JM. Programmed death ligand 1 expression in hepatocellular carcinoma: Relationship With clinical and pathological features. *Hepatology* 2016; **64**: 2038-2046 [PMID: 27359084 DOI: 10.1002/hep.28710]
36 **Long J**, Qu T, Pan XF, Tang X, Wan HH, Qiu P, Xu YH. Expression of programmed death ligand-1 and programmed death 1 in hepatocellular carcinoma and its clinical significance. *J Cancer Res Ther* 2018; **14**: S1188-S1192 [PMID: 30539869 DOI: 10.4103/0973-1482.204850]
37 **Dai X**, Xue J, Hu J, Yang SL, Chen GG, Lai PBS, Yu C, Zeng C, Fang X, Pan X, Zhang T. Positive Expression of Programmed Death Ligand 1 in Peritumoral Liver Tissue is Associated with Poor Survival after Curative Resection of Hepatocellular Carcinoma. *Transl Oncol* 2017; **10**: 511-517 [PMID: 28558264 DOI: 10.1016/j.tranon.2017.03.009]
38 **Xu F**, Jin T, Zhu Y, Dai C. Immune checkpoint therapy in liver cancer. *J Exp Clin Cancer Res* 2018; **37**: 110 [PMID: 29843754 DOI: 10.1186/s13046-018-0777-4]
39 **Sangro B**, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, Riezu-Boj JI, Larrea E, Alfaro C, Sarobe P, Lasarte JJ, Pérez-Gracia JL, Melero I, Prieto J. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; **59**: 81-88 [PMID: 23466307 DOI: 10.1016/j.jhep.2013.02.022]
40 **Duffy AG**, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, Davis JL, Hughes MS, Heller T, ElGindi M, Uppala A, Korangy F, Kleiner DE, Figg WD, Venzon D, Steinberg SM, Venkatesan

AM, Krishnasamy V, Abi-Jaoudeh N, Levy E, Wood BJ, Greten TF. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J Hepatol* 2017; **66**: 545-551 [PMID: 27816492 DOI: 10.1016/j.jhep.2016.10.029]

41 **El-Khoueiry AB**. Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

42 **Okusaka T**, Ikeda M. Immunotherapy for hepatocellular carcinoma: current status and future perspectives. *ESMO Open* 2018; **3**: e000455 [PMID: 30622744 DOI: 10.1136/esmoopen-2018-000455]

43 **Bogdanos DP**, Gao B, Gershwin ME. Liver immunology. *Compr Physiol* 2013; **3**: 567-598 [PMID: 23720323 DOI: 10.1002/cphy.c120011]

44 **Zhang R**, Zhang Z, Liu Z, Wei D, Wu X, Bian H, Chen Z. Adoptive cell transfer therapy for hepatocellular carcinoma. *Front Med* 2019; **13**: 3-11 [PMID: 30659408 DOI: 10.1007/s11684-019-0684-x]

45 **Wissniowski TT**, Hänsler J, Neureiter D, Frieser M, Schaber S, Esslinger B, Voll R, Strobel D, Hahn EG, Schuppan D. Activation of tumor-specific T lymphocytes by radio-frequency ablation of the VX2 hepatoma in rabbits. *Cancer Res* 2003; **63**: 6496-6500 [PMID: 14559842]

46 **Behm B**, Di Fazio P, Michl P, Neureiter D, Kemmerling R, Hahn EG, Strobel D, Gress T, Schuppan D, Wissniowski TT. Additive antitumour response to the rabbit VX2 hepatoma by combined radio frequency ablation and toll like receptor 9 stimulation. *Gut* 2016; **65**: 134-143 [PMID: 25524262 DOI: 10.1136/gutjnl-2014-308286]

47 **Park JH**, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, Sauter C, Wang Y, Santomasso B, Mead E, Roshal M, Maslak P, Davila M, Brentjens RJ, Sadelain M. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N Engl J Med* 2018; **378**: 449-459 [PMID: 29385376 DOI: 10.1056/NEJMoa1709919]

48 **Maude SL**, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, Qayed M, De Moerloose B, Hiramatsu H, Schlis K, Davis KL, Martin PL, Nemecek ER, Yanik GA, Peters C, Baruchel A, Boissel N, Mechinaud F, Balduzzi A, Krueger J, June CH, Levine BL, Wood P, Taran T, Leung M, Mueller KT, Zhang Y, Sen K, Lebwohl D, Pulsipher MA, Grupp SA. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med* 2018; **378**: 439-448 [PMID: 29385370 DOI: 10.1056/NEJMoa1709866]

49 **Locke FL**, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, Lin Y, Braunschweig I, Hill BT, Timmerman JM, Deol A, Reagan PM, Stiff P, Flinn IW, Farooq U, Goy A, McSweeney PA, Munoz J, Siddiqi T, Chavez JC, Herrera AF, Bartlett NL, Wiezorek JS, Navale L, Xue A, Jiang Y, Bot A, Rossi JM, Kim JJ, Go WY, Neelapu SS. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol* 2019; **20**: 31-42 [PMID: 30518502 DOI: 10.1016/S1470-2045(18)30864-7]

50 **Neelapu SS**, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, Braunschweig I, Oluwole OO, Siddiqi T, Lin Y, Timmerman JM, Stiff PJ, Friedberg JW, Flinn IW, Goy A, Hill BT, Smith MR, Deol A, Farooq U, McSweeney P, Munoz J, Avivi I, Castro JE, Westin JR, Chavez JC, Ghobadi A, Komanduri KV, Levy R, Jacobsen ED, Witzig TE, Reagan P, Bot A, Rossi J, Navale L, Jiang Y, Aycock J, Elias M, Chang D, Wiezorek J, Go WY. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med* 2017; **377**: 2531-2544 [PMID: 29226797 DOI: 10.1056/NEJMoa1707447]

51 **Spear TT**, Nagato K, Nishimura MI. Strategies to genetically engineer T cells for cancer immunotherapy. *Cancer Immunol Immunother* 2016; **65**: 631-649 [PMID: 27138532 DOI: 10.1007/s00262-016-1842-5]

52 **Long KB**, Young RM, Boesteanu AC, Davis MM, Melenhorst JJ, Lacey SF, DeGaramo DA, Levine BL, Fraietta JA. CAR T Cell Therapy of Non-hematopoietic Malignancies: Detours on the Road to Clinical Success. *Front Immunol* 2018; **9**: 2740 [PMID: 30559740 DOI: 10.3389/fimmu.2018.02740]

53 **Chen Y**, E CY, Gong ZW, Liu S, Wang ZX, Yang YS, Zhang XW. Chimeric antigen receptor-engineered T-cell therapy for liver cancer. *Hepatobiliary Pancreat Dis Int* 2018; **17**: 301-309 [PMID: 29861325 DOI: 10.1016/j.hbpd.2018.05.005]

54 **Haruyama Y**, Kataoka H. Glypican-3 is a prognostic factor and an immunotherapeutic target in hepatocellular carcinoma. *World J Gastroenterol* 2016; **22**: 275-283 [PMID: 26755876 DOI: 10.3748/wjg.v22.i1.275]

55 **Kaseb AO**, Hassan M, Lacin S, Abdel-Wahab R, Amin HM, Shalaby A, Wolff RA, Yao J, Rashid A, Vennapusa B, Feng J, Ohtomo T. Evaluating clinical and prognostic implications of Glypican-3 in hepatocellular carcinoma. *Oncotarget* 2016; **7**: 69916-69926 [PMID: 27655712 DOI: 10.18632/oncotarget.12066]

56 **Gao H**, Li K, Tu H, Pan X, Jiang H, Shi B, Kong J, Wang H, Yang S, Gu J, Li Z. Development of T cells redirected to glypican-3 for the treatment of hepatocellular carcinoma. *Clin Cancer Res* 2014; **20**: 6418-6428 [PMID: 25320357 DOI: 10.1158/1078-0432.CCR-14-1170]

57 **Dargel C**, Bassani-Sternberg M, Hasreiter J, Zani F, Bockmann JH, Thiele F, Bohne F, Wisskirchen K, Wilde S, Sprinzl MF, Schendel DJ, Krackhardt AM, Uckert W, Wohlleber D, Schiemann M, Stemmer K, Heikenwälder M, Busch DH, Richter G, Mann M, Protzer U. T Cells Engineered to Express a T-Cell Receptor Specific for Glypican-3 to Recognize and Kill Hepatoma Cells In Vitro and in Mice. *Gastroenterology* 2015; **149**: 1042-1052 [PMID: 26052074 DOI: 10.1053/j.gastro.2015.05.055]

58 **Zhai B**, Shi D, Gao H, Qi X, Jiang H, Zhang Y, Chi J, Ruan H, Wang H, Ru QC, Li Z. A phase I study of anti-GPC3 chimeric antigen receptor modified T cells (GPC3 CAR-T) in Chinese patients with refractory or relapsed GPC3+ hepatocellular carcinoma (r/r GPC3+ HCC). *J Clin Oncol* 2017; **35**: 3049 [DOI: 10.1200/JCO.2017.35.15_suppl.3049]

59 **Zhao J**, Gray SG, Greene CM, Lawless MW. Unmasking the pathological and therapeutic potential of histone deacetylases for liver cancer. *Expert Rev Gastroenterol Hepatol* 2019; **13**: 247-256 [PMID: 30791763 DOI: 10.1080/17474124.2019.1568870]

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

61 **Ocker M**, Schneider-Stock R. Histone deacetylase inhibitors: signalling towards p21cip1/waf1. *Int J Biochem Cell Biol* 2007; **39**: 1367-1374 [PMID: 17412634 DOI: 10.1016/j.biocel.2007.03.001]

62 **Di Fazio P**, Schneider-Stock R, Neureiter D, Okamoto K, Wissniowski T, Gahr S, Quint K, Meissnitzer M, Alinger B, Montalbano R, Sass G, Hohenstein B, Hahn EG, Ocker M. The pan-deacetylase inhibitor

panobinostat inhibits growth of hepatocellular carcinoma models by alternative pathways of apoptosis. *Cell Oncol* 2010; **32**: 285-300 [PMID: 20208142 DOI: 10.3233/CLO-2010-0511]
63 **Ocker M**. Deacetylase inhibitors - focus on non-histone targets and effects. *World J Biol Chem* 2010; **1**: 55-61 [PMID: 21540990 DOI: 10.4331/wjbc.v1.i5.55]
64 **Montalbano R**, Waldegger P, Quint K, Jabari S, Neureiter D, Illig R, Ocker M, Di Fazio P. Endoplasmic reticulum stress plays a pivotal role in cell death mediated by the pan-deacetylase inhibitor panobinostat in human hepatocellular cancer cells. *Transl Oncol* 2013; **6**: 143-157 [PMID: 23544167 DOI: 10.1593/tlo.12271]
65 **Di Fazio P**, Waldegger P, Jabari S, Lingelbach S, Montalbano R, Ocker M, Slater EP, Bartsch DK, Illig R, Neureiter D, Wissniowski TT. Autophagy-related cell death by pan-histone deacetylase inhibition in liver cancer. *Oncotarget* 2016; **7**: 28998-29010 [PMID: 27058414 DOI: 10.18632/oncotarget.8585]
66 **Gahr S**, Wissniowski T, Zopf S, Strobel D, Pustowka A, Ocker M. Combination of the deacetylase inhibitor panobinostat and the multi-kinase inhibitor sorafenib for the treatment of metastatic hepatocellular carcinoma - review of the underlying molecular mechanisms and first case report. *J Cancer* 2012; **3**: 158-165 [PMID: 22514558 DOI: 10.7150/jca.4211]
67 **Yeo W**, Chung HC, Chan SL, Wang LZ, Lim R, Picus J, Boyer M, Mo FK, Koh J, Rha SY, Hui EP, Jeung HC, Roh JK, Yu SC, To KF, Tao Q, Ma BB, Chan AW, Tong JH, Erlichman C, Chan AT, Goh BC. Epigenetic therapy using belinostat for patients with unresectable hepatocellular carcinoma: a multicenter phase I/II study with biomarker and pharmacokinetic analysis of tumors from patients in the Mayo Phase II Consortium and the Cancer Therapeutics Research Group. *J Clin Oncol* 2012; **30**: 3361-3367 [PMID: 22915658 DOI: 10.1200/JCO.2011.41.2395]
68 **Bitzer M**, Horger M, Giannini EG, Ganten TM, Wörns MA, Siveke JT, Dollinger MM, Gerken G, Scheulen ME, Wege H, Zagonel V, Cillo U, Trevisani F, Santoro A, Montesarchio V, Malek NP, Holzapfel J, Herz T, Ammendola AS, Pegoraro S, Hauns B, Mais A, Lauer UM, Henning SW, Hentsch B. Resminostat plus sorafenib as second-line therapy of advanced hepatocellular carcinoma - The SHELTER study. *J Hepatol* 2016; **65**: 280-288 [PMID: 26952006 DOI: 10.1016/j.jhep.2016.02.043]
69 **Tak WY**, Ryoo BY, Lim HY, Kim DY, Okusaka T, Ikeda M, Hidaka H, Yeon JE, Mizukoshi E, Morimoto M, Lee MA, Yasui K, Kawaguchi Y, Heo J, Morita S, Kim TY, Furuse J, Katayama K, Aramaki T, Hara R, Kimura T, Nakamura O, Kudo M. Phase I/II study of first-line combination therapy with sorafenib plus resminostat, an oral HDAC inhibitor, versus sorafenib monotherapy for advanced hepatocellular carcinoma in east Asian patients. *Invest New Drugs* 2018; **36**: 1072-1084 [PMID: 30198057 DOI: 10.1007/s10637-018-0658-x]
70 **Llovet JM**, Hernandez-Gea V. Hepatocellular carcinoma: reasons for phase III failure and novel perspectives on trial design. *Clin Cancer Res* 2014; **20**: 2072-2079 [PMID: 24589894 DOI: 10.1158/1078-0432.CCR-13-0547]
71 **Hong YK**, Li Y, Pandit H, Li S, Pulliam Z, Zheng Q, Yu Y, Martin RCG. Epigenetic modulation enhances immunotherapy for hepatocellular carcinoma. *Cell Immunol* 2019; **336**: 66-74 [PMID: 30626493 DOI: 10.1016/j.cellimm.2018.12.010]
72 **Llopiz D**, Ruiz M, Villanueva L, Iglesias T, Silva L, Egea J, Lasarte JJ, Pivette P, Trochon-Joseph V, Vasseur B, Dixon G, Sangro B, Sarobe P. Enhanced anti-tumor efficacy of checkpoint inhibitors in combination with the histone deacetylase inhibitor Belinostat in a murine hepatocellular carcinoma model. *Cancer Immunol Immunother* 2019; **68**: 379-393 [PMID: 30547218 DOI: 10.1007/s00262-018-2283-0]
73 **Bruix J**, Cheng AL, Meinhardt G, Nakajima K, De Sanctis Y, Llovet J. Prognostic factors and predictors of sorafenib benefit in patients with hepatocellular carcinoma: Analysis of two phase III studies. *J Hepatol* 2017; **67**: 999-1008 [PMID: 28687477 DOI: 10.1016/j.jhep.2017.06.026]
74 **Ocker M**. Biomarkers for hepatocellular carcinoma: What's new on the horizon? *World J Gastroenterol* 2018; **24**: 3974-3979 [PMID: 30254402 DOI: 10.3748/wjg.v24.i35.3974]
75 **Pinyol R**, Montal R, Bassaganyas L, Sia D, Takayama T, Chau GY, Mazzaferro V, Roayaie S, Lee HC, Kokudo N, Zhang Z, Torrecilla S, Moeini A, Rodriguez-Carunchio L, Gane E, Verslype C, Croitoru AE, Cillo U, de la Mata M, Lupo L, Strasser S, Park JW, Camps J, Solé M, Thung SN, Villanueva A, Pena C, Meinhardt G, Bruix J, Llovet JM. Molecular predictors of prevention of recurrence in HCC with sorafenib as adjuvant treatment and prognostic factors in the phase 3 STORM trial. *Gut* 2018 [PMID: 30108162 DOI: 10.1136/gutjnl-2018-316408]
76 **Teufel M**, Seidel H, Köchert K, Meinhardt G, Finn RS, Llovet JM, Bruix J. Biomarkers Associated With Response to Regorafenib in Patients With Hepatocellular Carcinoma. *Gastroenterology* 2019; **156**: 1731-1741 [PMID: 30738047 DOI: 10.1053/j.gastro.2019.01.261]
77 **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]
78 **Black AP**, Mehta AS. The search for biomarkers of hepatocellular carcinoma and the impact on patient outcome. *Curr Opin Pharmacol* 2018; **41**: 74-78 [PMID: 29772420 DOI: 10.1016/j.coph.2018.04.002]
79 **Lou J**, Zhang L, Lv S, Zhang C, Jiang S. Biomarkers for Hepatocellular Carcinoma. *Biomark Cancer* 2017; **9**: 1-9 [PMID: 28469485 DOI: 10.1177/1179299X16684640]
80 **Ng CKY**, Di Costanzo GG, Terracciano LM, Piscuoglio S. Circulating Cell-Free DNA in Hepatocellular Carcinoma: Current Insights and Outlook. *Front Med (Lausanne)* 2018; **5**: 78 [PMID: 29632864 DOI: 10.3389/fmed.2018.00078]
81 **Clark TA**, Chung JH, Kennedy M, Hughes JD, Chennagiri N, Lieber DS, Fendler B, Young L, Zhao M, Coyne M, Breese V, Young G, Donahue A, Pavlick D, Tsiros A, Brennan T, Zhong S, Mughal T, Bailey M, He J, Roels S, Frampton GM, Spoerke JM, Gendreau S, Lackner M, Schleifman E, Peters E, Ross JS, Ali SM, Miller VA, Gregg JP, Stephens PJ, Welsh A, Otto GA, Lipson D. Analytical Validation of a Hybrid Capture-Based Next-Generation Sequencing Clinical Assay for Genomic Profiling of Cell-Free Circulating Tumor DNA. *J Mol Diagn* 2018; **20**: 686-702 [PMID: 29936259 DOI: 10.1016/j.jmoldx.2018.05.004]
82 **Plagnol V**, Woodhouse S, Howarth K, Lensing S, Smith M, Epstein M, Madi M, Smalley S, Leroy C, Hinton J, de Kievit F, Musgrave-Brown E, Herd C, Baker-Neblett K, Brennan W, Dimitrov P, Campbell N, Morris C, Rosenfeld N, Clark J, Gale D, Platt J, Calaway J, Jones G, Forshew T. Analytical validation of a next generation sequencing liquid biopsy assay for high sensitivity broad molecular profiling. *PLoS One* 2018; **13**: e0193802 [PMID: 29543828 DOI: 10.1371/journal.pone.0193802]
83 **Kakehashi A**, Ishii N, Sugihara E, Gi M, Saya H, Wanibuchi H. CD44 variant 9 is a potential biomarker of tumor initiating cells predicting survival outcome in hepatitis C virus-positive patients with resected hepatocellular carcinoma. *Cancer Sci* 2016; **107**: 609-618 [PMID: 26882440 DOI: 10.1111/cas.12908]

84 **Zhang Y**, Liu Y, Duan J, Yan H, Zhang J, Zhang H, Fan Q, Luo F, Yan G, Qiao K, Liu J. Hippocalcin-like 1 suppresses hepatocellular carcinoma progression by promoting p21(Waf/Cip1) stabilization by activating the ERK1/2-MAPK pathway. *Hepatology* 2016; **63**: 880-897 [PMID: 26659654 DOI: 10.1002/hep.28395]

85 **Das V**, Bruzzese F, Konečný P, Iannelli F, Budillon A, Hajdúch M. Pathophysiologically relevant in vitro tumor models for drug screening. *Drug Discov Today* 2015; **20**: 848-855 [PMID: 25908576 DOI: 10.1016/j.drudis.2015.04.004]

86 **Sant S**, Johnston PA. The production of 3D tumor spheroids for cancer drug discovery. *Drug Discov Today Technol* 2017; **23**: 27-36 [PMID: 28647083 DOI: 10.1016/j.ddtec.2017.03.002]

87 **Khawar IA**, Park JK, Jung ES, Lee MA, Chang S, Kuh HJ. Three Dimensional Mixed-Cell Spheroids Mimic Stroma-Mediated Chemoresistance and Invasive Migration in hepatocellular carcinoma. *Neoplasia* 2018; **20**: 800-812 [PMID: 29981501 DOI: 10.1016/j.neo.2018.05.008]

88 **Jung HR**, Kang HM, Ryu JW, Kim DS, Noh KH, Kim ES, Lee HJ, Chung KS, Cho HS, Kim NS, Im DS, Lim JH, Jung CR. Cell Spheroids with Enhanced Aggressiveness to Mimic Human Liver Cancer In Vitro and In Vivo. *Sci Rep* 2017; **7**: 10499 [PMID: 28874716 DOI: 10.1038/s41598-017-10828-7]

89 **Yu C**, Mannan AM, Yvone GM, Ross KN, Zhang YL, Marton MA, Taylor BR, Crenshaw A, Gould JZ, Tamayo P, Weir BA, Tsherniak A, Wong B, Garraway LA, Shamji AF, Palmer MA, Foley MA, Winckler W, Schreiber SL, Kung AL, Golub TR. High-throughput identification of genotype-specific cancer vulnerabilities in mixtures of barcoded tumor cell lines. *Nat Biotechnol* 2016; **34**: 419-423 [PMID: 26928769 DOI: 10.1038/nbt.3460]

90 **Palma E**, Doornebal EJ, Chokshi S. Precision-cut liver slices: a versatile tool to advance liver research. *Hepatol Int* 2019; **13**: 51-57 [PMID: 30515676 DOI: 10.1007/s12072-018-9913-7]

91 **Kern MA**, Haugg AM, Eiteneuer E, Konze E, Drebber U, Dienes HP, Breuhahn K, Schirmacher P, Kasper HU. Ex vivo analysis of antineoplastic agents in precision-cut tissue slices of human origin: effects of cyclooxygenase-2 inhibition in hepatocellular carcinoma. *Liver Int* 2006; **26**: 604-612 [PMID: 16762006 DOI: 10.1111/j.1478-3231.2006.01268.x]

92 **Brown ZJ**, Heinrich B, Greten TF. Mouse models of hepatocellular carcinoma: an overview and highlights for immunotherapy research. *Nat Rev Gastroenterol Hepatol* 2018; **15**: 536-554 [PMID: 29904153 DOI: 10.1038/s41575-018-0033-6]

93 **Uehara T**, Pogribny IP, Rusyn I. The DEN and CCl4 -Induced Mouse Model of Fibrosis and Inflammation-Associated Hepatocellular Carcinoma. *Curr Protoc Pharmacol* 2014; **66**: 14.30.1-14.3010 [PMID: 25181010 DOI: 10.1002/0471141755.ph1430s66]

94 **Arboatti AS**, Lambertucci F, Sedlmeier MG, Pisani G, Monti J, Álvarez ML, Francés DEA, Ronco MT, Carnovale CE. Diethylnitrosamine enhances hepatic tumorigenic pathways in mice fed with high fat diet (Hfd). *Chem Biol Interact* 2019; **303**: 70-78 [PMID: 30826251 DOI: 10.1016/j.cbi.2019.02.024]

95 **Henderson JM**, Polak N, Chen J, Roediger B, Weninger W, Kench JG, McCaughan GW, Zhang HE, Gorrell MD. Multiple liver insults synergize to accelerate experimental hepatocellular carcinoma. *Sci Rep* 2018; **8**: 10283 [PMID: 29980757 DOI: 10.1038/s41598-018-28486-8]

96 **Arboatti AS**, Lambertucci F, Sedlmeier MG, Pisani G, Monti J, Álvarez ML, Francés DEA, Ronco MT, Carnovale CE. Diethylnitrosamine Increases Proliferation in Early Stages of Hepatic Carcinogenesis in Insulin-Treated Type 1 Diabetic Mice. *Biomed Res Int* 2018; **2018**: 9472939 [PMID: 29850590 DOI: 10.1155/2018/9472939]

97 **Sun Q**, Long Z, Wu H, Liu Y, Wang L, Zhang X, Wang X, Hai C. Effect of alcohol on diethylnitrosamine-induced hepatic toxicity: Critical role of ROS, lipid accumulation, and mitochondrial dysfunction. *Exp Toxicol Pathol* 2015; **67**: 491-498 [PMID: 26198575 DOI: 10.1016/j.etp.2015.07.003]

98 **Katz SF**, Lechel A, Obenauf AC, Begus-Nahrmann Y, Kraus JM, Hoffmann EM, Duda J, Eshraghi P, Hartmann D, Liss B, Schirmacher P, Kestler HA, Speicher MR, Rudolph KL. Disruption of Trp53 in livers of mice induces formation of carcinomas with bilineal differentiation. *Gastroenterology* 2012; **142**: 1229-1239.e3 [PMID: 22342966 DOI: 10.1053/j.gastro.2012.02.009]

99 **Dolezal JM**, Wang H, Kulkarni S, Jackson L, Lu J, Ranganathan S, Goetzman ES, Bharathi SS, Beezhold K, Byersdorfer CA, Prochownik EV. Sequential adaptive changes in a c-Myc-driven model of hepatocellular carcinoma. *J Biol Chem* 2017; **292**: 10068-10086 [PMID: 28432125 DOI: 10.1074/jbc.M117.782052]

100 **Yim SY**, Shim JJ, Shin JH, Jeong YS, Kang SH, Kim SB, Eun YG, Lee DJ, Conner EA, Factor VM, Moore DD, Johnson RL, Thorgeirsson SS, Lee JS. Integrated Genomic Comparison of Mouse Models Reveals Their Clinical Resemblance to Human Liver Cancer. *Mol Cancer Res* 2018; **16**: 1713-1723 [PMID: 30082483 DOI: 10.1158/1541-7786.MCR-18-0313]

101 **Mokkapati S**, Niopek K, Huang L, Cunniff KJ, Ruteshouser EC, deCaestecker M, Finegold MJ, Huff V. β-catenin activation in a novel liver progenitor cell type is sufficient to cause hepatocellular carcinoma and hepatoblastoma. *Cancer Res* 2014; **74**: 4515-4525 [PMID: 24848510 DOI: 10.1158/0008-5472.CAN-13-3275]

102 **Churin Y**, Roderfeld M, Stiefel J, Würger T, Schröder D, Matono T, Mollenkopf HJ, Montalbano R, Pompaiah M, Reifenberg K, Zahner D, Ocker M, Gerlich W, Glebe D, Roeb E. Pathological impact of hepatitis B virus surface proteins on the liver is associated with the host genetic background. *PLoS One* 2014; **9**: e90608 [PMID: 24594856 DOI: 10.1371/journal.pone.0090608]

103 **Montalbano R**, Honrath B, Wissniowski TT, Elxnat M, Roth S, Ocker M, Quint K, Churin Y, Roederfeld M, Schroeder D, Glebe D, Roeb E, Di Fazio P. Exogenous hepatitis B virus envelope proteins induce endoplasmic reticulum stress: involvement of cannabinoid axis in liver cancer cells. *Oncotarget* 2016; **7**: 20312-20323 [PMID: 26967385 DOI: 10.18632/oncotarget.7950]

104 **Kornek M**, Raskopf E, Guetgemann I, Ocker M, Gerceker S, Gonzalez-Carmona MA, Rabe C, Sauerbruch T, Schmitz V. Combination of systemic thioacetamide (TAA) injections and ethanol feeding accelerates hepatic fibrosis in C3H/He mice and is associated with intrahepatic up regulation of MMP-2, VEGF and ICAM-1. *J Hepatol* 2006; **45**: 370-376 [PMID: 16780996 DOI: 10.1016/j.jhep.2006.03.017]

105 **Kim YO**, Popov Y, Schuppan D. Optimized Mouse Models for Liver Fibrosis. *Methods Mol Biol* 2017; **1559**: 279-296 [PMID: 28063051 DOI: 10.1007/978-1-4939-6786-5_19]

106 **Hauff P**, Gottwald U, Ocker M. Early to Phase II drugs currently under investigation for the treatment of liver fibrosis. *Expert Opin Investig Drugs* 2015; **24**: 309-327 [PMID: 25547844 DOI: 10.1517/13543784.2015.997874]

107 **Santhekadur PK**, Kumar DP, Sanyal AJ. Preclinical models of non-alcoholic fatty liver disease. *J Hepatol* 2018; **68**: 230-237 [PMID: 29128391 DOI: 10.1016/j.jhep.2017.10.031]

108 **Charrez B**, Qiao L, Hebbard L. Hepatocellular carcinoma and non-alcoholic steatohepatitis: The state of

play. *World J Gastroenterol* 2016; **22**: 2494-2502 [PMID: 26937137 DOI: 10.3748/wjg.v22.i8.2494]
109 **Lau JKC**, Zhang X, Yu J. Animal Models of Non-alcoholic Fatty Liver Diseases and Its Associated Liver Cancer. *Adv Exp Med Biol* 2018; **1061**: 139-147 [PMID: 29956212 DOI: 10.1007/978-981-10-8684-7_11]
110 **Takakura K**, Oikawa T, Tomita Y, Mizuno Y, Nakano M, Saeki C, Torisu Y, Saruta M. Mouse models for investigating the underlying mechanisms of nonalcoholic steatohepatitis-derived hepatocellular carcinoma. *World J Gastroenterol* 2018; **24**: 1989-1994 [PMID: 29760542 DOI: 10.3748/wjg.v24.i18.1989]
111 **Kampschulte M**, Stöckl C, Langheinrich AC, Althöhn U, Bohle RM, Krombach GA, Stieger P, Churin Y, Kremer S, Dierkes C, Rath T, Roeb E, Roderfeld M. Western diet in ApoE-LDLR double-deficient mouse model of atherosclerosis leads to hepatic steatosis, fibrosis, and tumorigenesis. *Lab Invest* 2014; **94**: 1273-1282 [PMID: 25199052 DOI: 10.1038/labinvest.2014.112]
112 **Ma J**, Zhang L, Yang P, Zeng ZC, Xiang ZL. Integrated analysis of long noncoding RNA expression profiles in lymph node metastasis of hepatocellular carcinoma. *Gene* 2018; **676**: 47-55 [PMID: 29981417 DOI: 10.1016/j.gene.2018.07.001]
113 **Sang L**, Wang XM, Xu DY, Zhao WJ. Bioinformatics analysis of aberrantly methylated-differentially expressed genes and pathways in hepatocellular carcinoma. *World J Gastroenterol* 2018; **24**: 2605-2616 [PMID: 29962817 DOI: 10.3748/wjg.v24.i24.2605]
114 **Zhang L**, Huang Y, Ling J, Zhuo W, Yu Z, Shao M, Luo Y, Zhu Y. Screening and function analysis of hub genes and pathways in hepatocellular carcinoma via bioinformatics approaches. *Cancer Biomark* 2018; **22**: 511-521 [PMID: 29843214 DOI: 10.3233/CBM-171160]
115 **Huang S**, Cai N, Pacheco PP, Narrandes S, Wang Y, Xu W. Applications of Support Vector Machine (SVM) Learning in Cancer Genomics. *Cancer Genomics Proteomics* 2018; **15**: 41-51 [PMID: 29275361 DOI: 10.21873/cgp.20063]
116 **Chaudhary K**, Poirion OB, Lu L, Garmire LX. Deep Learning-Based Multi-Omics Integration Robustly Predicts Survival in Liver Cancer. *Clin Cancer Res* 2018; **24**: 1248-1259 [PMID: 28982688 DOI: 10.1158/1078-0432.CCR-17-0853]
117 **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]
118 **Chen J**, Zaidi S, Rao S, Chen JS, Phan L, Farci P, Su X, Shetty K, White J, Zamboni F, Wu X, Rashid A, Pattabiraman N, Mazumder R, Horvath A, Wu RC, Li S, Xiao C, Deng CX, Wheeler DA, Mishra B, Akbani R, Mishra L. Analysis of Genomes and Transcriptomes of Hepatocellular Carcinomas Identifies Mutations and Gene Expression Changes in the Transforming Growth Factor-β Pathway. *Gastroenterology* 2018; **154**: 195-210 [PMID: 28918914 DOI: 10.1053/j.gastro.2017.09.007]
119 **Désert R**, Nieto N, Musso O. Dimensions of hepatocellular carcinoma phenotypic diversity. *World J Gastroenterol* 2018; **24**: 4536-4547 [PMID: 30386103 DOI: 10.3748/wjg.v24.i40.4536]
120 **Lee JS**, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, Durnez A, Demetris AJ, Thorgeirsson SS. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology* 2004; **40**: 667-676 [PMID: 15349906 DOI: 10.1002/hep.20375]
121 **Boyault S**, Rickman DS, de Reyniès A, Balabaud C, Rebouissou S, Jeannot E, Hérault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007; **45**: 42-52 [PMID: 17187432 DOI: 10.1002/hep.21467]
122 **Chiang DY**, Villanueva A, Hoshida Y, Peix J, Newell P, Minguez B, LeBlanc AC, Donovan DJ, Thung SN, Solé M, Tovar V, Alsinet C, Ramos AH, Barretina J, Roayaie S, Schwartz M, Waxman S, Bruix J, Mazzaferro V, Ligon AH, Najfeld V, Friedman SL, Sellers WR, Meyerson M, Llovet JM. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. *Cancer Res* 2008; **68**: 6779-6788 [PMID: 18701503 DOI: 10.1158/0008-5472.CAN-08-0742]
123 **Hoshida Y**, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, Villanueva A, Newell P, Ikeda K, Hashimoto M, Watanabe G, Gabriel S, Friedman SL, Kumada H, Llovet JM, Golub TR. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009; **69**: 7385-7392 [PMID: 19723656 DOI: 10.1158/0008-5472.CAN-09-1089]
124 **Désert R**, Rohart F, Canal F, Sicard M, Desille M, Renaud S, Turlin B, Bellaud P, Perret C, Clément B, Lê Cao KA, Musso O. Human hepatocellular carcinomas with a periportal phenotype have the lowest potential for early recurrence after curative resection. *Hepatology* 2017; **66**: 1502-1518 [PMID: 28498607 DOI: 10.1002/hep.29254]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3151-3167

DOI: 10.3748/wjg.v25.i25.3151

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

REVIEW

Hepatocellular carcinoma: Mechanisms of progression and immunotherapy

Yu Jiang, Qiu-Ju Han, Jian Zhang

ORCID number: Yu Jiang (0000-0001-5058-3484); Qiu-Ju Han (0000-0002-6511-2308); Jian Zhang (0000-0001-5106-1397).

Author contributions: Jiang Y and Zhang J designed the structure of this review; Jiang Y contributed literature review, designed illustrations and tables and wrote the manuscript; Jiang Y, Han QJ and Zhang J revised the paper; All authors approved the final version.

Supported by the Natural Science Foundation of China, No. 81172789, 30972692 and 81373222; National Basic Research Program of China 973 Program, No. 2013CB531503; and National Mega Project on Major Infectious Diseases Prevention and Treatment, No. 2012ZX10002006.

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

Manuscript source: Invited manuscript

Yu Jiang, Qiu-Ju Han, Jian Zhang, Institute of Immunopharmaceutical Sciences, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, Shandong Province, China

Corresponding author: Jian Zhang, PhD, Professor, Institute of Immunopharmaceutical Sciences, School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan 250012, Shandong Province, China. zhangj65@sdu.edu.cn
Telephone: +86-531-88383781
Fax: +86-531-88383782

Abstract

Liver cancer is one of the most common malignancies, and various pathogenic factors can lead to its occurrence and development. Among all primary liver cancers, hepatocellular carcinoma (HCC) is the most common. With extensive studies, an increasing number of molecular mechanisms that promote HCC are being discovered. Surgical resection is still the most effective treatment for patients with early HCC. However, early detection and treatment are difficult for most HCC patients, and the postoperative recurrence rate is high, resulting in poor clinical prognosis of HCC. Although immunotherapy takes longer than conventional chemotherapy to produce therapeutic effects, it persists for longer. In recent years, the emergence of many new immunotherapies, such as immune checkpoint blockade and chimeric antigen receptor T cell therapies, has given new hope for the treatment of HCC.

Key words: Hepatocellular carcinoma; Mechanisms; Immunotherapy

Core tip: Among all primary liver cancers, hepatocellular carcinoma (HCC) is the most common and accounts for 90% of cases. Mechanisms related to HCC progression and treatment strategies have been extensively reported. In this paper, we review the molecular mechanisms involved in HCC progression and the latest advancements in immunotherapy by combining the research progress and results from our laboratory in recent years.

Citation: Jiang Y, Han QJ, Zhang J. Hepatocellular carcinoma: Mechanisms of progression and immunotherapy. *World J Gastroenterol* 2019; 25(25): 3151-3167
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3151.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3151

Received: March 6, 2019
Peer-review started: March 6, 2019
First decision: April 4, 2019
Revised: April 28, 2019
Accepted: May 18, 2019
Article in press: May 18, 2019
Published online: July 7, 2019

P-Reviewer: Al-Gayyar M, Mikulic D
S-Editor: Ma RY
L-Editor: Filipodia
E-Editor: Ma YJ

INTRODUCTION

Hepatocellular carcinoma (HCC) is predicted to be the sixth most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide in 2018, accounting for approximately 841,000 new cases and 782,000 deaths annually. Primary liver cancer includes HCC and intrahepatic cholangiocarcinoma as well as other rare types, with HCC accounting for 75%-85% of cases[1,2]. The main risk factors for HCC are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), aflatoxin-contaminated foodstuffs, heavy alcohol intake and type 2 diabetes[2]. Studies on the mechanisms of HCC processes have confirmed that the inactivation of multiple tumor suppressor genes (such as p53), abnormal activation of oncogenes (K-ras, *etc*.,) and multiple signaling pathways (PI3K, MAPK, JAK/STAT, NF-κB, Wnt/β-catenin, *etc*), abnormal regulation of epigenetic events (such as microRNAs), and even exosomes that deliver a large number of protumorigenic molecules are all involved in HCC development and progression[3]. The liver also acts as a special immune organ. In addition to the above carcinogenic factors, the immunological microenvironment in the liver is associated with HCC occurrence and development. In recent years, the interaction between various immune cells and tumor cells has attracted extensive attention. Many molecular mechanisms associated with the biological characteristics of tumor cells during hepatocarcinogenesis also have important effects on the immune system. Although improvements have been made in surgery, radiofrequency ablation and chemotherapy for HCC, the prognosis of HCC patients remains unsatisfactory due to the high rates of recurrence and metastasis. The emergence of many new immunotherapies, such as immune checkpoint blockade and chimeric antigen receptor (CAR) T-cell therapies, has given new hope for the treatment of HCC. Here, we review the molecular mechanisms that influence HCC progression and the latest advancements in immunotherapy by combining the latest research progress and results from our laboratory.

MOLECULAR MECHANISMS UNDERLYING HCC

The promotive effect of HBV on HCC

Chronic hepatitis caused by HBV infection is one of the main causes of HCC. Numerous studies have confirmed that HBV can activate a variety of signals to promote viral replication and inflammation progression and to accelerate hepatocarcinogenesis[4-7].

HBx and HCC: We also found that HBV further promoted viral replication by activating signal transducer and activator of transcription 3 (STAT3) signaling in HBV$^+$ HCC, while blocking STAT3 can inhibit HBV replication and proliferation and angiogenesis in HBV$^+$ HCC[8]. The HBV genome encodes four proteins, including the envelope protein (S/Pre-S), the core protein (C/pre-C), the polymerase (P), and the X protein (HBx). Among them, the multifunctional HBx protein has attracted substantial attention. HBx can block p53-mediated apoptosis and activate numerous signal transduction cascades (STAT, NF-κB, AP-1, *etc*.,) associated with cell proliferation and survival, promoting HCC occurrence and development[9-13]. HBx mutations in HCC patients haven been shown to be important for the development of HCC[14-16].

HBV and microRNAs: A large number of recent studies have shown that microRNAs play important roles in the occurrence and development of HBV-related HCC. Through the analysis of microRNA profiles, the expression levels of various microRNAs, such as miR-150 and miR-342-3p, were found to be changed in HBV-related HCC[17]. The analysis of a large number of clinical samples showed that microRNAs such as miR-375, miR-25, and let-7f are specific for HBV and have potential clinical value for the prediction and diagnosis of HBV$^+$ HCC[18-20]. Further studies have demonstrated that HBV promotes HCC by intervening with Wnt, MAPK, Notch and other signaling pathways through different microRNAs[17,21-24] (Figure 1). However, miR-122 expression is inhibited in HBV$^+$ HCC, which suggests that this microRNA likely plays an inhibitory role in HCC progression[25]. Mao *et al*[26] found that the tolerance of HBV$^+$ HCC patients to sorafenib was significantly higher than that of non-HBV-infected HCC patients, which was related to activated Mcl-1-mediated inhibitory effects on miR-193b, and restoration of miR-193b expression could increase the sensitivity of HBV$^+$ HCC to sorafenib. These phenomena indicate that the role of microRNAs in the progression of HBV-related HCC is complex and not simply promotive or suppressive.

HBV and immune tolerance: In addition to the above mechanisms, HBV-induced

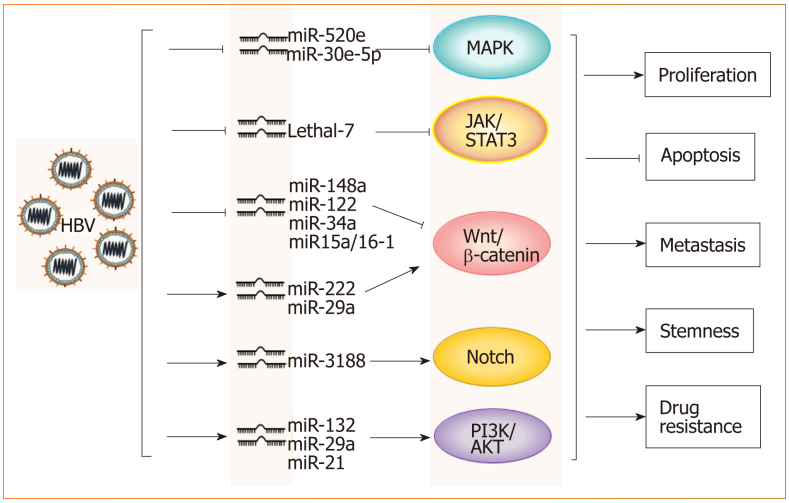


Figure 1 Hepatitis B virus promotes hepatocellular carcinoma by intervening various signal pathways through different microRNAs. Lines ending with arrows or bars indicate promotion or inhibitory effects, respectively. HBV: Hepatitis B virus.

suppressive effects on innate and adaptive immune cells promote the evolution from inflammation to tumorigenesis. In patients with chronic HBV hepatitis, the activation and function of natural killer (NK) cells are significantly inhibited, and these impaired NK cells cannot effectively clear HBV, which further accelerates the progression of hepatitis to HCC[27,28]. The TGF-β-miR-34a-CCL22 signal induces regulatory T (Treg) cell infiltration and promotes the metastasis of HBV^+ HCC[29], and the imbalance between helper T (Th)-17 and Treg cells is a risk factor for patients with HBV infection progressing to HCC[30]. In the course of chronic HBV infection, the expression of programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), CD244 and other inhibitory receptors on virus-specific $CD8^+$ T cells is increased, which mediates T cell depletion[27,31]. Recently, Zong *et al*[32] showed that in HBsAg-transgenic mice, the expression of TIGIT, a promising immune checkpoint in tumor immunotherapy, maintained the tolerance of $CD8^+$ T cells to HBV, and disrupting this tolerance by TIGIT blockade or deficiency could induce chronic hepatitis in HBsAg-transgenic mice and eventually lead to HCC development, suggesting that immune checkpoint therapy in HBV carriers might increase the risk of chronic hepatitis and liver cancer.

To date, the role of HBV in HCC progression has been widely studied, providing new ideas for the effective prevention and treatment of HCC.

STAT3 and related signaling pathways

As an important member of the STAT family, STAT3 is constitutively activated in many tumor and immune cells in the tumor microenvironment. The abnormal activation of STAT3 signaling is closely related to the occurrence, proliferation, drug resistance and stemness of various tumors, including HCC. STAT3 also plays an important role in the regulation of the complex network formed in the tumor microenvironment[33-36].

STAT3 and microRNAs: STAT3 deficiency prevents hepatocarcinogenesis in the thioacetamide-induced liver injury model[37]. Constitutive activation of STAT3 is observed in HCC cells and tissues, and STAT3 decoy oligodeoxynucleotides (STAT3-decoy ODN) can specifically block the activation of STAT3 signaling in HCC, resulting in the inhibition of proliferation and apoptosis in tumor cells[38]. MicroRNAs such as miR-589-5p and miR-500a-3p maintain the drug resistance and stemness of HCC by activating STAT3 signaling[39-41]. However, some microRNAs have been found to inhibit tumor development by interfering with STAT3. MiR-345 upregulation has been shown to inhibit epithelial-mesenchymal transition (EMT) in HCC by targeting interferon regulatory factor 1 (IRF1)-mediated mTOR/STAT3/AKT signaling[42]. Furthermore, miR-451 may function as a potential suppressor of tumor angiogenesis in HCC by targeting IL-6R/STAT3/VEGF signaling, indicating a promising therapeutic strategy for HCC[43]. Although different microRNAs have different effects on HCC, STAT3 plays a key role in the regulation of microRNA signaling during hepatocarcinogenesis.

TLR4 and STAT3: The relationship between inflammation and tumors has been well

established[44]. Approximately 70% of the blood supply to the liver comes from the outflow of intestinal veins, and the presence of the hepato-intestinal axis makes the liver the first line of defense against enterogenous antigens. Pathogen-associated molecular patterns (PAMPs) derived from the intestinal microbiota play a regulatory role in liver diseases by activating Toll-like receptors (TLRs). In a liver injury-cancer model induced by a combination of diethylnitrosamine (DEN) and the hepatotoxin carbon tetrachloride (CCl4), $TLR4^{-/-}$ mice showed a significant decrease in tumor number and volume formation compared to wild-type mice. Moreover, intestinal microbiota-derived lipopolysaccharides (LPS) can activate TLR4 signaling in hepatocytes to promote inflammation-induced hepatocarcinogenesis[45]. We also found that TLR4 was constitutively expressed in HCC, and further study demonstrated that TLR4 promoted HCC occurrence and progression depending on the activation of the Cox-2/PGE2/STAT3 axis and was associated with multiple drug resistance[46]. Significantly, sorafenib can inhibit HCC by blocking TLR4/STAT3/SUMO1 activation[47].

STAT3 and the tumor microenvironment: With advancements in research, the occurrence and development of tumors is thought to arise due to not only the deterioration and proliferation of tumor cells, but also the immunosuppressive tumor microenvironment. As a key transcription factor, STAT3 is constitutively activated in both tumor cells and immune cells in the microenvironment (Figure 2). We found that blocking STAT3 in HCC cells could effectively disrupt tumor-induced immune tolerance and induce an antitumor reaction in tumor-bearing mice, which might be related to the downregulation of transforming growth factor-beta (TGF-β) and interleukin(IL)-10 and the upregulation of type I interferons (IFNs)[48,49]. Alternatively, STAT3 could directly regulate miR-146a expression to upregulate the expression of TGF-β, IL-17 and VEGF and downregulate the expression of type I IFNs, mediating the inhibitory effect of NK cells on HCC[50]. More importantly, STAT3 could inhibit the Th1 immune response and promote the formation of an immunosuppressive microenvironment[51,52]. Studies have shown that tumor-associated macrophages (TAMs) promote HCC progression by secreting cytokines such as IL-8 and IL-6 to activate STAT3 in HCC[53,54]. HCC-associated fibroblasts induce the activation of STAT3 pathways in neutrophils and dendritic cells (DCs), and these STAT3-over-activated neutrophils and DCs display protumorigenic roles[55,56]. Blocking STAT3 activation in immune cells such as TAMs and DCs can inhibit HCC progression[56,57]. Meanwhile, blocking STAT3 not only inhibits HCC proliferation but also upregulates NKG2D ligand expression, such as ULBPs and MICA/B, in HCC cells to increase the sensitivity of HCC to NK cell-mediated cytolysis and enhance the anti-HCC activity of NK cells[48,49].

In summary, STAT3 signaling can interact with multiple pathways to promote HCC. STAT3 plays an important role in both the maintenance of HCC malignancy and the suppression of the immune microenvironment, which makes STAT3 an ideal target for HCC treatment. The development of STAT3 inhibitors used for clinical application is an attractive research topic. STAT3 inhibitors, including AZD9150 and TTI-101, have entered the clinical trial phase for HCC treatment (ClinicalTrials.gov identifier: NCT03195699 and NCT01839604, respectively). Several drugs, which were not initially applied for tumor treatment, have also been found to exert anticancer effects by blocking STAT3[58-60]. Thus, the identification of new STAT3-targeted inhibitors is still an important direction for drug development.

Homeobox genes

Homeobox genes were first discovered in the fruit fly Drosophila, which are divided into many subfamilies (Hox, PAX, NKX, *etc.*,) on the basis of the level of similarity among them[61]. Homologous homeobox genes have been found in mammals. Most homeobox genes are involved in regulating the expression of genes related to embryonic development and cell differentiation. Mutations in these genes can lead to abnormal organ development in eukaryotes[61-64]. In addition, in recent years, a variety of homeobox genes have been found to be involved in the occurrence and development of tumors[64,65], and different homeobox genes have been shown to play different roles in the progression of cancer such as HCC.

Hox and HCC: The Hox gene, an important member of the homeobox family, is abnormally expressed in multiple malignant solid tumors[65]. The role of Hox has also been studied in HCC over the years. Among HOX genes, HOXA13 has been reported to be the most deregulated in HCC. HOXA13 overexpression in HCC cell lines results in increased colony formation and migration but reduced sensitivity to sorafenib[66-68]. Knockdown of endogenous HOXA7 results in decreased proliferation of HCC cells by inhibiting cyclin E1/cyclin-dependent kinase-2[69]. HOXB7 can promote EMT and

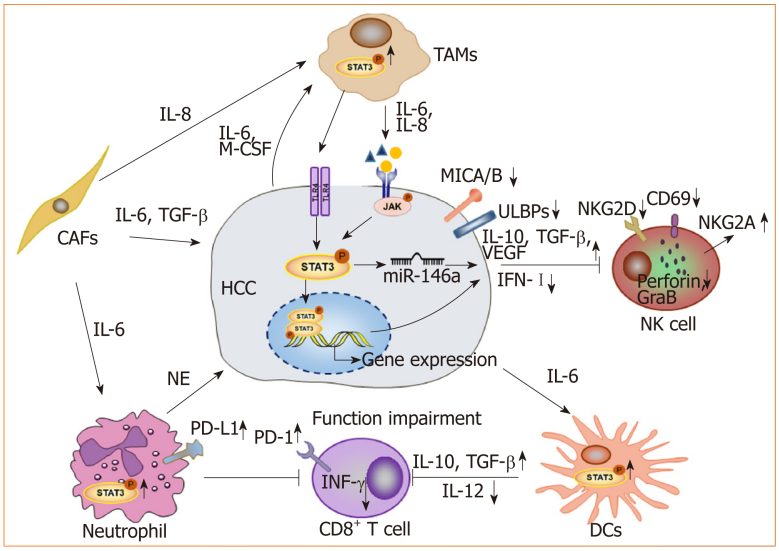


Figure 2 STAT3 signaling contributes to form an immunosuppressive microenvironment in hepatocellular carcinoma. Long lines ending with arrows or bars indicate activating or inhibitory effects, respectively. Short arrows pointing up or down indicate up-regulated or down-regulated, respectively. TAMs: Tumor-associated macrophages; CAFs: Cancer-associated fibroblasts; NK cell: Natural killer cell; DCs: Dendritic cells; NE: Neutrophil elastase; HCC: Hepatocellular carcinoma.

stemness formation by upregulating the expression of c-*Myc* and *Slug* in HCC[70].

HMBOX1 and HCC: Homeobox containing 1 (HMBOX1), a novel human homeobox gene, was first isolated from the human pancreatic cDNA library. HMBOX1 belongs to the HNF homeobox class of the homeobox family[63,71]. The expression of HMBOX1 is reported to be up- or downregulated in some tumors[72-75]. Our previous study revealed that the expression level of HMBOX1 in liver cancer was lower than that in adjacent noncancerous tissues[74]. Further study showed that HMBOX1 expression was negatively correlated with the differentiation and clinical stage of HCC, and HMBOX1 overexpression could inhibit HCC by promoting autophagy, inhibiting the cancer stem cell phenotype and increasing tumor cell sensitivity to NK cell-mediated cytolysis. The underlying mechanisms may be related to changes in the expression of Fas and programmed cell death ligand 1 (PD-L1) in HCC cells mediated by HMBOX1 overexpression[76]. Additionally, HMBOX1 expression in hepatocytes has been shown to prevent inflammation and liver injury by reducing macrophage infiltration and acti-vation, thereby blocking inflammation-related tumor development[77].

Other homeobox genes and HCC: Prospero-related homeobox 1 (PROX1) is closely related to proliferation, differentiation and prognosis in HCC. PROX1 can upregulate IL-8 expression and activate NF-κB and β-catenin signals to promote HCC angiogenesis and sorafenib resistance[78-80]. NK3 homeobox 1 (NKX3.1) was initially found to play an important role in the regulation of prostate development and tumorigenesis[81]. Recently, NKX3.1 expression was shown to be decreased in HCC tissues, and NKX3.1 overexpression induced G1/S phase arrest in HCC cells through upregulation of FOXO1[82]. HLXB9 is highly expressed in poorly differentiated HCC samples[83].

From the above, different homeobox genes exhibit different functions via related mechanisms to promote or inhibit HCC progression (Table 1). Because the homeobox family has a large number of genes, its role in HCC and other tumors remains to be further explored.

Wnt signaling

As a highly conserved signaling pathway during biological evolution, the Wnt signaling pathway plays an important role in a variety of physiological and pathological processes[84]. With advancements in research, the regulatory role of the Wnt signaling pathway in cancer progression and the emergence of stemness has attracted widespread attention. According to the different mechanisms of signal tran-sduction, the Wnt pathway is generally divided into the canonical Wnt/β-catenin and non-canonical β-catenin-independent pathways.

Wnt signaling and HCC: Aberrant activation of the Wnt/β-catenin signaling path-

Table 1 Homeobox genes show different roles in the progression of hepatocellular carcinoma

Homeobox genes	Involvement in HCC process	Target genes	Ref.
HOXA13	Colony formation (+), migration (+), Drug resistance (+)	–	[65,67]
HOXA7	Proliferation (-)	*Cyclin e1/cdk2*	[68]
HOXB7	Stemness (+), EMT (+)	*c-Myc, slug*	[69]
HMBOX1	Autophagy (+), Stemness (-), Immunosuppression (-)	*PD-L1, Fas*	[75]
PROX1	Drug resistance (+), angiogenesis (+)	*IL-8, NF-κB, β-catenin*	[77-79]
NKX3.1	Proliferation (-)	*FOXO1*	[80]

(-): Inhibit; (+): Promote; -: Unavailable; HCC: Hepatocellular carcinoma.

way has been observed in HCC patients, and various molecules, such as the protein components of HBV and HCV, as well as hypoxia-induced factor (commonly known as HIF), can activate the Wnt/β-catenin pathway in HCC[5,85-88]. The activation of Wnt/β-catenin signaling is closely related to the occurrence and development of HCC, the formation of stemness and drug resistance[79,89-91] (Figure 3). In addition, Wnt signals regulate HCC progression by interacting with Hippo and Notch signaling pathways[92-94]. In addition to mutations in CTNNB1, AXIN1 and other related genes[95-98], epigenetic regulation is involved in the aberrant activation of the Wnt signaling pathway. For instance, the long noncoding RNA lncTCF7 promotes stemness and dissemination in HCC by activating Wnt signaling[99]. In HBV-related HCC, HBx silences secreted frizzled-related proteins (SFRPs) by mediating DNA methylation to activate Wnt signaling[100]. Many microRNAs regulate the activation of Wnt signaling at the posttranscriptional level to affect HCC progression. For example, miR-542-3p can target the frizzled 7/Wnt signaling pathway to inhibit HCC[101], while Octamer 4/miR-1246 promotes stemness by inhibiting AXIN2 and GSK3 and thereby activating Wnt/β-catenin signaling in HCC[102].

Wnt signaling and the tumor microenvironment: In addition to the effect on tumor cells themselves, Wnt signaling has recently been found to play an important role in the formation of a tumor immunosuppressive microenvironment. β-catenin activation in DCs can inhibits the process of antigen cross presentation at $CD8^+$ T cells[103,104] and participates in the differentiation and activation of Treg cells[105]. Wnt/β-catenin activation in TAMs facilitates M2 polarization, which promotes HCC[106]. TAMs also activate β-catenin by secreting CCL17 to promote EMT in HCC[107]. Although many studies have demonstrated that Wnt activation plays an immunosuppressive role, the mechanism of Wnt activation in the tumor microenvironment remains to be further explored. A better understanding of the role of Wnt signaling in HCC progression is thus essential for the prevention and treatment of HCC.

Role of exosomes in HCC

Exosomes can be released by all cell types, including cancer cells and immune cells, and play important roles in intercellular communication[108]. As carriers and transporters, exosomes deliver a variety of biological molecules, including proteins, lipids, and nucleic acids. Exosomes have been shown to play important roles in most cancer-associated processes.

Exosomes promote HCC: We have reported that exosomes present in the sera of chronic hepatitis B patients contain both HBV-derived nucleic acids and HBV proteins and can transfer HBV to hepatocytes in an active manner. Moreover, exosomes mediate the transmission of HBV into NK cells, resulting in the impairment of NK cell functions[109]. This may contribute to the progression of chronic HBV infection to HCC. Exosomes derived from metastatic HCC cell lines carry a large number of protumorigenic RNAs and proteins, such as MET protooncogenes and S100 family members. These exosomes promote metastasis by triggering the PI3K/AKT and MAPK signaling pathways in hepatocytes and increasing the secretion of active matrix metalloproteinase (MMP) 2 and MMP-9[110].

Exosomes and chemoresistance: HCC is highly resistant to chemotherapy. Qu *et al*[111] found that exosomes derived from HCC cells can induce sorafenib resistance by activating the HGF/c-Met/Akt signaling pathway. Moreover, exosomes derived from highly invasive HCC cells have greater efficacy than exosomes derived from less invasive cells[111]. Exosomal miR-32-5p can induce multidrug resistance in HCC by activating the PI3K/Akt pathway[112]. Therefore, HCC cell-derived exosomes might be

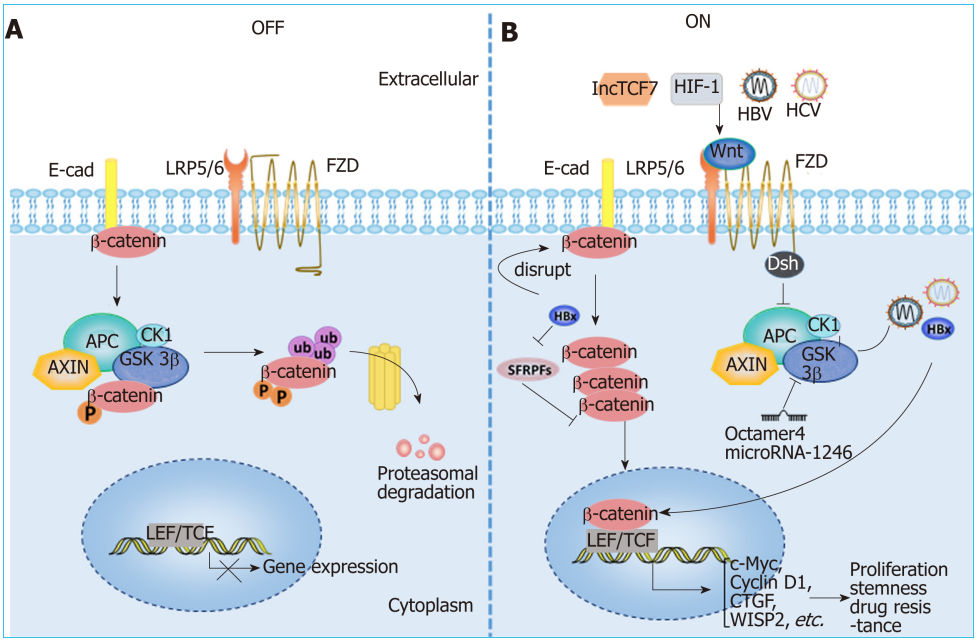


Figure 3 Aberrant activation of the Wnt/β-catenin signaling pathway in hepatocellular carcinoma. A: Wnt signaling is inactive in the absence of Wnt ligands (OFF); B: Wnt signaling can be activated by various molecules in HCC (ON). HBV and HCV can active Wnt/β-catenin signaling by activating TCF or inhibiting GSK3β; HBx can silence SFRPs to activate Wnt signaling; LncTCF7 triggers Wnt7a and TCF7 expression to activate Wnt signaling. Lines ending with arrows or bars indicate activating or inhibitory effects, respectively. HIF1α: Hypoxia-inducible factor 1α; LEF: Lymphoid enhancer-binding factor; LRP: Low-density lipoprotein receptor-related protein; TCF: T cell factor; FZD: Frizzled; E-cad: E-cadherin; SFRPs: Secreted frizzled-related proteins; CTGF: Connective tissue growth factor; WISP2: Wnt1 inducible signaling pathway protein 2.

an important target for reversing chemoresistance.

Exosomes and the tumor microenvironment: As carriers, exosomes play an important role in cell-cell interactions in the tumor microenvironment. Anticancer drugs induce the release of exosomes containing heat shock proteins (HSPs) from HCC cells, which elicit effective NK cell-mediated antitumor responses[113]. However, 14-3-3ζ proteins delivered by exosomes can be transmitted from HCC cells to tumor-infiltrating T cells, impairing the functions, proliferation and activation of T cells[114]. Additionally, HCC-derived exosomes containing lncRNA TUC339 can be taken up by macrophages to play important roles in macrophage activation and regulation of M1/M2 polarization[115]. Additionally, highly metastatic HCC cell-secreted exosomal miR-1247-3p directly targets B4GALT3, leading to the activation of β1-integrin-NF-κB signaling in fibroblasts, which converts normal fibroblasts to cancer-associated fibroblasts. Activated cancer-associated fibroblasts further promote cancer progression. Clinical data also show that high serum exosomal miR-1247-3p levels are correlated with lung metastasis in HCC patients[116].

Therefore, exosomes play important roles in the development of HCC (Figure 4). Nevertheless, more studies on how exosomes mediate HCC progression are still needed to promote the clinical utilization of exosomes.

HCC IMMUNOTHERAPY

Clinical treatment of HCC includes liver transplantation, surgical resection, chemotherapy, radiotherapy, interventional therapy and immunotherapy. Liver transplantation is the only treatment option for HCC patients with unresectable tumors or cirrhosis[117]. The 5-year survival rates can reach 60%–70% in patients with HCC after liver transplantation[118]. However, the number of patients that need liver transplantation exceeds the number of available donor organs[119]. Therefore, HCC patients must be selected very carefully in terms of tumor size and number of tumor nodules[120]. Surgical resection of early HCC is still the first choice for treatment, but the recurrence rate within five years is as high as 70%[2]. The multikinase inhibitor sorafenib is recognized as the most effective molecular targeted drug for the treatment of advanced HCC worldwide. Despite advancements in molecular therapy with the multikinase inhibitor sorafenib, the prognosis of advanced HCC cases remains poor, with five-year survival rates of 3%-11%[121]. The immune system plays a key role in

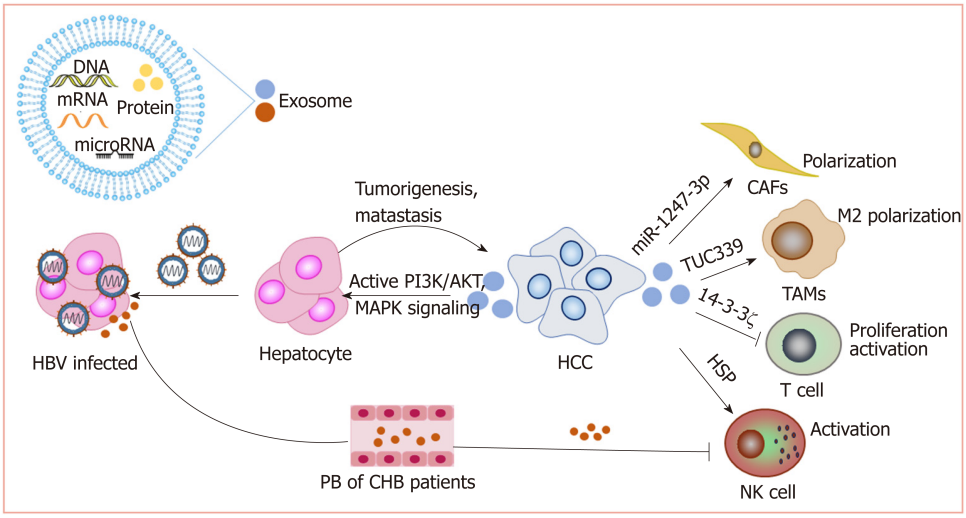


Figure 4 Exosomes play important roles in the development of hepatocellular carcinoma. Exosomes deliver a variety of biological molecules that have been proven to play important roles in hepatocellular carcinoma progression and immunosuppression. Lines ending with arrows or bars indicate activating or inhibitory effects, respectively. HCC: Hepatocellular carcinoma.

controlling and eradicating cancer. Therefore, immunotherapy has received much attention in recent years. Additionally, although immunotherapy takes longer than conventional chemotherapy to produce a therapeutic effect, it persists for longer. Earlier immunotherapy mainly included cytokine-mediated immunotherapy, oncolytic virus therapy, TLR agonist therapy and DC vaccine. In recent years, emerging immunotherapies, such as immune checkpoint blockade and CAR T cell therapies, have shown better therapeutic effects on some tumors, thus giving us new hope for the treatment of HCC.

Immune checkpoint blockade

Checkpoint blockade, currently the top candidate in immunotherapy, has been shown to be effective for the treatment of many cancers, especially for chemotherapy resistant malignant tumors. Among the available immune checkpoint inhibitors, CTLA-4 and PD-1 display the most pronounced effects and have shown remarkable efficacy in the treatment of malignant melanoma[122,123]. Nivolumab, pembrolizumab (PD-1 inhibitor) and tremelimumab (CTLA-4 inhibitor) have been demonstrated to be safe and effective in clinical trials[124-126], and nivolumab has been approved by the U.S. Food and Drug Administration (commonly known as the FDA) as a second-line treatment for HCC[127].

Although good results have been achieved in the treatment of HCC with checkpoint blockade, the response rate to the treatment is relatively low due to the formation of an immunosuppressive microenvironment. The combination of checkpoint blockade inhibitors and other methods may enhance the efficacy[128,129]. Recently, Zhou *et al*[130] found that the combined use of checkpoint inhibitors and myeloid-derived suppressor cell infiltration blockers can augment the therapeutic effect of anti-PD-L1 antibody in HCC. The use of tremelimumab combined with radio-frequency ablation to treat advanced HCC promoted the accumulation of $CD8^+$ T cells in tumor tissues, suggesting that this combined treatment might be a new therapeutic method[131]. The combined use of anti-CTLA-4 antibodies, anti-PD-L1 antibodies and the histone deacetylase inhibitor belinostat completely eliminated tumor load in HCC tumor-bearing mice[132]. An increasing number of researchers are investigating the therapeutic effect of combination therapy when immune checkpoint blockade alone is not effective. Thus, combined therapy may be a new strategy for the future application of checkpoint blockade in solid tumors, including liver cancer, but large amounts of clinical trial data are still needed to support the development of specific treatment strategies.

Adoptive transfer of genetically modified lymphocytes

Adoptive cell transfer is the most representative tumor immunotherapy at present, and it is mediated by cytolytic activity against tumor cells by the transfer of lymphocytes from the patient themselves or from donors. Before the emergence of CAR T cells, adoptive transfer therapy in HCC mainly focused on tumor-infiltrating lymphocytes and cytokine induced killer cells[133-135]. With the recent discovery of CAR

T cells and their amazing therapeutic effect on hematological tumors[136], the effect of CAR T cell therapy in solid tumors such as HCC has attracted increasing attention.

CAR T cells: CARs provide T cells with the ability to directly recognize tumor antigens independent of the human leukocyte antigen. This allows CAR engineered T cells to recognize a wider range of targets than natural T cells. At present, the most widely used CAR structure consists of a single-chain antibody extracellular domain that recognizes and binds specific antigens, an extracellular hinge region, a transmembrane region, and an intracellular domain that provides proliferation and activation signals. Throughout the entirety of the immunotherapy process, the design and integration of CARs into T cells to generate CAR T cells are the most critical steps[137]. Multiple studies have demonstrated that glypican 3 (GPC3) is an attractive liver cancer-specific target, because its expression is high in HCC tissues but limited in normal tissues[138]. GPC3-specific CAR T cell therapy for HCC exhibits a strong killing effect on GPC3-positive HCC cells both *in vivo* and *in vitro*[139]. Furthermore, a relevant phase 1 clinical trial study (ClinicalTrials.gov identifier: NCT02395250) showed that autologous T cells bearing GPC3-specific CARs were safe and effective in patients with relapsed or refractory HCC. Meanwhile, another phase 1 clinical trial (ClinicalTrials.gov identifier: NCT02541370) involving CD133-directed CAR T cells for advanced HCC demonstrated feasibility, controllable toxicities and effectiveness[140]. However, immunosuppressive microenvironments can hinder the infiltration of CAR T cells into tumor tissues, thereby reducing CAR T cell-mediated antitumor effects[141]. Interestingly, Guo *et al*[142] showed that further inhibition of PD-1 expression in GPC3-specific CAR T cells can enhance the killing effect of CAR T cells on HCC cells.

Treatment of HCC based on NK cells: Similar to T cells, NK cells can be modified with CARs that recognize antigens expressed by tumors and combine with signaling components that enhance NK cell activity. At present, clinical studies on CAR NK cells mostly focus on the treatment of lymphoma and hematological tumors, and only a few studies exist regarding the treatment of solid tumors such as HCC. Yu *et al*[143] found that GPC3-specific CAR NK cells constructed with NK-92 cells could effectively inhibit proliferation and promote apoptosis in HCC cells. Furthermore, CAR NK cells display lower toxicity than CAR T cells and do not need patient matching, which makes CAR NK cells more promising for cancer treatment[144]. Previously, we constructed gene-modified NK cells to augment NK cell activity and found that IL-15- or IFN-α-gene modification increased the production of TNF-α and IFN-γ by NK-92 or NKL cells, promoting apoptosis in HCC cells by upregulating the expression of NKG2D ligands and Fas on HCC cells. These NK cells also exerted enhanced antitumor effects *in vivo*[145-147].

TLR agonists

The role of TLR agonists as a vaccine adjuvant and tumor immunotherapeutic agent has been recently noted[148-150]. As a vaccine adjuvant, TLR agonists trigger antigen presentation by promoting the maturation of DCs. Multiple TLRs, such as TLR3 and TLR9, have been confirmed to be expressed on HCC cells[151-153], and the role of TLR agonists in tumor therapy has received much attention. The TLR2/4 agonist OM-174 has potential roles in the prevention of invasion and metastasis in HCC[154]. We also found that both TLR3 agonist poly (I:C) and TLR9 agonist ODN M362 can exert antitumor effects on HCC cells. Surprisingly, we found that simultaneous transfection of poly (I:C) and ODN M362 exhibits a lower proapoptotic effect on HCC than transfection of poly (I:C) alone. Further investigation demonstrated that ODN M362 blocks the entrance of poly (I:C) when simultaneously used to treat HCC cells and then decreases the activation of poly (I:C)-triggered cellular apoptosis; however, poly (I:C)-mediated proapoptotic effects could be enhanced by pretreating HCC cells with CpG ODN[155]. TLR agonists can also work as adjuvants to stimulate the immune system during tumor treatment, but their effects on tumor cells cannot be ignored. These therapeutic effects may thus be the overall outcome of various mechanisms.

Tumor vaccine

DCs are central regulators of the adaptive immune response and are thus necessary for T-cell-mediated antitumor immunity. DC vaccines have the characteristics of low complication rates and good tolerance, and DC-based tumor vaccines have been used for a variety of solid tumors[156]. Currently, there are many clinical studies on the use of DC vaccines for HCC treatment. The injection of DCs prestimulated by HCC-specific antigens can increase the number of $CD8^+$ T cells, promote antitumor immune responses and improve liver function[157-159]. The combined use of DC vaccines and other treatments, such as radiotherapy, can induce immunogenic death of tumor cells, which can prolong the overall survival of patients[160].

Exosomes display an array of HCC antigens. Rao *et al*[161] demonstrated that tumor cell-derived exosomes could trigger a stronger DC-mediated immune response than cell lysates and improve the HCC tumor microenvironment. Exosomes derived from α-fetoprotein (AFP)-expressing DCs (DEX_{AFP}) elicited strong antigen-specific immune responses, resulting in significantly delayed tumor growth and prolonged survival rates in mice with HCC tumors[162]. Therefore, DEX_{AFP} might be a promising vaccine for HCC immunotherapy. AFP, a carcinoembryonic antigen, is highly expressed in HCC and serves as an important marker in the diagnosis of HCC, as well as a potential immunotherapy target for HCC. Zhang *et al*[163] prepared a mouse AFP recom-binant vaccine by genetic engineering and found that it could induce cellular and humoral immune responses in tumor-bearing mice and show obvious antitumor effects. Additionally, with the use of HCC cell lysates derived from STAT3-inhibited HCC cells to immunize healthy mice, we found that a variety of immune cells, such as T cells and NK cells, were significantly activated after challenge with murine HCC cells in these immunized mice, showing effective inhibition characteristics against the transplanted tumor and resulting in the formation of immune memory[164]. Therefore, as a target for HCC treatment and prevention, blocking STAT3 not only prevents tumor growth but also exerts an important effect on immune system activation.

Although immunotherapy for HCC has made significant progress, the clinical efficacy still needs to be further improved. Finding new targets for the treatment of HCC is still the direction of scientific researchers in the next few years. In recent years, studies about the roles of epigenetics and metabolomics on HCC progression have also become hot spots. Related drug development is also ongoing. A single treatment may not bring satisfactory therapeutic effect. Individual differences need to be more considered. Combined therapy and individualized therapy may be a promising option in HCC treatment.

CONCLUSION

The development of HCC results from the accumulation of many factors and the interaction among many mechanisms. Exploring the molecular mechanisms underlying the occurrence and development of HCC is important for us to obtain a more comprehensive understanding of the disease process and to identify more effective therapeutic targets and strategies. With continuous breakthroughs in research, in addition to traditional therapies, immunotherapies have shown good efficacy for HCC in both preclinical and clinical trials, offering hope for curing this disease. Thus, the combination of drugs acting on various pathways, targets and treatment methods might be effective strategies to achieve greater clinical benefits for the treatment of HCC.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Llovet JM**, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016; **2**: 16018 [PMID: 27158749 DOI: 10.1038/nrdp.2016.18]

3 **Aravalli RN**, Cressman EN, Steer CJ. Cellular and molecular mechanisms of hepatocellular carcinoma: an update. *Arch Toxicol* 2013; **87**: 227-247 [PMID: 23007558 DOI: 10.1007/s00204-012-0931-2]

4 **Wang M**, Xi D, Ning Q. Virus-induced hepatocellular carcinoma with special emphasis on HBV. *Hepatol Int* 2017; **11**: 171-180 [PMID: 28097530 DOI: 10.1007/s12072-016-9779-5]

5 **Daud M**, Rana MA, Husnain T, Ijaz B. Modulation of Wnt signaling pathway by hepatitis B virus. *Arch Virol* 2017; **162**: 2937-2947 [PMID: 28685286 DOI: 10.1007/s00705-017-3462-6]

6 **Gao J**, Xiong Y, Wang Y, Wang Y, Zheng G, Xu H. Hepatitis B virus X protein activates Notch signaling by its effects on Notch1 and Notch4 in human hepatocellular carcinoma. *Int J Oncol* 2016; **48**: 329-337 [PMID: 26530164 DOI: 10.3892/ijo.2015.3221]

7 **Yoneda M**, Hyun J, Jakubski S, Saito S, Nakajima A, Schiff ER, Thomas E. Hepatitis B Virus and DNA Stimulation Trigger a Rapid Innate Immune Response through NF-κB. *J Immunol* 2016; **197**: 630-643 [PMID: 27288535 DOI: 10.4049/jimmunol.1502677]

8 **Yang Y**, Zheng B, Han Q, Zhang C, Tian Z, Zhang J. Targeting blockage of STAT3 inhibits hepatitis B virus-related hepatocellular carcinoma. *Cancer Biol Ther* 2016; **17**: 449-456 [PMID: 26934469 DOI: 10.1080/15384047.2016.1156257]

9 **Arbuthnot P**, Capovilla A, Kew M. Putative role of hepatitis B virus X protein in hepatocarcinogenesis: effects on apoptosis, DNA repair, mitogen-activated protein kinase and JAK/STAT pathways. *J Gastroenterol Hepatol* 2000; **15**: 357-368 [PMID: 10824878]

10 **Diao J**, Khine AA, Sarangi F, Hsu E, Iorio C, Tibbles LA, Woodgett JR, Penninger J, Richardson CD. X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with up-regulation of the SAPK/JNK pathway. *J Biol Chem* 2001; **276**: 8328-8340 [PMID: 11099494 DOI: 10.1074/jbc.M006026200]

Martin-Lluesma S, Schaeffer C, Robert EI, van Breugel PC, Leupin O, Hantz O, Strubin M. Hepatitis B

11 virus X protein affects S phase progression leading to chromosome segregation defects by binding to damaged DNA binding protein 1. *Hepatology* 2008; **48**: 1467-1476 [PMID: 18781669 DOI: 10.1002/hep.22542]

12 **Diao J**, Garces R, Richardson CD. X protein of hepatitis B virus modulates cytokine and growth factor related signal transduction pathways during the course of viral infections and hepatocarcinogenesis. *Cytokine Growth Factor Rev* 2001; **12**: 189-205 [PMID: 11325602]

13 **Geng M**, Xin X, Bi LQ, Zhou LT, Liu XH. Molecular mechanism of hepatitis B virus X protein function in hepatocarcinogenesis. *World J Gastroenterol* 2015; **21**: 10732-10738 [PMID: 26478665 DOI: 10.3748/wjg.v21.i38.10732]

14 **Chen GG**, Li MY, Ho RL, Chak EC, Lau WY, Lai PB. Identification of hepatitis B virus X gene mutation in Hong Kong patients with hepatocellular carcinoma. *J Clin Virol* 2005; **34**: 7-12 [PMID: 16087118 DOI: 10.1016/j.jcv.2005.01.006]

15 **Kim JK**, Chang HY, Lee JM, Baatarkhuu O, Yoon YJ, Park JY, Kim DY, Han KH, Chon CY, Ahn SH. Specific mutations in the enhancer II/core promoter/precore regions of hepatitis B virus subgenotype C2 in Korean patients with hepatocellular carcinoma. *J Med Virol* 2009; **81**: 1002-1008 [PMID: 19382267 DOI: 10.1002/jmv.21501]

16 **Benhenda S**, Cougot D, Buendia MA, Neuveut C. Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. *Adv Cancer Res* 2009; **103**: 75-109 [PMID: 19854353 DOI: 10.1016/S0065-230X(09)03004-8]

17 **Wang G**, Dong F, Xu Z, Sharma S, Hu X, Chen D, Zhang L, Zhang J, Dong Q. MicroRNA profile in HBV-induced infection and hepatocellular carcinoma. *BMC Cancer* 2017; **17**: 805 [PMID: 29191172 DOI: 10.1186/s12885-017-3816-1]

18 **Zhu HT**, Liu RB, Liang YY, Hasan AME, Wang HY, Shao Q, Zhang ZC, Wang J, He CY, Wang F, Shao JY. Serum microRNA profiles as diagnostic biomarkers for HBV-positive hepatocellular carcinoma. *Liver Int* 2017; **37**: 888-896 [PMID: 28061012 DOI: 10.1111/liv.13356]

19 **Chen S**, Chen H, Gao S, Qiu S, Zhou H, Yu M, Tu J. Differential expression of plasma microRNA-125b in hepatitis B virus-related liver diseases and diagnostic potential for hepatitis B virus-induced hepatocellular carcinoma. *Hepatol Res* 2017; **47**: 312-320 [PMID: 27152955 DOI: 10.1111/hepr.12739]

20 **Li LM**, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF, Shen HB, Zhang CY, Zen K. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. *Cancer Res* 2010; **70**: 9798-9807 [PMID: 21098710 DOI: 10.1158/0008-5472.CAN-10-1001]

21 **Tian JH**, Liu WD, Zhang ZY, Tang LH, Li D, Tian ZJ, Lin SW, Li YJ. Influence of miR-520e-mediated MAPK signalling pathway on HBV replication and regulation of hepatocellular carcinoma cells via targeting EphA2. *J Viral Hepat* 2019; **26**: 496-505 [PMID: 30521133 DOI: 10.1111/jvh.13048]

22 **Qin X**, Li C, Guo T, Chen J, Wang HT, Wang YT, Xiao YS, Li J, Liu P, Liu ZS, Liu QY. Upregulation of DARS2 by HBV promotes hepatocarcinogenesis through the miR-30e-5p/MAPK/NFAT5 pathway. *J Exp Clin Cancer Res* 2017; **36**: 148 [PMID: 29052520 DOI: 10.1186/s13046-017-0618-x]

23 **Zhou SJ**, Deng YL, Liang HF, Jaoude JC, Liu FY. Hepatitis B virus X protein promotes CREB-mediated activation of miR-3188 and Notch signaling in hepatocellular carcinoma. *Cell Death Differ* 2017; **24**: 1577-1587 [PMID: 28574502 DOI: 10.1038/cdd.2017.87]

24 **Xie KL**, Zhang YG, Liu J, Zeng Y, Wu H. MicroRNAs associated with HBV infection and HBV-related HCC. *Theranostics* 2014; **4**: 1176-1192 [PMID: 25285167 DOI: 10.7150/thno.8715]

25 **Liang HW**, Wang N, Wang Y, Wang F, Fu Z, Yan X, Zhu H, Diao W, Ding Y, Chen X, Zhang CY, Zen K. Hepatitis B virus-human chimeric transcript HBx-LINE1 promotes hepatic injury via sequestering cellular microRNA-122. *J Hepatol* 2016; **64**: 278-291 [PMID: 26409216 DOI: 10.1016/j.jhep.2015.09.013]

26 **Mao K**, Zhang J, He C, Xu K, Liu J, Sun J, Wu G, Tan C, Zeng Y, Wang J, Xiao Z. Restoration of miR-193b sensitizes Hepatitis B virus-associated hepatocellular carcinoma to sorafenib. *Cancer Lett* 2014; **352**: 245-252 [PMID: 25034398 DOI: 10.1016/j.canlet.2014.07.004]

27 **Tjwa ET**, van Oord GW, Hegmans JP, Janssen HL, Woltman AM. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. *J Hepatol* 2011; **54**: 209-218 [PMID: 21095036 DOI: 10.1016/j.jhep.2010.07.009]

28 **Sun C**, Sun H, Zhang C, Tian Z. NK cell receptor imbalance and NK cell dysfunction in HBV infection and hepatocellular carcinoma. *Cell Mol Immunol* 2015; **12**: 292-302 [PMID: 25308752 DOI: 10.1038/cmi.2014.91]

29 **Yang P**, Li QJ, Feng Y, Zhang Y, Markowitz GJ, Ning S, Deng Y, Zhao J, Jiang S, Yuan Y, Wang HY, Cheng SQ, Xie D, Wang XF. TGF-β-miR-34a-CCL22 signaling-induced Treg cell recruitment promotes venous metastases of HBV-positive hepatocellular carcinoma. *Cancer Cell* 2012; **22**: 291-303 [PMID: 22975373 DOI: 10.1016/j.ccr.2012.07.023]

30 **Li K**, Liu H, Guo T. Th17/Treg imbalance is an indicator of liver cirrhosis process and a risk factor for HCC occurrence in HBV patients. *Clin Res Hepatol Gastroenterol* 2017; **41**: 399-407 [PMID: 28169127 DOI: 10.1016/j.clinre.2016.12.004]

31 **Raziorrouh B**, Schraut W, Gerlach T, Nowack D, Grüner NH, Ulsenheimer A, Zachoval R, Wächtler M, Spannagl M, Haas J, Diepolder HM, Jung MC. The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology* 2010; **52**: 1934-1947 [PMID: 21064032 DOI: 10.1002/hep.23936]

32 **Zong L**, Peng H, Sun C, Li F, Zheng M, Chen Y, Wei H, Sun R, Tian Z. Breakdown of adaptive immunotolerance induces hepatocellular carcinoma in HBsAg-tg mice. *Nat Commun* 2019; **10**: 221 [PMID: 30644386 DOI: 10.1038/s41467-018-08096-8]

33 **Yu H**, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat Rev Cancer* 2014; **14**: 736-746 [PMID: 25342631 DOI: 10.1038/nrc3818]

34 **Wörmann SM**, Song L, Ai J, Diakopoulos KN, Kurkowski MU, Görgülü K, Ruess D, Campbell A, Doglioni C, Jodrell D, Neesse A, Demir IE, Karpathaki AP, Barenboim M, Hagemann T, Rose-John S, Sansom O, Schmid RM, Protti MP, Lesina M, Algül H. Loss of P53 Function Activates JAK2-STAT3 Signaling to Promote Pancreatic Tumor Growth, Stroma Modification, and Gemcitabine Resistance in Mice and Is Associated With Patient Survival. *Gastroenterology* 2016; **151**: 180-193.e12 [PMID: 27003603 DOI: 10.1053/j.gastro.2016.03.010]

35 **Jones LM**, Broz ML, Ranger JJ, Ozcelik J, Ahn R, Zuo D, Ursini-Siegel J, Hallett MT, Krummel M, Muller WJ. STAT3 Establishes an Immunosuppressive Microenvironment during the Early Stages of Breast Carcinogenesis to Promote Tumor Growth and Metastasis. *Cancer Res* 2016; **76**: 1416-1428

[PMID: 26719528 DOI: 10.1158/0008-5472.CAN-15-2770]
36 **Yu H**, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* 2007; **7**: 41-51 [PMID: 17186030 DOI: 10.1038/nri1995]
37 **Abe M**, Yoshida T, Akiba J, Ikezono Y, Wada F, Masuda A, Sakaue T, Tanaka T, Iwamoto H, Nakamura T, Sata M, Koga H, Yoshimura A, Torimura T. STAT3 deficiency prevents hepatocarcinogenesis and promotes biliary proliferation in thioacetamide-induced liver injury. *World J Gastroenterol* 2017; **23**: 6833-6844 [PMID: 29085226 DOI: 10.3748/wjg.v23.i37.6833]
38 **Sun X**, Zhang J, Wang L, Tian Z. Growth inhibition of human hepatocellular carcinoma cells by blocking STAT3 activation with decoy-ODN. *Cancer Lett* 2008; **262**: 201-213 [PMID: 18248786 DOI: 10.1016/j.canlet.2007.12.009]
39 **Long J**, Jiang C, Liu B, Dai Q, Hua R, Chen C, Zhang B, Li H. Maintenance of stemness by miR-589-5p in hepatocellular carcinoma cells promotes chemoresistance via STAT3 signaling. *Cancer Lett* 2018; **423**: 113-126 [PMID: 29196128 DOI: 10.1016/j.canlet.2017.11.031]
40 **Jiang C**, Long J, Liu B, Xu M, Wang W, Xie X, Wang X, Kuang M. miR-500a-3p promotes cancer stem cells properties via STAT3 pathway in human hepatocellular carcinoma. *J Exp Clin Cancer Res* 2017; **36**: 99 [PMID: 28750679 DOI: 10.1186/s13046-017-0568-3]
41 **Li T**, Li M, Hu S, Cheng X, Gao Y, Jiang S, Yu Q, Zhang C, Sun P, Xian W, Song Z, Zhang Y, Zheng Q. MiR-221 mediates the epithelial-mesenchymal transition of hepatocellular carcinoma by targeting AdipoR1. *Int J Biol Macromol* 2017; **103**: 1054-1061 [PMID: 28539268 DOI: 10.1016/j.ijbiomac.2017.05.108]
42 **Yu M**, Xue H, Wang Y, Shen Q, Jiang Q, Zhang X, Li K, Jia M, Jia J, Xu J, Tian Y. miR-345 inhibits tumor metastasis and EMT by targeting IRF1-mediated mTOR/STAT3/AKT pathway in hepatocellular carcinoma. *Int J Oncol* 2017; **50**: 975-983 [PMID: 28098858 DOI: 10.3892/ijo.2017.3852]
43 **Liu X**, Zhang A, Xiang J, Lv Y, Zhang X. miR-451 acts as a suppressor of angiogenesis in hepatocellular carcinoma by targeting the IL-6R-STAT3 pathway. *Oncol Rep* 2016; **36**: 1385-1392 [PMID: 27461244 DOI: 10.3892/or.2016.4971]
44 **Morrison WB**. Inflammation and cancer: a comparative view. *J Vet Intern Med* 2012; **26**: 18-31 [PMID: 22151229 DOI: 10.1111/j.1939-1676.2011.00836.x]
45 **Dapito DH**, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, Khiabanian H, Adeyemi A, Bataller R, Lefkowitch JH, Bower M, Friedman R, Sartor RB, Rabadan R, Schwabe RF. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* 2012; **21**: 504-516 [PMID: 22516259 DOI: 10.1016/j.ccr.2012.02.007]
46 **Lin A**, Wang G, Zhao H, Zhang Y, Han Q, Zhang C, Tian Z, Zhang J. TLR4 signaling promotes a COX-2/PGE_2/STAT3 positive feedback loop in hepatocellular carcinoma (HCC) cells. *Oncoimmunology* 2015; **5**: e1074376 [PMID: 27057441 DOI: 10.1080/2162402X.2015.1074376]
47 **Li J**, Zhou Y, Liu Y, Dai B, Zhang YH, Zhang PF, Shi XL. Sorafenib inhibits caspase-1 expression through suppressing TLR4/stat3/SUMO1 pathway in hepatocellular carcinoma. *Cancer Biol Ther* 2018; 1-8 [PMID: 30277836 DOI: 10.1080/15384047.2018.1480280]
48 **Sun X**, Sui Q, Zhang C, Tian Z, Zhang J. Targeting blockage of STAT3 in hepatocellular carcinoma cells augments NK cell functions via reverse hepatocellular carcinoma-induced immune suppression. *Mol Cancer Ther* 2013; **12**: 2885-2896 [PMID: 24107450 DOI: 10.1158/1535-7163.MCT-12-1087]
49 **Sui Q**, Zhang J, Sun X, Zhang C, Han Q, Tian Z. NK cells are the crucial antitumor mediators when STAT3-mediated immunosuppression is blocked in hepatocellular carcinoma. *J Immunol* 2014; **193**: 2016-2023 [PMID: 25015826 DOI: 10.4049/jimmunol.1302389]
50 **Sun X**, Zhang J, Hou Z, Han Q, Zhang C, Tian Z. miR-146a is directly regulated by STAT3 in human hepatocellular carcinoma cells and involved in anti-tumor immune suppression. *Cell Cycle* 2015; **14**: 243-252 [PMID: 25607648 DOI: 10.4161/15384101.2014.977112]
51 **Takeda K**, Clausen BE, Kaisho T, Tsujimura T, Terada N, Förster I, Akira S. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 1999; **10**: 39-49 [PMID: 10023769]
52 **Kortylewski M**, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mulé J, Kerr WG, Jove R, Pardoll D, Yu H. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med* 2005; **11**: 1314-1321 [PMID: 16288283 DOI: 10.1038/nm1325]
53 **Fu XT**, Dai Z, Song K, Zhang ZJ, Zhou ZJ, Zhou SL, Zhao YM, Xiao YS, Sun QM, Ding ZB, Fan J. Macrophage-secreted IL-8 induces epithelial-mesenchymal transition in hepatocellular carcinoma cells by activating the JAK2/STAT3/Snail pathway. *Int J Oncol* 2015; **46**: 587-596 [PMID: 25405790 DOI: 10.3892/ijo.2014.2761]
54 **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]
55 **Cheng Y**, Li H, Deng Y, Tai Y, Zeng K, Zhang Y, Liu W, Zhang Q, Yang Y. Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma. *Cell Death Dis* 2018; **9**: 422 [PMID: 29556041 DOI: 10.1038/s41419-018-0458-4]
56 **Cheng JT**, Deng YN, Yi HM, Wang GY, Fu BS, Chen WJ, Liu W, Tai Y, Peng YW, Zhang Q. Hepatic carcinoma-associated fibroblasts induce IDO-producing regulatory dendritic cells through IL-6-mediated STAT3 activation. *Oncogenesis* 2016; **5**: e198 [PMID: 26900950 DOI: 10.1038/oncsis.2016.7]
57 **Yin Z**, Ma T, Lin Y, Lu X, Zhang C, Chen S, Jian Z. IL-6/STAT3 pathway intermediates M1/M2 macrophage polarization during the development of hepatocellular carcinoma. *J Cell Biochem* 2018; **119**: 9419-9432 [PMID: 30015355 DOI: 10.1002/jcb.27259]
58 **Zhao T**, Jia H, Cheng Q, Xiao Y, Li M, Ren W, Li C, Feng Y, Feng Z, Wang H, Zheng J. Nifuroxazide prompts antitumor immune response of TCL-loaded DC in mice with orthotopically-implanted hepatocarcinoma. *Oncol Rep* 2017; **37**: 3405-3414 [PMID: 28498414 DOI: 10.3892/or.2017.5629]
59 **Ma H**, Yan D, Wang Y, Shi W, Liu T, Zhao C, Huo S, Duan J, Tao J, Zhai M, Luo P, Guo J, Tian L, Mageta L, Jou D, Zhang C, Li C, Lin J, Lv J, Li S, Lin L. Bazedoxifene exhibits growth suppressive activity by targeting interleukin-6/glycoprotein 130/signal transducer and activator of transcription 3 signaling in hepatocellular carcinoma. *Cancer Sci* 2019; **110**: 950-961 [PMID: 30648776 DOI: 10.1111/cas.13940]
60 **Sharma D**, Wang J, Fu PP, Sharma S, Nagalingam A, Mells J, Handy J, Page AJ, Cohen C, Anania FA,

Saxena NK. Adiponectin antagonizes the oncogenic actions of leptin in hepatocellular carcinogenesis. *Hepatology* 2010; **52**: 1713-1722 [PMID: 20941777 DOI: 10.1002/hep.23892]
61 **Abate-Shen C**. Deregulated homeobox gene expression in cancer: cause or consequence? *Nat Rev Cancer* 2002; **2**: 777-785 [PMID: 12360280 DOI: 10.1038/nrc907]
62 **Bienz M**. Homeotic genes and positional signalling in the Drosophila viscera. *Trends Genet* 1994; **10**: 22-26 [PMID: 7908470]
63 **Holland PW**, Booth HA, Bruford EA. Classification and nomenclature of all human homeobox genes. *BMC Biol* 2007; **5**: 47 [PMID: 17963489 DOI: 10.1186/1741-7007-5-47]
64 **Samuel S**, Naora H. Homeobox gene expression in cancer: insights from developmental regulation and deregulation. *Eur J Cancer* 2005; **41**: 2428-2437 [PMID: 16199152 DOI: 10.1016/j.ejca.2005.08.014]
65 **Bhatlekar S**, Fields JZ, Boman BM. HOX genes and their role in the development of human cancers. *J Mol Med (Berl)* 2014; **92**: 811-823 [PMID: 24996520 DOI: 10.1007/s00109-014-1181-y]
66 **Quagliata L**, Matter MS, Piscuoglio S, Arabi L, Ruiz C, Procino A, Kovac M, Moretti F, Makowska Z, Boldanova T, Andersen JB, Hämmerle M, Tornillo L, Heim MH, Diederichs S, Cillo C, Terracciano LM. Long noncoding RNA HOTTIP/HOXA13 expression is associated with disease progression and predicts outcome in hepatocellular carcinoma patients. *Hepatology* 2014; **59**: 911-923 [PMID: 24114970 DOI: 10.1002/hep.26740]
67 **Pan TT**, Jia WD, Yao QY, Sun QK, Ren WH, Huang M, Ma J, Li JS, Ma JL, Yu JH, Ge YS, Liu WB, Zhang CH, Xu GL. Overexpression of HOXA13 as a potential marker for diagnosis and poor prognosis of hepatocellular carcinoma. *Tohoku J Exp Med* 2014; **234**: 209-219 [PMID: 25341685]
68 **Quagliata L**, Quintavalle C, Lanzafame M, Matter MS, Novello C, di Tommaso L, Pressiani T, Rimassa L, Tornillo L, Roncalli M, Cillo C, Pallante P, Piscuoglio S, Ng CK, Terracciano LM. High expression of HOXA13 correlates with poorly differentiated hepatocellular carcinomas and modulates sorafenib response in in vitro models. *Lab Invest* 2018; **98**: 95-105 [PMID: 29035381 DOI: 10.1038/labinvest.2017.107]
69 **Li Y**, Yang XH, Fang SJ, Qin CF, Sun RL, Liu ZY, Jiang BY, Wu X, Li G. HOXA7 stimulates human hepatocellular carcinoma proliferation through cyclin E1/CDK2. *Oncol Rep* 2015; **33**: 990-996 [PMID: 25501982 DOI: 10.3892/or.2014.3668]
70 **Huan HB**, Yang DP, Wen XD, Chen XJ, Zhang L, Wu LL, Bie P, Xia F. HOXB7 accelerates the malignant progression of hepatocellular carcinoma by promoting stemness and epithelial-mesenchymal transition. *J Exp Clin Cancer Res* 2017; **36**: 86 [PMID: 28646927 DOI: 10.1186/s13046-017-0559-4]
71 **Chen S**, Saiyin H, Zeng X, Xi J, Liu X, Li X, Yu L. Isolation and functional analysis of human HMBOX1, a homeobox containing protein with transcriptional repressor activity. *Cytogenet Genome Res* 2006; **114**: 131-136 [PMID: 16825764 DOI: 10.1159/000093328]
72 **Yu YL**, Diao NN, Li YZ, Meng XH, Jiao WL, Feng JB, Liu ZP, Lu N. Low expression level of HMBOX1 in high-grade serous ovarian cancer accelerates cell proliferation by inhibiting cell apoptosis. *Biochem Biophys Res Commun* 2018; **501**: 380-386 [PMID: 29709478 DOI: 10.1016/j.bbrc.2018.04.203]
73 **Gong J**, Liu R, Zhuang R, Zhang Y, Fang L, Xu Z, Jin L, Wang T, Song C, Yang K, Wei Y, Yang A, Jin B, Chen L. miR-30c-1* promotes natural killer cell cytotoxicity against human hepatoma cells by targeting the transcription factor HMBOX1. *Cancer Sci* 2012; **103**: 645-652 [PMID: 22320217 DOI: 10.1111/j.1349-7006.2012.02207.x]
74 **Dai J**, Wu L, Zhang C, Zheng X, Tian Z, Zhang J. Recombinant expression of a novel human transcriptional repressor HMBOX1 and preparation of anti-HMBOX1 monoclonal antibody. *Cell Mol Immunol* 2009; **6**: 261-268 [PMID: 19728927 DOI: 10.1038/cmi.2009.35]
75 **Zhang P**, Liu Q, Yan S, Yuan G, Shen J, Li G. Homeoboxcontaining protein 1 loss is associated with clinicopathological performance in glioma. *Mol Med Rep* 2017; **16**: 4101-4106 [PMID: 28731165 DOI: 10.3892/mmr.2017.7050]
76 **Zhao H**, Jia H, Han Q, Zhang J. Homeobox containing 1 inhibits liver cancer progression by promoting autophagy as well as inhibiting stemness and immune escape. *Oncol Rep* 2018; **40**: 1657-1665 [PMID: 30015890 DOI: 10.3892/or.2018.6551]
77 **Zhao H**, Han Q, Lu N, Xu D, Tian Z, Zhang J. HMBOX1 in hepatocytes attenuates LPS/D-GalN-induced liver injury by inhibiting macrophage infiltration and activation. *Mol Immunol* 2018; **101**: 303-311 [PMID: 30032072 DOI: 10.1016/j.molimm.2018.07.021]
78 **Liu Y**, Zhang Y, Wang S, Dong QZ, Shen Z, Wang W, Tao S, Gu C, Liu J, Xie Y, Qin LX. Prospero-related homeobox 1 drives angiogenesis of hepatocellular carcinoma through selectively activating interleukin-8 expression. *Hepatology* 2017; **66**: 1894-1909 [PMID: 28646551 DOI: 10.1002/hep.29337]
79 **Liu Y**, Ye X, Zhang JB, Ouyang H, Shen Z, Wu Y, Wang W, Wu J, Tao S, Yang X, Qiao K, Zhang J, Liu J, Fu Q, Xie Y. PROX1 promotes hepatocellular carcinoma proliferation and sorafenib resistance by enhancing β-catenin expression and nuclear translocation. *Oncogene* 2015; **34**: 5524-5535 [PMID: 25684142 DOI: 10.1038/onc.2015.7]
80 **Shimoda M**, Takahashi M, Yoshimoto T, Kono T, Ikai I, Kubo H. A homeobox protein, prox1, is involved in the differentiation, proliferation, and prognosis in hepatocellular carcinoma. *Clin Cancer Res* 2006; **12**: 6005-6011 [PMID: 17062673 DOI: 10.1158/1078-0432.CCR-06-0712]
81 **Bhatia-Gaur R**, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Cardiff RD, Cunha GR, Abate-Shen C, Shen MM. Roles for Nkx3.1 in prostate development and cancer. *Genes Dev* 1999; **13**: 966-977 [PMID: 10215624]
82 **Jiang J**, Liu Z, Ge C, Chen C, Zhao F, Li H, Chen T, Yao M, Li J. NK3 homeobox 1 (NKX3.1) up-regulates forkhead box O1 expression in hepatocellular carcinoma and thereby suppresses tumor proliferation and invasion. *J Biol Chem* 2017; **292**: 19146-19159 [PMID: 28972178 DOI: 10.1074/jbc.M117.793760]
83 **Wilkens L**, Jaggi R, Hammer C, Inderbitzin D, Giger O, von Neuhoff N. The homeobox gene HLXB9 is upregulated in a morphological subset of poorly differentiated hepatocellular carcinoma. *Virchows Arch* 2011; **458**: 697-708 [PMID: 21484430 DOI: 10.1007/s00428-011-1070-5]
84 **Clevers H**, Nusse R. Wnt/β-catenin signaling and disease. *Cell* 2012; **149**: 1192-1205 [PMID: 22682243 DOI: 10.1016/j.cell.2012.05.012]
85 **Aicher S**, Kakkanas A, Cohen L, Blumen B, Oprisan G, Njouom R, Meurs EF, Mavromara P, Martin A. Differential regulation of the Wnt/β-catenin pathway by hepatitis C virus recombinants expressing core from various genotypes. *Sci Rep* 2018; **8**: 11185 [PMID: 30046100 DOI: 10.1038/s41598-018-29078-2]
86 **Wang W**, Pan Q, Fuhler GM, Smits R, Peppelenbosch MP. Action and function of Wnt/β-catenin signaling in the progression from chronic hepatitis C to hepatocellular carcinoma. *J Gastroenterol* 2017; **52**: 419-431 [PMID: 28035485 DOI: 10.1007/s00535-016-1299-5]

87 **Xu W**, Zhou W, Cheng M, Wang J, Liu Z, He S, Luo X, Huang W, Chen T, Yan W, Xiao J. Hypoxia activates Wnt/β-catenin signaling by regulating the expression of BCL9 in human hepatocellular carcinoma. *Sci Rep* 2017; **7**: 40446 [PMID: 28074862 DOI: 10.1038/srep40446]

88 **Chen Z**, Tang J, Cai X, Huang Y, Gao Q, Liang L, Tian L, Yang Y, Zheng Y, Hu Y, Tang N. HBx mutations promote hepatoma cell migration through the Wnt/β-catenin signaling pathway. *Cancer Sci* 2016; **107**: 1380-1389 [PMID: 27420729 DOI: 10.1111/cas.13014]

89 **Yamashita T**, Budhu A, Forgues M, Wang XW. Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in hepatocellular carcinoma. *Cancer Res* 2007; **67**: 10831-10839 [PMID: 18006828 DOI: 10.1158/0008-5472.CAN-07-0908]

90 **Mokkapati S**, Niopek K, Huang L, Cunniff KJ, Ruteshouser EC, deCaestecker M, Finegold MJ, Huff V. β-catenin activation in a novel liver progenitor cell type is sufficient to cause hepatocellular carcinoma and hepatoblastoma. *Cancer Res* 2014; **74**: 4515-4525 [PMID: 24848510 DOI: 10.1158/0008-5472.CAN-13-3275]

91 **Huang M**, Chen C, Geng J, Han D, Wang T, Xie T, Wang L, Wang Y, Wang C, Lei Z, Chu X. Targeting KDM1A attenuates Wnt/β-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma. *Cancer Lett* 2017; **398**: 12-21 [PMID: 28377178 DOI: 10.1016/j.canlet.2017.03.038]

92 **Kim W**, Khan SK, Gvozdenovic-Jeremic J, Kim Y, Dahlman J, Kim H, Park O, Ishitani T, Jho EH, Gao B, Yang Y. Hippo signaling interactions with Wnt/β-catenin and Notch signaling repress liver tumorigenesis. *J Clin Invest* 2017; **127**: 137-152 [PMID: 27869648 DOI: 10.1172/JCI88486]

93 **Wang R**, Sun Q, Wang P, Liu M, Xiong S, Luo J, Huang H, Du Q, Geller DA, Cheng B. Notch and Wnt/β-catenin signaling pathway play important roles in activating liver cancer stem cells. *Oncotarget* 2016; **7**: 5754-5768 [PMID: 26735577 DOI: 10.18632/oncotarget.6805]

94 **Kim W**, Khan SK, Yang Y. Interacting network of Hippo, Wnt/β-catenin and Notch signaling represses liver tumor formation. *BMB Rep* 2017; **50**: 1-2 [PMID: 27881216]

95 **Monga SP**. β-Catenin Signaling and Roles in Liver Homeostasis, Injury, and Tumorigenesis. *Gastroenterology* 2015; **148**: 1294-1310 [PMID: 25747274 DOI: 10.1053/j.gastro.2015.02.056]

96 **Amaddeo G**, Cao Q, Ladeiro Y, Imbeaud S, Nault JC, Jaoui D, Gaston Mathe Y, Laurent C, Laurent A, Bioulac-Sage P, Calderaro J, Zucman-Rossi J. Integration of tumour and viral genomic characterizations in HBV-related hepatocellular carcinomas. *Gut* 2015; **64**: 820-829 [PMID: 25021421 DOI: 10.1136/gutjnl-2013-306228]

97 **Perugorria MJ**, Olaizola P, Labiano I, Esparza-Baquer A, Marzioni M, Marin JJG, Bujanda L, Banales JM. Wnt-β-catenin signalling in liver development, health and disease. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 121-136 [PMID: 30451972 DOI: 10.1038/s41575-018-0075-9]

98 **Abitbol S**, Dahmani R, Coulouarn C, Ragazzon B, Mlecnik B, Senni N, Savall M, Bossard P, Sohier P, Drouet V, Tournier E, Dumont F, Sanson R, Calderaro J, Zucman-Rossi J, Vasseur-Cognet M, Just PA, Terris B, Perret C, Gilgenkrantz H. AXIN deficiency in human and mouse hepatocytes induces hepatocellular carcinoma in the absence of β-catenin activation. *J Hepatol* 2018; **68**: 1203-1213 [PMID: 29525529 DOI: 10.1016/j.jhep.2017.12.018]

99 **Wang Y**, He L, Du Y, Zhu P, Huang G, Luo J, Yan X, Ye B, Li C, Xia P, Zhang G, Tian Y, Chen R, Fan Z. The long noncoding RNA lncTCF7 promotes self-renewal of human liver cancer stem cells through activation of Wnt signaling. *Cell Stem Cell* 2015; **16**: 413-425 [PMID: 25842979 DOI: 10.1016/j.stem.2015.03.003]

100 **Xie Q**, Chen L, Shan X, Shan X, Tang J, Zhou F, Chen Q, Quan H, Nie D, Zhang W, Huang AL, Tang N. Epigenetic silencing of SFRP1 and SFRP5 by hepatitis B virus X protein enhances hepatoma cell tumorigenicity through Wnt signaling pathway. *Int J Cancer* 2014; **135**: 635-646 [PMID: 24374650 DOI: 10.1002/ijc.28697]

101 **Wu W**, Dang S, Feng Q, Liang J, Wang Y, Fan N. MicroRNA-542-3p inhibits the growth of hepatocellular carcinoma cells by targeting FZD7/Wnt signaling pathway. *Biochem Biophys Res Commun* 2017; **482**: 100-105 [PMID: 27815069 DOI: 10.1016/j.bbrc.2016.10.136]

102 **Chai S**, Ng KY, Tong M, Lau EY, Lee TK, Chan KW, Yuan YF, Cheung TT, Cheung ST, Wang XQ, Wong N, Lo CM, Man K, Guan XY, Ma S. Octamer 4/microRNA-1246 signaling axis drives Wnt/β-catenin activation in liver cancer stem cells. *Hepatology* 2016; **64**: 2062-2076 [PMID: 27639189 DOI: 10.1002/hep.28821]

103 **Fu C**, Liang X, Cui W, Ober-Blöbaum JL, Vazzana J, Shrikant PA, Lee KP, Clausen BE, Mellman I, Jiang A. β-Catenin in dendritic cells exerts opposite functions in cross-priming and maintenance of CD8+ T cells through regulation of IL-10. *Proc Natl Acad Sci USA* 2015; **112**: 2823-2828 [PMID: 25730849 DOI: 10.1073/pnas.1414167112]

104 **Liang X**, Fu C, Cui W, Ober-Blöbaum JL, Zahner SP, Shrikant PA, Clausen BE, Flavell RA, Mellman I, Jiang A. β-catenin mediates tumor-induced immunosuppression by inhibiting cross-priming of CD8 T cells. *J Leukoc Biol* 2014; **95**: 179-190 [PMID: 24023259 DOI: 10.1189/jlb.0613330]

105 **Hong Y**, Manoharan I, Suryawanshi A, Majumdar T, Angus-Hill ML, Koni PA, Manicassamy B, Mellor AL, Munn DH, Manicassamy S. β-catenin promotes regulatory T-cell responses in tumors by inducing vitamin A metabolism in dendritic cells. *Cancer Res* 2015; **75**: 656-665 [PMID: 25568183 DOI: 10.1158/0008-5472.CAN-14-2377]

106 **Yang Y**, Ye YC, Chen Y, Zhao JL, Gao CC, Han H, Liu WC, Qin HY. Crosstalk between hepatic tumor cells and macrophages via Wnt/β-catenin signaling promotes M2-like macrophage polarization and reinforces tumor malignant behaviors. *Cell Death Dis* 2018; **9**: 793 [PMID: 30022048 DOI: 10.1038/s41419-018-0818-0]

107 **Zhu F**, Li X, Chen S, Zeng Q, Zhao Y, Luo F. Tumor-associated macrophage or chemokine ligand CCL17 positively regulates the tumorigenesis of hepatocellular carcinoma. *Med Oncol* 2016; **33**: 17 [PMID: 26781124 DOI: 10.1007/s12032-016-0729-9]

108 **Ruivo CF**, Adem B, Silva M, Melo SA. The Biology of Cancer Exosomes: Insights and New Perspectives. *Cancer Res* 2017; **77**: 6480-6488 [PMID: 29162616 DOI: 10.1158/0008-5472.CAN-17-0994]

109 **Yang Y**, Han Q, Hou Z, Zhang C, Tian Z, Zhang J. Exosomes mediate hepatitis B virus (HBV) transmission and NK-cell dysfunction. *Cell Mol Immunol* 2017; **14**: 465-475 [PMID: 27238466 DOI: 10.1038/cmi.2016.24]

110 **He M**, Qin H, Poon TC, Sze SC, Ding X, Co NN, Ngai SM, Chan TF, Wong N. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncogenic proteins and RNAs. *Carcinogenesis* 2015; **36**: 1008-1018 [PMID: 26054723 DOI: 10.1093/carcin/bgv081]

111 **Qu Z**, Wu J, Wu J, Luo D, Jiang C, Ding Y. Exosomes derived from HCC cells induce sorafenib

resistance in hepatocellular carcinoma both in vivo and in vitro. *J Exp Clin Cancer Res* 2016; **35**: 159 [PMID: 27716356 DOI: 10.1186/s13046-016-0430-z]
112 **Fu X**, Liu M, Qu S, Ma J, Zhang Y, Shi T, Wen H, Yang Y, Wang S, Wang J, Nan K, Yao Y, Tian T. Exosomal microRNA-32-5p induces multidrug resistance in hepatocellular carcinoma via the PI3K/Akt pathway. *J Exp Clin Cancer Res* 2018; **37**: 52 [PMID: 29530052 DOI: 10.1186/s13046-018-0677-7]
113 **Lv LH**, Wan YL, Lin Y, Zhang W, Yang M, Li GL, Lin HM, Shang CZ, Chen YJ, Min J. Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. *J Biol Chem* 2012; **287**: 15874-15885 [PMID: 22396543 DOI: 10.1074/jbc.M112.340588]
114 **Wang X**, Shen H, Zhangyuan G, Huang R, Zhang W, He Q, Jin K, Zhuo H, Zhang Z, Wang J, Sun B, Lu X. 14-3-3ζ delivered by hepatocellular carcinoma-derived exosomes impaired anti-tumor function of tumor-infiltrating T lymphocytes. *Cell Death Dis* 2018; **9**: 159 [PMID: 29415983 DOI: 10.1038/s41419-017-0180-7]
115 **Li X**, Lei Y, Wu M, Li N. Regulation of Macrophage Activation and Polarization by HCC-Derived Exosomal lncRNA TUC339. *Int J Mol Sci* 2018; **19** [PMID: 30274167 DOI: 10.3390/ijms19102958]
116 **Fang T**, Lv H, Lv G, Li T, Wang C, Han Q, Yu L, Su B, Guo L, Huang S, Cao D, Tang L, Tang S, Wu M, Yang W, Wang H. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nat Commun* 2018; **9**: 191 [PMID: 29335551 DOI: 10.1038/s41467-017-02583-0]
117 **Mazzola A**, Costantino A, Petta S, Bartolotta TV, Raineri M, Sacco R, Brancatelli G, Cammà C, Cabibbo G. Recurrence of hepatocellular carcinoma after liver transplantation: an update. *Future Oncol* 2015; **11**: 2923-2936 [PMID: 26414336 DOI: 10.2217/fon.15.239]
118 **Jemal A**, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277-300 [PMID: 20610543 DOI: 10.3322/caac.20073]
119 **Sapisochin G**, Bruix J. Liver transplantation for hepatocellular carcinoma: outcomes and novel surgical approaches. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 203-217 [PMID: 28053342 DOI: 10.1038/nrgastro.2016.193]
120 **Kollmann D**, Selzner N, Selzner M. Bridging to liver transplantation in HCC patients. *Langenbecks Arch Surg* 2017; **402**: 863-871 [PMID: 28755240 DOI: 10.1007/s00423-017-1609-2]
121 **Llovet JM**, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015; **12**: 408-424 [PMID: 26054909 DOI: 10.1038/nrclinonc.2015.103]
122 **Hodi FS**, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711-723 [PMID: 20525992 DOI: 10.1056/NEJMoa1003466]
123 **Ott PA**, Hodi FS, Robert C. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clin Cancer Res* 2013; **19**: 5300-5309 [PMID: 24089443 DOI: 10.1158/1078-0432.CCR-13-0143]
124 **El-Khoueiry AB**. Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]
125 **Sangro B**, Gomez-Martin C, de la Mata M, Iñarrairaegui M, Garralda E, Barrera P, Riezu-Boj JI, Larrea E, Alfaro C, Sarobe P, Lasarte JJ, Pérez-Gracia JL, Melero I, Prieto J. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; **59**: 81-88 [PMID: 23466307 DOI: 10.1016/j.jhep.2013.02.022]
126 **Zhu AX**, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, Verslype C, Zagonel V, Fartoux L, Vogel A, Sarker D, Verset G, Chan SL, Knox J, Daniele B, Webber AL, Ebbinghaus SW, Ma J, Siegel AB, Cheng AL, Kudo M; KEYNOTE-224 investigators. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol* 2018; **19**: 940-952 [PMID: 29875066 DOI: 10.1016/S1470-2045(18)30351-6]
127 **Kudo M**. Systemic Therapy for Hepatocellular Carcinoma: 2017 Update. *Oncology* 2017; **93** Suppl 1: 135-146 [PMID: 29258077 DOI: 10.1159/000481244]
128 **Greten TF**, Wang XW, Korangy F. Current concepts of immune based treatments for patients with HCC: from basic science to novel treatment approaches. *Gut* 2015; **64**: 842-848 [PMID: 25666193 DOI: 10.1136/gutjnl-2014-307990]
129 **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]
130 **Zhou J**, Liu M, Sun H, Feng Y, Xu L, Chan AWH, Tong JH, Wong J, Chong CCN, Lai PBS, Wang HK, Tsang SW, Goodwin T, Liu R, Huang L, Chen Z, Sung JJ, Chow KL, To KF, Cheng AS. Hepatoma-intrinsic CCRK inhibition diminishes myeloid-derived suppressor cell immunosuppression and enhances immune-checkpoint blockade efficacy. *Gut* 2018; **67**: 931-944 [PMID: 28939663 DOI: 10.1136/gutjnl-2017-314032]
131 **Duffy AG**, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, Davis JL, Hughes MS, Heller T, ElGindi M, Uppala A, Korangy F, Kleiner DE, Figg WD, Venzon D, Steinberg SM, Venkatesan AM, Krishnasamy V, Abi-Jaoudeh N, Levy E, Wood BJ, Greten TF. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J Hepatol* 2017; **66**: 545-551 [PMID: 27816492 DOI: 10.1016/j.jhep.2016.10.029]
132 **Llopiz D**, Ruiz M, Villanueva L, Iglesias T, Silva L, Egea J, Lasarte JJ, Pivette P, Trochon-Joseph V, Vasseur B, Dixon G, Sangro B, Sarobe P. Enhanced anti-tumor efficacy of checkpoint inhibitors in combination with the histone deacetylase inhibitor Belinostat in a murine hepatocellular carcinoma model. *Cancer Immunol Immunother* 2019; **68**: 379-393 [PMID: 30547218 DOI: 10.1007/s00262-018-2283-0]
133 **Pan K**, Li YQ, Wang W, Xu L, Zhang YJ, Zheng HX, Zhao JJ, Qiu HJ, Weng DS, Li JJ, Wang QJ, Huang LX, He J, Chen SP, Ke ML, Wu PH, Chen MS, Li SP, Xia JC, Zeng YX. The efficacy of cytokine-induced killer cell infusion as an adjuvant therapy for postoperative hepatocellular carcinoma patients. *Ann Surg Oncol* 2013; **20**: 4305-4311 [PMID: 23892527 DOI: 10.1245/s10434-013-3144-x]
134 **Jiang SS**, Tang Y, Zhang YJ, Weng DS, Zhou ZG, Pan K, Pan QZ, Wang QJ, Liu Q, He J, Zhao JJ, Li J,

Chen MS, Chang AE, Li Q, Xia JC. A phase I clinical trial utilizing autologous tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma. *Oncotarget* 2015; **6**: 41339-41349 [PMID: 26515587 DOI: 10.18632/oncotarget.5463]

135 **Yu X**, Zhao H, Liu L, Cao S, Ren B, Zhang N, An X, Yu J, Li H, Ren X. A randomized phase II study of autologous cytokine-induced killer cells in treatment of hepatocellular carcinoma. *J Clin Immunol* 2014; **34**: 194-203 [PMID: 24337625 DOI: 10.1007/s10875-013-9976-0]

136 **Brentjens RJ**, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M, Sadelain M. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* 2013; **5**: 177ra38 [PMID: 23515080 DOI: 10.1126/scitranslmed.3005930]

137 **June CH**, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science* 2018; **359**: 1361-1365 [PMID: 29567707 DOI: 10.1126/science.aar6711]

138 **Baumhoer D**, Tornillo L, Stadlmann S, Roncalli M, Diamantis EK, Terracciano LM. Glypican 3 expression in human nonneoplastic, preneoplastic, and neoplastic tissues: a tissue microarray analysis of 4,387 tissue samples. *Am J Clin Pathol* 2008; **129**: 899-906 [PMID: 18480006 DOI: 10.1309/HCQWPWD50XHD2DW6]

139 **Gao H**, Li K, Tu H, Pan X, Jiang H, Shi B, Kong J, Wang H, Yang S, Gu J, Li Z. Development of T cells redirected to glypican-3 for the treatment of hepatocellular carcinoma. *Clin Cancer Res* 2014; **20**: 6418-6428 [PMID: 25320357 DOI: 10.1158/1078-0432.CCR-14-1170]

140 **Wang Y**, Chen M, Wu Z, Tong C, Dai H, Guo Y, Liu Y, Huang J, Lv H, Luo C, Feng KC, Yang QM, Li XL, Han W. CD133-directed CAR T cells for advanced metastasis malignancies: A phase I trial. *Oncoimmunology* 2018; **7**: e1440169 [PMID: 29900044 DOI: 10.1080/2162402X.2018.1440169]

141 **Bagley SJ**, Desai AS, Linette GP, June CH, O'Rourke DM. CAR T-cell therapy for glioblastoma: recent clinical advances and future challenges. *Neuro Oncol* 2018; **20**: 1429-1438 [PMID: 29509936 DOI: 10.1093/neuonc/noy032]

142 **Guo X**, Jiang H, Shi B, Zhou M, Zhang H, Shi Z, Du G, Luo H, Wu X, Wang Y, Sun R, Li Z. Disruption of PD-1 Enhanced the Anti-tumor Activity of Chimeric Antigen Receptor T Cells Against Hepatocellular Carcinoma. *Front Pharmacol* 2018; **9**: 1118 [PMID: 30327605 DOI: 10.3389/fphar.2018.01118]

143 **Yu M**, Luo H, Fan M, Wu X, Shi B, Di S, Liu Y, Pan Z, Jiang H, Li Z. Development of GPC3-Specific Chimeric Antigen Receptor-Engineered Natural Killer Cells for the Treatment of Hepatocellular Carcinoma. *Mol Ther* 2018; **26**: 366-378 [PMID: 29339014 DOI: 10.1016/j.ymthe.2017.12.012]

144 **Li Y**, Hermanson DL, Moriarity BS, Kaufman DS. Human iPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-tumor Activity. *Cell Stem Cell* 2018; **23**: 181-192.e5 [PMID: 30082067 DOI: 10.1016/j.stem.2018.06.002]

145 **Jiang W**, Zhang C, Tian Z, Zhang J. hIFN-α gene modification augments human natural killer cell line anti-human hepatocellular carcinoma function. *Gene Ther* 2013; **20**: 1062-1069 [PMID: 23759701 DOI: 10.1038/gt.2013.31]

146 **Jiang W**, Zhang C, Tian Z, Zhang J. hIL-15 gene-modified human natural killer cells (NKL-IL15) augments the anti-human hepatocellular carcinoma effect in vivo. *Immunobiology* 2014; **219**: 547-553 [PMID: 24721706 DOI: 10.1016/j.imbio.2014.03.007]

147 **Jiang W**, Zhang J, Tian Z. Functional characterization of interleukin-15 gene transduction into the human natural killer cell line NKL. *Cytotherapy* 2008; **10**: 265-274 [PMID: 18418772 DOI: 10.1080/14653240801965156]

148 **Dubensky TW**, Reed SG. Adjuvants for cancer vaccines. *Semin Immunol* 2010; **22**: 155-161 [PMID: 20488726 DOI: 10.1016/j.smim.2010.04.007]

149 **Coffman RL**, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity* 2010; **33**: 492-503 [PMID: 21029960 DOI: 10.1016/j.immuni.2010.10.002]

150 **Xu A**, Zhang L, Yuan J, Babikr F, Freywald A, Chibbar R, Moser M, Zhang W, Zhang B, Fu Z, Xiang J. TLR9 agonist enhances radiofrequency ablation-induced CTL responses, leading to the potent inhibition of primary tumor growth and lung metastasis. *Cell Mol Immunol* 2018 [PMID: 30467420 DOI: 10.1038/s41423-018-0184-y]

151 **Preiss S**, Thompson A, Chen X, Rodgers S, Markovska V, Desmond P, Visvanathan K, Li K, Locarnini S, Revill P. Characterization of the innate immune signalling pathways in hepatocyte cell lines. *J Viral Hepat* 2008; **15**: 888-900 [PMID: 18673429 DOI: 10.1111/j.1365-2893.2008.01001.x]

152 **Zhang Y**, Lin A, Zhang C, Tian Z, Zhang J. Phosphorothioate-modified CpG oligodeoxynucleotide (CpG ODN) induces apoptosis of human hepatocellular carcinoma cells independent of TLR9. *Cancer Immunol Immunother* 2014; **63**: 357-367 [PMID: 24452201 DOI: 10.1007/s00262-014-1518-y]

153 **Khvalevsky E**, Rivkin L, Rachmilewitz J, Galun E, Giladi H. TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. *J Cell Biochem* 2007; **100**: 1301-1312 [PMID: 17243100 DOI: 10.1002/jcb.21119]

154 **Yu P**, Cheng X, Guo J, Wang X. Toll-like receptors 2/4 agonists: a potential strategy for preventing invasion and metastasis of hepatocellular carcinoma. *Gut* 2010; **59**: 1447-8; author reply 1448-9 [PMID: 20675699 DOI: 10.1136/gut.2009.190835]

155 **Zhang Y**, Lin A, Sui Q, Zhang C, Tian Z, Zhang J. Phosphorothioate modification of the TLR9 ligand CpG ODN inhibits poly(I:C)-induced apoptosis of hepatocellular carcinoma by entry blockade. *Cancer Lett* 2014; **355**: 76-84 [PMID: 25224571 DOI: 10.1016/j.canlet.2014.09.013]

156 **Gardner A**, Ruffell B. Dendritic Cells and Cancer Immunity. *Trends Immunol* 2016; **37**: 855-865 [PMID: 27793569 DOI: 10.1016/j.it.2016.09.006]

157 **Butterfield LH**, Ribas A, Dissette VB, Lee Y, Yang JQ, De la Rocha P, Duran SD, Hernandez J, Seja E, Potter DM, McBride WH, Finn R, Glaspy JA, Economou JS. A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. *Clin Cancer Res* 2006; **12**: 2817-2825 [PMID: 16675576 DOI: 10.1158/1078-0432.CCR-05-2856]

158 **Tada F**, Abe M, Hirooka M, Ikeda Y, Hiasa Y, Lee Y, Jung NC, Lee WB, Lee HS, Bae YS, Onji M. Phase I/II study of immunotherapy using tumor antigen-pulsed dendritic cells in patients with hepatocellular carcinoma. *Int J Oncol* 2012; **41**: 1601-1609 [PMID: 22971679 DOI: 10.3892/ijo.2012.1626]

159 **El Ansary M**, Mogawer S, Elhamid SA, Alwakil S, Aboelkasem F, Sabaawy HE, Abdelhalim O. Immunotherapy by autologous dendritic cell vaccine in patients with advanced HCC. *J Cancer Res Clin Oncol* 2013; **139**: 39-48 [PMID: 22886490 DOI: 10.1007/s00432-012-1298-8]

160 **Chi KH**, Liu SJ, Li CP, Kuo HP, Wang YS, Chao Y, Hsieh SL. Combination of conformal radiotherapy

and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. *J Immunother* 2005; **28**: 129-135 [PMID: 15725956]

161 **Rao Q**, Zuo B, Lu Z, Gao X, You A, Wu C, Du Z, Yin H. Tumor-derived exosomes elicit tumor suppression in murine hepatocellular carcinoma models and humans in vitro. *Hepatology* 2016; **64**: 456-472 [PMID: 26990897 DOI: 10.1002/hep.28549]

162 **Lu Z**, Zuo B, Jing R, Gao X, Rao Q, Liu Z, Qi H, Guo H, Yin H. Dendritic cell-derived exosomes elicit tumor regression in autochthonous hepatocellular carcinoma mouse models. *J Hepatol* 2017; **67**: 739-748 [PMID: 28549917 DOI: 10.1016/j.jhep.2017.05.019]

163 **Zhang W**, Liu J, Wu Y, Xiao F, Wang Y, Wang R, Yang H, Wang G, Yang J, Deng H, Li J, Wen Y, Wei Y. Immunotherapy of hepatocellular carcinoma with a vaccine based on xenogeneic homologous alpha fetoprotein in mice. *Biochem Biophys Res Commun* 2008; **376**: 10-14 [PMID: 18725206 DOI: 10.1016/j.bbrc.2008.08.061]

164 **Han Q**, Wang Y, Pang M, Zhang J. STAT3-blocked whole-cell hepatoma vaccine induces cellular and humoral immune response against HCC. *J Exp Clin Cancer Res* 2017; **36**: 156 [PMID: 29115974 DOI: 10.1186/s13046-017-0623-0]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3168-3182

DOI: 10.3748/wjg.v25.i25.3168

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

MINIREVIEWS

Epidemiology of hepatitis E in South-East Europe in the "One Health" concept

Anna Mrzljak, Petra Dinjar-Kujundzic, Lorena Jemersic, Jelena Prpic, Ljubo Barbic, Vladimir Savic, Vladimir Stevanovic, Tatjana Vilibic-Cavlek

ORCID number: Anna Mrzljak (0000-0001-6270-2305); Petra Dinjar-Kujundzic (0000-0002-6505-0821); Lorena Jemersic (0000-0002-8379-4787); Jelena Prpic (0000-0001-7333-267X); Ljubo Barbic (0000-0002-5170-947X); Vladimir Savic (0000-0003-0398-5346); Vladimir Stevanovic (0000-0002-9572-8760); Tatjana Vilibic-Cavlek (0000-0002-1877-5547).

Author contributions: Mrzljak A and Dinjar-Kujundzic P made contributions to conception and design of the study, acquisition of the data, involved in drafting the manuscript. Jemersic L, Prpic J, Barbic L, Savic V and Stevanovic V made contributions in acquisition of the data, involved in drafting the manuscript. Vilibic-Cavlek T made contributions to design, involved in drafting and revising the manuscript critically. All authors read and approved the final manuscript.

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

Anna Mrzljak, Department of Medicine, Merkur University Hospital; School of Medicine, University of Zagreb, Zagreb 10000, Grad Zagreb, Croatia

Petra Dinjar-Kujundzic, Department of Medicine, Merkur University Hospital, Zagreb 10000, Grad Zagreb, Croatia

Lorena Jemersic, Jelena Prpic, Vladimir Savic, Croatian Veterinary Institute, Zagreb 10000, Grad Zagreb, Croatia

Ljubo Barbic, Vladimir Stevanovic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb 10000, Grad Zagreb, Croatia

Tatjana Vilibic-Cavlek, Department of Virology, Croatian Institute of Public Health; School of Medicine, University of Zagreb, Zagreb 10000, Grad Zagreb, Croatia

Corresponding author: Anna Mrzljak MD, PhD, Assistant Professor, Department of Medicine, Merkur University Hospital, Zajceva 19, Zagreb 10000, Grad Zagreb, Croatia.
anna.mrzljak@mef.hr
Telephone: +385-1-2431390
Fax: +385-1-2431393

Abstract

The significance of hepatitis E virus (HEV) as an important public health problem is rising. Until a decade ago, cases of HEV infection in Eur-ope were mainly confined to returning travelers, but nowadays, hepatitis E represents an emerging zoonotic infection in many European countries. The aim of this manuscript is to perform a systematic review of the published literature on hepatitis E distribution in humans, animals and environmental samples ("One Health" concept) in the South-Eastern European countries. Comparison of the available data showed that the anti-HEV seroprevalence in the South-Eastern Europe varies greatly, depending on the population studied, geographical area and methods used. The IgG seroprevalence rates in different population groups were found to be 1.1%-24.5% in Croatia, up to 20.9% in Bulgaria, 5.9-%17.1% in Romania, 15% in Serbia, up to 9.7% in Greece and 2%-9.7% in Albania. Among possible risk factors, older age was the most significant predictor for HEV seropositivity in most studies. Higher seroprevalence rates were found in animals. HEV IgG antibodies in domestic pigs were detected in 20%-54.5%, 29.2%-50%, 38.94%-50% and 31.1%-91.7% in Serbia, Bulgaria, Romania and Croatia, respectively. In wild boars seroprevalence rates were up to 10.3%, 30.3% and 31.1% in Romania, Slovenia and Croatia, respectively. A high HEV RNA prevalence in wild boars in some

Manuscript source: Invited manuscript

Received: March 11, 2019
Peer-review started: March 11, 2019
First decision: May 9, 2019
Revised: May 16, 2019
Accepted: May 31, 2019
Article in press: June 1, 2019
Published online: July 7, 2019

P-Reviewer: Komatsu H, Sergi C
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

countries (Croatia and Romania) indicated that wild boars may have a key role in the HEV epidemiology. There are very few data on HEV prevalence in environmental samples. HEV RNA was detected in 3.3% and 16.7% surface waters in Slovenia and Serbia, respectively. There is no evidence of HEV RNA in sewage systems in this region. The available data on genetic characterization show that human, animal and environmental HEV strains mainly belong to the genotype 3.

Key words: Hepatitis E virus; "One-Health"; Humans; Animals; Environment; South-East Europe

Core tip: In South-East Europe, the hepatitis E virus (HEV) prevalence as in other parts of Europe varies greatly, depending on the studied population, geographical area and methods used. Seroprevalence rates were found to be 0%-36% in humans and 10.3%-54.5% in animals. Human studies showed sporadic detection of HEV RNA in patients with acute hepatitis and in transplant population. HEV RNA was detected in up to 31.6% pigs and 16.7% environmental samples. Studies on phylogenetic characterization in human, animal and environmental samples showed that HEV strains from the south-eastern European countries mainly belong to the genotype 3.

Citation: Mrzljak A, Dinjar-Kujundzic P, Jemersic L, Prpic J, Barbic L, Savic V, Stevanovic V, Vilibic-Cavlek T. Epidemiology of hepatitis E in South-East Europe in the "One Health" concept. *World J Gastroenterol* 2019; 25(25): 3168-3182
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3168.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3168

INTRODUCTION

Hepatitis E represents an important public health problem in many parts of the world. The World Health Organization estimates that 20 million hepatitis E virus (HEV) infections occur worldwide leading to an estimated 3.3 million symptomatic cases and 44000 deaths related to hepatitis E. Until a decade ago, cases of HEV infection in Europe were mainly confined to travelers returning from endemic areas, whereas nowadays, hepatitis E is endemic in many European countries. It is estimated that 5%-15% of all acute hepatitis infections of unknown origin in Europe are caused by HEV[1]. The seroprevalence ranges from 0.6% to 52.5%, depending on the population group tested and geographical region. In addition to differences in seroprevalence rates between countries, there are also differences in HEV seropositivity within the same country[2]. Changes in HEV epidemiology could be explained by the confirmation of zoonotic nature of the disease. To date, three zoonotic HEV genotypes have been confirmed to infect humans. In addition to well-known HEV-3 and HEV-4 genotypes, a new genotype HEV-7 has also been recently described to infect humans[3,4]. Domestic pigs and wild boars represent the most important animal reservoirs for HEV-3 and HEV-4 worldwide with usually high seroprevalence rates ranging between 23%-100%[5]. However, zoonotic transmission of HEV has also been reported from some other animal reservoirs such as rabbits, deer and camels[3,6-8]. In addition, HEV must be considered as a food-borne pathogen as well, since food-borne cases of hepatitis E in humans are increasingly reported. Food-borne HEV-3 and HEV-4 infections due to consumption of undercooked meat and meat products from infected animal reservoirs have been repeatedly described[4,6,9-11]. Furthermore, HEV-3 has also been found in mollusks and human consumption of contaminated shellfish has been implicated as the cause of sporadic cases of acute hepatitis E[12,13]. Other types of food such as berry fruit have rarely been suspected to act as vehicles for HEV transmission after environmental contamination with animal feces[14,15].

In this manuscript, a bibliographic review of the literature on hepatitis E was performed with the aim of gathering the latest data regarding the prevalence, risk factors and HEV genotype distribution in the South-East Europe in the multi-disciplinary "One Health" concept (Tables 1-3).

Table 1 Prevalence of hepatitis E in different population groups

Country	Population	Sample size	Method	anti-HEV	HEV RNA (%)/genotype (subtype)	Ref.
Albania	Refugees in Greece (pregnant women)	500	EIA	2%[1]	NT	Malamitsi-Puchner *et al*[16]
	Refugees in Greece (adult population)	350	EIA/IB	4.85%[1]	NT	Dalekos *et al*[17]
	General population	ND	ND	IgG 9.7%	NT	Adhami *et al*[18]
	Thalassemic children	ND	ND	IgG 0%	NT	
	Patients with chronic liver disease	109	EIA	36.6%[1]	NT	Kondili *et al*[19]
	Patients with no apparent liver disease	190	EIA	12.1%[1]	NT	
Bulgaria	Patients with symptoms of acute hepatitis	806	EIA	IgM/IgG 2.48%	NT	Baymakova *et al*[23]
	Hospitalized patients with clinical symptoms of hepatitis and outpatients with laboratory data of liver dysfunction	325	EIA	IgM 13.2% IgG 20.9%	NT NT	Stoykova *et al*[25]
	Patients with acute hepatitis E (IgM positive)	105	RT-PCR	NT	G3 98% (3e 62%; 3f 24%; 3c 13%); G1 2%	Bruni *et al*[26]
	Patients conducting ambulatory examination due to various reasons	741	EIA	IgM/IgG 1.48% IgG 9.04%		Teoharov *et al*[27]
Croatia	Patients with clinical symptoms of hepatitis negative for HAV/HBV/HCV	504	EIA/IB/RT-PCR	IgM/IgG 10.7%	IgM positive 35.7%	Đaković Rode *et al*[31]
	HIV-infected patients	88	EIA/IB	IgG 1.1%	NT	
	Liver transplant recipients	242	EIA	IgG 24.5%	NT	Mrzljak *et al*[32]
	Alcohol abusers	56	EIA/IB	IgG 8.9%	NT	Vilibic-Cavlek *et al*[33]
	Patients with PTSD	35	EIA/IB	IgG 8.1%	NT	
	Injecting drug users	49	EIA/IB	IgG 6.1%	NT	
	Persons with risk sexual behaviour	37	EIA/IB	IgG 0%	NT	
	Forest workers	37	EIA/IB	IgG 8.1%	NT	Jeličić *et al*[34]
	Healthcare workers	50	EIA/IB	IgG 2.0%	NT	Jeličić *et al*[34]
	Pregnant women	68	EIA/IB	IgG 2.9%	NT	Jeličić *et al*[35]
	Hunters	25	EIA/IB	IgG 4.0%	NT	Jeličić *et al*[35]
	General population	87	EIA/IB	IgG 3.4%	NT	Jeličić *et al*[34]
	Blood donors	1036	EIA/IB EIA	IgM 1.7% IgG 20.3%	NT	Miletic Lovric *et al*[36]
Greece	Transplant patients	76	RT-PCR	NT	1 positive/1.3% G3	Sinakos *et al*[45]
	Transfusion dependent thalassaemia	96	EIA	IgG 0%	0%	Klonizakis *et al*[44]
	Blood donors	1 200	EIA	IgG 2.9%	NT	Zervou *et al*[49]
	HIV patients	243	EIA	IgG 7.3%	NT	Politou *et al*[48]
	Blood donors (South Greece)	265	EIA	IgG 9.43	NT	Pittaras *et al*[47]
	Patients after open-heart surgery	204	EIA	IgG 5.4%	NT	Zervou *et al*[43]

	Patients on haemodialysis	351	EIA	IgG 4.8%	NT	Stefanidis *et al*[46]
	Non-A,-B hepatitis patients	198	EIA/RT-PCR	IgG 7.6% IgM 1%	1 positive	Psichogiou *et al*[42]
	Healthy controls	316	EIA/RT-PCR	IgM 0%/IgG 2.2%	NT	
Epirus region	Healthy blood donors	2636	EIA/IB	IgG 0.23%	NT	Dalekos *et al*[17]
	Refugees from southern Albania	350	EIA/IB	IgG 4.85%	NT	
	Children	165	EIA/IB	IgG 0%	NT	
	Injecting drug users	65	EIA/IB	IgG 0%	NT	
	Multiply transfused patients	62	EIA/IB	IgG 0%	NT	
	Patients with chronic viral hepatitis	75	EIA/IB	IgG 5.30%	NT	
	Chronic haemodialysis patients	149	EIA/IB	IgG 1.34%	NT	
Agrinion area	Healthy blood donors	380	EIA/IB	IgG 0.53%	NT	
	Chronic hemodialysis patients	62	EIA/IB	IgG 9.7%	NT	
Kosovo	Kosovar refugees	104	EIA/RT-PCR	IgM 7.7%	0%	Rey *et al*[56]
Montenegro	Patients with acute viral hepatitis	400	EIA	IgM 6%	NT	Terzić *et al*[57]
Romania	Patients with hepatitis B or C	25	EIA	IgG 12%	NT	Anita *et al*[59]
	Students	40	EIA	IgG 12.5%	NT	Voiculescu *et al*[60]
	Doctors and nurses	93	EIA	IgG 13.98%	NT	Voiculescu *et al*[60]
	Persons undergoing routine hematological tests	148	EIA	IgG 14.86%	NT	Anita *et al*[61]
	General population	67	EIA	IgG 5.9%	NT	Savuta G *et al*[62]
Serbia	Blood donors	200	EIA RT-PCR	IgG 15%	0%	Petrović *et al*[66]
Slovenia	Acute/recent hepatitis E (IgM antibodies)	10	RT-PCR	NT	3/10 (G3e and G1)	Steyer *et al*[74]

[1]Total anti-hepatitis E virus antibodies. HEV: Hepatitis E virus; EIA: Enzyme immunoassay; IB: Immunoblot; RT-PCR: Reverse-transcriptase polymerase chain reaction; NT: Not tested; ND: No data.

Search strategy and selection criteria

A literature search was conducted in the following electronic databases: PubMed, Web of Science, Medline and Scopus along with hand-searching references of key articles and a ResearchGate and Google search with no limitations placed on year of publication and language restriction. The search included articles in peer-reviewed journals and grey literature. Books, dissertations, review articles and unpublished reports were excluded. In addition, non-relevant studies by the review of the abstracts were also excluded. Keywords searched were: Hepatitis E virus, epidemiology, human, animals, pigs, wild boars, environment, waste water, vegetables, seroprevalence, risk factors, HEV RNA, genotype. Once a comprehensive list of abstracts has been retrieved and reviewed, any studies appearing to meet inclusion criteria were reviewed in full.

Out of 11 south-eastern European countries, data for Albania, Bulgaria, Croatia, Greece, Kosovo, Montenegro, Romania, Serbia and Slovenia are available and presented in this review (Figure 1). So far, there have been no data published on HEV for Bosnia and Herzegovina and the Republic of North Macedonia.

ALBANIA

There are few studies on hepatitis E in Albania published in 1990s and 2000s. In the

Table 2 Prevalence of hepatitis E in different animal species

Country	Population	Sample size	Method	anti-HEV	HEV RNA (%)/genotype (subtype)	Ref.
Bulgaria	Piglets	44	EIA	IgG 50%	NT	Pishmisheva *et al*[28]
	Fattening pigs	41	EIA	IgG 29.2%	NT	
Croatia	Domestic pigs	848	RT-PCR	NT	24.5% (G3)	Prpić *et al*[40]
	Wild boars	536	RT-PCR	NT	12.3% (G3)	
	Molluscs (mussels, oysters)	538	RT-PCR	NA	0%	
	Cattle	32	RT-PCR	NT	0%	
	Red fox	50	RT-PCR	NT	0%	
	Deer	320	RT-PCR	NT	0%	
	Muflons	12	RT-PCR	NT	0%	
	Ferrets	8	RT-PCR	NT	0%	
	Martens	10	RT-PCR	NT	0%	
	Pigs (serum samples)	60	EIA/RT-PCR	IgG 91.7%	13.3% 8.1%	Lipej *et al*[39]
	Pigs (bile samples)	60	RT-PCR	NA	8.1%	
	Pigs, domestic	1424	EIA/RT-PCR	IgG 32.94%	0%	Jemeršić *et al*[41]
	Wild boars	1000	EIA/RT-PCR	IgG 31.10%	11.33%	
Greece	Mussels	51	RT-PCR	NT	0%	Diez-Valcarce *et al*[55]
	Black rats/Norway rats	20	RT-PCR	NT	10%	Ryll *et al*[50]
Romania	Farm pigs	50	EIA/IB	IgG 50%	NT	Savuta *et al*[62]
	Backyard pigs	95	EIA/IB	IgG 38.94%		
	Backyard pigs	112	EIA	IgG 49.27%	NT	Savuta *et al*[58]
	Pigs (2-4 mo) stool samples	19	RT-PCR	NT	31.58% (G3)	Anita *et al*[61]
	Wild boars	52	EIA	9.61%[1]	NT	Porea *et al*[63]
	Wild boars	68	EIA	IgG 10.29%	NT	Porea *et al*[63]
	Wild boars	50	RT-PCR	NT	18% (G3)	Porea *et al*[65]
Serbia	Farm pigs (pooled stool samples)	30	RT-PCR	NT	30%	Petrovic *et al*[67]
	Farm pigs (polled tissue samples)	20	RT-PCR	NT	45%	
	Backyard pigs (pooled tissue samples)	15	RT-PCR	NT	0%	
	Wild boars (pooled stool samples)	10	RT-PCR	NT	0%	
	Backyard pigs	315	EIA	IgG 34.6%	NT	Lupulovic *et al*[68]
	Pigs (liver samples)	50	NT	NT	26%	Savic *et al*[69]
	Fattening pigs	95	NT	NT	7.37%	Petrović *et al*[71]
	Piglets (8 wk)	50	NT	NT	64%	
	Pigs (blood samples)	55	EIA	IgG 54.54%	NT	Lupulovic *et al*[70]
	Pigs (meat juice samples)	55	EIA	IgG 20%	NT	
Slovenia	Black rats/Norway rats	17/1	RT-PCR	NT	0%	Ryll *et al*[50]
	Domestic pigs	85	RT-PCR	NT	20.3%	Steyer *et al*[74]
	Suckling pigs (0-3 wk)	38	RT-PCR	NT	5.3%	
	Weanling pigs (3-10 wk)	21	RT-PCR	NT	28.6%	
	Fattening pigs (> 10 wk)	26	RT-PCR	NT	26.9%	
	Wild boars	288	EIA/RT-PCR	30.21%[1]	0.35%	Žele *et al*[75]
	Pigs (stool samples)	811	RT-PCR	NT	5.4%	Raspor Lainšček *et al*[76]
	Pigs (bile samples)	811	RT-PCR	NT	4.9%	
	Pigs (liver samples)	811	RT-PCR	NT	5.3% (G3a, 3b, 3c, 3e)	

[1]Total anti-hepatitis E virus antibodies. HEV: Hepatitis E virus; EIA: Enzyme immunoassay; IB: Immunoblot; RT-PCR: Reverse-transcriptase polymerase chain reaction; NT: Not tested; NA: Not applicable.

studies conducted among Albanian refugees in Greece, the anti-HEV prevalence was 2% among pregnant women[16] and 4.85% in the adult population from southern Albania[17]. In 2000s, acute HEV in Albania made up 2.4% of all acute viral hepatitis cases. HEV infections occurred more frequently in women (female to male ratio 2:1),

Table 3 Prevalence of hepatitis E virus RNA in different environmental samples

Country	Sample	Sample size	HEV RNA (%)/ genotype (subtype)	Ref.
Greece	Vegetable leafy greens	Pooled samples	4.76%[1] 3.2%[1]	Kokkinos *et al*[51]
	Sewage	48	0%	Kokkinos *et al*[54]
	Sewage	5	0%	Clemente-Casares *et al*[53]
Serbia	Vegetable leafy greens	Pooled samples	4.76%[1] 3.2%[1]	Kokkinos *et al*[51]
	Berry fruits	Pooled samples	2.5%[1]	Maunula *et al*[15]
	Surface waters	60	16.67%	Lazić *et al*[73]
	Urban sewage	6	0%	
Slovenia	Waste water treatment plant	12	0%	Steyer *et al*[77]
	Swabs from the different site on the slaughter line	62	3.2% (G3)	Raspor Lainšček *et al*[76]
	Minced meat	22	0%	
	Bratwurst	30	0%	
	Surface water	60	3.3% (G3)	Steyer *et al*[74]

[1]Pooled samples. HEV: Hepatitis E virus; EIA: Enzyme immunoassay; IB: Immunoblot; NT: Not tested.

as well as in adults over 35 years (73.3%). In the general population, the prevalence of anti-HEV antibodies was 9.7% and increased progressively with age, from 1.2% in children less than 9 years to 17.7% in persons older than 60 years. There was no difference in the prevalence between the general population and patients with chronic liver disease (10.5%). The prevalence of anti-HEV antibodies in pregnant women was lower than in the general population (1.1%). No positive cases were found among children with thalassemia receiving multiple transfusions[18]. In contrast, in the other Albanian case-control study, HEV antibodies were found in 36.6% patients with chronic liver disease compared to 12.1% in patients with no apparent liver disease. In the univariate analysis, anti-HEV prevalence was found to be associated with the age (> 50 years), lower education status (≤ 8 years of formal education) and positivity for hepatitis B surface antigen (HBsAg), while no association was observed in people who lived in villages and/or those who were occupationally involved with handling animals. In the multivariate analysis, significant associations remained for age and HBsAg positivity[19].

Since there are no recent data on HEV infection in Albania, the burden of diseases caused by HEV is not known. Moreover, no data on the role of animals in the epidemiology of HEV infections in Albania is currently available. Therefore, more research should be done to evaluate a local HEV epidemiology in Albania.

BULGARIA

In Bulgaria, the first human cases of hepatitis E were reported in 1995[20]. Several studies reported cases and hospitalized patients with acute hepatitis E[21,22]. A retrospective study of acute viral hepatitis performed in Sofia (2004-2012) found anti-HEV IgM/IgG antibodies in 2.48% patients[23]. HEV infection more frequently affected males (61%-69%), with the highest incidence in persons over 50 years of age[23,24]. Another study in the North-Eastern Bulgaria (2012-2016) analyzed the prevalence of HEV infection in hospitalized patients with clinical symptoms of acute hepatitis and outpatients with laboratory data of liver dysfunction. Acute infection was documented in 13.2% patients, while 20.9% patients showed only IgG antibodies indicating past HEV infection[25]. A study conducted from 2013 to 2015 analyzed HEV genotypes in patients with acute hepatitis E. Phylogenetic analysis showed HEV-3 in 98% and HEV-1 in 2% of cases. Subtyping of HEV-3 sequences showed 3e (62%), 3f (24%) and 3c (13%) subtypes. There were differences in the geographical distribution of genotypes. Subtypes 3f and 3c were scattered throughout the country, while 3e subtype was restricted to the South-West area[26]. A seroepidemiological study conducted in the general Bulgarian population from Plovdiv region during 2012-2013 found anti-HEV IgG seroprevalence of 9.04%, while 1.48% participants showed both IgM and IgG antibodies. Seropositivity did not differ significantly between males (9.87%) and females (8.47%), but increased with the age from 3.53% in the age group 1-9 years to 19.23% in the group over 60 years[27].

In 2016, serum samples from clinically healthy pigs from five industrial farms in two districts in South Bulgaria were collected and tested for the presence of anti-HEV

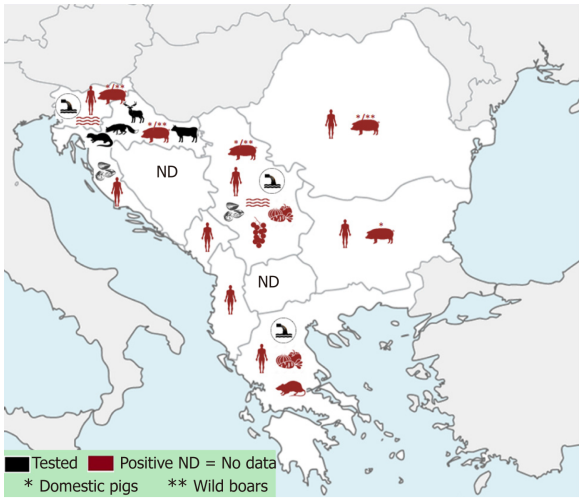


Figure 1 Data on hepatitis E prevalence in South-East Europe in humans, animals and environmental samples ("One health" concept).

antibodies. The overall HEV seroprevalence was 40.0% with 50.0% seropositive piglets and 29.2% fattening pigs. Seropositivity differed significantly among regions from 0% (Peshtera) to 100% (Nova Zagora)[28]. In a recently published study, genetic diversity and the phylogenetic relationships among different strains of human HEV genotype 3 were analyzed to estimate the date of origin and the demographic history of HEV epidemic in Bulgaria. The root of the Bayesian tree showed at least two different epidemic entrances for HEV genotype 3e strains[29].

Seroprevalence data and the high prevalence of clinical cases registered indicate that HEV infection in Bulgaria is endemic. Phylogenetic analysis showed that human HEV isolates in 98% of cases belonged to HEV 3 genotype.

CROATIA

In Croatia, the first autochthonous human case of hepatitis E was reported in 2012[30]. A study conducted from 2011 to 2013 in patients with clinical symptoms of hepatitis and elevated liver enzymes who tested negative for hepatitis A-C, detected HEV IgM/IgG antibodies in 10.7% patients. Among IgM positive patients, 35.7% were positive for HEV RNA[31].

Several studies analyzed the seroprevalence of HEV infection in different population groups in Croatia. HEV IgG antibodies were detected in 24.5% liver transplant recipients[32], 8.9% alcohol abusers[33], 8.6% patients with war-related posttraumatic stress disorder[33], 8.1% forest workers[34], 6.1% injecting drug users[33], 4.0% hunters[35], 3.4% general population[34], 2.9% pregnant women[35], 2.0% healthcare workers[34] and 1.1% HIV-infected patients[31]. No person with high-risk sexual behavior was HEV seropositive[33]. One study conducted among blood donors in 2016 showed a high IgG seroprevalence rate of 20.3% with 1.7% IgM positive. None of IgM positive samples was positive for HEV RNA[36]. HEV IgG positivity increased significantly with the age[33,37]. In addition, seroprevalence rates were higher in residents of suburban and rural areas compared with residents of urban areas, sub-jects living in families with more household members, persons who use wells as a source of drinking water[33] and those who use a sewage system connected to a septic tank[37]. Gender, marital status, educational level, history of blood transfusions, surgical procedures, tattooing and traveling were not associated with HEV ser-opositivity[33]. In 2018, the first documented case of HEV induced acute-on-chronic liver failure was reported with severe clinical course considered for liver tran-splantation. The patient was treated with ribavirin and subsequently recovered[38].

Several studies addressed the HEV distribution in domestic animals and wildlife in Croatia[39,40]. During 2009-2010, a comprehensive survey was carried out based on HEV RNA detection in blood, spleen and liver samples originating from different domestic and wild animals from all Croatian counties. Furthermore, digestive gland samples from molluscs were also analyzed. A high HEV RNA prevalence was found in domestic pigs (24.5%) and wild boars (12.3%), whereas cattle, molluscs, ruminant and carnivore wildlife samples tested negative. Molecular characterization confirmed the phylogenetic clustering of the obtained sequences into HEV genotype 3[40]. In 2012, the

epidemiology of naturally occurring hepatitis E was investigated in swine herds from three large pig farms in continental Croatia. Nearly all animals (91.7%) tested seropositive for HEV. In addition, active infection was detected in all age groups by detection of HEV RNA in 13.3% serum samples and 8.1% bile samples[39]. In 2016, the overall HEV seroprevalence was shown to be 32.94% (range 8.33%-60.00%) in domestic pigs from 11 counties and 31.10% (range 7.70%-50.60%) in wild boars from six Croatian counties. While no positive HEV RNA samples were detected in domestic pigs, 11.33% seropositive wild boars were found to be HEV RNA positive indicating that wild boars may have a key role in HEV epidemiology[41].

Data on genetic characterization of HEV are available only for animal species, confirming genotype 3 in pigs and wild boars. It is important to note a relatively small sample size of some tested population groups in humans. Therefore, studies on larger samples as well as species comparison studies on HEV genotypes are needed to obtain better insight into HEV epidemiology/molecular epidemiology in Croatia.

GREECE

In 1995, autochthonous hepatitis E virus was documented in Greece. Several studies investigated HEV infection in various population groups in different regions in Greece. A study conducted among patients with clinical signs of hepatitis showed a significantly higher anti-HEV IgG seroprevalence among acute non-A, non-B hepatitis patients (7.6%) compared to healthy controls (2.2%). Acute HEV infection (IgM antibodies) was confirmed in 1.0% of acute non-A, non-B hepatitis patients, while one patient was HEV RNA positive. None of anti-HEV IgM positive patients reported any possible risk factors[42].

In the north-western Greece (Epirus and Agrinion region), a very low anti-HEV IgG prevalence was reported in various populations. In Epirus region, none of different population groups such as children, injecting drug users or multiply transfused patients tested positive for HEV antibodies. Seroprevalence in healthy blood donors was only 0.23%. In contrast, higher anti-HEV prevalence was found among patients with chronic viral hepatitis (5.3%) and patients on hemodialysis (HD) (1.34%). Even higher anti-HEV prevalence was found among HD patients in the Agrinion region (9.7%). No association between the seroprevalence and duration of hemodialysis, seropositivity for hepatitis B or C, history of hepatitis, increased alanine aminotransferase, renal transplantation, history of transfusion or number of units transfused was detected[17]. Another study from the Epirus region (Ioannina) analyzed the risk of blood borne HEV transmission among patients after open-heart surgery who had received more than three blood units perioperatively. Past HEV infections were documented in 5.2% of surgical patients and 0% of healthy controls[43].

Another study from northern Greece analyzed the presence of antibodies and HEV RNA in transfusion dependent thalassaemia patients. The study confirmed no past or acute HEV infection in this multi-transfused population[44].

The most recent data from northern Greece (Thessaloniki) found that 1.3% of liver transplant patients were positive for HEV RNA. Phylogenetic analysis showed that the sequences clustered into the HEV genotype 3 clade. The only patient that tested RNA positive experienced acute hepatitis flare without progression into chronic form. The HEV RNA prevalence in the Greek transplant population (1.3%) corresponds with previously reported data in immunocompromised patients from other European countries[45].

Study from the semi-rural region of central Greece (Thessalia region) on HD patients in three different HD units found a total seroprevalence rate of 4.8% (varying from 1.8%-9.8% according to the unit). The highest anti-HEV prevalence was found in Karditsa unit (9.8%), which finding the authors assume to have been a result of a local infection in the past. Risk factors for HEV seropositivity (age, sex, duration of HD, hepatitis B or C virus infection markers, previously elevated aminotransferase levels or history of transfusion) were not identified[46].

Data from southern Greece showed even higher seroprevalence rates. In 2014, a study among blood donors from urban population of Athens demonstrated a seroprevalence of 9.43%[47]. Furthermore, a study on HIV patients in Athens indicated 7.3% anti-HEV IgG seroprevalence[48].

A recent study from 11 Blood Services throughout Greece revealed 2.9% and 3.6% of anti-HEV IgG seroprevalence among blood donors and multi-transfused patients, respectively. The highest seroprevalence rate (13.3%) had male blood donors from Heraklion, Crete. The seroprevalence was higher in persons older than 50 years (5.9%) compared to younger group (1.8%)[49].

There is only one study on HEV infection among animals in Greece. The animals

tested included Norway rats (*Rattus norvegicus*) and Black rats (*R. rattus*) from northern Greece, with the total prevalence of 10% in both species[50].

HEV was reported in a leafy green vegetable supply chain in Greece, showing that HEV RNA was found in 4.76% and 3.2% samples from the primary production and point-of-sale phases, respectively[51]. Later, the same authors investigated the presence of HEV in irrigation water samples used for the leafy green vegetables production and showed that one out of 20 samples tested positive[52]. The sample which was found HEV positive was a groundwater sample collected from the depth of 100 m, indicating well contamination unlikely[51].

Further two studies tested sewage samples from Patras (western Greece) for the presence of HEV. HEV RNA was not found in any of the samples[53,54]. Likewise, commercial mussels tested at the retail in Greece showed no samples positive for HEV RNA[55].

In conclusion, higher seroprevalence rates were detected in southern parts of Greece, the highest so far being in Crete, in contrary to the seroprevalence rates in the northern regions. Moreover, the prevalence of anti-HEV in risk populations (transplant, HIV, HD patients) was higher than in healthy blood donors. Phylogenetic analysis of human HEV isolates in Greece showed that the sequences clustered into the HEV genotype 3 clade. Further researches are needed, especially in animals and environmental samples in individual regions in Greece to gain a better insight in local HEV epidemiology.

KOSOVO

An outbreak of acute hepatitis E occurred among the Kosovar refugees in July 1999, after the return to their country. Several field surveys were undertaken as well as a serological study in patients with symptoms of hepatitis to assess the possible risk factors for HEV infection. A higher incidence of HEV infection was found among well-water consumers compared to those drinking network waters. An acute HEV infection (anti-HEV IgM positive) was found in 7.7% of refugees that had been tested. Only four persons had clinical symptoms, while the others were asymptomatic. There are no other published data on HEV prevalence/seroprevalence in Kosovo[56].

MONTENEGRO

The first available investigation about HEV infection in Montenegro was published in 2009. Data included hospitalized and out-patients with acute viral hepatitis collected from 2000 to 2007, showing that 6% of patients had acute hepatitis E. Although a higher seropositivity was found in males (62.5%) than in females (37.5%), the difference was not statistically significant. Epidemiological data showed that the majority of patients had never travelled out of the country, indicating an autochthonous HEV infection in Montenegro. An asymptomatic form of the disease was serologically confirmed in 7/24 patients, whereas the mild or short course of subclinical disease in 5/7 patients. No one patient had severe, fulminant or chronic course of disease[57].

ROMANIA

There are several studies on HEV infection in humans in Romania. The earliest human studies analyzed anti-HEV IgG prevalence in eastern Romania. The seroprevalence rate of 5.9% was found in the general population[58] and of 12% among patients with hepatitis B or C[59].

A study from 2010 analyzed the prevalence rates in low risk population groups in South and South-Eastern Romania. The seroprevalence of anti-HEV IgG among students (mean age: 22.91 ± 0.56 years) was 12.5%, whereas among doctors and nurses (mean age: 36.71 ± 8.88 years) was 13.98%[60].

A following study from the North-Eastern Romania among persons undergoing routine haematological tests with no signs of hepatitis showed anti-HEV IgG seroprevalence of 17.1% and 12.82% in 2011 and 2012, respectively. In 2011, the youngest age group (9-20 years) tested negative, whereas as older age groups (> 20 and > 40 years) had seroprevalence of 28.6% and 12.9%, respectively. In 2012, the youngest age group (18-45 years) had seroprevalence of 6.25%, whereas the older groups had the seroprevalence up to 28%. These results indicate that HEV infections in this region mainly affect middle-aged adults. Moreover, anti-HEV IgG was more prevalent in

women (15.78%) than in men (10%)[61].

One of the earliest animal studies investigated swine population for the presence of anti-HEV IgG in farm and backyard pigs. The immunoblot results for the backyard pigs showed seroprevalence rates of 50%[58,62] and for the farm pigs of 38.94%[62].

A more recent study tested fecal samples from five swine farms (pigs aged between 2 and 4 mo) for HEV RNA, and 6 out of 19 samples tested positive. Phylogenetic analysis showed that Romanian swine isolates grouped into genotype 3 and were closely related to the swine and human HEV isolates identified in other European countries[61]. In 2015 and 2016, serological studies investigated HEV prevalence in wild boars from eastern Romania. The seroprevalence rates were 9.61% (5/52)[63] and 10.29% (7/68), respectively[64]. A recent study on molecular detection of HEV in liver and spleen samples from wild boars aged > 1 year from eastern Romania showed an overall prevalence of 18%. All isolates belonged to the genotype 3 subtypes 3a and 3h[65].

Studies in humans are scarce and new data are needed to define the seroprevalence of HEV infection. Studies conducted in animal species show high seroprevalence rates. Phylogenetic analyses show that Romanian swine and wild boar isolates grouped into genotype 3.

SERBIA

There is only one published study on the HEV seroprevalence in humans in Serbia. The study was conducted among volunteer blood donors aged 19 to 65 years (average age 39.3 years), of whom 15% tested positive for anti-HEV IgG. No significant difference in anti-HEV IgG seropositivity was found between men and women (14.6% and 16.7%, respectively). HEV seroprevalence increased with age, as higher rates were recorded in subjects older than 51 years (21.5%) when compared with younger age groups (< 50 years or < 30 years)[66].

Several studies on HEV prevalence were conducted in Serbia in different animal species. The first study on HEV in swine population in 2007, tested pooled swine stool and tissue samples (spleen, mesenteric lymph node and liver). Thirty percent of stool and 45% of tissue samples tested HEV positive. HEV RNA was detected in four out of five pig farms examined. Simultaneously, all samples from backyard pigs and wild boars were negative[67]. Further studies also confirmed the high prevalence of HEV among pigs in Serbia. The first HEV serology testing in pigs was done on 315 serum samples collected from 3-4 mo old backyard pigs in 63 herds from 28 towns and villages of four different districts in northern Serbia (Vojvodina province), demonstrating seroprevalence rate of 34.6%. The prevalence of anti-HEV antibodies varied widely between municipalities (range 16.7%-75.0%) and herds (range (0-100%)[68].

Furthermore, a study in 2010 analyzed liver tissue samples from 50 dead farm pigs aged 7 to 15 wk which died on pig farms from different regions in Serbia, and detected HEV RNA in 13 of samples (26%)[69]. In 2013, a study on 55 serum samples and meat juice samples collected from three slaughterhouses were analyzed for the presence of anti-HEV IgG antibodies. The mean seroprevalence in the pig serum and meat juice samples was 54.54% and 20%, re-spectively[70]. In addition, stool, liver, bile and meat samples from 145 animals (95 fatteners and 50 eight weeks piglets) collected on during slaughter were tested for HEV RNA. Among fatteners, HEV has been detected in 7.37% of the stool samples. In piglets, HEV RNA prevalence was high and detected in 54%, 26%, 16% and 10% samples of stool, bile, liver and meat, respectively[71]. The presence of HEV IgG and HEV RNA was examined in 201 blood and 298 liver samples from wild boars culled during hunting season from January 2010 until February 2011. The samples were collected from 27 hunting grounds located on the territory of 7 counties of the country. The overall seroprevalence rate was 34.33%. HEV RNA prevalence was 9.40% with marked regional differences. A high proportion of adult wild sow and wild boars were found positive for HEV RNA[72].

In addition to animal studies, there are few data regarding the environment reporting the contamination of vegetable supply chain in Serbia. Namely, HEV RNA was found in 4.76 % and 3.2% of samples of leafy green vegetable from the primary production and point-of-sale phases, respectively[51]. Another report also documented the presence of HEV RNA in frozen raspberries (2.6%)[15]. Lately, HEV RNA has also been detected in surface waters in northern Serbia (Vojvodina Province) where 16.67% of samples tested positive for HEV RNA, during summer sampling occasion, whereas none of the tested urban sewage systems tested positive[73].

In Serbia, different population groups should be studied in future in order to reflect

local epidemiology, modes of transmission and risk factors for HEV infection. Animal and environmental studies on larger samples as well as studies on HEV genotyping are needed to obtain better insight into HEV epidemiology.

SLOVENIA

Human studies on HEV infection in Slovenia are scarce. Steyer *et al*[74] tested 10 serum samples of patients with the diagnosis of acute/recent hepatitis E based on detection of HEV IgM antibodies, and three samples tested HEV RNA positive. One sample was typed as genotype 3, clustered in the lineage "e" and the second was genotype 1 strain. Slovenian human HEV strains were not related to porcine HEV strains identified in this study[74].

There are several studies in different animal species in Slovenia. In 2011[74], 20.3% stool samples of domestic pigs were positive for HEV RNA, of which 5.3%, 28.6% and 26.9% were from suckling, weanling and fattening pigs, respectively. All HEV strains were analyzed at 5′ ORF1 and 5′ ORF2 regions and both genome regions confirmed that Slovenian HEV strains represent a distinct genotype 3 lineage. All but one HEV strains detected in pigs in Slovenia represent a monophyletic branch in phylogenetic trees, with a high degree of sequence identity. One human HEV strain belonged to genotype 1 and two to genotype 3 but did not match the new genotype 3 lineage detected in Slovenian pig herds.

HEV RNA was tested in Norway rat and Black rats from one site close to Ljubljana and none of the rats tested HEV-RNA positive[50]. The HEV presence in the wild boar population in Slovenia was first documented in 2016, showing HEV antibodies in 30.2% of animals tested, whereas HEV RNA was detected in only one sample (1/288)[75].

Recently, the possibility of HEV entering into the food supply chain was investigated in a large study analyzing pigs entering a slaughterhouse. The study covered three different age groups and three different samples (feces, bile and liver), showing the overall HEV RNA (sample) prevalence of 5.4%, 4.9% and 5.3%, respectively[76]. In the group of three months old pigs, 13.7% of feces, 13.0% of bile and 2.1% of liver samples were HEV RNA positive. The youngest group originating from Slovenia (none imported), was proven to be a group for highest risk for HEV infection. In the group of six months old pigs (imported from Austria), only one liver and one bile sample out of 400 tested positive. In the category of sows, no positive samples were found. The same study analyzed swabs collected at three different sites on the slaughter line (the hooks that were used to hang the liver and the lung, the containers in which livers were stored and the hooks for hanging carcasses in the cooling room). Of 62 swab samples, two were positive (a swab from the hook that carried liver and one from the liver container). However, all minced meat and bratwurst samples from these studies tested HEV RNA negative[76]. The phylogenetic analysis revealed that detected strains were clustered into subtypes 3a, 3b, 3c and 3e.

Further environmental studies investigated surface water and waste water treatment plant. Out of 60 surface water samples tested throughout the country, only two (3.3%) were HEV RNA positive, one of them in the near vicinity of a pig farm. HEV sequences detected in the surface water belonged to genotype 3[74]. In another study, the waste water treatment plant samples were collected from the effluent on a monthly basis from January to December 2012 and tested for HEV RNA. No HEV RNA was detected during the whole length of the study, neither before nor after the concentration step[77].

There are several studies in different animal species showing high prevalence of HEV RNA in Slovenia, with the genotype 3 being the most common. On the other hand, studies in humans are lacking.

CONCLUSION

In the South-East Europe, anti-HEV seroprevalence as in other parts of Europe varies greatly, depending on the population studied, geographical area and assays used for the detection. Studies on HEV RNA detection and phylogenetic characterization in human samples showed that human HEV strains from the South-East European countries mainly belong to the genotype 3. Detection of the same genotype in animals as well as in the environment emphasizes the need of the multidisciplinary collaboration ("One Health" approach) in the surveillance and control of this emerging infection.

Although significant progress in HEV epidemiology has been made in the past

decade, many important questions remain[9]. The origin of HEV is still largely unknown. Swine and wild boars were proven to be the primary natural HEV reservoir, but recent studies indicate that rabbits may serve as an additional reservoir of HEV[78]. Some hepatitis E cases observed in immunocompromised patients clustered with rabbit HEV strains confirming their contribution to zoonotic HEV transmission[79,80]. It is still unclear whether HEV strains present in other animals can cross the species barrier and infect humans. However, it is likely that the known host range of HEV has increased and novel strains continue to be identified. Transmission of HEV-3 from deer to humans has been described, although deer most probably undergoes spillover infections from wild boars, rather than being a natural HEV reservoir[7]. It seems that recently described new HEV-7 has been widely distributed in dromedary camels from the Middle East[3,8], but was also detected in an immunocompromised transplant patient who regularly consumed camel milk and meat[4]. While HEV transmission by breast milk was confirmed in humans[81], a recent study from China indicates that viral RNA of HEV-4 can be excreted by cow milk[82]. Although cattle have been rarely described to be infected with HEV, these findings implicate possible HEV transmission through milk or milk products. Human contacts with other animals, especially pet animals should be investigated in future for better understanding of HEV epidemiology. Rabbits are commonly farmed in many countries for meat consumption and fur production but also as pet and possible transmission from pet house rabbits to humans should be considered as another possible transmission route[83]. Infection of dogs and cats with HEV is confirmed serologically[84], but their importance in HEV epidemiology need to be further investigated.

Although HEV infection is not an economically important pig disease, development of a vaccine against the zoonotic genotypes 3 and 4 and vaccination of swine should be considered as a possible public health measure. Such vaccine should also be useful for high-risk populations such as organ transplant recipients since the majority of the chronic hepatitis E are caused by the zoonotic HEV-3[9].

REFERENCES

1 **World Health Organization**. Hepatitis E. Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-e

2 **Hartl J**, Otto B, Madden RG, Webb G, Woolson KL, Kriston L, Vettorazzi E, Lohse AW, Dalton HR, Pischke S. Hepatitis E Seroprevalence in Europe: A Meta-Analysis. *Viruses* 2016; 8 [PMID: 27509518 DOI: 10.3390/v8080211]

3 **Woo PC**, Lau SK, Teng JL, Tsang AK, Joseph M, Wong EY, Tang Y, Sivakumar S, Xie J, Bai R, Wernery R, Wernery U, Yuen KY. New hepatitis E virus genotype in camels, the Middle East. *Emerg Infect Dis* 2014; **20**: 1044-1048 [PMID: 24856611 DOI: 10.3201/eid2006.140140]

4 **Lee GH**, Tan BH, Teo EC, Lim SG, Dan YY, Wee A, Aw PP, Zhu Y, Hibberd ML, Tan CK, Purdy MA, Teo CG. Chronic Infection With Camelid Hepatitis E Virus in a Liver Transplant Recipient Who Regularly Consumes Camel Meat and Milk. *Gastroenterology* 2016; **150**: 355-7.e3 [PMID: 26551551 DOI: 10.1053/j.gastro.2015.10.048]

5 **Meng XJ**. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol* 2010; **140**: 256-265 [PMID: 19361937 DOI: 10.1016/j.vetmic.2009.03.017]

6 **Abravanel F**, Lhomme S, El Costa H, Schvartz B, Peron JM, Kamar N, Izopet J. Rabbit Hepatitis E Virus Infections in Humans, France. *Emerg Infect Dis* 2017; **23**: 1191-1193 [PMID: 28628452 DOI: 10.3201/eid2307.170318]

7 **Anheyer-Behmenburg HE**, Szabo K, Schotte U, Binder A, Klein G, Johne R. Hepatitis E Virus in Wild Boars and Spillover Infection in Red and Roe Deer, Germany, 2013-2015. *Emerg Infect Dis* 2017; **23**: 130-133 [PMID: 27983488 DOI: 10.3201/eid2301.161169]

8 **Rasche A**, Saqib M, Liljander AM, Bornstein S, Zohaib A, Renneker S, Steinhagen K, Wernery R, Younan M, Gluecks I, Hilali M, Musa BE, Jores J, Wernery U, Drexler JF, Drosten C, Corman VM. Hepatitis E Virus Infection in Dromedaries, North and East Africa, United Arab Emirates, and Pakistan, 1983-2015. *Emerg Infect Dis* 2016; **22**: 1249-1252 [PMID: 27315454 DOI: 10.3201/eid2207.160168]

9 **Yugo DM**, Meng XJ. Hepatitis E virus: foodborne, waterborne and zoonotic transmission. *Int J Environ Res Public Health* 2013; **10**: 4507-4533 [PMID: 24071919 DOI: 10.3390/ijerph10104507]

10 **Li TC**, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, Kurata Y, Ishida M, Sakamoto S, Takeda N, Miyamura T. Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis* 2005; **11**: 1958-1960 [PMID: 16485490 DOI: 10.3201/eid1112.051041]

11 **Choi JY**, Lee JM, Jo YW, Min HJ, Kim HJ, Jung WT, Lee OJ, Yun H, Yoon YS. Genotype-4 hepatitis E in a human after ingesting roe deer meat in South Korea. *Clin Mol Hepatol* 2013; **19**: 309-314 [PMID: 24133670 DOI: 10.3350/cmh.2013.19.3.309]

12 **Gao S**, Li D, Zha E, Zhou T, Wang S, Yue X. Surveillance of hepatitis E virus contamination in shellfish in China. *Int J Environ Res Public Health* 2015; **12**: 2026-2036 [PMID: 25689991 DOI: 10.3390/ijerph120202026]

13 **Crossan C**, Baker PJ, Craft J, Takeuchi Y, Dalton HR, Scobie L. Hepatitis E virus genotype 3 in shellfish, United Kingdom. *Emerg Infect Dis* 2012; **18**: 2085-2087 [PMID: 23171845 DOI: 10.3201/eid1812.120924]

14 **Brassard J**, Gagné MJ, Généreux M, Côté C. Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. *Appl Environ Microbiol* 2012; **78**: 3763-3766 [PMID: 22427499 DOI: 10.1128/AEM.00251-12]

15 **Maunula L**, Kaupke A, Vasickova P, Söderberg K, Kozyra I, Lazic S, van der Poel WH, Bouwknegt M, Rutjes S, Willems KA, Moloney R, D'Agostino M, de Roda Husman AM, von Bonsdorff CH, Rzeżutka A, Pavlik I, Petrovic T, Cook N. Tracing enteric viruses in the European berry fruit supply chain. *Int J Food Microbiol* 2013; **167**: 177-185 [PMID: 24135674 DOI: 10.1016/j.ijfoodmicro.2013.09.003]

16 **Malamitsi-Puchner A**, Papacharitonos S, Sotos D, Tzala L, Psichogiou M, Hatzakis A, Evangelopoulou A, Michalas S. Prevalence study of different hepatitis markers among pregnant Albanian refugees in Greece. *Eur J Epidemiol* 1996; **12**: 297-301 [PMID: 8884198 DOI: 10.1007/BF00145420]

17 **Dalekos GN**, Zervou E, Elisaf M, Germanos N, Galanakis E, Bourantas K, Siamopoulos KC, Tsianos EV. Antibodies to hepatitis E virus among several populations in Greece: increased prevalence in an hemodialysis unit. *Transfusion* 1998; **38**: 589-595 [PMID: 9661693 DOI: 10.1046/j.1537-2995.1998.38698326339.x]

18 **Adhami JE**, Angoni R. [Hepatitis E virus infection in Albania]. *Sante* 2001; **11**: 13-15 [PMID: 11313227]

19 **Kondili LA**, Chionne P, Porcaro A, Madonna E, Taffon S, Resuli B, Taliani G, Rapicetta M. Seroprevalence of hepatitis E virus (HEV) antibody and the possible association with chronic liver disease: a case-control study in Albania. *Epidemiol Infect* 2006; **134**: 95-101 [PMID: 16409655 DOI: 10.1017/S095026880500470X]

20 **Teoharov P**, Tiholova M, Draganov P. First cases of hepatitis E virus infection in Bulgaria. *Infectol (Sofia)* 1995; **32**: 17-18

21 **Teoharov P**, Pishmisheva M, Kovaleva V. Hepatitis E virus infection: a case report. *Infectol (Sofia)* 2008; **45**: 43-44

22 **Rouseva A**, Nikolovska D. Two cases of hepatitis E in Bulgaria. *Pediatr Infect Dis (Sofia)* 2010; **2**: 26-28

23 **Baymakova M**, Sakem B, Plochev K, Popov GT, Mihaylova-Garnizova R, Kovaleva V, Kundurdjiev T. Epidemiological characteristics and clinical manifestations of hepatitis E virus infection in Bulgaria: A report on 20 patients. *Srp Arh Celok Lek* 2016; **144**: 63-68 [PMID: 27276860]

24 **Baymakova M**, Popov GT, Pepovich R, Tsachev I. Hepatitis E virus infection in Bulgaria: A brief analysis of the situation in the country. *Open Access Maced J Med Sci* 2019; **7**: 458-460 [DOI: 10.3889/oamjms.2019.073]

25 **Stoykova Z**, Ivanova L, Tsaneva-Damyanova D, Kostadinova T. Hepatitis E virus infection in Northeastern Bulgaria. *Med Review* 2017; **53**: 30-34

26 **Bruni R**, Villano U, Equestre M, Chionne P, Madonna E, Trandeva-Bankova D, Peleva-Pishmisheva M, Tenev T, Cella E, Ciccozzi M, Pisani G, Golkocheva-Markova E, Ciccaglione AR. Hepatitis E virus genotypes and subgenotypes causing acute hepatitis, Bulgaria, 2013-2015. *PLoS One* 2018; **13**: e0198045 [PMID: 29879148 DOI: 10.1371/journal.pone.0198045]

27 **Teoharov P**, Kevorkyan A, Raycheva R, Golkocheva-Markova E, Trandeva-Bankova D, Andonov A. Data on the prevalence of hepatitis E in Bulgaria. *C R Acad Bulg Sci* 2014; **67**: 1429-1432

28 **Pismisheva M**, Baymakova M, Golkocheva-Markova E, Kundurzhiev T, Pepovich R, Popov GT, Tsachev I. First serological study of hepatitis E virus infection in pigs in Bulgaria. *C R Acad Bulg Sci* 2018; **71**: 1001-1008 [DOI: 10.7546/CRABS.2018.07.18]

29 **Cella E**, Golkocheva-Markova E, Sagnelli C, Scolamacchia V, Bruni R, Villano U, Ciccaglione AR, Equestre M, Sagnelli E, Angeletti S, Ciccozzi M. Human hepatitis E virus circulation in Bulgaria: Deep Bayesian phylogenetic analysis for viral spread control in the country. *J Med Virol* 2019; **91**: 132-138 [PMID: 30168583 DOI: 10.1002/jmv.25296]

30 **Čivljak R**, Đaković-Rode O, Jemeršić L, Topić A, Turalija M, Čačić M, Kuzman I. Autochthonous hepatitis E in a patient from Zagreb: a case report. *Croatian J Infect* 2013; **33**: 35–39

31 **Đaković Rode O**, Jemeršić L, Brnić D, Pandak N, Mikulić R, Begovac J, Vince A. Hepatitis E in patients with hepatic disorders and HIV-infected patients in Croatia: is one diagnostic method enough for hepatitis E diagnosis? *Eur J Clin Microbiol Infect Dis* 2014; **33**: 2231-2236 [PMID: 25005459 DOI: 10.1007/s10096-014-2187-7]

32 **Mrzljak A**, Djakovic Rode O, Dinjar Kujundžić P, Perkov S, Vince A. Seroprevalence of hepatitis E virus among liver transplant patients in Croatia: preliminary pilot study data. 27th ECCMID, Vienna, 22-25 April, 2017, EV0274.

33 **Vilibic-Cavlek T**, Vilibic M, Kolaric B, Jemersic L, Kucinar J, Barbic L, Bagaric A, Stevanovic V, Tabain I, Sviben M, Jukic V, Mlinaric-Galinovic G. Seroepidemiology of Hepatitis E in Selected Population Groups in Croatia: A Prospective Pilot Study. *Zoonoses Public Health* 2016; **63**: 494-502 [PMID: 26776465 DOI: 10.1111/zph.12254]

34 **Jeličić P**, Jemeršić L, Brumen V, Janev-Holcer N, Prohić A, Barbić Lj, Tabain I, Stevanović V, Vilibić-Čavlek V. Seroprevalence of hepatitis E in professionally exposed groups in Croatia: preliminary results. 7th International Congress "Veterinary Science and Profession", Zagreb, 5-7 October, 2017, p.55.

35 **Jeličić P**, Vilibić-Čavlek T, Vilibić M, Jemeršić L, Kolarić B, Jemeršić L, Kučinar J, Barbić Lj, Stevanović V, Janev-Holcer N, Tabain I, Brumen V, Djaković I, Prohić A, Košec V, Kaić B. Seroprevalence of hepatitis E in different population groups in Croatia. 7th Congress of Slovenian Microbiological Society, Bled, Slovenia, 20-22 September, 2017, p.155.

36 **Miletic Lovric M**, Stojic Vidovic M, Hecimovic A, Mihaljevic I, Jemersic L, Strauss-Patko M, Jukic I. Seroprevalence of hepatitis E among Croatian blood donors. *Vox Sang* 2016; **111**: 309 [DOI: 10.1111/vox.12429]

37 **Mrzljak A**, Dinjar Kujundžić P, Djakovic Rode O, Kolarić B, Vince A. Socio-demografic risk factors for high seroprevalence among liver transplant recipients in Croatia. 4th Central and Eastern European Meeting on Hepatitis and HIV, Prague, 10-12. October;. *Rev Antivir Ther Infect Dis* 2018; **12**: 6

38 **Dinjar Kujundžić P**, PavičićŠarić J, Betica Radić Lj, Mrzljak A. Fulminant hepatitis E on a liver transplantation list. Proceeding from the "Emerging and neglected zoonoses in the One Health context", Zagreb, 18-19 October. 2018; 83

39 **Lipej Z**, Novosel D, Vojta L, Roić B, Simpraga M, Vojta A. Detection and characterisation of hepatitis E virus in naturally infected swine in Croatia. *Acta Vet Hung* 2013; **61**: 517-528 [PMID: 23974940 DOI: 10.1556/AVet.2013.031]

40 **Prpić J**. Černi S, Škorić D, Keros T, Brnić D, Cvetnić Ž, Jemeršić L. Distribution and Molecular Characterization of Hepatitis E virus in Domestic Animals and Wildlife in Croatia. *Food Environ Virol* 2015; **7**: 195-205 [PMID: 25820413 DOI: 10.1007/s12560-015-9193-5]

41 **Jemeršić L**. Keros T, Maltar Lj, Barbić Lj, Vilibić-Čavlek T, Jeličić P, Rode Đaković O, Prpić J. Differences in hepatitis E virus (HEV) presence in naturally infected seropositive domestic pigs and wild boars - confirmation of wild boars having a key role in HEV epidemiology. *Vet Arh* 2017; **87**: 651-663 [DOI: 10.24099/vet.arhiv.170208]

42 **Psichogiou MA**, Tassopoulos NC, Papatheodoridis GV, Tzala E, Klarmann R, Witteler H, Schlauder GG, Troonen H, Hatzakis A. Hepatitis E virus infection in a cohort of patients with acute non-A, non-B hepatitis. *J Hepatol* 1995; **23**: 668-673 [PMID: 8750165 DOI: 10.1016/0168-8278(95)80032-8]

43 **Zervou EK**, Georgiadou SP, Liapi GK, Karabini F, Giogiakas V, Zisiadis K, Gatselis NK, Goudevenos I, Dalekos GN. Markers of hepatitis viruses and human T-lymphotropic virus types I/II in patients who have undergone open-heart surgery: evidence of increased risk for exposure to HBV and HEV. *Eur J Intern Med* 2005; **16**: 424-428 [PMID: 16198902 DOI: 10.1016/j.ejim.2005.02.020]

44 **Klonizakis P**, Gioula G, Exindari M, Apostolou C, Kotsiafti A, Vlachaki E. Hepatitis E in transfusion-dependent thalassaemia patients, in Greece: a single centre experience. *Vox Sang* 2017; **112**: 678-679 [PMID: 28891120 DOI: 10.1111/vox.12572]

45 **Sinakos E**. Gioula G, Liava C, Papa A, Papadopoulou E, Tsakni E, Fouzas I, Akriviadis E. Prevalence of hepatitis E in liver transplant recipients in Greece. *Epidemiol Infect* 2018; **146**: 1619-1621 [PMID: 29974836 DOI: 10.1017/S0950268818001887]

46 **Stefanidis I**, Zervou EK, Rizos C, Syrganis C, Patsidis E, Kyriakopoulos G, Sdrakas L, Tsianas N, Rigopoulou EI, Liakopoulos V, Dalekos GN. Hepatitis E virus antibodies in hemodialysis patients: an epidemiological survey in central Greece. *Int J Artif Organs* 2004; **27**: 842-847 [PMID: 15560678 DOI: 10.1177/039139880402701005]

47 **Pittaras T**, Valsami S, Mavrouli M, Kapsimali V, Tsakris A, Politou M. Seroprevalence of hepatitis E virus in blood donors in Greece. *Vox Sang* 2014; **106**: 387 [PMID: 24387713 DOI: 10.1111/vox.12122]

48 **Politou M**, Boti S, Androutsakos T, Valsami S, Pittaras T, Kapsimali V. Seroprevalence of hepatitis E in HIV infected patients in Greece. *J Med Virol* 2015; **87**: 1517-1520 [PMID: 25965115 DOI: 10.1002/jmv.24214]

49 **Zervou EZ**, Politis CP, Hassapopoulou EH, Vini MV, Parara MP, Kavallierou LK, Fountouli KF, Zaxarioudaki AZ, Hatzitaki MH, Martinis GM, Katopi DK, Megalou AM, Avrami DA, Halkia PH, Aggelou EA, Tsironi ET, Lafiatis IL, Richardson CR. Prevalence of hepatitis E virus (HEV) infection in blood donors and multi-transfused patients in Greece. *Vox Sang* 2015; **109**: 242-243

50 **Ryll R**. Bernstein S, Heuser E, Schlegel M, Dremsek P, Zumpe M, Wolf S, Pépin M, Bajomi D, Müller G, Heiberg AC, Spahr C, Lang J, Groschup MH, Ansorge H, Freise J, Guenther S, Baert K, Ruiz-Fons F, Pikula J, Knap N, Tsakmakidis I, Dovas C, Zanet S, Imholt C, Heckel G, Johne R, Ulrich RG. Detection of rat hepatitis E virus in wild Norway rats (Rattus norvegicus) and Black rats (Rattus rattus) from 11 European countries. *Vet Microbiol* 2017; **208**: 58-68 [PMID: 28888650 DOI: 10.1016/j.vetmic.2017.07.001]

51 **Kokkinos P**, Kozyra I, Lazic S, Bouwknegt M, Rutjes S, Willems K, Moloney R, de Roda Husman AM, Kaupke A, Legaki E, D'Agostino M, Cook N, Rzeżutka A, Petrovic T, Vantarakis A. Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. *Food Environ Virol* 2012; **4**: 179-191 [PMID: 23412890 DOI: 10.1007/s12560-012-9087-8]

52 **Kokkinos P**, Kozyra I, Lazic S, Söderberg K, Vasickova P, Bouwknegt M, Rutjes S, Willems K, Moloney R, de Roda Husman AM, Kaupke A, Legaki E, D'Agostino M, Cook N, von Bonsdorff CH, Rzeżutka A, Petrovic T, Maunula L, Pavlik I, Vantarakis A. Virological Quality of Irrigation Water in Leafy Green Vegetables and Berry Fruits Production Chains. *Food Environ Virol* 2017; **9**: 72-78 [PMID: 27709435 DOI: 10.1007/s12560-016-9264-2]

53 **Clemente-Casares P**, Pina S, Buti M, Jardi R, MartIn M, Bofill-Mas S, Girones R. Hepatitis E virus epidemiology in industrialized countries. *Emerg Infect Dis* 2003; **9**: 448-454 [PMID: 12702225 DOI: 10.3201/eid0904.020351]

54 **Kokkinos P**, Ziros P, Meri D, Filippidou S, Kolla S, Galanis A, Vantarakis A. Environmental surveillance. An additional/alternative approach for virological surveillance in Greece? *Int J Environ Res Public Health* 2011; **8**: 1914-1922 [PMID: 21776209 DOI: 10.3390/ijerph8061914]

55 **Diez-Valcarce M**, Kokkinos P, Söderberg K, Bouwknegt M, Willems K, de Roda-Husman AM, von Bonsdorff CH, Bellou M, Hernández M, Maunula L, Vantarakis A, Rodríguez-Lázaro D. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. *Food Environ Virol* 2012; **4**: 73-80 [PMID: 23412813 DOI: 10.1007/s12560-012-9078-9]

56 **Rey JL**, Ramadani Q, Soarès JL, Nicand E, Ibrahime D, Preteni E, Buisson Y, Teyssou R. [Sero-epidemiological study of the hepatitis epidemic in Mitrovica in the aftermath of the war in Kosovo (1999)]. *Bull Soc Pathol Exot* 2002; **95**: 3-7 [PMID: 12012960]

57 **Terzic D**, Dupanovic B, Mugosa B, Draskovic N, Svirtlih N. Acute hepatitis E in Montenegro: epidemiology, clinical and laboratory features. *Ann Hepatol* 2009; **8**: 203-206 [PMID: 19841498]

58 **Savuţa G**, Aniţă A, Aniţă D, Ludu L, Duca E, Pavio N. Seroepidemiological researches regarding swine and human hepatits E in Romania. *Lucr St Med Vet USAMVB Timisoara* 2008; **XLI**: 309-313

59 **Anita A**, Anita D, Ludu L, Savuta G. Seroepidemiological investigation of human and swine hepatitis in Botosani County. *Bull UASVM Vet Med* 2010; **67**: 19-22

60 **Voiculescu M**, Iliescu L, Ionescu C, Micu L, Ismail G, Zilisteanu D, Radasan A, Micu G, Pertache I. A cross-sectional epidemiological study of HBV, HCV, HDV and HEV prevalence in the SubCarpathian and South-Eastern regions of Romania. *J Gastrointestin Liver Dis* 2010; **19**: 43-48 [PMID: 20361074]

61 **Aniţă A**, Gorgan L, Aniţă D, Oşlobanu L, Pavio N, Savuţa G. Evidence of hepatitis E infection in swine and humans in the East Region of Romania. *Int J Infect Dis* 2014; **29**: 232-237 [PMID: 25447729 DOI: 10.1016/j.ijid.2014.10.018]

62 **Savuţa G**, Aniţă A, Aniţă D, Ludu L, Pavio N. Preliminary epidemiological investigations regarding hepatitis E virus infection in swine form the North-east of Romania. *Bull USAMV-CN* 2007; **64**: 356-358

63 **Porea D**, Anita A, Paslaru A, Savuta G. Serological evidence of wild boar hepatitis E infection in three counties from eastern Romania. *Lucr St Med Vet USAMVB Timisoara* 2015; **48**: 174-178

64 **Porea D**, Anita A, Paslaru A, Savuta G. Wild boar hepatitis E seroprevalence in hunting funds from Buzau and Galati counties. *Bull UASVM Vet Med* 2016; **73**: 44-48 [DOI: 10.15835/buasvmcn-vm:11506]

65 **Porea D**, Anita A, Demange A, Raileanu C, Oslobanu Ludu L, Anita D, Savuta G, Pavio N. Molecular detection of hepatitis E virus in wild boar population in eastern Romania. *Transbound Emerg Dis* 2018; **65**: 527-533 [PMID: 29027370 DOI: 10.1111/tbed.12736]

66 **Petrović T**, Lupulović D, Jiménez de Oya N, Vojvodić S, Blázquez AB, Escribano-Romero E, Martín-Acebes MA, Potkonjak A, Milošević V, Lazić S, Saiz JC. Prevalence of hepatitis E virus (HEV) antibodies in Serbian blood donors. *J Infect Dev Ctries* 2014; **8**: 1322-1327 [PMID: 25313610 DOI: 10.3855/jidc.4369]

67 **Petrovic T**, Prodanov J, Lazic S. First preliminary results on the presence of Hepatitis E virus in swine

population in Serbia. Symposium on Current Developments in Food and Environmental Virology, Pisa, Italy 2008 October 9-11, pp. 52-53.
68 **Lupulovic D**, Lazic S, Prodanova Radulovic J, Jimenez de Oya N, Escribano-Romero E, Saiz Juan-Carslos, Petrovic R. First Serological Study of Hepatitis E Virus Infection in Backyard Prigs from Serbia. *Food Environ Virol* 2010; **2**: 110-113 [DOI: 10.1007/s12560-010-9033-6]
69 **Savic B**, Milicevic V, Bojkovski J, Kureljusic B, Ivetic V, Pavlovic I. Detection rates of the swine torque teno viruses (TTVs), porcine circovirus type 2 (PCV2) and hepatitis E virus (HEV) in the livers of pigs with hepatitis. *Vet Res Commun* 2010; **34**: 641-648 [PMID: 20676762 DOI: 10.1007/s11259-010-9432-z]
70 **Lupulovic D**, Grgic Z, Lazic G, Prodanov-Radulovic J, Potkonjak A, Lazic S, Petrovic T. Detection of hepatitis E virus antibodies in blood and meat juice samples in slaughtered pigs in Serbia. Proceedings of the 16th International Symposiom on the World Association of Veterinary Laboratory Diagnosticians (WAVLD), 2013, June 5-8; Berlin, Germany. 2013; 372
71 **Petrovic T**, Lupulovic D, Zivoslav G, Lazic G, Vidanovic D, Dosen R, Savic S. The preliminary survey of HEV presence in samples of pigs – pork production in slaughthouses in Serbia. Proceedings of the 16th International Symposiom on the World Association of Veterinary Laboratory Diagnosticians (WAVLD), 2013, June 5-8; Berlin, Germany. 2013; 310
72 **Petrović T**, Lupulović D, Lazić S, Lazić G, Blázquez AB, Escribano-Romero E, Saiz JC. Presence of hepatitis E virus infection in wild board population in Serbia. Proceedings of the IX International Congress of Veternary Virology. 2012; September 4-7, Madrid, Spain; p241
73 **Lazić G**, Grubač S, Lupulović D, Bugarski D, Lazić S, Knežević P, Petrović T. Presence of Human and Animal Viruses in Surface Waters in Vojvodina Province of Serbia. *Food Environ Virol* 2015 [PMID: 25687987 DOI: 10.1007/s12560-015-9187-3]
74 **Steyer A**, Naglič T, Močilnik T, Poljšak-Prijatelj M, Poljak M. Hepatitis E virus in domestic pigs and surface waters in Slovenia: prevalence and molecular characterization of a novel genotype 3 lineage. *Infect Genet Evol* 2011; **11**: 1732-1737 [PMID: 21802527 DOI: 10.1016/j.meegid.2011.07.007]
75 **Žele D**, Barry AF, Hakze-van der Honing RW, Vengušt G, van der Poel WH. Prevalence of Anti-Hepatitis E Virus Antibodies and First Detection of Hepatitis E Virus in Wild Boar in Slovenia. *Vector Borne Zoonotic Dis* 2016; **16**: 71-74 [PMID: 26757050 DOI: 10.1089/vbz.2015.1819]
76 **Raspor Lainšček P**, Toplak I, Kirbiš A. A comprehensive study of hepatitis E virus infection in pigs entering a slaughterhouse in Slovenia. *Vet Microbiol* 2017; **212**: 52-58 [PMID: 29173588 DOI: 10.1016/j.vetmic.2017.11.002]
77 **Steyer A**, Gutiérrez-Aguirre I, Rački N, Beigot Glaser S, Brajer Humar B, Stražar M, Škrjanc I, Poljšak-Prijatelj M, Ravnikar M, Rupnik M. The Detection Rate of Enteric Viruses and Clostridium difficile in a Waste Water Treatment Plant Effluent. *Food Environ Virol* 2015 [PMID: 25663146 DOI: 10.1007/s12560-015-9183-7]
78 **Lhomme S**, Dubois M, Abravanel F, Top S, Bertagnoli S, Guerin JL, Izopet J. Risk of zoonotic transmission of HEV from rabbits. *J Clin Virol* 2013; **58**: 357-362 [PMID: 23474012 DOI: 10.1016/j.jcv.2013.02.006]
79 **Izopet J**, Dubois M, Bertagnoli S, Lhomme S, Marchandeau S, Boucher S, Kamar N, Abravanel F, Guérin JL. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. *Emerg Infect Dis* 2012; **18**: 1274-1281 [PMID: 22840216 DOI: 10.3201/eid1808.120057]
80 **Kaiser M**, Delaune D, Chazouillères O, Blümel J, Roque-Afonso AM, Baylis SA. A World Health Organization Human Hepatitis E Virus Reference Strain Related to Similar Strains Isolated from Rabbits. *Genome Announc* 2018; **6** [PMID: 29674549 DOI: 10.1128/genomeA.00292-18]
81 **Rivero-Juarez A**, Frias M, Rodriguez-Cano D, Cuenca-López F, Rivero A. Isolation of Hepatitis E Virus From Breast Milk During Acute Infection. *Clin Infect Dis* 2016; **62**: 1464 [PMID: 27025819 DOI: 10.1093/cid/ciw186]
82 **Huang F**, Li Y, Yu W, Jing S, Wang J, Long F, He Z, Yang C, Bi Y, Cao W, Liu C, Hua X, Pan Q. Excretion of infectious hepatitis E virus into milk in cows imposes high risks of zoonosis. *Hepatology* 2016; **64**: 350-359 [PMID: 27286751 DOI: 10.1002/hep.28668]
83 **Caruso C**, Modesto P, Prato R, Scaglione FE, De Marco L, Bollo E, Acutis PL, Masoero L, Peletto S. Hepatitis E Virus: First Description in a Pet House Rabbit. A New Transmission Route for Human? *Transbound Emerg Dis* 2015; **62**: 229-232 [PMID: 25773737 DOI: 10.1111/tbed.12348]
84 **Liang H**, Chen J, Xie J, Sun L, Ji F, He S, Zheng Y, Liang C, Zhang G, Su S, Li S. Hepatitis E virus serosurvey among pet dogs and cats in several developed cities in China. *PLoS One* 2014; **9**: e98068 [PMID: 24896257 DOI: 10.1371/journal.pone.0098068]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3183-3195

DOI: 10.3748/wjg.v25.i25.3183

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

MINIREVIEWS

Infections with *Helicobacter pylori* and challenges encountered in Africa

Stella Smith, Muinah Fowora, Rinaldo Pellicano

ORCID number: Stella Smith (0000-0003-2163-1189); Muinah Fowora (0000-0001-7140-2689); Rinaldo Pellicano (0000-0003-3438-0649).

Author contributions: Smith S and Pellicano R contributed equally to the concept and design of the study; while all authors were involved in the literature review and analysis, drafting and critical revision and editing and final approval of the final version.

Conflict-of-interest statement: No potential conflict of interest and no financial support.

Manuscript source: Invited manuscript

Received: March 17, 2019
Peer-review started: March 18, 2019
First decision: April 4, 2019
Revised: May 2, 2019
Accepted: May 31, 2019
Article in press: June 1, 2019
Published online: July 7, 2019

Stella Smith, Muinah Fowora, Department of Molecular Biology and Biotechnology, Nigerian Institute of Medical Research, Lagos PMB 2013, Nigeria

Rinaldo Pellicano, Unit of Gastroenterology, Molinette Hospital, Turin 10126, Italy

Corresponding author: Stella Smith, PhD, Director (Research), Department of Molecular Biology and Biotechnology, Nigerian Institute of Medical Research, 6 Edmond Crescent, Yaba, Lagos PMB 2013, Nigeria. stellasmith@nimr.gov.ng
Telephone: +234-803-7058989

Abstract

Helicobacter pylori (*H. pylori*) is the causative agent of gastritis, peptic ulcer disease, mucosa associated lymphoid tissue lymphoma and gastric cancer (GC). While this bacterium infects 50% of the world's population, in Africa its prevalence reach as high as 80% as the infection is acquired during childhood. Risk factors for *H. pylori* acquisition have been reported to be mainly due to overcrowding, to have infected siblings or parent and to unsafe water sources. Despite this high *H. pylori* prevalence there still does not exist an African guideline, equivalent to the Maastricht V/Florence Consensus Report of the European Helicobacter and Microbiota Study Group for the management of this infection. In this continent, although there is a paucity of epidemiologic data, a contrast between the high prevalence of *H. pylori* infection and the low incidence of GC has been reported. This phenomenon is the so-called "African Enigma" and it has been hypothesized that it could be explained by environmental, dietary and genetic factors. A heterogeneity of data both on diagnosis and on therapy have been published. In this context, it is evident that in several African countries the increasing rate of bacterial resistance, mainly to metronidazole and clarithromycin, requires continental guidelines to recommend the appropriate management of *H. pylori*. The aim of this manuscript is to review current literature on *H. pylori* infection in Africa, in terms of prevalence, risk factors, impact on human health, treatment and challenges encountered so as to proffer possible solutions to reduce *H. pylori* transmission in this continent.

Key words: *Helicobacter pylori*; Africa; Risk factors; African enigma; Prevalence; Treatment; Diagnosis

Core tip: Africa has the highest rates of global prevalence of *Helicobacter pylori* (*H.*

P-Reviewer: Abadi ATB, Nishida T, Yücel O
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

pylori) infection worldwide. Nevertheless, scarce data are available, describing in some cases both inappropriate diagnostic approaches and therapeutic regimens. This probably depends on the lack of continental consensus guideline for the management of *H. pylori* infection. As a consequence, there is an increasing number of papers reporting, in several countries, a high rate of bacterial resistance to the most commonly used antibiotics for *H. pylori* treatment. This manuscript gives an update on the African literature about *H. pylori* infection and on the present and future challenges in this context.

Citation: Smith S, Fowora M, Pellicano R. Infections with *Helicobacter pylori* and challenges encountered in Africa. *World J Gastroenterol* 2019; 25(25): 3183-3195
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3183.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3183

IMPACT OF *HELICOBACTER PYLORI* INFECTION ON HUMAN HEALTH

Helicobacter pylori (*H. pylori*) infection is mostly asymptomatic in its carriers, but when it affects human health, gastritis, gastric ulcers and duodenal ulcers (DU) can be induced. About 90% to 100% of all DU and 70% to 80% of all gastric ulcers are caused by *H. pylori* infection[1,2]. After bacterial eradication the recurrence rate of peptic ulcer is dramatically reduced to 5%-10%[3]. In 1994, The International Agency for Research on Cancer, an arm of the World Health Organization classified *H. pylori* as a class I carcinogen for gastric cancer (GC), a definition given for the highest cancer causing agent[4]. As a consequence, *H. pylori* eradication has also been identified and adopted from several studies as a potential strategy for the primary prevention of GC[5]. Thereafter, *H. pylori* was identified as the causative agent of mucosa associated lymphoid tissue lymphoma[6].

Beyond its role in several gastroduodenal disorders, *H. pylori* has been involved in many extra-gastroduodenal manifestations, like idiopathic thrombocytopenic purpura, cardiovascular diseases, chronic liver diseases, iron-deficiency anaemia, and diabetes mellitus (DM)[7-9]. However, the Maastricht V/Florence Consensus Report of the European *Helicobacter* and Microbiota Study Group actually consider causal association with *H. pylori* only in the case of unexplained iron-deficiency anemia and idiopathic thrombocytopenic purpura[10]. Our objective was to review the current literature on *H. pylori* prevalence, impact on human health, treatment and challenges encountered in Africa.

On the bases of our aim, the main inclusion criteria of the articles considered were that these must have been published within 10 years (from 2009 onwards), that they were published in peer-reviewed journals and in English language and that they were performed on Africans, resident/located in Africa. Articles not meeting these inclusion criteria were excluded. Also, articles published as correspondence, letters, and conference proceedings were not considered. When no article was found in a particular African region, an exception of extending the publication year for more than 10 years was made. The search databases included MEDLINE, PUBMED, Web of Science, Scopus, the Cochrane Database of Systematic Reviews and Google scholar.

PREVALENCE OF *HELICOBACTER PYLORI* IN AFRICA

There is a huge paucity of data on *H. pylori* prevalence in the general population across different regions of Africa. The majority of data published on the prevalence of *H. pylori* included patients presenting with symptoms of gastroduodenal diseases. *H. pylori* infects over 50% of the world's population. The distribution of *H. pylori* is influenced by age, sex, geographical location, ethnicity, and socio economic factors[11-13]. The geographical distribution of *H. pylori* shows higher prevalence in the developing countries when compared to the developed countries especially in younger ages. With a majority of countries in Africa, classified as developing or underdeveloped, *H. pylori* is therefore mainly ubiquitous in this continent.

A systematic review with meta-analysis, carried out by Hooi *et al*[11], included 184 studies, from 62 different countries, published between 1970 to 2016 on the prevalence of *H. pylori* infection and its worldwide distribution. Africa had the highest rate of *H.*

pylori infection with a prevalence of 70.1%, followed by South America and Western Asia with prevalence of 69.4% and 66.6%, respectively. The authors re-ported that Nigeria had the highest *H. pylori* prevalence at 87.7% followed closely by Portugal and Estonia with a *H. pylori* prevalence of 86.4% and 82.5%, respectively[11]. In contrast, Zamani *et al*[13] in a recent meta-analysis evaluating the global prevalence of *H. pylori* infection found Latin America and the Caribbean to have the highest prevalence of *H. pylori* worldwide with a prevalence of 59.3%. Nevertheless, these authors also reported that Nigeria had the highest prevalence of *H. pylori* infection with a rate of 89.7%. The high prevalence of *H. pylori* in Africa is presumed to be influenced by sociodemographic and geographical factors[14].

In Rwanda, Southern Africa, Walker *et al*[15] reported 75% positivity rate to *H. pylori* in patients attending the University Hospital Butare over a period of 12 mo, which was found to be similar to the prevalence of other sub-Saharan African countries. A study on genomic evolution *H. pylori* in two South African families revealed that transmission episodes were significantly more frequent between individuals living in the same house and close relatives, however transmission did not always occur within families[16]. Comparing horizontal and familial transmission of *H. pylori* in Africa, Schwarz *et al*[17] reported that horizontal transmission occurred often between persons who do belong to a core-family, hence tainting the typical pattern of familial transmission in developed countries. This is substantiated by the work of Nell *et al*[18], from Central Africa who reported the acquisition of *H. pylori* by Baka pygmies of Cameroon through secondary contact with their non-Baka agriculturist neighbours. The prevalence rate of *H. pylori* infection amongst asymptomatic patients from Harare, Zimbabwe was 67.7%[19].

In Northern Africa, a recent study in Egypt compared the prevalence of *H. pylori* antibodies in patients with idiopathic thrombocytopenic purpura and in the general Egyptian population[20]. Seropositivity of anti-*Helicobacter* IgM was higher in the general population (54.4%) when compared with patients with idiopathic thrombocytopenic purpura (28.9%). Also, seropositivity of anti-*Helicobacter* IgG was higher in the general population (79.8%) when compared with the controls[20]. In Morocco, Bounder *et al*[21] assessed *H. pylori* prevalence in subjects with and without symptoms of gastric disorders. The authors reported an overall *H. pylori* seropositivity prevalence of 92.6% among asymptomatic Moroccans and 89.6% among patients with gastric disorders.

In Eastern Africa, a study from Kenya, among patients who presented with dyspepsia, showed a prevalence of *H. pylori* infection of 73.3% in children *vs* 54.8% in adults[22]. Taddesse *et al*[23] reported a *H. pylori* prevalence of 53% in dyspeptic patients in Addis Ababa with an estimated prevalence peak in patients aged between 54-61 years. Another study by Hestvik *et al*[24], in Uganda, reported a prevalence of 44.3% of *H. pylori* in healthy children aged 0-12 years, with identified factors of increased infection risk including source of drinking water, use of pit latrine and wealth index driving transmission. These factors coupled with re-crudescence or reinfection from multiple sources accounts for the continuous high prevalence of *H. pylori* infection in Africa[25]. Though the route of transmission of this infection is not well established; possible routes of transmission such as person-person, oral-oral and faecal-oral have been suggested. The ability of the pathogen to survive for some days in water buttressed the fact of possible water transmission[26,27].

Melese *et al*[28] evaluated the prevalence of *H. pylori* in Ethiopia by different studies carried out on different populations and different geographical areas of the country. The results of their meta-analysis showed an overall pooled prevalence of *H. pylori* as 52.2%. The authors also reported that the prevalence of *H. pylori* was highest in Somali (71%) and lowest in Oromia (39.9%).

In Nigeria, West Africa, the issue of differing prevalence based on geographical location was encountered. Aje *et al*[29], in a study conducted in south-west Nigeria, on dyspeptic patients, reported a 67.4% prevalence of *H. pylori*. In a similar study, Jemilohun *et al*[30] in Ibadan, reported a prevalence of 63.5% in patients with gastritis. However, Etukudo *et al*[31] in a study from Uyo, south-south Nigeria, reported a lower seroprevalence rate (30.9%) in children with a peak (40.7%) for the 6-10 years age group. Factors associated with high seroprevalence were increased household population (P = 0.009), source of drinking water (P = 0.014), low social class (P = 0.038), type of convenience used (P = 0.019) and method of household waste disposal (P = 0.043).

Ophori *et al*[32], including healthy volunteers in Delta State in South South Nigeria, reported a prevalence of *H. pylori* infection of 89.7% in their study population. Ishaleku and Ihiabe reported a *H. pylori* infection prevalence of 54% amongst healthy university students in Nassarawa state, North Central Nigeria[33]. In contrast, Ezeigbo and Ezeigbo reported 39.7% *H. pylori* prevalence amongst apparently healthy adults residing in Aba, Abia State, South Eastern Nigeria[34]. A current report on the

prevalence of *H. pylori* from patients with and without type 2 DM in South West and South South Nigeria showed a prevalence of 68.4% amongst those with type 2 DM[35].

Awuku *et al*[36], using a lateral flow immunochromatographic assay for the qualitative detection of *H. pylori* antigen in a fecal specimen, reported a prevalence of *H. pylori* of 14.2% among asymptomatic children in a rural setting in Ghana. Table 1 shows the prevalence rate of *H. pylori* in Africa. An observation reported in all African studies was that *H. pylori* prevalence increased with age and that factors such as location, access to potable water and hygiene, and socio-economic status influenced the variability seen in *H. pylori* prevalence within and between countries.

Helicobacter pylori and socio-economic status

A study from Zambia, Southern Africa, performed by McLaughlin *et al*[37] showed no correlation between *H. pylori* infection and socio-economic factors. In Kano, North-West Nigeria, Bello *et al*[38] showed high prevalence of *H. pylori* particularly amongst subjects with low socioeconomic status. Factors such as unclean water source, overcrowding and cigarette smoking were significant risk factors for *H. pylori* infection. In contrast, in a study from South–West Nigeria, Smith *et al*[39] reported that most characteristics studied such as smoking, alcohol consumption and sources of drinking water were not significantly associated with *H. pylori*. Rather, prior antibiotic use, overcrowding, having siblings/parents with history of ulcer/gastritis had significant association. Thus, overcrowding was the main common risk factor in both studies in Nigeria.

These reports were also corroborated by Aguemon *et al*[40] in a study performed in Benin republic, Western African too, who reported that overcrowding and family contact with infected persons were as-sociated risk factors for *H. pylori* acquisition and slightly in support of these findings were reports from Cameroon, Central Africa, by Kouitcheu Mabeku *et al*[41] where risk factors for *H. pylori* acquisition were low income and family history of GC.

A study from Ghana showed that low socio-economic class and farming profession accounted for higher *H. pylori* prevalence[25]. Another study from the same country, including children, reported increasing household numbers, open-air defaecation and other sources of drinking water with the exception of pipe and borehole as risk factors of *H. pylori*[36]. A study from Egypt, Northern Africa, by El-Sharouny *et al*[42] reported the isolation of *H. pylori* from drinking water by culture and polymerase chain reaction (PCR) although at low prevalence (3.8%).

PECULIARITY OF THE IMPACT OF *H. PYLORI* INFECTION ON HUMAN HEALTH IN AFRICA (THE AFRICAN ENIGMA)

The natural history of *H. pylori* in Africa seems to differ from those in the developed countries. In fact, in this continent, the most common gastroduodenal disease associated with *H. pylori* infection is gastritis. Kuipers and Meijer[43] suggested that the progression of *H. pylori* infection to atrophic gastritis in the African population is quite similar to that reported in the Western countries or other regions, but unidentified factors could inhibit the evolution to GC. As a consequence, despite the worldwide reported association between gastric adenocarcinoma and *H. pylori*, the development of this malignancy is rare in Africans, a phenomenon that has been referred to as the "African enigma"[44]. This occurs even when risk factors (positivity for *cag*A and *vac*A genes) for development of cancers are ubiquitous in *H. pylori* isolates of African origin[45].

The term "African Enigma" was first coined by Holcombe describing the phenomenon of high prevalence of *H. pylori* in Africa but without a corresponding severe pathology such as GC[46]. According to this observation is the fact that the infection has different patterns from that of the Western countries, in term of age of acquisition, environmental and dietary factors, and genetics. Holcombe identified gastritis as the main health problem caused by *H. pylori* infection in Africa and this has been largely confirmed[46].

In Northern Africa, despite the scarcity of publications on *H. pylori* prevalence among patients with gastroduodenal diseases or in the general population, the few studies showed that gastritis was the most common disease associated with *H. pylori* infection followed by peptic ulcer. In Morocco, Boukhris *et al*[47] found a significant association between *H. pylori* infection and gastritis followed by that with peptic ulcer disease, but no significant association was seen between *H. pylori* and GC. Similarly, in the same country, Bounder *et al*[21] found chronic gastritis as the most gastric disease associated with *H. pylori* infection[21]. In Egypt, although GC is rare, studies with small sample size including patients with GC, found the presence of *H. pylori* in all ca-

Table 1 Prevalence rate of *Helicobacter pylori* in Africa

Region	Includedsample	Prevalence (%)	Ref.
North Africa			
Morocco	Asymptomatic	92.6	[21]
	Gastric disorder	89.6	
Egypt	[1]General	79.8	[20]
West Africa			
Nigeria	[1]General	87.7-89.7	[11,13]
Ghana	Children	14.2	[36]
East Africa			
Ethiopia	Dyspeptic	52.2-53.0	[23]
Kenya	Children	73.3	[22]
	Adults	54.8	
Uganda	Children	44.3	[24]
Southern Africa			
South Africa	Gastric related morbidities	66.1	[16]
Rwanda	[1]General	75.0	[16]
Zimbabwe	Asymptomatic	67.7	[19]

[1]General: People chosen without stating whether symptomatic or asymptomatic.

ses[48,49]. Thus, this contrasts with the above-suggested pattern but the above reported limitation could explain the difference with the general literature.

In Eastern Africa, gastritis was the most reported common disease associated with *H. pylori* infection[50-52]. In Ethiopia, Alebie and Kaba found a high prevalence of *H. pylori* infection (71.0%) among students with gastritis[53]. Ayana *et al*[50], in Tanzania, reported the prevalence of *H. pylori* in patients with gastroduodenal disorders. In this cohort, the authors found gastritis (61.1%), gastroesophageal reflux disease (57%), peptic ulcer disease (24.1%), and GC (6.7%). However, only gastritis and DU were significantly associated with *H. pylori* infection. Similarly, Oling *et al*[51], in a tertiary hospital that served both Kenyans and Ugandans, evaluated the prevalence of *H. pylori* in dyspeptic patients. The authors found chronic non-active gastritis as the most common gastroduodenal disorder and reported a strong association between gastritis and DU with *H. pylori*.

There are scarce data on the prevalence of *H. pylori* infection among patients with gastroduodenal diseases or the general population in Central Africa. The majority of the current articles on *H. pylori* were reported from Cameroon, and similarly to the African literature, a strong association between gastritis and *H. pylori* was found. Ankouane *et al*[53] observed in their study that 71.2% of patients with atrophic gastritis were positive for *H. pylori* infection. The authors also found a statistically significant association between the severity of atrophic gastritis and *H. pylori* infection. This result was corroborated by Ebule *et al*[54] also in Cameroon, who reported that 72.8% of patients with superficial gastritis were infected with *H. pylori*.

In Ghana, Western Africa, a study by Afihene *et al*[55] found that the most common ailment among patients with gastroduodenal disorders was gastritis. However, the authors observed that the only gastroduodenal disorder significantly associated with *H. pylori* infection was DU. Similarly, Darko *et al*[56], even in Ghana, found gastritis and DU as the two most common endoscopic findings in patients with *H. pylori* infection. In Nigeria, Harrison *et al*[57] found a significant correlation between *H. pylori* infection (identified by urea breath test) and chronic gastritis but did not find such association with other disease outcomes.

Tanih *et al*[58], in the Eastern Cape Province of Southern Africa, reported that the prevalence of *H. pylori*, in patients suffering from gastric-related morbidities, was 66.1% and highest in patients with nonulcer dyspepsia. Though the pathogenicity of the circulating *H. pylori* strain may influence its clinical manifestation, nonetheless, mild pathogenicity and low incidence of GC in Africa is recorded even in the face of high prevalence of highly pathogenic *H. pylori* strains[57]. This further corroborates the African enigma. Probably, the latter results from a combination of three major causes: The cancer cause potential of the specific strains of *H. pylori*, the modulation by coinfections of the immune response towards a Th-2 type, and the preponderance of antioxidants in the diet. In the traditional epidemiologic model the combination of

these factors forms a web of causation whose dynamics most likely differs in the groups where the enigma has been reported. The modulation of the immune system by coinfections, such as with helminthes, plays a prominent role influencing the impact of *H. pylori* infection[59].

CHALLENGES ON THE PREVALENCE OF *HELICOBACTER PYLORI* IN AFRICA

Some of the challenges in estimating the prevalence of *H. pylori* in Africa have already been mentioned above. Prevalence of *H. pylori* is variable within and between countries, different population groups, and the testing method used[60]. Problems of paucity of data or lack of recent articles, even in the last ten years were encountered for some regions in Africa. Studies such as the meta-analysis by Melese *et al*[28] identified that, in Ethiopia, despite the high prevalence of *H. pylori* there was a decreasing pattern in the trend of infection over the years[28]. Hence, using such old data for some African regions may not provide a good representative of the current prevalence of *H. pylori* in such regions and in the continent.

Another challenge faced in reporting the prevalence of *H. pylori* in Africa is that a majority of the studies focused on patients with symptoms of gastroduodenal disease. Considering that *H. pylori* has been identified as the main causative organism for gastric disorders, using data from such studies would overestimate the prevalence of *H. pylori* in Africa. Even studies that evaluated the prevalence of *H. pylori* in asymptomatic people were hospital-based cross-sectional studies and not community studies or population data. Carrying out a random selection of people in the community or even cross-sectional studies of people in the different African regions will provide a more accurate prevalence of *H. pylori* in Africa.

Another major challenge in estimating the prevalence of *H. pylori* in Africa is the different choice of the screening test used to identify the presence of the bacterium. A majority of the studies used blood serology to test for antibodies against *H. pylori*. This greatly influenced the estimated prevalence of *H. pylori* in Africa. In fact, according to Hanafi *et al*[20] getting a positive result when testing for the presence of *H. pylori* antibodies in the blood or serum does not distinguish between previous contact and active infection.

Several studies comparing the use of different tests on the estimated prevalence of *H. pylori* infection showed that the method significantly affects the prevalence of *H. pylori* infection. Asrat *et al*[61] compared *H. pylori* culture, rapid urease test, PCR-denaturing gel electrophoresis, histopathology, silver staining, stool antigen test and enzyme immunoassay assay. The authors found that *H. pylori* infection prevalence varied significantly based on the detection method used. They reported a prevalence of 69%, 71%, 91%, 81%, 75%, 81% and 80% using culture, rapid urease test, PCR-denaturing gel electrophoresis, histopathology, silver staining, stool antigen test and enzyme immunoassay assay, respectively[61]. Similar disparities was seen in the study by Harrison *et al*[57] with varying results using urea breath test, culture and a rapid urease test. Seid and Demsiss compared the stool antigen test with a serum anti-*H. pylori* IgG test. The authors found a detection rate of 30.4% and 60.5%, respectively[60].

Another challenge that affects estimating the prevalence of *H. pylori* infection in Africa is its dynamics. In most Western countries, *H. pylori* infection prevalence reduces with age. However, this has not been documented in most African countries where the reverse has been observed. Some of the studies found on the prevalence of *H. pylori* in asymptomatic patients or done in the community focused on children[36]. Melese *et al*[28], in their systematic review and meta-analysis, noted that in Ethiopia the prevalence of *H. pylori* increased with age[28]. A similar trend was reported by Mungazi *et al*[19] in Zimbabwe. This could reflect the so-called cohort-effect but considering that the prevalence of *H. pylori* may change from childhood to adulthood, using reports on a population group may under or overestimate the real epidemiology in that region.

TREATMENT OF *H. PYLORI* IN AFRICA: THE ACTUAL SCENARIO

Literature data recommend the use of a combination of proton pump inhibitors (PPIs) and antimicrobials to treat *H. pylori*. The Maastricht V Consensus Report recommends that the first-line regimen should be based on PPI drugs, clarithromycin and amoxicillin or metronidazole for 14 d in regions with low clarithromycin resistance. In

areas where a resistance rates of over 15%-20% for clarithromycin is recorded, the bismuth-containing quadruple therapy or concomitant therapy (including 3 antibiotics and a PPI) are recommended. Second line treatment should be based on the need to carry out an endoscopy. When this approach is requested, culture and standard antimicrobial susceptibility testing (AST) should be performed to lead to the more appropriate therapy. When endoscopy is not requested or is not possible, the rationale of the second-line treatment is to drop the empirical use of clarithromycin, due to the high possibility that strains of *H. pylori*-resistant to clarithromycin have developed. The use of levofloxacin-containing triple therapy, as a rescue therapy following the failure of the standard triple therapy, is a reasonable alternative when local fluoroquinolone resistance is < 10%. Third line therapy should be guided by AST only[10].

In Africa, there are no guidelines addressing *H. pylori* treatment in all countries of the continent. Nevertheless, the actual African scenario is enriched year by year by publications reporting the results of randomized clinical trials (RCT) or real-world data as well as the results of microbiological and genotypic analysis of bacterial resistance to antimicrobials.

STUDIES ON CLINICAL EFFICACY OF ANTIMICROBIALS FOR *H. PYLORI* ERADICATION IN AFRICA

The African pattern of *H. pylori* eradication rates could be described analyzing the macro-regions of this continent. In Tunisia, North Africa, the eradication rate has been reported significantly higher among patients treated by omeprazole, amoxicillin and clarithromycin (69.6%) compared to those treated with omeprazole, amoxicillin and metronidazole (48.7%)[62]. In Morocco, the results of two clinical trials based on the treatment with a clarithromycin-based triple therapy, indicated a high rate of *H. pylori*-resistance to cla-rithromycin. The first showed an eradication rate inferior than 80% [intention to treat (ITT) analysis: 78.2; per protocol (PP) analysis: 79.6][63]. The second showed a reduction in terms of eradication rates obtained by this regimen (ITT: 65.9; PP: 71)[64], indirectly suggesting an increased resistance rate to clarithromycin.

In Egypt, an attempt to found new antibiotic regimens has been reported. Two hundred and 24 patients with dyspeptic symptoms and *H. pylori*–infection were enrolled in a randomized study. Patients in group 1 received nitazoxanide (an antibiotic with characteristics similar to metronidazole) 500 mg twice daily, clarithromycin 500 mg twice daily, and omeprazole 40 mg twice daily for 14 d. Patients in group 2 received metronidazole 500 mg twice daily, clarithromycin 500 mg twice daily, and omeprazole 40 mg twice daily for 14 d. To assess the eradication rate, the stool antigen test was performed 6 wk after cessation of these treatments. The eradication rate was significantly higher in group 1 than in group 2. According to PP analysis, 106 cases (94.6%) of 112 patients who completed the study in the former group obtained complete cure *vs* 60.6% of 104 patients who completed the study in group 2, ($P < 0.001$). Thus, nitazoxanide is a promising antibiotic for the first-line therapy of *H. pylori* eradication[65].

In Nigeria, West Africa, a RCT comparing a 7-d *vs* a 10-d regimen of rabeprazole, amoxicillin and clarithromycin was carried out in 50 *H. pylori* positive patients with several gastroduodenal symptoms. The average eradication rate was 87.2% without significant difference between the two regimens[66].

In Kenya, East Africa, a RCT including 120 *H. pylori* positive dyspeptic patients, compared the efficacy of a 7-d *vs* a 14-d regimen using esomeprazole, amoxicillin and clarithromycin. The eradication rates, according to the ITT analysis, were 76.7% and 73.3% for 7 and 14 d respectively, while the eradication rates by PP analysis were 92% and 93.6% for 7 and 14 d, respectively. Thus, there was no significant difference between these two regimens[67].

In Rwanda, Central Africa, Kabakambira *et al*[68] conducted a RCT from November 2015 to October 2016. The authors enrolled 299 dyspeptic patients with several gastroduodenal diseases and *H. pylori* infection. All subjects were randomized to either a triple therapy, for 10 d, containing omeprazole, amoxicillin and one among clarithromycin/ciprofloxacin/metronidazole or a quadruple therapy containing omeprazole, amoxicillin, ciprofloxacin and doxycycline. The rate of *H. pylori* eradication was 80% in the total population and 78% in patients without a history of previous triple therapy treatment. Considered globally, the results showed a higher risk of failure in the metronidazole-based group although this was insignificant (36%, $P = 0.086$).

STUDIES ON LABORATORY-BASED ANTIMICROBIAL RESISTANCE OF *H. PYLORI* IN AFRICA

Antimicrobial resistance of H. pylori is a key factor associated with eradication failure. The prevalence of such resistance varies amongst different geographical areas and has increased globally.

Focusing on North Africa, in Egypt AST showed high phenotypic metronidazole resistance (100%) of *H. pylori* to metronidazole and low resistance to other tested antimicrobials[69]. Recently, the Cairo's University Hospital investigated the same issue, using molecular methods, in a study including 70 *H. pylori* positive biopsies of patients never treated for this infection. In 62.9% of the samples, the *rdxA* gene deletion (marker of metronidazole re-sistance) was detected, while analyzing the *H. pylori* 23S rRNA V domain, the A2142G mutation (marker of clarithromycin resistance) was shown in 55.7% of the cases[70]. The difference of results between these two studies may be explained by at least two factors. The first is the different age of patients included. In the latter, patients were > 18 years old while in the former patients were 2-17 years old, a group at higher risk of exposition to metronidazole for the treatment of parasitic infections. The second factor is the method used. In fact, in the former study the antibiotic resistance was evaluated by culture and *in vitro* AST[69], a method that have the tendency to overestimate resistance. In the latter study, metronidazole resistance was detected through genetic analysis[70]. In Tunisia, using both E-test and real-time PCR with Scorpion primers, resistances to clarithromycin and metronidazole were 15.4% and 51.3% respectively, with 0% resistance to amoxicillin. No discrepancy between the two methods was reported[71]. In Algeria, the prevalence of *H. pylori* resistance to clarithromycin was 33%[72]. In another study, in the same country, *H. pylori* isolates were sensitive to amoxicillin, tetracycline, rifampicin, but exhibited a high rate of resistance to metronidazole (61.1%) and a lower rate of resistance to clarithromycin (22.8%) and ciprofloxacin (16.8%). There was no statistically significant relationship found between *vac*A and *cag*A genotypes and antibiotic resistance results with the exception of metronidazole, for which there was a statistically significant relationship with *cag*A genotype (P = 0.001)[73]. In another prospective study carried out in Algeria between November 2015 and August 2016, isolation of *H. pylori* by culture was performed on antral and fundic gastric biopsies of adult patients from 3 hospitals. Additionally, real-time PCR using the fluorescence resonance energy transfer principle for the detection of *H. pylori* followed by a melting curve analysis to detect mutations associated with resistance to clarithromycin was employed. The prevalence of *H. pylori* infection was 57% using this technique with primary and secondary resistance rates to clarithromycin being 23% and 36%, respectively, and to metronidazole, 45% and 71%, respectively. All isolates were sensitive to amoxicillin, tetracycline, and rifampicin while only one isolate was resistant to levofloxacin[74]. In a study from Morocco, the primary resistance of *H. pylori* to clarithromycin was 28.2%. It was noted that women more often than men were infected with a resistant strain of *H. pylori* (38% *vs* 18%, P = 0.044). Using Scorpion PCR among 22 biopsies positive for *H. pylori*, 15 (68%) harbored an A2142G mutation, 6 (27%) an A2143G mutation, and 2 (9%) an A2142C mutation. The remaining *H. pylori* positive biopsy harbored a mixture of both A2142G and A2143G mutations. In 16 (77%) biopsies, a mixed infection with a susceptible and a resistant strain was reported[75].

Hence, considering that the resistance rate to clarithromycin is largely over the 15%-20% threshold put forward by the Maastricht V Consensus Report[10], it is appropriate to quit the clarithromycin-based treatment as a first-line strategy in several areas of North Africa.

In Senegal, West Africa, it was reported that the bacterial isolates showed a high rate of resistance to metronidazole (85%), low rate of resistance to clarithromycin (1%), and no resistance to amoxicillin and tetracycline[76]. In another study from Senegal, *H. pylori* resistance to metronidazole ranged from 85%-90%, that of clarithromycin ranged from 0-1% and the reported susceptibility to amoxicillin and ciprofloxacin was 100%[77]. In Nigeria, Aboderin *et al*[78] in the year 2007 reported multiple *H. pylori* resistance to amoxicillin (100%), clarithromycin (100%) and metronidazole (100%), as determined by disc diffusion test. Resistance to rifampicin, tetracycline and ciprofloxacin were 93.5%, 87.1% and 15.6% respectively. A total of 5 distinct antibiograms were encountered in the 32 *H. pylori* strains and the patterns varied with resistance ranging from 4 to 6 antimicrobial agents. Interesting, the profile of ciprofloxacin resistance was significantly different from that reported in 2005, when 0% of resistance was published by Albdulrasheed *et al*[79]. Another Nigerian study, published in 2017, reported by phenotypic evaluation a high resistance rate to metronidazole (99.1%), and decreasing resistance rates to amoxicillin (33.3%),

clarithromycin (14.4%) and tetracycline (4.5%)[57]. In Gambia, using DNA transformation and sequencing, Secka *et al*[80] investigated the role of the gene *rdxA* in metronidazole susceptibility. Metronidazole-resistant strains of *H. pylori* were rendered metronidazole susceptible by transformation with a functional *rdxA* gene; conversely, metronidazole-susceptible strains were rendered metronidazole resistant by *rdxA* inactivation. *RdxA* sequencing revealed many mutations amongst their *H. pylori* strains, which probably explains inactivation of *rdxA* in metronidazole-resistant strains. None of the isolates were resistant to cla-rithromycin and erythromycin while amoxicillin and tetracycline resistances were rare. These data suggest that in Gambia, the use of metronidazole-based therapies for *H. pylori* eradication should be considered with caution[80].

In Congo, Central Africa, by using molecular methods, *H. pylori* resistance to clarithromycin and tetracycline were surprisingly low (1.7 and 2.5% respectively) but a high rate of resistance (50%) to fluoroquinolones was reported[81]. A study from Cameroon reported high resistance rates to tetracycline, clarithromycin and metronidazole (44.7%, 85.6% and 93.2%, respectively)[82]. In Uganda, analyzing by molecular methods stool samples of patients with peptic ulcer disease, among samples positive for *H. pylori* infection, 29% were resistant to clarithromycin and 42% to fluoroquinolones[83].

In East Africa, *H. pylori* strains had resistance rates of 0-6.4% to clarithromycin, 0-6% to amoxicillin, 0-1.9% to tetracycline but had 4.6% to 100% resistance to metronidazole[22,84,85]. Hence, in these countries a clarithromycin-based therapy could represent an appropriate therapeutic strategy.

In South Africa, *H. pylori* resistance rates were 0% for ciprofloxacin, 2.5% for amoxicillin, 20% and 27.5% for clarithromycin and gentamicin, while the resistance rate to metronidazole was very high (95.5%)[86]. The antibiotic resistance profile changed analyzing data region by region and over the time. In another study, published 3 years after, the same group reported a similar *H. pylori* resistance to clarithromycin (15.3%) but a higher resistance rate (10.2%) to fluoroquinolones[87].

Finally, in a systematic review with meta-analysis Jaka *et al*[88] investigated the magnitude of *H. pylori* antibiotic resistance in Africa. Considering 26 articles, the overall *H. pylori* resistance rates to fluoroquinolones, clarithromycin, tetracycline, metronidazole and amoxicillin were: 17.4%, 29.2%, 48.7%, 75.8%, and 72.6%, respectively. As expected, the commonest mutation detected for resistant strains were A2143G for clarithromycin, RdxA for metronidazole and D87I for fluoroquinolones. Figure 1 shows an overview of the laboratory-based antimicrobial resistance of *H. pylori* in Africa. Taking together these data suggest that in several African countries, a clarithromycin-based first-line regimen should be abandoned and the surveillance of antibiotic resistance should lead empirical treatments where an AST is not available.

CONCLUSION

H. pylori remains highly prevalent in Africa. However, even in the face of highly pathogenic *H. pylori*, the clinical manifestations of this infection are still mild. Several factors, which have not been fully studied, fuel this intriguing issue and there is a need for further research to identify host factors that may explain the "African enigma" . The current prevalence of *H. pylori* in the different African regions does not seemingly provide an accurate view of the prevalence of *H. pylori* in the continent. It is necessary to carry out studies that will provide population data, and that will use highly accurate methods to correctly detect the presence of *H. pylori*. However, considering that such research may be expensive in a low resource continent like Africa, meta-analyses grouping data of the different African countries or of the different regions could provide a pooled and better estimate of the prevalence of *H. pylori* infection. Since *H. pylori* infection represents a major challenge in Africa, two major issues should be considered, in terms of prevention and cure. First, the possibility of preventing *H. pylori* acquisition is linked to the availability of a vaccine and to the amelioration of hygienic conditions of the general population[89-91]. However, the fact that *H. pylori* infection induces strong humoral and cellular immune responses, and that the latter are not able to eliminate the bacterium, raises doubts about the possibility of developing an effective vaccine. Second, in terms of cure, it should be detailed in each country the pattern of *H. pylori* antibiotic resistance. This is mandatory to establish the more appropriate treatment. Where these data would be available, the epidemiologic surveillance over time should lead clinicians to the choice of the best option.

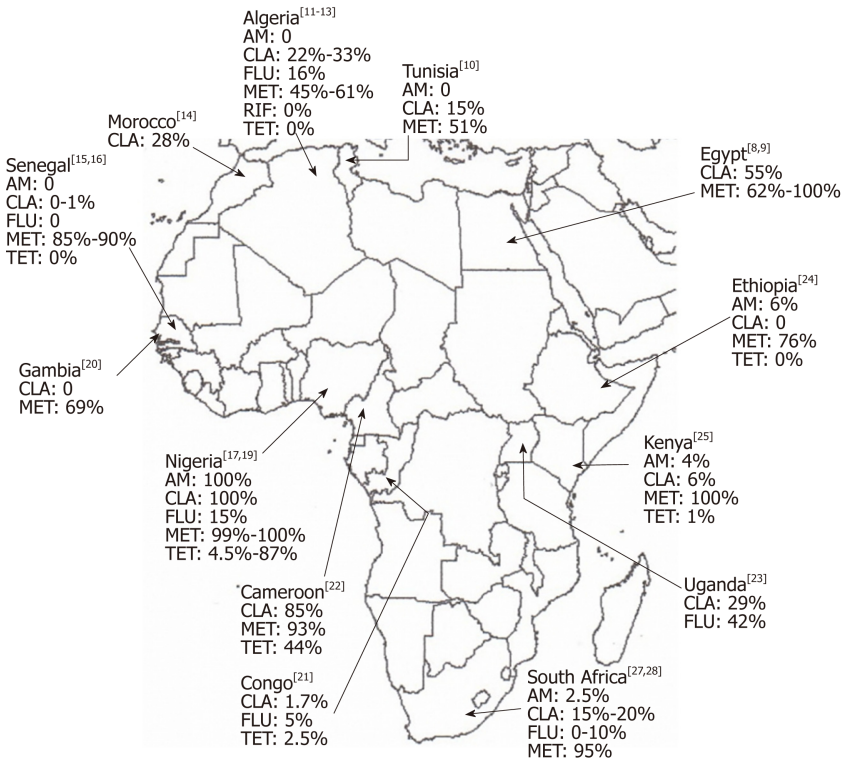


Figure 1 Laboratory-based antimicrobial resistance of *Helicobacter pylori* in Africa. AM: Amoxicillin; CLA: Clarithromycin; FLU: Fluoroquinolones; MET: Metronidazole; RIF: Rifampicin; TET: Tetracycline.

REFERENCES

1 **Peek RM**, Crabtree JE. Helicobacter infection and gastric neoplasia. *J Pathol* 2006; **208**: 233-248 [PMID: 16362989 DOI: 10.1002/path.1868]
2 **Pizzorno JE**, Murray MT, Joiner-Bey H, The clinicians handbook of Natural Medicine. Peptic Ulcers. The clinicians handbook of Natural Medicine. *3rd ed*. St Lois, Missouri: Elsevier 2016; 779-786 [DOI: 10.1016/B978-0-7020-5514-0.00072-5]
3 **Jonaitis L**, Pellicano R, Kupcinskas L. Helicobacter pylori and nonmalignant upper gastrointestinal diseases. *Helicobacter* 2018; **23** Suppl 1: e12522 [PMID: 30203583 DOI: 10.1111/hel.12522]
4 **IARC**. *Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans*. Lyon: WHO press 1994; 1-241
5 **Rugge M**, Fassan M, Graham DY, Strong V. Epidemiology of gastric cancer. Strong V. *Gastric Cancer*. Cham: Springer 2015; 23-32
6 **Graham DY**. History of Helicobacter pylori, duodenal ulcer, gastric ulcer and gastric cancer. *World J Gastroenterol* 2014; **20**: 5191-5204 [PMID: 24833849 DOI: 10.3748/wjg.v20.i18.5191]
7 **Pellicano R**, Ménard A, Rizzetto M, Mégraud F. Helicobacter species and liver diseases: Association or causation? *Lancet Infect Dis* 2008; **8**: 254-260 [PMID: 18353266 DOI: 10.1016/S1473-3099(08)70066-5]
8 **Ribaldone DG**, Fagoonee S, Hickman I, Altruda F, Saracco GM, Pellicano R. Helicobacter pylori infection and ischemic heart disease: Could experimental data lead to clinical studies? *Minerva Cardioangiol* 2016; **64**: 686-696 [PMID: 27603552]
9 **de Korwin JD**, Ianiro G, Gibiino G, Gasbarrini A. Helicobacter pylori infection and extragastric diseases in 2017. *Helicobacter* 2017; 22 Suppl 1 [PMID: 28891133 DOI: 10.1111/hel.12411]
10 **Malfertheiner P**, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
11 **Hooi JKY**, Lai WY, Ng WK, Suen MMY, Underwood FE, Tanyingoh D, Malfertheiner P, Graham DY, Wong VWS, Wu JCY, Chan FKL, Sung JJY, Kaplan GG, Ng SC. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology* 2017; **153**: 420-429 [PMID: 28456631 DOI: 10.1053/j.gastro.2017.04.022]
12 World gastroenterology organisation global guideline: Helicobacter pylori in developing countries. *J Dig Dis* 2011; **12**: 319-326 [PMID: 21955424 DOI: 10.1111/j.1751-2980.2011.00529.x]
13 **Zamani M**, Ebrahimtabar F, Zamani V, Miller WH, Alizadeh-Navaei R, Shokri-Shirvani J, Derakhshan MH. Systematic review with meta-analysis: The worldwide prevalence of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2018; **47**: 868-876 [PMID: 29430669 DOI: 10.1111/apt.14561]
14 **Tanih NF**, Dube C, Green E, Mkwetshana N, Clarke AM, Ndip LM, Ndip RN. An African perspective on Helicobacter pylori: Prevalence of human infection, drug resistance, and alternative approaches to treatment. *Ann Trop Med Parasitol* 2009; **103**: 189-204 [PMID: 19341534 DOI: 10.1179/136485909X398311]
15 **Walker TD**, Karemera M, Ngabonziza F, Kyamanywa P. Helicobacter pylori status and associated

gastroscopic diagnoses in a tertiary hospital endoscopy population in Rwanda. *Trans R Soc Trop Med Hyg* 2014; **108**: 305-307 [PMID: 24598794 DOI: 10.1093/trstmh/tru029]
16 **Didelot X**, Nell S, Yang I, Woltemate S, van der Merwe S, Suerbaum S. Genomic evolution and transmission of Helicobacter pylori in two South African families. *Proc Natl Acad Sci U S A* 2013; **110**: 13880-13885 [PMID: 23898187 DOI: 10.1073/pnas.1304681110]
17 **Schwarz S**, Morelli G, Kusecek B, Manica A, Balloux F, Owen RJ, Graham DY, van der Merwe S, Achtman M, Suerbaum S. Horizontal versus familial transmission of Helicobacter pylori. *PLoS Pathog* 2008; **4**: e1000180 [PMID: 18949030 DOI: 10.1371/journal.ppat.1000180]
18 **Nell S**, Eibach D, Montano V, Maady A, Nkwescheu A, Siri J, Elamin WF, Falush D, Linz B, Achtman M, Moodley Y, Suerbaum S. Recent acquisition of Helicobacter pylori by Baka pygmies. *PLoS Genet* 2013; **9**: e1003775 [PMID: 24068950 DOI: 10.1371/journal.pgen.1003775]
19 **Mungazi SG**, Chihaka OB, Muguti GI. Prevalence of Helicobacter pylori in asymptomatic patients at surgical outpatient department: Harare hospitals. *Ann Med Surg (Lond)* 2018; **35**: 153-157 [PMID: 30302246 DOI: 10.1016/j.amsu.2018.09.040]
20 **Hanafi NF**, Mikhael IL, Younan DN. Prevalence of Helicobacter pylori Antibodies in Egyptians with Idiopathic Thrombocytopenic purpura and in the General Egyptian Population: A Comparative Study. *Int J Curr Microbiol Appl Sci* 2017; **6**: 2482-2492 [DOI: 10.20546/ijcmas.2017.605.278]
21 **Bounder G**, Boura H, Nadifiyine S, Jouimyi MR, Bensassi M, Kadi M, Eljihad M, Badre, W, Benomar H, Kettani A, Lebrazi H, Maaachi F. Epidemiology of Helicobacter pylori infection and related gastric pathologies in Moroccan population. *J Life Sci* 2017; **11**: 211-218 [DOI: 10.17265/1934-7391/2017.05.001]
22 **Kimang'a AN**, Revathi G, Kariuki S, Sayed S, Devani S. Helicobacter pylori: Prevalence and antibiotic susceptibility among Kenyans. *S Afr Med J* 2010; **100**: 53-57 [PMID: 20429490 DOI: 10.1016/j.revmed.2009.02.027]
23 **Taddesse G**, Habteselassie A, Desta K, Esayas S, Bane A. Association of dyspepsia symptoms and Helicobacter pylori infections in private higher clinic, Addis Ababa, Ethiopia. *Ethiop Med J* 2011; **49**: 109-116 [PMID: 21796910]
24 **Hestvik E**, Tylleskar T, Kaddu-Mulindwa DH, Ndeezi G, Grahnquist L, Olafsdottir E, Tumwine JK. Helicobacter pylori in apparently healthy children aged 0-12 years in urban Kampala, Uganda: A community-based cross sectional survey. *BMC Gastroenterol* 2010; **10**: 62 [PMID: 20553588 DOI: 10.1186/1471-230X-10-62]
25 **Archampong TN**, Asmah RH, Wiredu EK, Gyasi RK, Nkrumah KN, Rajakumar K. Epidemiology of Helicobacter pylori infection in dyspeptic Ghanaian patients. *Pan Afr Med J* 2015; **20**: 178 [PMID: 26430475 DOI: 10.11604/pamj.2015.20.178.5024]
26 **Dube C**, Tanih NF, Ndip RN. Helicobacter pylori in water sources: A global environmental health concern. *Rev Environ Health* 2009; **24**: 1-14 [PMID: 19476289 DOI: 10.1515/REVEH.2009.24.1.1]
27 **Dube C**, Tanih NF, Clarke AM, Mkwetshana N, Green E, Ndip RN. Helicobacter pylori infection and transmission in Africa: Household hygiene and water sources are plausible factor exacerbating spread-. *Afr J Biotechnol* 2009; **8**: 6028-6035 [DOI: 10.5897/AJB09.824]
28 **Melese A**, Genet C, Zeleke B, Andualem T. Helicobacter pylori infections in Ethiopia; prevalence and associated factors: A systematic review and meta-analysis. *BMC Gastroenterol* 2019; **19**: 8 [PMID: 30630433 DOI: 10.1186/s12876-018-0927-3]
29 **Aje AO**, Otegbayo JA, Odaibo GN, Bojuwoye BJ. Comparative study of stool antigen test and serology for Helicobacter pylori among Nigerian dyspeptic patients--a pilot study. *Niger J Clin Pract* 2010; **13**: 120-124 [PMID: 20499740 DOI: 10.1007/s00063-010-1076-9]
30 **Jemilohun AC**, Otegbayo JA, Ola SO, Oluwasola OA, Akere A. Prevalence of Helicobacter pylori among Nigerian patients with dyspepsia in Ibadan. *Pan Afr Med J* 2010; **6**: 18 [PMID: 21734925 DOI: 10.4314/pamj.v6i1.69090]
31 **Etukudo OM**, Ikpeme EE, Ekanem EE. Seroepidemiology of Helicobacter pylori infection among children seen in a tertiary hospital in Uyo, southern Nigeria. *Pan Afr Med J* 2012; **12**: 39 [PMID: 22891097]
32 **Ophori EA**, Isibor C, Onemu SO, Johnny EJ. Immunological response to Helicobacter pylori among healthy volunteers in Agbor, Nigeria. *Asian Pac J Trop Dis* 2011; **1**: 38-40
33 **Ishaleku D**, Ihiabe, H. Seroprevalence of Helicobacter pylori infection among students of a Nigerian University. *Asian Pac J Trop Med* 2010; **3**: 584-585 [DOI: 10.1016/S1995-7645(10)60142-3]
34 **Ezeigbo RO**, Ezeigbo CI. Prevalence of Helicobacter pylori and its associated peptic ulcer infection among adult residents of Aba, Southeastern, Nigeria. *Int J Curr Microbiol Appl Sci* 2016; **5**: 16-21 [DOI: 10.20546/ijcmas.2016.506.002]
35 **Smith SI**, Jolaiya T, Onyekwere C, Fowora M, Ugiagbe R, Agbo I, Cookey C, Lesi O, Ndububa D, Adekanle O, Palamides P, Adeleye I, Njom H, Idowu A, Clarke A, Pellicano R. Prevalence of Helicobacter pylori infection among dyspeptic patients with and without type 2 diabetes mellitus in Nigeria. *Minerva Gastroenterol Dietol* 2019; **65**: 36-41 [PMID: 30293417 DOI: 10.23736/S1121-421X.18.02528-X]
36 **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]
37 **McLaughlin NJ**, McLaughlin DI, Lefcort H. The influence of socio-economic factors on Helicobacter pylori infection rates of students in rural Zambia. *Cent Afr J Med* 2003; **49**: 38-41 [PMID: 14562589]
38 **Bello AK**, Umar AB, Borodo MM. Prevalence and risk factors for Helicobacter pylori infection in gastroduodenal diseases in Kano, Nigeria. *Afr J Med Health Sci* 2018; **17**: 41-46 [DOI: 10.4103/ajmhs.ajmhs_36_17]
39 **Smith S**, Jolaiya T, Fowora M, Palamides P, Ngoka F, Bamidele M, Lesi O, Onyekwere C, Ugiagbe R, Agbo I, Ndububa D, Adekanle O, Adedeji A, Adeleye I, Harrison U. Clinical and Socio- Demographic Risk Factors for Acquisition of Helicobacter pylori Infection in Nigeria. *Asian Pac J Cancer Prev* 2018; **19**: 1851-1857 [PMID: 30049197 DOI: 10.22034/APJCP.2018.19.7.1851]
40 **Aguemon BD**, Struelens MJ, Massougbodji A, Ouendo EM. Prevalence and risk-factors for Helicobacter pylori infection in urban and rural Beninese populations. *Clin Microbiol Infect* 2005; **11**: 611-617 [PMID: 16008612 DOI: 10.1111/j.1469-0691.2005.01189.x]
41 **Kouitcheu Mabeku LB**, Noundjeu Ngamga ML, Leundji H. Potential risk factors and prevalence of Helicobacter pylori infection among adult patients with dyspepsia symptoms in Cameroon. *BMC Infect Dis* 2018; **18**: 278 [PMID: 29907086 DOI: 10.1186/s12879-018-3146-1]

42 **El-Sharouny E**, El-Shazli H, Olama Z. Detection of Helicobacter pylori DNA in Some Egyptian Water Systems and Its Incidence of Transmission to Individuals. *Iran J Public Health* 2015; **44**: 203-210 [PMID: 25905054]

43 **Kuipers EJ**, Meijer GA. Helicobacter pylori gastritis in Africa. *Eur J Gastroenterol Hepatol* 2000; **12**: 601-603 [PMID: 10912474 DOI: 10.1097/00042737-200012060-00003]

44 **Kidd M**, Louw JA, Marks IN. Helicobacter pylori in Africa: Observations on an 'enigma within an enigma'. *J Gastroenterol Hepatol* 1999; **14**: 851-858 [PMID: 10535465 DOI: 10.1046/j.1440-1746.1999.01975.x]

45 **Asombang AW**, Kelly P. Gastric cancer in Africa: What do we know about incidence and risk factors? *Trans R Soc Trop Med Hyg* 2012; **106**: 69-74 [PMID: 22136952 DOI: 10.1016/j.trstmh.2011.11.002]

46 **Holcombe C**. Helicobacter pylori: The African enigma. *Gut* 1992; **33**: 429-431 [PMID: 1582581 DOI: 10.1136/gut.33.4.429]

47 **Alaoui Boukhris S**, Benajah DA, El Rhazi K, Ibrahimi SA, Nejjari C, Amarti A, Mahmoud M, El Abkari M, Souleimani A, Bennani B. Prevalence and distribution of Helicobacter pylori cagA and vacA genotypes in the Moroccan population with gastric disease. *Eur J Clin Microbiol Infect Dis* 2012; **31**: 1775-1781 [PMID: 22160824 DOI: 10.1007/s10096-011-1501-x]

48 **Al-Eraky DM**, Helmy OM, Ragab YM, Abdul-Khalek Z, El-Seidi EA, Ramadan MA. Prevalence of CagA and antimicrobial sensitivity of H. pylori isolates of patients with gastric cancer in Egypt. *Infect Agent Cancer* 2018; **13**: 24 [PMID: 30026792 DOI: 10.1186/s13027-018-0198-1]

49 **Ezzat AHH**, Ali MH, El-Seidi, EA, Wali IE, Sedky NA, Naguib SMM. Genotypic characterization of Helicobacter pylori isolates among Egyptian patients with upper gastrointestinal diseases. *Chinese-German J Clin Oncol* 2012; **11**: 15-23 [DOI: 10.1007/s10330-011-0880-x]

50 **Ayana SM**, Swai B, Maro VP, Kibiki GS. Upper gastrointestinal endoscopic findings and prevalence of Helicobacter pylori infection among adult patients with dyspepsia in northern Tanzania. *Tanzan J Health Res* 2014; **16**: 16-22 [PMID: 26867268 DOI: 10.4314/thrb.v16i1.3]

51 **Oling M**, Odongo J, Kituuka O, Galukande M. Prevalence of Helicobacter pylori in dyspeptic patients at a tertiary hospital in a low resource setting. *BMC Res Notes* 2015; **8**: 256 [PMID: 26100113 DOI: 10.1186/s13104-015-1184-y]

52 **Alebie G**, Kaba D. Prevalence of Helicobacter pylori infection and associated factors among gastritis students in Jigjiga University, jigiiga, somali regional state of Ethiopia. *J Bacteriol Mycol* 2016; **3**: 234-239 [DOI: 10.15406/jbmoa.2016.03.00060]

53 **Ankouane F**, Noah DN, Enyime FN, Ndjollé CM, Djapa RN, Nonga BN, Njoya O, Ndam EC. Helicobacter pylori and precancerous conditions of the stomach: The frequency of infection in a cross-sectional study of 79 consecutive patients with chronic antral gastritis in Yaoundé, Cameroon. *Pan Afr Med J* 2015; **20**: 52 [PMID: 26090010 DOI: 10.11604/pamj.2015.20.52.5887]

54 **Ebule IA**, Djune FAK, Sitedjeya MIL, Tanni B, Heugueu C, Longdoh AN, Noah ND, Okomo AMC, Paloheimo L, Njoya O, Syrjanen K. Prevalence of H. pylori Infection and Atrophic Gastritis among dyspeptic subjects in Cameroon using a Panel of Serum Biomarkers (PGI, PGII, G-17, HpIgG). *Sch J Appl Med Sci* 2017; **5**: 1230-1239 [DOI: 10.21276/sjams]

55 **Afihene MKY**, Denyer M, Amuasi JJ, Boakye I, Nkrumah K. Prevalence of Helicobacter pylori and endoscopic findings among dyspeptics in Kumasi, Ghana. *Open Sci J Clin Med* 2014; **2**: 63-68

56 **Darko R**, Yawson AE, Osei V, Owusu-Ansah J, Aluze-Ele S. Changing Patterns of the Prevalence of Helicobacter Pylori Among Patients at a Corporate Hospital in Ghana. *Ghana Med J* 2015; **49**: 147-153 [PMID: 26693189 DOI: 10.4314/gmj.v49i3.4]

57 **Harrison U**, Fowora MA, Seriki AT, Loell E, Mueller S, Ugo-Ijeh M, Onyekwere CA, Lesi OA, Otegbayo JA, Akere A, Ndububa DA, Adekanle O, Anomneze E, Abdulkareem FB, Adeleye IA, Crispin A, Rieder G, Fischer W, Smith SI, Haas R. Helicobacter pylori strains from a Nigerian cohort show divergent antibiotic resistance rates and a uniform pathogenicity profile. *PLoS One* 2017; **12**: e0176454 [PMID: 28463973 DOI: 10.1371/journal.pone.0176454]

58 **Tanih NF**, Okeleye BI, Ndip LM, Clarke AM, Naidoo N, Mkwetshana N, Green E, Ndip RN. Helicobacter pylori prevalence in dyspeptic patients in the Eastern Cape province - race and disease status. *S Afr Med J* 2010; **100**: 734-737 [PMID: 21081026 DOI: 10.7196/SAMJ.4041]

59 **Ghoshal UC**, Chaturvedi R, Correa P. The enigma of Helicobacter pylori infection and gastric cancer. *Indian J Gastroenterol* 2010; **29**: 95-100 [PMID: 20585917 DOI: 10.1007/s12664-010-0024-1]

60 **Seid A**, Demsiss W. Feco-prevalence and risk factors of Helicobacter pylori infection among symptomatic patients at Dessie Referral Hospital, Ethiopia. *BMC Infect Dis* 2018; **18**: 260 [PMID: 29879914 DOI: 10.1186/s12879-018-3179-5]

61 **Asrat D**, Nilsson I, Mengistu Y, Ashenafi S, Ayenew K, Al-Soud WA, Wadström T, Kassa E. Prevalence of Helicobacter pylori infection among adult dyspeptic patients in Ethiopia. *Ann Trop Med Parasitol* 2004; **98**: 181-189 [PMID: 15035728 DOI: 10.1179/000349804225003190]

62 **Loghmari H**, Bdioui F, Bouhlel W, Melki W, Hellara O, Ben Chaabane N, Safer L, Zakhama A, Saffar H. Clarithromycin versus metronidazole in first-line Helicobacter pylori eradication. Prospective randomized study of 85 Tunisian adults. *Tunis Med* 2012; **90**: 31-35 [PMID: 22311445]

63 **Lahbabi M**, Alaoui S, El Rhazi K, El Abkari M, Nejjari C, Amarti A, Bennani B, Mahmoud M, Ibrahimi A, Benajah DA. Sequential therapy versus standard triple-drug therapy for Helicobacter pylori eradication: Result of the HPFEZ randomised study. *Clin Res Hepatol Gastroenterol* 2013; **37**: 416-421 [PMID: 23168228 DOI: 10.1016/j.clinre.2012.10.002]

64 **Seddik H**, Ahid S, El Adioui T, El Hamdi FZ, Hassar M, Abouqal R, Cherrah Y, Benkirane A. Sequential therapy versus standard triple-drug therapy for Helicobacter pylori eradication: A prospective randomized study. *Eur J Clin Pharmacol* 2013; **69**: 1709-1715 [PMID: 23695545 DOI: 10.1007/s00228-013-1524-6]

65 **Shehata MA**, Talaat R, Soliman S, Elmesseri H, Soliman S, Abd-Elsalam S. Randomized controlled study of a novel triple nitazoxanide (NTZ)-containing therapeutic regimen versus the traditional regimen for eradication of Helicobacter pylori infection. *Helicobacter* 2017; 22 [PMID: 28524341 DOI: 10.1111/hel.12395]

66 **Onyekwere CA**, Odiagah JN, Igetei R, Emanuel AO, Ekere F, Smith S. Rabeprazole, clarithromycin, and amoxicillin Helicobacter pylori eradication therapy: report of an efficacy study. *World J Gastroenterol* 2014; **20**: 3615-3619 [PMID: 24707145 DOI: 10.3748/wjg.v20.i13.3615]

67 **Sokwala A**, Shah MV, Devani S, Yonga G. Helicobacter pylori eradication: A randomised comparative trial of 7-day versus 14-day triple therapy. *S Afr Med J* 2012; **102**: 368-371 [PMID: 22668909 DOI: 10.7196/SAMJ.5302]

68 **Kabakambira JD**, Hategeka C, Page C, Ntirenganya C, Dusabejambo V, Ndoli J, Ngabonziza F, Hale D,

Bayingana C, Walker T. Efficacy of Helicobacter pylori eradication regimens in Rwanda: A randomized controlled trial. *BMC Gastroenterol* 2018; **18**: 134 [PMID: 30165823 DOI: 10.1186/s12876-018-0863-2]
69 **Sherif M**, Mohran Z, Fathy H, Rockabrand DM, Rozmajzl PJ, Frenck RW. Universal high-level primary metronidazole resistance in Helicobacter pylori isolated from children in Egypt. *J Clin Microbiol* 2004; **42**: 4832-4834 [PMID: 15472354 DOI: 10.1128/JCM.42.10.4832-4834.2004]
70 **Ramzy I**, Elgarem H, Hamza I, Ghaith D, Elbaz T, Elhosary W, Mostafa G, Elzahry MA. Genetic mutations affecting the first line eradication therapy of helicobacter pylori-infected egyptian patients. *Rev Inst Med Trop Sao Paulo* 2016; **58**: 88 [PMID: 27982354 DOI: 10.1590/S1678-9946201658088]
71 **Ben Mansour K**, Burucoa C, Zribi M, Masmoudi A, Karoui S, Kallel L, Chouaib S, Matri S, Fekih M, Zarrouk S, Labbene M, Boubaker J, Cheikh I, Hriz MB, Siala N, Ayadi A, Filali A, Mami NB, Najjar T, Maherzi A, Sfar MT, Fendri C. Primary resistance to clarithromycin, metronidazole and amoxicillin of Helicobacter pylori isolated from Tunisian patients with peptic ulcers and gastritis: A prospective multicentre study. *Ann Clin Microbiol Antimicrob* 2010; **9**: 22 [PMID: 20707901 DOI: 10.1186/1476-0711-9-22]
72 **Djennane-Hadibi F**, Bachtarzi M, Layaida K, Ali Arous N, Nakmouche M, Saadi B, Tazir M, Ramdani-Bouguessa N, Burucoa C. High-Level Primary Clarithromycin Resistance of Helicobacter pylori in Algiers, Algeria: A Prospective Multicenter Molecular Study. *Microb Drug Resist* 2016; **22**: 223-226 [PMID: 26554340 DOI: 10.1089/mdr.2015.0209]
73 **Bachir M**, Allem R, Tifrit A, Medjekane M, Drici AE, Diaf M, Douidi KT. Primary antibiotic resistance and its relationship with cagA and vacA genes in Helicobacter pylori isolates from Algerian patients. *Braz J Microbiol* 2018; **49**: 544-551 [PMID: 29452847 DOI: 10.1016/j.bjm.2017.11.003]
74 **Raaf N**, Amhis W, Saoula H, Abid A, Nakmouche M, Balamane A, Ali Arous N, Ouar-Korichi M, Vale FF, Bénéjat L, Mégraud F. Prevalence, antibiotic resistance, and MLST typing of Helicobacter pylori in Algiers, Algeria. *Helicobacter* 2017; 22 [PMID: 29035009 DOI: 10.1111/hel.12446]
75 **Bouilhat N**, Burucoa C, Benkirane A, El Idrissi-Lamghari A, Al Bouzidi A, El Feydi A, Elouennas M, Benouda A. High-level primary clarithromycin resistance of Helicobacter pylori in Morocco: A prospective multicenter molecular study. *Helicobacter* 2015; **20**: 422-423 [PMID: 25735573 DOI: 10.1111/hel.12219]
76 **Seck A**, Burucoa C, Dia D, Mbengue M, Onambele M, Raymond J, Breurec S. Primary antibiotic resistance and associated mechanisms in Helicobacter pylori isolates from Senegalese patients. *Ann Clin Microbiol Antimicrob* 2013; **12**: 3 [PMID: 23298145 DOI: 10.1186/1476-0711-12-3]
77 **Seck A**, Mbengue M, Gassama-Sow A, Diouf L, Ka MM, Boye CS. Antibiotic susceptibility of Helicobacter pylori isolates in Dakar, Senegal. *J Infect Dev Ctries* 2009; **3**: 137-140 [PMID: 19755744 DOI: 10.3855/jidc.512]
78 **Aboderin OA**, Abdu AR, Odetoyin B', Okeke IN, Lawal OO, Ndububa DA, Agbakwuru AE, Lamikanra A. Antibiotic resistance of Helicobacter pylori from patients in Ile-Ife, South-west, Nigeria. *Afr Health Sci* 2007; **7**: 143-147 [PMID: 18052867]
79 **Abdulrasheed A**, Lawal OO, Abioye-Kuteyi EA, Lamikanra A. Antimicrobial susceptibility of Helicobacter pylori isolates of dyspeptic Nigerian patients. *Trop Gastroenterol* 2005; **26**: 85-88 [PMID: 16225052]
80 **Secka O**, Berg DE, Antonio M, Corrah T, Tapgun M, Walton R, Thomas V, Galano JJ, Sancho J, Adegbola RA, Thomas JE. Antimicrobial susceptibility and resistance patterns among Helicobacter pylori strains from The Gambia, West Africa. *Antimicrob Agents Chemother* 2013; **57**: 1231-1237 [PMID: 23263004 DOI: 10.1128/AAC.00517-12]
81 **Ontsira Ngoyi EN**, Atipo Ibara BI, Moyen R, Ahoui Apendi PC, Ibara JR, Obengui O, Ossibi Ibara RB, Nguimbi E, Niama RF, Ouamba JM, Yala F, Abena AA, Vadivelu J, Goh KL, Menard A, Benejat L, Sifre E, Lehours P, Megraud F. Molecular Detection of Helicobacter pylori and its Antimicrobial Resistance in Brazzaville, Congo. *Helicobacter* 2015; **20**: 316-320 [PMID: 25585658 DOI: 10.1111/hel.12204]
82 **Ndip RN**, Malange Takang AE, Ojongokpoko JE, Luma HN, Malongue A, Akoachere JF, Ndip LM, MacMillan M, Weaver LT. Helicobacter pylori isolates recovered from gastric biopsies of patients with gastro-duodenal pathologies in Cameroon: Current status of antibiogram. *Trop Med Int Health* 2008; **13**: 848-854 [PMID: 18384477 DOI: 10.1111/j.1365-3156.2008.02062.x]
83 **Angol DC**, Ocama P, Ayazika Kirabo T, Okeng A, Najjingo I, Bwanga F. Helicobacter pylori from Peptic Ulcer Patients in Uganda Is Highly Resistant to Clarithromycin and Fluoroquinolones: Results of the GenoType HelicoDR Test Directly Applied on Stool. *Biomed Res Int* 2017; **2017**: 5430723 [PMID: 28555193 DOI: 10.1155/2017/5430723]
84 **Asrat D**, Kassa E, Mengistu Y, Nilsson I, Wadström T. Antimicrobial susceptibility pattern of Helicobacter pylori strains isolated from adult dyspeptic patients in Tikur Anbassa University Hospital, Addis Ababa, Ethiopia. *Ethiop Med J* 2004; **42**: 79-85 [PMID: 16895024 DOI: 10.1128/JCM.42.6.2682-2684.2004]
85 **Lwai-Lume L**, Ogutu EO, Amayo EO, Kariuki S. Drug susceptibility pattern of Helicobacter pylori in patients with dyspepsia at the Kenyatta National Hospital, Nairobi. *East Afr Med J* 2005; **82**: 603-608 [PMID: 16619703 DOI: 10.4314/eamj.v82i12.9364]
86 **Tanih NF**, Okeleye BI, Naidoo N, Clarke AM, Mkwetshana N, Green E, Ndip LM, Ndip RN. Marked susceptibility of South African Helicobacter pylori strains to ciprofloxacin and amoxicillin: Clinical implications. *S Afr Med J* 2010; **100**: 49-52 [PMID: 20429489]
87 **Tanih NF**, Ndip RN. Molecular Detection of Antibiotic Resistance in South African Isolates of Helicobacter pylori. *Gastroenterol Res Pract* 2013; **2013**: 259457 [PMID: 23710166 DOI: 10.1155/2013/259457]
88 **Jaka H**, Rhee JA, Östlundh L, Smart L, Peck R, Mueller A, Kasang C, Mshana SE. The magnitude of antibiotic resistance to Helicobacter pylori in Africa and identified mutations which confer resistance to antibiotics: Systematic review and meta-analysis. *BMC Infect Dis* 2018; **18**: 193 [PMID: 29699490 DOI: 10.1186/s12879-018-3099-4]
89 **Ferreira J**, Moss SF. Current Paradigm and Future Directions for Treatment of Helicobacter pylori Infection. *Curr Treat Options Gastroenterol* 2014; **12**: 373-384 [PMID: 25187235 DOI: 10.1007/s11938-014-0027-6]
90 **Talebi Bezmin Abadi A**. Vaccine against Helicobacter pylori: Inevitable approach. *World J Gastroenterol* 2016; **22**: 3150-3157 [PMID: 27003991 DOI: 10.3748/wjg.v22.i11.3150]
91 **Maleki Kakelar H**, Barzegari A, Dehghani J, Hanifian S, Saeedi N, Barar J, Omidi Y. Pathogenicity of Helicobacter pylori in cancer development and impacts of vaccination. *Gastric Cancer* 2019; 23-36 [PMID: 30145749 DOI: 10.1007/s10120-018-0867-1]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3196-3206

DOI: 10.3748/wjg.v25.i25.3196

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

ORIGINAL ARTICLE

Basic Study

Sporamin suppresses growth of xenografted colorectal carcinoma in athymic BALB/c mice by inhibiting liver β-catenin and vascular endothelial growth factor expression

Chun Yang, Jing-Jie Zhang, Xiao-Peng Zhang, Rong Xiao, Peng-Gao Li

ORCID number: Chun Yang (0000-0001-9463-444X); Jing-Jie Zhang (0000-0001-7261-5125); Xiao-Peng Zhang (0000-0003-1389-5838); Rong Xiao (0000-0002-0603-9518); Peng-Gao Li (0000-0001-9134-5116).

Author contributions: Yang C and Zhang JJ contributed equally to the work and should be regarded as co-first authors; Li PG designed the research; Zhang JJ and Zhang XP performed the research; Yang C and Zhang JJ analyzed the data and wrote the paper; Xiao R revised the manuscript; Li PG has primary responsibility for the final content.

Supported by the National Natural Science Foundation of China, No. 81573128 and No. 81703216.

Institutional animal care and use committee statement: This study was approved by the institutional animal care and use committee of Capital Medical University.

Conflict-of-interest statement: The authors declare no conflicts of interest.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Chun Yang, Jing-Jie Zhang, Xiao-Peng Zhang, Rong Xiao, Peng-Gao Li, School of Public Health, Capital Medical University, Beijing Key Laboratory of Clinical Epidemiology, Beijing 100069, China

Corresponding author: Peng-Gao Li, PhD, Associate Professor, School of Public Health, Capital Medical University, Beijing Key Laboratory of Clinical Epidemiology, 10 Xitoutiao, You An Men Wai, Fengtai District, Beijing 100069, China. penggao@ccmu.edu.cn
Telephone: +86-10-83911651
Fax: +86-10-83911651

Abstract

BACKGROUND

Colorectal cancer (CRC) is the third most common malignancy of the digestive tract and the fifth leading cause of cancer-related mortality in China. Sporamin, a Kunitz-type trypsin inhibitor isolated from sweet potato, is a potential anti-cancer agent with activities against a number of malignant tumor cells *in vitro*. The liver secretes a myriad of endocrine factors that may facilitate the growth and transformation of tumors in the development of CRC.

AIM

To investigate the effects of sporamin on liver morphology and biomarkers of xenografted CRC in the liver of athymic BALB/c mice.

METHODS

Twenty-seven male BALB/c nude mice were randomly divided into control, vehicle, and sporamin groups. Mice in the latter two groups were intraperitoneally xenografted with LoVo colorectal carcinoma cells and intragastrically infused with saline or sporamin (0.5 g/kg body weight/d), respectively, for 3 wk. Hematoxylin and eosin (HE) staining of the sections was performed to observe morphological changes in hepatic tissue and real-time fluorescent quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) were used to measure the expression of β-catenin and vascular endothelial growth factor (VEGF) in the liver.

RESULTS

Sporamin significantly reduced the number and weight of tumor nodules formed in the abdominal cavity. Compared with the vehicle group, the mean tumor weight (± SD) in the sporamin group was significantly reduced (0.44 ± 0.10 g *vs*

Manuscript source: Unsolicited manuscript

Received: February 23, 2019
Peer-review started: February 23, 2019
First decision: April 4, 2019
Revised: May 14, 2019
Accepted: May 31, 2019
Article in press: June 1, 2019
Published online: July 7, 2019

P-Reviewer: Demir Y, Mohamed SY
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Ma YJ

0.26 ± 0.15 g) and the total number of tumors decreased from 93 to 55. HE staining showed that enlargement of the nucleus and synthesis of proteins within hepatocytes, as well as infiltration of inflammatory cells into the liver, were attenuated by sporamin. Immunohistochemical staining and ELISA showed that the concentrations of β-catenin and VEGF in the liver were significantly reduced by sporamin. Compared with the vehicle group, the expression of β-catenin measured in integrated optical density units per area was reduced in the sporamin group (47.29 ± 9.10 *vs* 26.14 ± 1.72; $P = 0.003$). Expression of VEGF was also reduced after sporamin intervention from 20.78 ± 2.06 in the vehicle group to 15.80 ± 1.09 in the sporamin group ($P = 0.021$). Compared with the vehicle group, the concentration of β-catenin decreased from 134.42 ± 22.04 pg/mL to 109.07 ± 9.65 pg/mL after sporamin intervention ($P = 0.00002$). qPCR indicated that compared to the vehicle group, relative mRNA expression of β-catenin and VEGF in the liver of mice in the sporamin-treated group was significantly reduced to 71% ± 1% ($P = 0.000001$) and 23% ± 7% ($P = 0.00002$), respectively, of the vehicle group levels.

CONCLUSION
Sporamin down-regulates the expression and secretion of β-catenin and VEGF in the liver, which subsequently inhibits the transcription of downstream genes involved in cancer progression and angiogenesis.

Key words: Sporamin; Colorectal cancer; Liver; Vascular endothelial growth factor; β-catenin

Core tip: Sporamin, a Kunitz-type trypsin inhibitor, restrains the growth of intraperitoneally xenografted LoVo [also known as colorectal cancer (CRC) cells] in athymic BALB/c mice. The mechanism determined by changes in morphology and tumor biomarkers in the liver involves sporamin-induced down-regulation of β-catenin secretion and vascular endothelial growth factor expression. This suppresses the formation of xenografted tumor nodules *in vivo* and subsequently inhibits the transcription of downstream genes involved in cancer progression and angiogenesis. The anti-cancer effects of sporamin against CRC are closely associated with its inhibitory effect on these tumor biomarkers. Further studies are warranted to elucidate the corresponding signal transduction events that mediate this process.

Citation: Yang C, Zhang JJ, Zhang XP, Xiao R, Li PG. Sporamin suppresses growth of xenografted colorectal carcinoma in athymic BALB/c mice by inhibiting liver β-catenin and vascular endothelial growth factor expression. *World J Gastroenterol* 2019; 25(25): 3196-3206
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3196.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3196

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy of the digestive tract and the fifth leading cause of cancer-related mortality in China[1,2]. The age-standardized incidence rate of CRC in China has increased from 12.8 per 100000 in 2003 to 14.2 per 100000 in 2017, while the mortality rate has risen from 5.9 to 7.4 per 100000. China has lower rates of CRC incidence and mortality than most developed countries, but has a higher case-fatality ratio (14.0%) and mortality/incidence ratio (52.1%)[3].

Treatment for CRC generally consists of surgery, adjuvant radiation, and chemotherapy as well as immunotherapy. Due to the low survival rate of CRC patients, there is an urgent need for new agents to combat this malignancy[4]. Plants are a rich source of various phytochemicals that may exert anti-oxidative, proapoptotic, anti-proliferative, anti-metastatic, and anti-angiogenic effects, depending on tumor type[5-8]. Sporamin is a Kunitz-type trypsin inhibitor that is found in the dico-

tyledonous plant, sweet potato (*Ipomoea batatas*), which belongs to the Convolvulaceae family. The tuberous roots contain 0.49%-2.24% crude sporamin protein on a fresh-weight basis[9,10]. Previous studies have identified sporamin as a potential anti-cancer agent against a number of malignant tumor cells, including HT29, HCT116, and SW480 colorectal cancer cells[11], TCA8113 tongue carcinoma cells[12] as well as PANC-1 and BxPC-3 pancreatic cancer cells[13].

The liver plays a vital role in the development of digestive tract cancers. It secretes a myriad of endocrine factors that may facilitate the growth and transformation of tumors, including β-catenin and vascular endothelial growth factor (VEGF). It is also the main metastatic target of CRC[14-16]. Thus, substances that can reduce the levels of tumor biomarkers in the liver may have the potential to become promising anti-cancer agents in the future. However, the effects of sporamin on the expression and secretion of tumor biomarkers in the liver are currently unknown. Therefore, in the present study, LoVo colorectal carcinoma cells were intraperitoneally xenografted into athymic BALB/c nude mice and sporamin was given orally to the mice to observe its effect on the growth of tumors, with a focus on changes in the structure and function of the liver, especially the expression and secretion of β-catenin and VEGF.

MATERIALS AND METHODS

Materials

Sporamin was extracted from sweet potatoes as previously reported[8]. Reagents used in these experiments were obtained from the following suppliers: SYBR Green and cDNA Reverse Transcription Kit, Thermo Scientific, Shanghai, China; TRIzol and diethyl pyrocarbonate, Invitrogen, Shanghai, China; anhydrous ethanol, chloroform, and isopropanol, Beijing Chemical Reagent Company, Beijing, China; Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, and PBS buffer, Corning, NY, United States; penicillin-streptomycin mixture and trypsin, Keygen Biotech, Nanjing, China; VEGF and β-catenin ELISA kits, Mecenbio, Beijing, China; xylene, gradient ethanol, 1% hydrochloric acid ethanol, and chloral hydrate dry powder, Sinopharm Science and Technology, Beijing, China; distilled water, Experimental Platform of Capital Medical University, Beijing, China; neutral formalin fixative, Leagene, Beijing, China; 3% hydrogen peroxide, BSA, hematoxylin dyeing solution, neutral gum, DAB chromogenic reagent, and β-catenin polyclonal antibody, Solarbio, Shanghai, China; VEGF polyclonal antibody, Abcam, Beijing, China; HRP-conjugated goat anti-rabbit antibody, KPL, Wuhan, China; RNAlater RNA stabilization reagent, Sigma, St. Louis, MO, United States.

Cells

The colon cancer LoVo cell line was purchased from the Tumor Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). The cells were grown in DMEM high-glucose medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2. The medium was changed every 48 h. Single cell suspensions containing 5×10^6 cells in 0.2 mL PBS were prepared during the logarithmic growth phase.

Animal experiments

All experiments were approved by the local Ethics Committee for Animal Research Studies at Capital Medical University, Beijing, China (animal experiment ethics review number: AEEI-2016-018).

Twenty-seven male BALB/c nude mice aged 4-6 wk old and weighing 13-15 g were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and maintained under specific pathogen-free conditions with free access to drinking water throughout the experiments. Animals were housed in a restricted access room under a 12-h/12-h light/dark cycle with a controlled temperature of 18-29 °C, daily range of temperature ≤ 3 °C, relative humidity of 40%-70%, airflow velocity ≤ 0.18 m/s, and room air pressure gradient of 20-50 Pa.

After one week of acclimation, animals were randomly divided into three groups, with nine animals in each group, and we verified that there was no significant differences in body weight between the groups. The groups were as follows: (1) Control group; (2) Vehicle group; and (3) Sporamin group. In the latter two groups, 5×10^6 LoVo cells in 0.2 mL PBS were xenografted into the abdominal cavity of the mice. Mice in the control group were injected with an equal volume of PBS to mimic the operation in the other groups. Sporamin was dissolved in distilled water and given through intragastric infusion to the mice at a dose of 0.5 g/kg body weight/d. Mice in the control and vehicle groups were given the same amount of distilled water by

intragastric infusion. The body weights of the mice were recorded every three days during the experiment. After three weeks of sporamin treatment, the mice were anesthetized with 10% chloral hydrate (0.1 mL/10 g body weight) and sacrificed by cervical dislocation. An autopsy of each mouse was carried out and the liver and the tumor nodes formed in the abdominal cavity were carefully counted, collected, and weighed. Then, the tissues samples were divided into three portions. Samples for pathological examinations were fixed in 10% neutral formalin and stored at room temperature. Samples for quantitative PCR (qPCR) were preserved in RNA stabilization reagent and stored at -20 °C. Samples for ELISA assays were directly stored at -80 °C in cryotubes.

Pathological examinations

Tissue samples fixed with formalin were subjected to paraffin embedding and sectioned at 5 μm for observation. Hematoxylin and eosin (HE) staining of the sections was performed to observe morphological changes in the tissues. After dewaxing and antigen retrieval, immunochemical assays were performed using specific polyclonal antibodies against VEGF and β-catenin. HRP-conjugated goat anti-rabbit antibody was then applied to the sections to label the primary antibodies. DAB chromogenic reagent and hematoxylin were then added and coverslips were added to sections after color development. The expression of VEGF and β-catenin in liver tissue was qualitatively analyzed with a high-magnification inverted fluorescence phase-contrast microscope (40×; DMIL, Leica, Germany) and photographs were taken. ImageJ was used for image analysis to compare the average optical density (AOD) of positive staining sites in each group.

Real-time fluorescent qPCR

Total RNA was extracted from the liver with TRIzol reagent. The mRNA was reverse-transcribed into cDNA and SYBR Green PCR Master Mix was used to determine the transcriptional expression of specific genes. The primer sequences were as follows: β-catenin forward, 5′-TCT GAG GAC AAG CCA CAG GA-3′ and reverse, 5′-GCA CCA ATG TCC AGT CCA AG-3′; VEGF forward, 5′-CTT CAG CTC GCT CCT CCA CT-3′ and reverse, 5′-CAG GCC TCT TCT TCC ACC AC-3′; β-actin forward, 5′-GTG CTA TGT TGC TCT AGA CTT CG-3′ and reverse, 5′-ATG CCA CAG GAT TCC ATA CC-3′. Amplification of the housekeeping gene beta-actin from the same samples was used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method[17] .

ELISA

Liver samples were ground into homogenates and assayed according to the VEGF and β-catenin ELISA kit manufacturer's instructions. The OD value of each sample well was measured with a microplate reader at a wavelength of 450 nm, and the concentrations of VEGF and β-catenin in the liver homogenates were calculated and presented in pg/mL. Reproducibility was evaluated in three independent experiments, and two standard curves were run on each plate.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software (IBM, Armonk, NY, United States). Image analysis was performed using ImageJ (https://imagej.nih.gov). Statistical charts were created using Prism5.0 (Graphpad, San Diego, CA, United States). All data are expressed as the mean ± standard deviation (SD). The differences between any two groups were tested by independent sample *t*-tests and comparisons of multiple groups were made by one-way ANOVA; Tukey's test was used for multiple pairwise comparisons of means. A P-value < 0.05 was considered statistically significant.

RESULTS

Body weight, liver weight, and tumor burden

The body weight of the animals in all the three groups showed an upward trend as the experiment progressed. From day 9-15, body weight increased rapidly, but slowed from day 16-21. In comparison with the control and vehicle groups, it was noteworthy that the body weight of animals in the sporamin group was highest although there were no significant differences among these groups when the increase in body weight ceased on day 15 (P = 0.12; Figure 1). There were also no significant differences in liver weights or liver/body weight ratios among the three groups (Table 1).

All of the nude mice that were inoculated with cancer cells developed tumor nodules in their abdominal cavities. Figure 2 shows that the tumors grew mainly on

Table 1 Liver weights and the liver/body weight ratios of the BALB/c nude mice

Group	Liver weight (g)	Liver/body weight
Control	1.15 ± 0.17	0.06 ± 0.01
Vehicle	1.10 ± 0.24	0.06 ± 0.01
Sporamin	1.04 ± 0.24	0.06 ± 0.01
F	0.50	0.10
P	0.61	0.76

the mesentery instead of the intestine. They were rough, hard, and grayish white nodules differing in number and volume. Compared to the vehicle group, the total weight of the tumor nodules in the sporamin group was reduced by 51.15% (95% confidence interval: 0.16-0.31) and the total number of tumors was reduced from 93 to 55 (Figure 3). Compared with the vehicle group (0.44 ± 0.10 g), the mean tumor weight in the sporamin group (0.26 ± 0.15 g) was significantly reduced (P = 0.04).

Pathological examinations

HE staining of liver histological sections showed that, compared to the control group, the size of hepatocytes in the vehicle group was enlarged, and nuclei were also enlarged and stained deeply blue. The cytoplasm displayed a basophilic change. Cell membranes and the borders between them became blurred. As a result, the hepatic sinusoid was compressed by the enlarged hepatocytes and the structure of the hepatic plate became irregular. There were also many lymphocytes which had infiltrated into the hepatic tissues, but no metastatic tumor cells were found. Compared to the vehicle group, the histomorphology of livers from animals in the sporamin group was similar to that of the control group with a relatively small cell size, clearer hepatic plate, and a reduced degree of blue staining of the cytoplasm, indicating that sporamin had restored the normal structure of the liver in these animals (Figure 4).

Immunohistochemistry

To investigate functional changes in the liver, antibodies against β-catenin and VEGF were applied to the liver sections to show the levels and intracellular locations of these tumor biomarkers. After antibody incubation, the nuclei of the liver cells were stained blue and biomarkers were stained brownish yellow or light yellow. As shown in Figure 5, high concentrations of β-catenin were found in the cytoplasm and also translocated to the nucleus, indicating that it had bound to its target genes and had initiated the transcription process. Quantitative analysis of the AOD of the positively stained areas in the liver tissue sections showed that, compared with the vehicle group (47.29 ± 9.10), the expression of β-catenin was significantly reduced by sporamin to 26.14 ± 1.72 (P = 0.003; Figure 6). In line with the increase in β-catenin expression and nuclear translocation in the vehicle group, the expression of the angiogenic factor VEGF, which is also a downstream target of β-catenin, was increased in the cytoplasm of hepatocytes in the vehicle group. Similarly, expression of the VEGF protein was also reduced after sporamin intervention from 20.78 ± 2.06 in the vehicle group to 15.80 ± 1.09 in the sporamin group (P = 0.021; Figure 6), sug-gesting that sporamin had an anti-angiogenic effect in these animals.

ELISA

The concentrations of β-catenin and VEGF protein in liver tissue were assessed by ELISA. Figure 7 shows that the concentration of β-catenin increased, albeit non-significantly, from 134.42 ± 22.04 pg/mL in the control group to 143.33 ± 5.06 pg/mL in the vehicle group (P = 0.35). However, the concentration of VEGF in liver tissue was significantly increased from 132.05 ± 7.96 pg/mL in the control group to 158.73 ± 6.23 pg/mL in the vehicle group (P = 0.00007). Compared with the vehicle group, the concentration of β-catenin after sporamin intervention decreased to 109.07 ± 9.65 pg/mL (P = 0.00002). The concentration of VEGF also decreased to 150.90 ± 10.38 pg/mL but it was not significant (P = 0.14). These results are consistent with changes observed in the immunohistochemistry analysis.

qPCR

To further verify the effects of sporamin on the expression of β-catenin and VEGF at the transcriptional level, qPCR was conducted to detect the relative abundance of these mRNAs in the liver samples. Figure 8 shows that compared with the control group, relative expression of β-catenin mRNA in the vehicle group was significantly increased (P = 0.0004), but the increase was not significant for VEGF (P = 0.26).

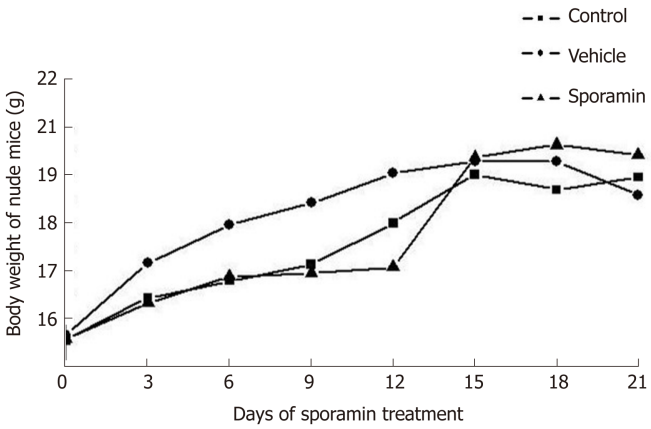


Figure 1 Body weight changes during sporamin treatment.

Compared with the vehicle group, the relative abundance of β-catenin and VEGF mRNA in the sporamin group was significantly reduced to 71% ± 1% (P = 0.000001) and 23% ± 7% (P = 0.00002) of the vehicle group levels, respectively.

DISCUSSION

CRC is one of the most common malignancies of the digestive tract in both developing and developed countries[18]. Metastasis and recurrence of the primary tumor are the main reasons for the high mortality rate of this disease. At present, chemotherapy still has severe side effects, which limits its use in many circumstances. In recent years, many studies have indicated that phytochemicals may be a promising source of new anti-cancer agents against CRC which will play an important role in chemoprevention of the disease (for review see[19]). In the current study, compared with the vehicle group, the number and total weight of tumor nodules formed in the abdominal cavities of the mice were significantly reduced by treatment with sporamin, a Kunitz type trypsin inhibitor obtained from the sweet potato. This is in line with previous studies which showed that sporamin can suppress the growth of a variety of cancer cell lines including human esophageal squamous cell carcinoma cells[20], human pancreatic cancer cells[13], and human tongue carcinoma cells[12], both *in vitro* and *in vivo*.

Considering that the liver is usually the first target organ of CRC metastasis, the present study mainly focused on changes in the structure and function of the liver in the tumor-bearing mice. The liver is the largest endocrine gland in the body which can secrete a great number of hormones, growth factors, and cytokines and plays a crucial role in the development and transformation of many malignant tumors. The liver is also the most common anatomical site for hematogenous metastases of CRC, which are one of the most difficult and challenging obstacles in the treatment of CRC [21]. In our study, although the weights of the body and the liver and the ratio of liver weight to body weight were not significantly different among the control, vehicle, and sporamin groups, we found that sporamin increased body weight and reduced liver weight compared with vehicle treatment, implying that sporamin was possibly beneficial for the general health status of the mice. This effect may be partially attributed to the nutritional effects of sporamin because it is a dietary protein with many biological activities[22].

In accordance with previous findings that colon cancer cells can induce the liver to synthesize and secrete a variety of hormones, growth factors, and cytokines, which facilitate the growth of the tumor and induce a systematic inflammatory status[23], our studies showed that after intraperitoneal tumor cell inoculation, the histomorphology and function of the liver were all significantly changed . In contrast, it was noteworthy that, compared with the vehicle group, sporamin demonstrated the ability to restore the normal structure and function of the liver. Hepatocyte enlargement, nuclear pyknosis, cell membrane blurring, and blue staining of the cytoplasm were all improved, suggesting that sporamin had attenuated protein synthesis within the cell and alleviated the hypertrophic status of the hepatocytes. Functionally, the accumulation of β-catenin in the cytoplasm as well as its translocation to the nucleus of the hepatocytes was both significantly attenuated by sporamin. As is well known, the activation of the Wnt/β-catenin signaling pathway plays a key role in the de-

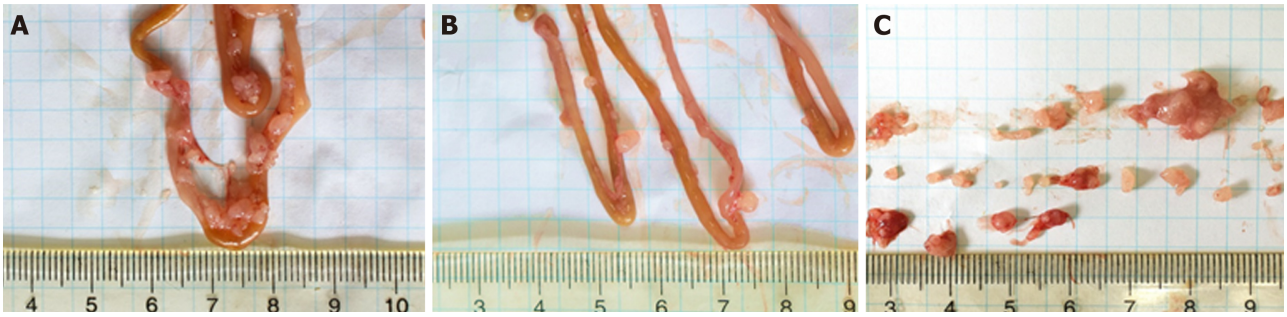


Figure 2 Establishment of a colorectal cancer *in vivo* model. Photos taken of intestines from the vehicle group (A) and sporamin-treated group (B). C: Tumor shapes are shown.

velopment of CRC[24]. As a transcriptional activator, the translocation of β-catenin from the cytoplasm to the nucleus initiates the transcription of a number of target genes[25] which participate in the malignant transformation of CRC[26]. Therefore, any agents that can reduce the level of β-catenin within the tumor cells and hepatocytes may exhibit a potent effect against CRC. For example, in ade-nomatous polyposis coli (APC) tumor suppressor gene-mutated $APC^{min/+}$ mice, the formation of colorectal adenomas is inhibited by suppressing the activation of the Wnt/β-catenin signaling pathway[27]. Therefore, our findings suggest that sporamin may partially exert its effect by inhibiting the synthesis and function of β-catenin in the liver.

VEGF is one of the most potent angiogenic factors that can be synthesized by tumor cells as well as hepatocytes after stimulation by various environmental factors such as hypoxia. VEGF is also a downstream target of the β-catenin pathway, and expression of VEGF is usually positively correlated with the expression of β-catenin in tumor cells and with the progression of the tumor[28]. Consequently, if the expression of β-catenin in tumor cells is reduced, the expression of VEGF usually decreases as well[29]. Our results are consistent with these previous findings and show that sporamin has an anti-angiogenic effect against CRC. As to the specific mechanisms by which sporamin inhibits the expression of β-catenin and VEGF as well as signaling events during this process, further investigation will be required.

In conclusion, our study suggests that sporamin can suppress the growth of xenografted colorectal tumor nodules in mice by restoring the normal structure of the liver and downregulating the expression and secretion of β-catenin and VEGF in the liver. These anti-cancer effects of sporamin against CRC are closely associated with its inhibitory effect on these tumor biomarkers. Further studies are warranted to elucidate the corresponding signal transduction events mediating this process.

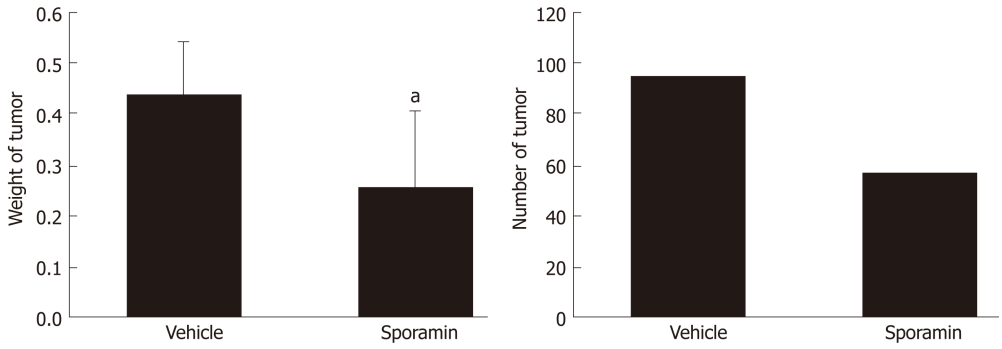


Figure 3 Changes in the weight and number of tumors following sporamin administration. [a]*P* < 0.05 *vs* vehicle.

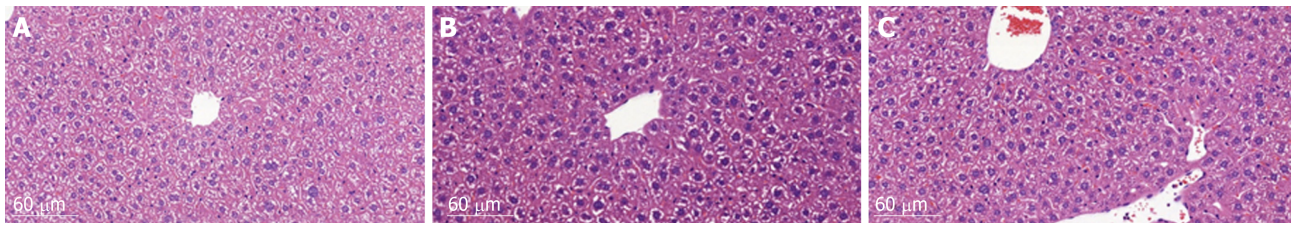


Figure 4 Liver histological changes after sporamin treatment. Photomicrographs of hematoxylin and eosin staining of liver sections from the normal control group (A), vehicle group (B), and sporamin group (C). Magnification, 20×.

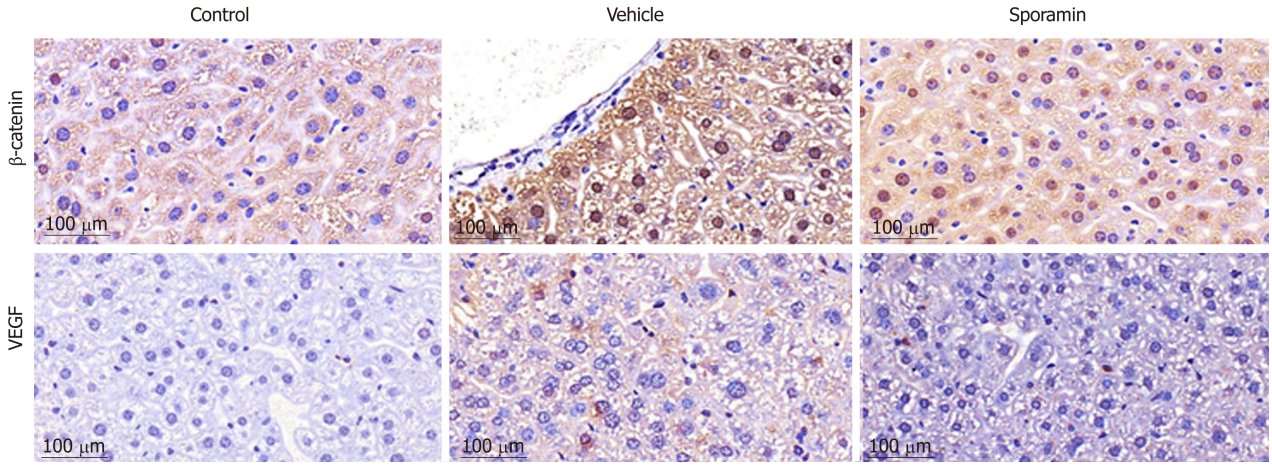


Figure 5 Immunohistochemical staining for vascular endothelial growth factor and β-catenin in the liver of nude mice after sporamin treatment. Photomicrographs of liver sections from the control group (A), vehicle group (B), and sporamin group (C). Magnification, 40×. VEGF: Vascular endothelial growth factor.

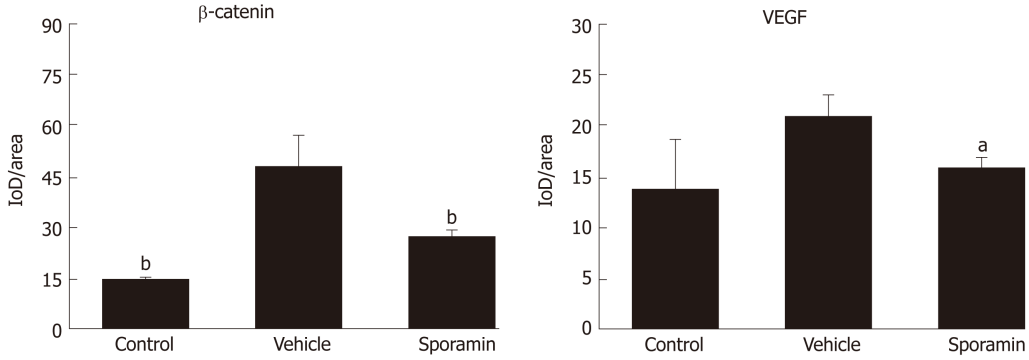


Figure 6 Average optical density of vascular endothelial growth factor and β-catenin in the liver of nude mice after sporamin treatment. Measurements were made using ImageJ; *n* = 3 per group. [a]*P* < 0.05 *vs* vehicle, [b]*P* < 0.01 *vs* vehicle. VEGF: Vascular endothelial growth factor.

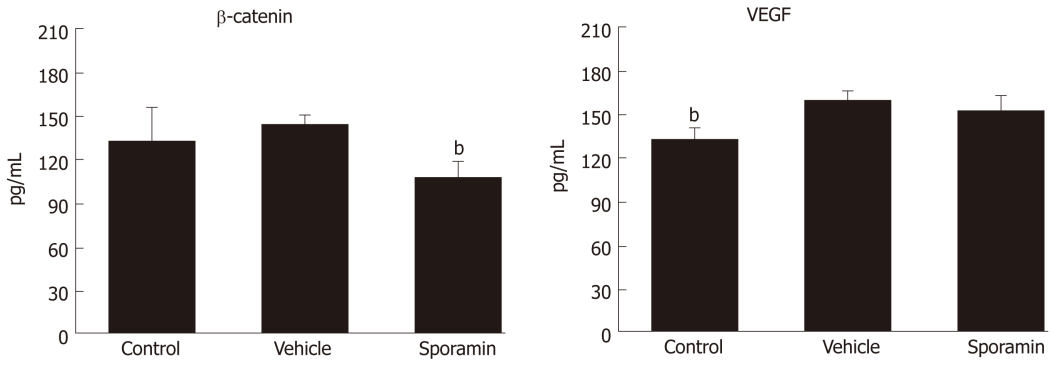


Figure 7 Concentrations of β-catenin and vascular endothelial growth factor in the liver of nude mice after sporamin treatment. $^{b}P < 0.01$ *vs* vehicle. VEGF: Vascular endothelial growth factor.

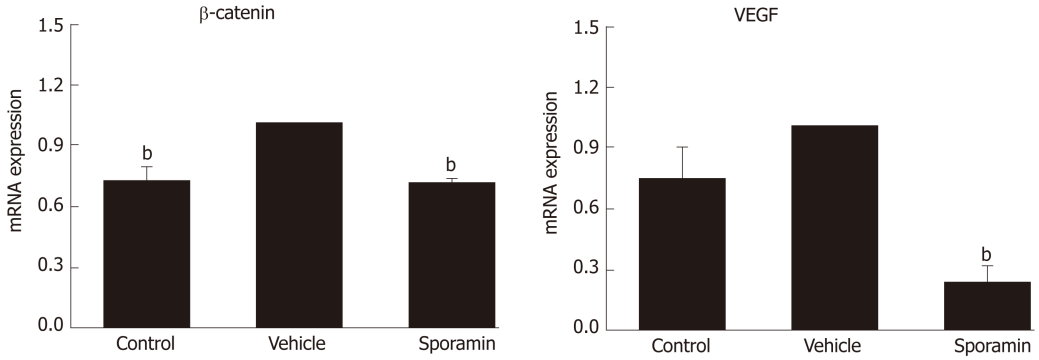


Figure 8 Relative mRNA expression of β-catenin and vascular endothelial growth factor. $^{b}P < 0.01$ *vs* vehicle.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the third most common malignancy of the digestive tract and the fifth leading cause of cancer-related mortality in China. Sporamin, a Kunitz-type trypsin inhibitor isolated from sweet potato, is a potential anti-cancer agent with activity against a number of malignant tumor cells *in vitro*. The liver secretes a myriad of endocrine factors that may facilitate the growth and transformation of tumors in the development of CRC.

Research motivation

Sporamin as a potential anti-cancer agent against a number of malignant tumor cells, including HT29, HCT116, and SW480 colorectal cancer cells, TCA8113 tongue carcinoma cells as well as PANC-1 and BxPC-3 pancreatic cancer cells. However, the effects of sporamin on the expression and secretion of tumor biomarkers in the liver are currently unknown. Therefore, in the present study, LoVo colorectal carcinoma cells were intraperitoneally xenografted into athymic BALB/c nude mice and sporamin was given orally to the mice to observe its effect on the growth of tumors, with a focus on changes in the structure and function of the liver, especially the expression and secretion of β-catenin and vascular endothelial growth factor (VEGF).

Research objectives

To investigate the effects of sporamin on liver morphology and biomarkers of xenografted CRC in the liver of BALB/c athymic mice.

Research methods

Twenty-seven male BALB/c nude mice were randomly divided into control, vehicle, and sporamin groups. Mice in the latter two groups were intraperitoneally xenografted with LoVo colorectal carcinoma cells and intragastrically infused with saline or sporamin (0.5 g/kg body weight/d), respectively, for 3 weeks. Hematoxylin and eosin (HE) staining of the sections was performed to observe morphological changes in hepatic tissue and real-time fluorescent quantitative PCR and enzyme-linked immunosorbent assays (ELISA) were used to measure the expression of β-catenin and VEGF in the liver.

Research results

Sporamin significantly reduced the number and weight of tumor nodules formed in the abdominal cavity. Compared with the vehicle group, the mean tumor weight (± SD) in the

sporamin group was significantly reduced (0.26 ± 0.15 g *vs* 0.44 ± 0.10 g) and the total number of tumors decreased from 93 to 55. HE staining showed that enlargement of the nucleus and synthesis of proteins within hepatocytes, as well as infiltration of inflammatory cells into the liver, were attenuated by sporamin. Immunohistochemical staining and ELISA showed that the concentrations of β-catenin and VEGF in the liver were significantly reduced by sporamin. Compared with the vehicle group, the expression of β-catenin measured in integrated optical density units per area was reduced in the sporamin group (47.29 ± 9.10 *vs* 26.14 ± 1.72; $P = 0.003$). Expression of VEGF was also reduced after sporamin intervention from 20.78 ± 2.06 in the vehicle group to 15.80 ± 1.09 in the sporamin group ($P = 0.021$). The secretion of VEGF and β-catenin in the liver was also assessed by ELISA, which showed that the concentration of VEGF in liver tissue increased significantly from 132.05 ± 7.96 pg/mL in the control group to 158.73 ± 6.23 pg/mL in the sporamin-treated group ($P = 0.00007$). Compared with the vehicle group, the concentration of β-catenin decreased from 134.42 ± 22.04 pg/mL to 109.07 ± 9.65 pg/mL after sporamin intervention ($P = 0.00002$). Quantitative PCR (qPCR) indicated that compared to the vehicle group, relative mRNA expression of β-catenin and VEGF in the liver of the sporamin-treated group was significantly reduced to 71% ± 1% ($P = 0.000001$) and 23% ± 7% ($P = 0.00002$), respectively, of the vehicle group levels.

Research conclusions

Sporamin down-regulates the expression and secretion of β-catenin and VEGF in the liver, which subsequently inhibits the transcription of downstream genes involved in cancer progression and angiogenesis.

Research perspectives

Our study suggests that sporamin can suppress the growth of xenografted colorectal tumor nodules in mice by restoring the normal structure of the liver and downregulating the expression and secretion of β-catenin and VEGF in the liver. These anti-cancer effects of sporamin against CRC are closely associated with its inhibitory effect on these tumor biomarkers. Further studies are warranted to elucidate the corresponding signal transduction events mediating this process.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; **66**: 7-30 [PMID: 26742998 DOI: 10.3322/caac.21332]
2 **Chen W**. Cancer statistics: updated cancer burden in China. *Chin J Cancer Res* 2015; **27**: 1 [PMID: 25717219 DOI: 10.3978/j.issn.1000-9604.2015.02.07]
3 **Zhu J**, Tan Z, Hollis-Hansen K, Zhang Y, Yu C, Li Y. Epidemiological Trends in Colorectal Cancer in China: An Ecological Study. *Dig Dis Sci* 2017; **62**: 235-243 [PMID: 27796769 DOI: 10.1007/s10620-016-4362-4]
4 **Xie G**, Raufman JP. Role of the Aryl Hydrocarbon Receptor in Colon Neoplasia. *Cancers (Basel)* 2015; **7**: 1436-1446 [PMID: 26264025 DOI: 10.3390/cancers7030847]
5 **Ho JW**, Leung YK, Chan CP. Herbal medicine in the treatment of cancer. *Curr Med Chem Anticancer Agents* 2002; **2**: 209-214 [PMID: 12678744]
6 **Scarpa ES**, Ninfali P. Phytochemicals as Innovative Therapeutic Tools against Cancer Stem Cells. *Int J Mol Sci* 2015; **16**: 15727-15742 [PMID: 26184171 DOI: 10.3390/ijms160715727]
7 **Shu L**, Cheung KL, Khor TO, Chen C, Kong AN. Phytochemicals: cancer chemoprevention and suppression of tumor onset and metastasis. *Cancer Metastasis Rev* 2010; **29**: 483-502 [PMID: 20798979 DOI: 10.1007/s10555-010-9239-y]
8 **Crispin BJ**, Seghi RR, Globe H. Effect of different metal ceramic alloys on the color of opaque and dentin porcelain. *J Prosthet Dent* 1991; **65**: 351-356 [PMID: 2056453]
9 **Maeshima M**, Sasaki T, Asahi T. Characterization of major proteins in sweet potato tuberous roots. *Phytochemistry* 1985; **166**: 515-523 [DOI: 10.1016/S0031-9422(00)83088-5]
10 **Li PG**, Mu TH. Recovery of sporamin from naturally fermented sweet potato starch slurry by foam fractionation. *Int J Food Sci Tech* 2012; **47**: 1889-1895 [DOI: 10.1111/j.1365-2621.2012.03046.x]
11 **Li PG**, Mu TH, Deng L. Anticancer effects of sweet potato protein on human colorectal cancer cells. *World J Gastroenterol* 2013; **19**: 3300-3308 [PMID: 23745032 DOI: 10.3748/wjg.v19.i21.3300]
12 **Yao J**, Qian C. Sporamin induce apoptosis in human tongue carcinoma cells by down-regulating Akt/GSK-3 signaling. *Fundam Clin Pharmacol* 2011; **25**: 229-236 [PMID: 20408878 DOI: 10.1111/j.1472-8206.2010.00830.x]
13 **Qian C**, Chen X, Qi Y, Zhong S, Gao X, Zheng W, Mao Z, Yao J. Sporamin induces apoptosis and inhibits NF-κB activation in human pancreatic cancer cells. *Tumour Biol* 2017; **39**: 1010428317706917 [PMID: 28714369 DOI: 10.1177/1010428317706917]
14 **Adam R**, Lucidi V, Bismuth H. Hepatic colorectal metastases: methods of improving resectability. *Surg Clin North Am* 2004; **84**: 659-671 [PMID: 15062667 DOI: 10.1016/j.suc.2003.12.005]
15 **Nguyen DX**, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 2009; **9**: 274-284 [PMID: 19308067 DOI: 10.1038/nrc2622]
16 **Rudmik LR**, Magliocco AM. Molecular mechanisms of hepatic metastasis in colorectal cancer. *J Surg Oncol* 2005; **92**: 347-359 [PMID: 16299807 DOI: 10.1002/jso.20393]
17 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408 [PMID: 11846609 DOI: 10.1006/meth.2001.1262]
18 **Karsa LV**, Lignini TA, Patnick J, Lambert R, Sauvaget C. The dimensions of the CRC problem. *Best Pract Res Clin Gastroenterol* 2010; **24**: 381-396 [PMID: 20833343 DOI: 10.1016/j.bpg.2010.06.004]
19 **Mahadevappa R**, Kwok HF. Phytochemicals - A Novel and Prominent Source of Anti-cancer Drugs Against Colorectal Cancer. *Comb Chem High Throughput Screen* 2017; **20**: 376-394 [PMID: 28078982 DOI: 10.2174/1386207320666170112141833]
20 **Qian CJ**, Qi YX, Chen XY, Zeng JP, Yao J. Sporamin suppresses growth of human esophageal squamous

cell carcinoma cells by inhibition of NFκB via an AKTindependent pathway. *Mol Med Rep* 2017; **16**: 9620-9626 [PMID: 29039512 DOI: 10.3892/mmr.2017.7772]

21 **Xu J**, Qin X, Wang J, Zhang S, Zhong Y, Ren L, Wei Y, Zeng S, Wan D, Zheng S; Society of Surgery; Chinese Medical Association; Committee of Colorectal Cancer, Chinese Anti-cancer Association. Chinese guidelines for the diagnosis and comprehensive treatment of hepatic metastasis of colorectal cancer. *J Cancer Res Clin Oncol* 2011; **137**: 1379-1396 [PMID: 21796415 DOI: 10.1007/s00432-011-0999-8]

22 **Senthilkumar R**, Yeh KW. Multiple biological functions of sporamin related to stress tolerance in sweet potato (Ipomoea batatas Lam). *Biotechnol Adv* 2012; **30**: 1309-1317 [PMID: 22306516 DOI: 10.1016/j.biotechadv.2012.01.022]

23 **Xu J**, Ren L. [China Guideline for Diagnosis and Comprehensive Treatment of Colorectal Liver Metastases (Version 2018)]. *Zhonghua Wei Chang Wai Ke Za Zhi* 2018; **21**: 601-626 [PMID: 29968235]

24 **Larriba MJ**, González-Sancho JM, Barbáchano A, Niell N, Ferrer-Mayorga G, Muñoz A. Vitamin D Is a Multilevel Repressor of Wnt/b-Catenin Signaling in Cancer Cells. *Cancers (Basel)* 2013; **5**: 1242-1260 [PMID: 24202444 DOI: 10.3390/cancers5041242]

25 **Deep G**, Panigrahi GK. Hypoxia-Induced Signaling Promotes Prostate Cancer Progression: Exosomes Role as Messenger of Hypoxic Response in Tumor Microenvironment. *Crit Rev Oncog* 2015; **20**: 419-434 [PMID: 27279239 DOI: 10.1615/CritRevOncog.v20.i5-6.130]

26 **Ying Y**, Zhu H, Liang Z, Ma X, Li S. GLP1 protects cardiomyocytes from palmitate-induced apoptosis via Akt/GSK3b/b-catenin pathway. *J Mol Endocrinol* 2015; **55**: 245-262 [PMID: 26386043 DOI: 10.1530/JME-15-0155]

27 **Gao C**, Chen G, Kuan SF, Zhang DH, Schlaepfer DD, Hu J. FAK/PYK2 promotes the Wnt/β-catenin pathway and intestinal tumorigenesis by phosphorylating GSK3β. *Elife* 2015; **4** [PMID: 26274564 DOI: 10.7554/eLife.10072]

28 **Dılek FH**. Topak N, Tokyol Ç, Akbulut G, Dılek ON. β-Catenin and its relation to VEGF and cyclin D1 expression in pT3 rectosigmoid cancers. *Turk J Gastroenterol* 2010; **21**: 365-371 [PMID: 21331989]

29 **Zhang K**, Guo J, Ge Z, Zhang J. Nanosecond pulsed electric fields (nsPEFs) regulate phenotypes of chondrocytes through Wnt/β-catenin signaling pathway. *Sci Rep* 2014; **4**: 5836 [PMID: 25060711 DOI: 10.1038/srep05836]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3207-3217

DOI: 10.3748/wjg.v25.i25.3207

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

ORIGINAL ARTICLE

Basic Study

Silicone-covered biodegradable magnesium stent for treating benign esophageal stricture in a rabbit model

Kai Yang, Jun Cao, Tian-Wen Yuan, Yue-Qi Zhu, Bi Zhou, Ying-Sheng Cheng

ORCID number: Kai Yang (0000-0003-4804-9666); Jun Cao (0000-0001-5235-1223); Tian-Wen Yuan (0000-0001-7610-2626); Yue-Qi Zhu (0000-0001-5476-9075); Bi Zhou (0000-0001-7685-6372); Ying-Sheng Cheng (0000-0002-1107-0667).

Author contributions: Yang K and Cao J contributed equally to this study; Yang K, Cheng YS, and Cao J designed all the experiments; Yang K, Yuan TW, and Zhu YQ performed the research; Zhou B contributed analytic tools; Yang K and Cao J wrote the paper.

Supported by the National Natural Science Foundation of China, No. 81371659, No. 81571773, and No. 81771943; and Shanghai Municipal Health and Family Planning Commission, No. 201640191.

Institutional animal care and use committee statement: All experimental protocols were approved by the Animal Research Council of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University and followed the guidelines of the International Committee of Animal Care (US National Institutes of Health and European Commission).

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE

Kai Yang, Bi Zhou, Ying-Sheng Cheng, Department of Radiological Intervention, Shanghai Sixth People's Hospital East Campus Affiliated to Shanghai University of Medicine & Health Sciences, Shanghai 201306, China

Kai Yang, Bi Zhou, Ying-Sheng Cheng, Department of Radiological Intervention, Shanghai Sixth People's Hospital East Campus Affiliated to Shanghai Jiao Tong University, Shanghai 201306, China

Jun Cao, Tian-Wen Yuan, Department of Tumor Intervention, Dahua Hospital, Shanghai 200237, China

Yue-Qi Zhu, Department of Radiological Intervention, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China

Corresponding author: Ying-Sheng Cheng, MD, PhD, Academic Fellow, Doctor, Department of Radiological Intervention, Shanghai Sixth People's Hospital East Campus Affiliated to Shanghai Jiao-tong University, No. 222, West Huanhu No. 3 Road, Shanghai 201306, China.
chengyingsheng@hotmail.com
Telephone: +86-21-38297858
Fax: +86-21-38297706

Abstract

BACKGROUND

Stent insertion can effective alleviate the symptoms of benign esophageal strictures (BES). Magnesium alloy stents are a good candidate because of biological safety, but show a poor corrosion resistance and a quick loss of mechanical support *in vivo*.

AIM

To test the therapeutic and adverse effects of a silicone-covered magnesium alloy biodegradable esophageal stent.

METHODS

Fifteen rabbits underwent silicone-covered biodegradable magnesium stent insertion into the benign esophageal stricture under fluoroscopic guidance (stent group). The wall reconstruction and tissue reaction of stenotic esophagus in the stent group were compared with those of six esophageal stricture models (control group). Esophagography was performed at 1, 2, and 3 weeks. Four, six, and five rabbits in the stent group and two rabbits in the control groups were euthanized, respectively, at each time point for histological examination.

guidelines.

Manuscript source: Unsolicited manuscript

Received: March 6, 2019
Peer-review started: March 7, 2019
First decision: April 8, 2019
Revised: May 14, 2019
Accepted: June 7, 2019
Article in press: June 8, 2019
Published online: July 7, 2019

P-Reviewer: Garbuzenko DV, Su CC, Uygun I
S-Editor: Gong ZM
L-Editor: Wang TQ
E-Editor: Ma YJ

RESULTS

All stent insertions were well tolerated. The esophageal diameters at immediately, 1, 2 and 3 wk were 9.8 ± 0.3 mm, 9.7 ± 0.7 mm, 9.4 ± 0.8 mm, and 9.2 ± 0.5 mm, respectively (*vs* 4.9 ± 0.3 mm before stent insertion; $P < 0.05$). Magnesium stents migrated in eight rabbits [one at 1 wk (1/15), three at 2 wk (3/11), and four at 3 wk (4/5)]. Esophageal wall remodeling (thinner epithelial and smooth muscle layers) was found significantly thinner in the stent group than in the control group ($P < 0.05$). Esophageal injury and collagen deposition following stent insertion were similar and did not differ compared to rabbits with esophageal stricture and normal rabbits ($P > 0.05$).

CONCLUSION

Esophageal silicone-covered biodegradable magnesium stent insertion is feasible for BES without causing severe injury or tissue reaction. Our study suggests that insertion of silicone-covered magnesium esophageal stent is a promising approach for treating BES.

Key words: Benign esophageal stricture; Biodegradable stent; Magnesium; Silicone membrane

Core tip: Stent insertion can be a safe, easy, and effective way to alleviate the symptoms of benign esophageal strictures (BES). However, metallic stent implantation is associated with some severe complications, such as migration, tissue ingrowth, and in-stent restenosis. Biodegradable stent has been used as an effective and accepted method to treat BES. We fabricated a silicone-covered biodegradable magnesium stent, and evaluated technical feasibility, tissue reaction, and stent degradation for treating benign esophageal stricture in a rabbit model. We found that implantation of silicone-covered magnesium stent provided reliable support for at least two weeks, suggesting that it is a promising strategy to treat benign esophageal stricture.

Citation: Yang K, Cao J, Yuan TW, Zhu YQ, Zhou B, Cheng YS. Silicone-covered biodegradable magnesium stent for treating benign esophageal stricture in a rabbit model. *World J Gastroenterol* 2019; 25(25): 3207-3217
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3207.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3207

INTRODUCTION

Esophageal stricture is the abnormally stenotic segment of the esophagus, and benign esophageal stricture (BES) indicates a narrowing or tightening of the esophagus caused by non-cancerous reasons[1,2]. BES can be caused by non-operative factors like reflux, radiation, infection, sclerotherapy, and corrosion, as well as operative factors including surgical anastomosis and minimally invasive surgery for early esophageal neoplasms[3,4]. As one of the most common gastrointestinal conditions impacting patients on a day-to-day basis, BES can seriously degrade the quality of life and result in many problems, such as dysphagia, malnutrition, weight loss, aspiration, and respiratory failure[5-7]. Therefore, the development of effective therapeutic strategies for BES is a critical medical need.

Esophageal stenosis can be alleviated through esophageal stent insertion, which has been widely used as an effective means to improve the quality of life for patients with BES[8-11]. Implantation of stents fabricated from many materials can be applied for treating BES. However, metallic stent implantation is associated with some severe complications, such as migration, tissue ingrowth, and in-stent restenosis[8-11], which significantly limits the use of metallic stents in BES. In addition, the temporary recyclable stent represents a simple and feasible approach to provide sufficient support in 10-14 days for treating BES, but it needs to be removed after use and frequently causes pain, significant foreign body reaction, as well as potential risks including perforation and bleeding[11]. With the advancements in biological medical material technology, stents made from biodegradable alloy or polymer are extensively

improved to be able to provide enough force to tear the BES and reduce complications caused by stents[8,12,13].

Recently, biodegradable stents (BDS) have been used as an effective and accepted method to treat BES patients to improve their quality of life[14,15]. For example, polymer polylactic L-acid (PLLA) BDS exhibited a low complication rate in BES patients, but the high rate (77%) of early stent migration greatly limited its wider clinical application[16]. PLLA esophageal BDS manufactured with polydioxanone can reduce stent migration risk, but results in significant hyperblastosis than PLLA-BDS[17]. Therefore, esophageal BDS with prominent therapeutic effects and minimal adverse effects is still the great challenge in the field. More recently, Yuan *et al*[18] used a poly (ε-caprolactone) (PCL) and poly (trimethylene carbonate) (PTMC)-covered magnesium alloy stent to treat BES in a rabbit model. The stent can provide sufficient support for at least 4 wk, and did not result in damage or collagen loss in the esophageal wall[18]. With acceptable stent migration rates, the stent covered with biodegradable PCL-PTMC significantly prevented serious corrosion of magnesium alloy in the corrosive environment[18].

In this study, we integrated the biocompatibility of silicone membrane[19] and corrosion resistant property of magnesium alloy[20] into the design of an esophageal stent, and applied the silicone-covered magnesium stent into a rabbit model of BES. We determined the feasibility of this stent, and monitored the *in vivo* tissue reaction and stent degradation after stent insertion. Our study suggests that silicone-covered magnesium alloy is an ideal biodegradable material for fabricating esophageal stents to treat patients with BES.

MATERIALS AND METHODS

Fabrication of silicone-covered magnesium stents

Silicone-covered magnesium stents are magnesium stents coated with a silicone membrane. The commercial magnesium alloy was purchased from Sanming Biomedical Company (Yangzhou, China). The bare stent was constructed with 0.20 mm magnesium alloy, as previously described[18,21]. The skeleton of the stent is cylindrical, which was made of the magnesium alloy wires through cross-linked mesh. The diameter and length of the stent were 10 mm and 30 mm in the entire expansion state, respectively. The stents had 5 mm cydariform and tubiform shapes at both ends to prevent their migration (Figure 1).

The surface of the entire magnesium stent was covered with a silicon membrane through the dipping and spinning method developed in our laboratory[18,21]. Briefly, silicone rubber A and rubber B in the same amount (Shanghai Yanchen Industrial Company, Shanghai, China) were thoroughly mixed with n-octane (Shanghai Aladdin Biochemical Technology Company, Shanghai, China). The mixed silicone was impregnated on the stent mold and solidified for 6 h at 80 °C for drying. The molds were cooled in ambient conditions, and the magnesium stents were peeled off. Since the magnesium alloy and the silicon film were transparent and not developed under the X-ray, marks on both ends of the stents were placed to facilitate their positioning under fluoroscopy. The silicone-covered magnesium stents were compressed and loaded through a 6-mm-wide (18 French) delivery system.

Property evaluation of silicone-covered magnesium stents

The mechanical properties of the silicone-covered magnesium stents were tested by the mechanical compression curve analysis and the tensile stress (46 compressions), as previously described[18,21]. The degradation behaviors of the silicone-covered magnesium stents in terms of the magnesium mass lost were determined by incubating in two phosphate-buffered solutions with pH values of 7.4 and 4.0, as previously described[18,21].

The biological safety of silicone-covered magnesium stent was evaluated by testing its impact on the proliferation of human smooth muscle cell line HITC6 that was purchased from the Cell Bank of Type Culture Collection Committee of the Chinese Academy of Sciences, Shanghai, China. Cells were maintained in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, United States) supplemented with 10% fetal bovine serum (TransGen Biotech, Inc., Beijing, China), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, United States), and grown in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were seeded in 96 well-plates at a density of 5×10^3/well and incubated with or without silicone-coated magnesium stents. On days 1, 3, 6 and 9, cell proliferation was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay[22].

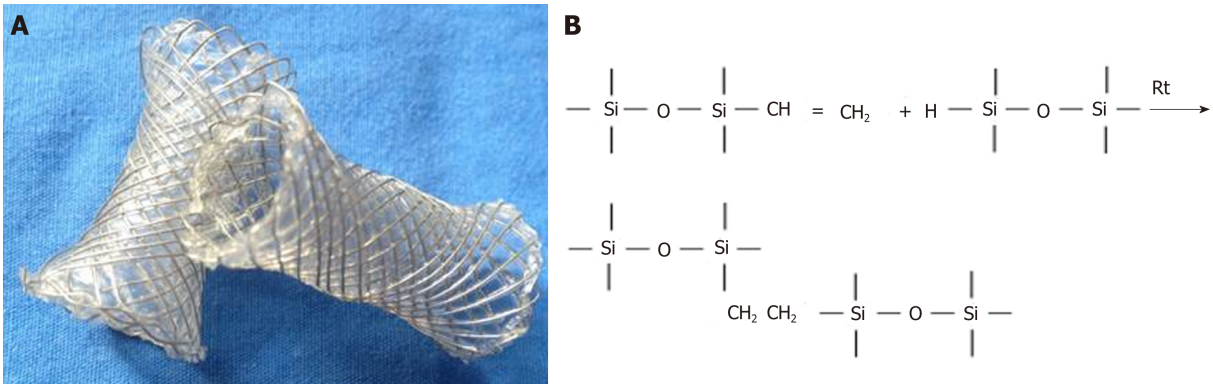


Figure 1 Photographs of the fully opened silicon coated magnesium-stent (A) and molecular formula of cross-linked silica gel (B).

Esophageal stenosis model

All experimental protocols were approved by the Animal Research Council of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University and followed the guidelines of the International Committee of Animal Care (US National Institutes of Health and European Commission).

New Zealand rabbits were provided by the Experimental Animals of the Public Health Center of Shanghai Jiao Tong University, Shanghai, China. The rabbits were housed in the Experimental Animal Center of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University. Rabbits were kept in cages with free access to food in an animal room with a relative humidity of 40%-50%, temperature of 22-25 °C, and 12 h/12 h light and dark alternates. Healthy New Zealand rabbits (weight, 2.3-3.8 kg and age, 10-11 wk) were used for constructing a rabbit esophageal stenosis model using the esophageal sewing method, similarly as previously described[23].

After anesthesia with 5% pentobarbital via the ear vein, rabbits were fixed in the supine position. A longitudinal incision about 3 cm away to the thoracic entrance was made to separate the subcutaneous tissue and muscle and expose the trachea. From the left side of the trachea, the esophagus was truncated about 3 cm, the distal segment of the esophagus was connected with an infusion tube, and the esophagus was further separated from the thorax about 3 cm. The 4.0 operation suture was applied to suture the esophageal lumen, and the position of stitches was adjusted to control the stenosis of esophagus by 50% to 60% under digital subtraction angiography (DSA; GE Medical Systems, United States).

Stent implantation

There were 15 rabbits in the stent group, while 5 rabbits in the control group did not receive any intervention. The silicone-covered magnesium stent was implanted into the esophageal stricture of the rabbits under DSA guidance, as previously described[18,21]. After the rabbits were anesthetized with 5% pentobarbital via the ear vein, a stiff wire (0.035-inch, 260-cm-long, Terumo, Tokyo, Japan) was implanted through the mouth to the stomach under DSA guidance, and used as a guidewire to deliver the silicone-coated magnesium stent into the esophageal stricture. After the release of the silicone-coated magnesium stent, a balloon catheter (10 mm × 40 mm, Changhong Medical Instrument Co., Ltd., Changzhou, China) was inflated within the silicone-covered magnesium stent to make it fully expanded. The expansion time of balloon was 20-30 s to ensure complete expansion of the stent. The diet and water intake in the experiment and control groups were indiscriminate.

Follow-up

Esophageal angiography was performed at 1, 2, and 3 wk after the stent implantation. The migration, patency of the stents, and the diameter of the esophagus between the stent group and the control group was compared.

Histological examination

Two rabbits each time in the control group, and 4, 6, and 5 rabbits in the stent group at the indicated time points (at 1, 2, and 3 wk after the stent implantation) were euthanized to evaluate the response and reconstruction of the esophagus wall. The silicone-covered magnesium stent was removed from the esophagus or gastrointestinal tract. The degradation rate of silicone-covered magnesium stents was assessed by calculating the percentage of destroyed mesh. If one of its four edges broke down under a microscope, a mesh was considered degraded. Mild, moderate,

and severe degradations were defined as below 25%, 25%-50%, and over 50% in degradation rate.

Histology evaluation of the samples was performed as previously described[18,21]. Hematoxylin and eosin (HE) staining was used to assess the inflammation responses to obtain an inflammatory score. Evaluation of submucosal collagen deposition was conducted by Mason's trichrome staining. The Elivision immunohistochemical technique was used to stain the esophageal samples. Mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:100 dilution; NeoMarkers, Thermo Fisher Scientific Inc., Fremont, CA, United States) and α-smooth muscle actin (α-SMA) antibody (1:50 dilution; Santa Cruz Biotechnology Inc., Dallas, TX, United States) were used as primary antibodies. Specimens were evaluated by two pathologists independently in a blinded manner.

Statistical analysis

GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States) was used for data analyses. One-way or two-way analysis of variance (ANOVA) was used to compare the overall changes in esophageal diameter, PCNA proliferation index, and collagen area between the control and stent groups at 1, 2, and 3 wk after stent insertion. The Shapiro-Wilk test was used to evaluate the variance and normal distribution of the dependent variables before one-way ANOVA. Statistical significance was set at $P < 0.05$.

RESULTS

Silicone-covered magnesium stent evaluation

The mechanical properties of the silicone-covered magnesium stent were tested by the tensile stress and strain (Figure 2). The silicone membrane was tightly wrapped and fixed to the cross-linked, knitted, bare magnesium mesh tube (Figure 1), which could maintain its size and morphology because of its prominent elasticity and flexibility. The silicone-covered magnesium stent showed good elastic deformation properties with no tearing or breakdown, thus was able to provide enough support against *in vivo* lesion compression. The magnesium mass lost was used to determine the degradation of the silicone-covered magnesium stent in buffered solutions with pH values of 4.0 and 7.4. As shown in Figure 3, the silicone-covered magnesium stent showed excellent biodegradable ability and maintained the main structure even at 4 wk after incubation in the acidic environment. Therefore, the silicone-covered magnesium stent had a retention time as long as 4 weeks. The biological safety of magnesium-silicone gel was evaluated by its impact on the proliferation of smooth muscle cells. As shown in Figure 4, the cells demonstrated similar proliferation ratios at the tested time points in the presence and absence of magnesium-silicone gel, which suggested that the silicone-covered magnesium stent had no cellular cytotoxicity against the growth of host cells.

Intervention with silicone-covered magnesium stents in the rabbit esophageal stenosis model

Esophageal angiography was performed to verify the stent expansion and the absence of esophageal perforation. No obvious aspiration, asphyxia, or death occurred during the procedures of stent implantation in rabbits. No stent migration occurred during the procedure period. Stent-related complications, such as perforation and bleeding of the esophagus, did not occur during the process or follow-up period. Esophagography demonstrated that stent expansion was good and the contrast agent can pass the stented esophagus smoothly [Figure 5A (a-c)].

Overview of the follow-up

After successful modeling, a total of 15 rabbits were implanted with silicone-covered magnesium stents, while 6 rabbits were left untreated. All of these rabbits underwent regular esophagography during follow-up and no animal died. At 1, 2, and 3 wk after stent implantation, 4, 6, and 5 rabbits in the stent group, respectively, were euthanized for examining the migration and location of silicone-covered magnesium stents. Stent migration was observed in 1 (25%) of 4 rabbits at 1 wk, 3 (50%) of 6 rabbits at 2 wk, and 4 (80%) of 5 rabbits at 3 wk after therapy. The esophageal diameter was 4.9 ± 0.3 mm before silicone-covered magnesium stent insertion and 9.8 ± 0.3 mm right after the stent implantation. The diameter was measured as 9.7 ± 0.7 mm, 9.8 ± 0.8 mm, and 9.2 ± 0.5 mm after 1, 2, and 3 wk, respectively ($P < 0.05$). In the stent group, in-stent stenosis did not occur in the follow-up period [Figure 5A (d-e)]. The weight was measured as 3.56 ± 0.3 kg before stent insertion and 3.48 ± 0.4 kg, 3.23 ± 0.3 kg, and

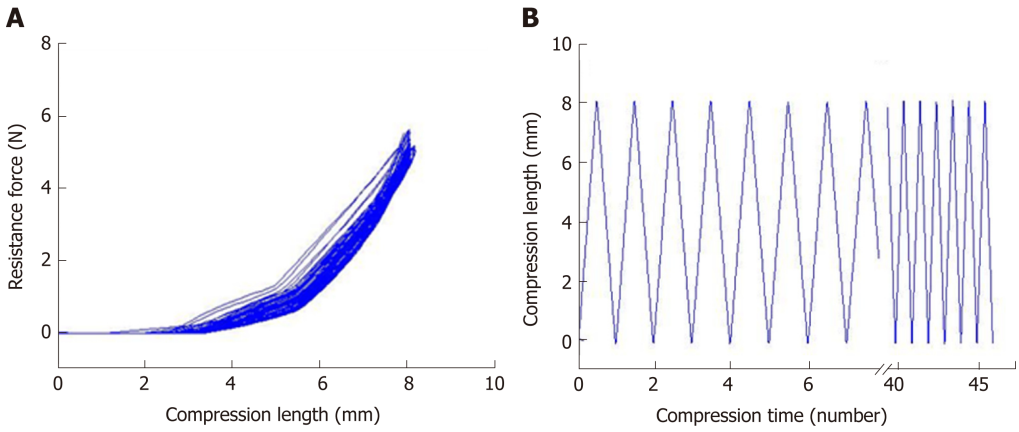


Figure 2 Evaluation of the mechanical properties of the silicone-coated magnesium stent. A: Mechanical compression curve analysis of the stent; B: compression-recovery curves of length and time in repeated compression tests (n = 5, constant pressure = 10 N).

2.89 ± 0.2 kg after 1, 2, and 3 wk, while the weight of controls was 3.53 ± 0.3 kg.

Stent morphological retention and location

Under microscopic examination, the degradation rates of silicone-covered magnesium stents, in terms of the number of degraded mesh units, in the rabbits without stent migration were 5.23% (minor degradation; 5.0%, 5.2%, and 5.5% for three individual rabbits) at 1 wk, 17.06% (minor degradation; 15.1%, 18.6%, and 17.5% for three individual rabbits) at 2 wk, and 88.0% for 1 rabbit at 3 wk after stent implantation (Figure 5B). Three stents (1 found at the first week and 2 at the second week) were in the stomach with partial degradation. These stents had about 40% remaining and a large amount of food residue in the cavity. Three stents (1 found in the second week and 2 in the third week) were located in the stomach, and they were almost completely degraded with only 1.5% ± 2.3% residual identified. In addition, two stents were excreted in the third week after stent insertion.

Histological study

The inflammation scores were 0.25 ± 0.4, 0.40 ± 0.5, and 0.22 ± 0.4 at 1, 2, and 3 wk after stent implantation, respectively, in the stent group, and no difference was identified between the stent group and the normal control group (P > 0.05). The proliferation index by quantitative analysis of the PCNA-positive cells revealed a significant difference between the two groups (P < 0.05). As indicated by the distribution of PCNA-positive cells (Figure 6A), the epithelial layer in the stent group was obviously thinner than that in the normal control group (141.2 ± 30.5 μm *vs* 261.5 ± 17.2 μm; P < 0.05). As revealed by immunostaining, the SMA layer in the muscle layer was slightly thicker than that in the control group (129.0 ± 9.5 μm *vs* 90.5 ± 17.0 μm; P > 0.05) (Figure 6B). The thickness of the epithelial and SMA layers at 1, 2, and 3 wk after stent insertion in the stent group had no difference during the follow-up period (P > 0.05), which indicated that the reconstruction of the esophageal wall was completed within 1 week. Moreover, the amount of collagen did not display differences between these two groups at 1, 2, and 3 wk during follow-up (P > 0.05; data not shown), indicating the absence of adverse tissue responses caused by stent dilatation injury and degradation.

DISCUSSION

Currently, two types of degradable stents, biodegradable metal stent and high polymer stent, are widely used in clinical practice. Due to the absence of chronic inflammation and SMC hyperplasia after complete biodegradation, biodegradable stents have already been used as an effective and accepted method to treat BES patients to improve their quality of life[8,15,24,25]. However, there are no ideal stents that can provide enough and long-lasting force to tear the benign stricture of the esophagus as well as completely reduce complications caused by stent implantation. Here, we determined the feasibility of silicone-covered magnesium alloy for fabricating esophageal stents, and analyzed the mechanical property, degradation behaviors, and biological safety of silicone-covered magnesium stents. Through *in vivo* study using a rabbit model of BES, we found that the inserted silicone-covered magnesium stents can provide

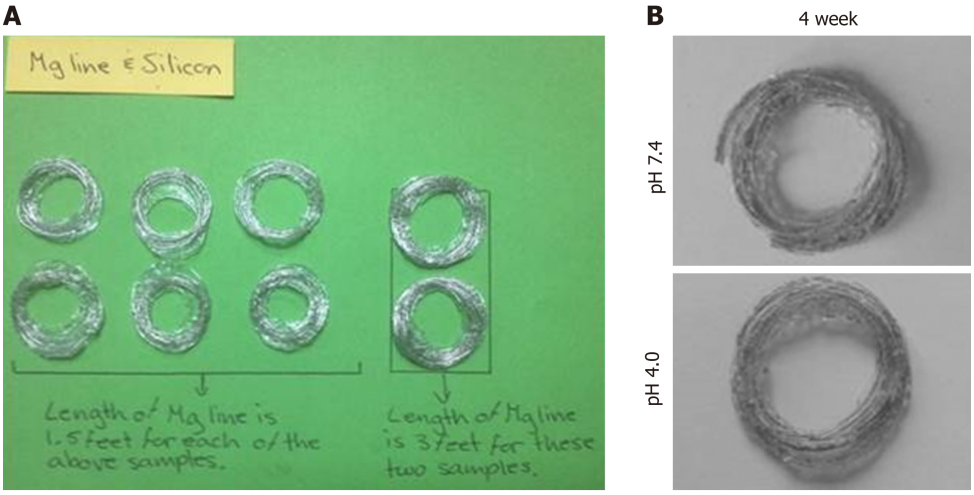


Figure 3 Evaluation of the degradation behaviors of the magnesium alloy wire. A: Topography of magnesium alloy wire with indicated length (left, 1.5 feet; and right, 3 feet); B and C: Degradation topography of magnesium alloy wire at 4 weeks after incubation in phosphate-buffered saline with pH values of 7.4 (B) and 4.0 (C).

reliable support for at least two weeks, and did not cause severe injury or collagen deposition in rabbits.

Magnesium alloy is a fully biodegradable and ideal material for *in vivo* stents. It provides sufficient radial support force for moderate or severe stenosis and reduces tissue hyperplasia[26,27]. However, the rapid degradation rate of magnesium alloy makes it very difficult to maintain radial support force as long as 4 wk after its insertion for treating BES[21,28,29]. Silicone has numerous advantages like excellent elasticity, prominent coating performance, and remarkable resistance to degradation. We designed a new biodegradable esophageal stent with bare magnesium wire stent coated with a silicone membrane, which was named silicone-covered magnesium stent. This stent was proven to bear many advantages including outstanding degradability, elasticity, flexibility, and biocompatibility, which meets the general needs in clinical practice.

The silicone-covered magnesium stent in our study can provide a reliable radial force. After 46 compressions, the silicone-covered magnesium stent could still maintain sufficient effective mechanical compression performance. This demonstrates that silicone can stabilize the mechanism of magnesium alloy tube and maintain its structural stability, thus providing a sufficient support radial force during the expected time *in vivo*. In addition, the silicone membrane isolated magnesium alloy in direct contact with esophageal digestive juice, and significantly reduced the degradation rate of magnesium alloy. Our *in vitro* degradation experiments demonstrated that silicone membrane significantly reduced the degradation rate of magnesium wire. The degradation rates were 5.2% and 16.1% after 1 and 2 wk in the environment with a low pH value of 4.0, respectively. This suggests that the silicone-covered magnesium stent could provide sufficient support within 2 weeks in the esophagus stenosis.

The basic concept of BES treatment is that the stent could tear the hyperplastic smooth muscle layer and provide sufficient support during esophageal repair[12]. In our model, silicone-covered magnesium stent implantation was successful in all the rabbits with esophagus stenosis, and early complications like esophageal perforation, bleeding, and stent migration did not occur. Through pathological examination, we found that the reconstruction of the esophageal wall was completed in 1 and 2 wk after stent insertion. Analyses of the inflammation scores, PCNA-positive cells in the epithelial and SMA layers demonstrated that silicone-covered magnesium stent did not induce severe injury, but caused a very slight inflammatory reaction to the esophageal wall. Some studies showed that the best time of temporary stenting for achalasia was usually 1-2 wk[30,31]. In our experiment, the effective support time could last 2 weeks in the BES model, and esophageal wall reconstruction can be completed successfully during this period. Therefore, our *in vivo* experiments confirmed that the silicone-coated magnesium stent exhibited satisfactory therapeutic effects for treating BES in rabbits. Moreover, the silicone-covered magnesium stent displayed reliable biocompatibility without adverse effects on the growth of SMCs, while the excretion of silicone membrane through the digestive tract further supports its biological safety. Taken together, our study on the silicone-covered magnesium stent provides a theoretical basis for the treatment of BES, and also inspires new ideas to treat other

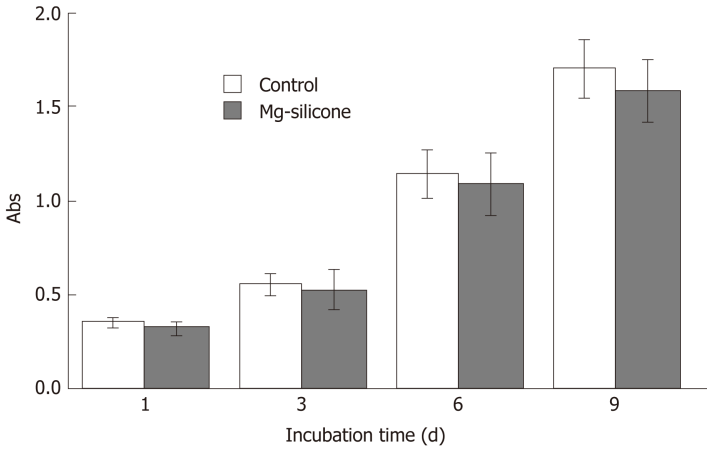


Figure 4 Biological safety evaluation of the magnesium-silicone material by testing the impact on proliferation of human smooth muscle cells. Smooth muscle cells were cultured in the presence and absence of silicone-magnesium gel, and cell proliferation was assayed at 1, 3, 6, and 9 d after culturing (n = 3 for each group at each time point). Control, cultured without silicon-magnesium gel; Mg-silicone, cultured with silicone-magnesium gel.

stenoses.

Although the experimental results are encouraging, there are still some limitations in this study. For example, there is still room for extending the *in vivo* retention time of the stent, as the effective support time is 2 wk. Further study is needed to reduce the biodegradation rate and prolong the support time. In addition, a longer follow-up study is required for determining the efficacy, optimal insertion time, and tissue responses.

In conclusion, the silicone-covered magnesium stent designed in this study can meet the requirements for clinical esophageal stents, in terms of tensile strength, biological safety, and complications. The silicone-covered magnesium esophageal stent exhibited good therapeutic effects in a rabbit model of BES. Injury to the esophagus and stent migration during sustained strength expansion did not occur after the insertion of the silicone-covered magnesium stent in rabbits. As a simple, controllable approach, implantation of the biodegradable silicone-covered magnesium stent is a promising strategy in treating BES.

ACKNOWLEDGEMENTS

We thank Wen-Guo Cui and his team at the Research Laboratory Center, the Animal Experiment Center of Shanghai Sixth People's Hospital for providing experiment support.

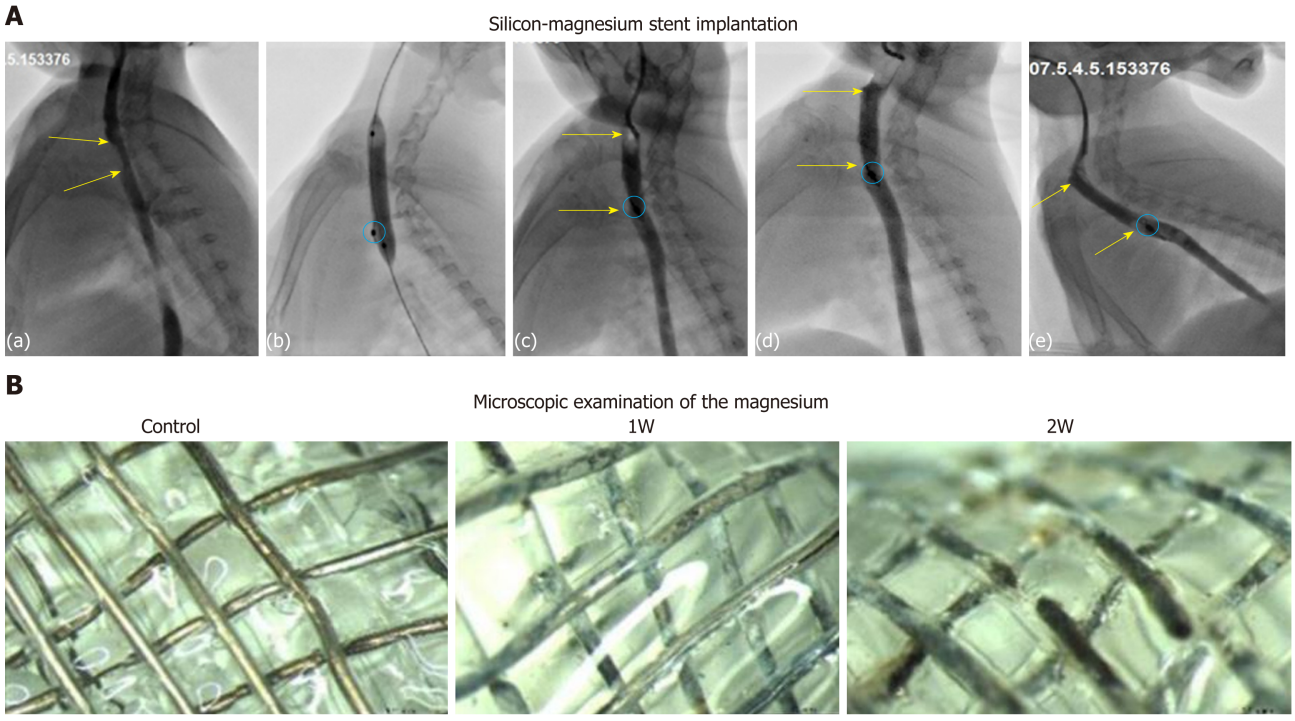


Figure 5 Implantation, follow-up, and *in vivo* degradation of silicone-coated magnesium stents. A: Representative esophagography images show the procedure of stent insertion in rabbits. (a) Upper-middle esophageal stenosis (yellow arrow); (b) Balloon expansion before stent implantation; (c) Esophagography after stent implantation. Stenotic esophagus expansion and in place (yellow arrow) are shown, and positioning mark in the bottom of stent is clearly visible (red oval); (d-e) Follow-up at 1 wk (d) and 2 wk (e) after stent insertion. Stenotic esophagus expansion and in place (yellow arrow) are shown, with positioning mark clearly visible (red oval). B: Microscopic examination of the magnesium to track its retention before (control) and at 1 wk (1W) and 2 wk (2W) after stent implantation.

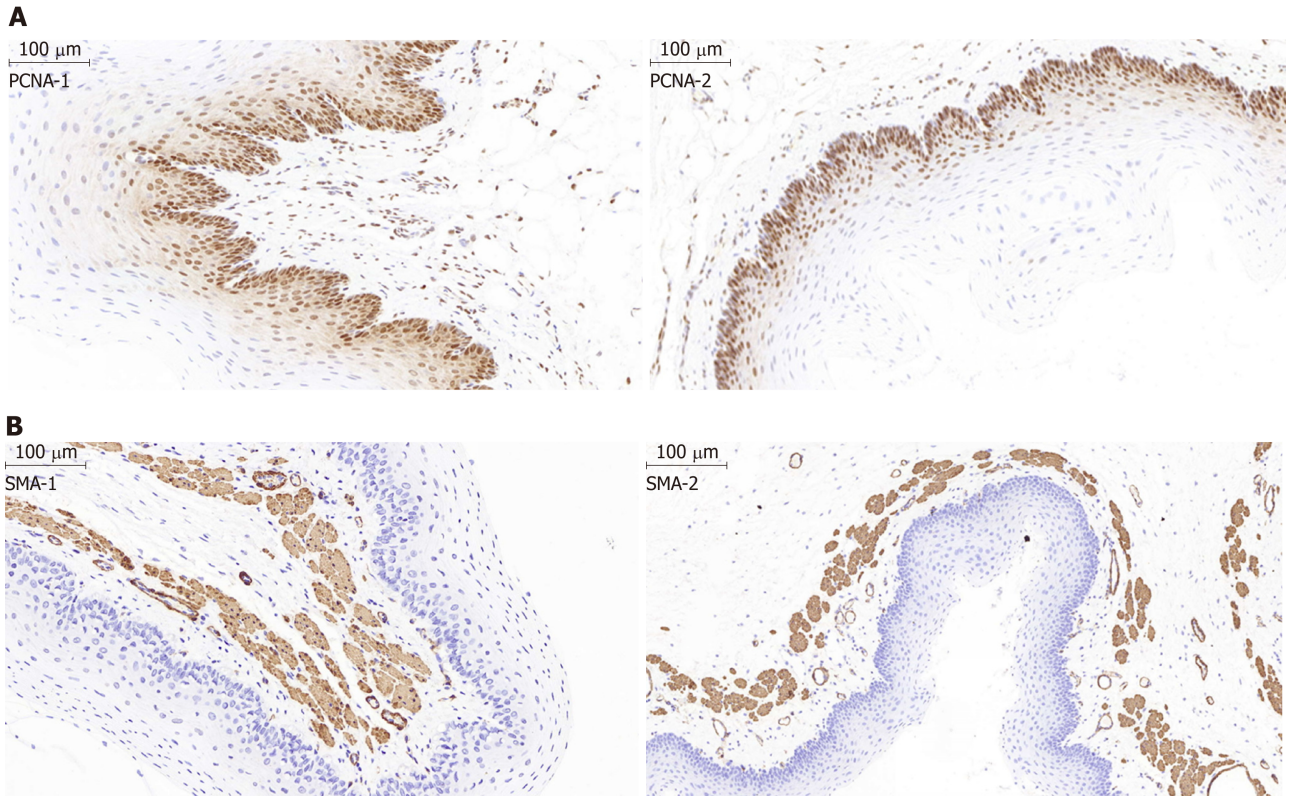


Figure 6 Histological examination of the epithelial layer and the α-smooth muscle layer of the esophagus after stent insertion. A: Representative images show the proliferating cell nuclear antigen (PCNA) staining in the epithelial layer of the esophagus from the stent group and the normal control group. The percentage of PCNA-positive proliferating cells in the epithelial layer in the control group (PCNA-1) was similar to that in the magnesium-silicone stent group (PCNA-2) (magnification, ×100). B: Representative images showing the α-smooth muscle (SMA) staining. The thickness of the epithelial and SMA-positive layers did not differ between the normal control group (SMA-1) and magnesium-silicone stent group (SMA-2) (magnification, ×200).

ARTICLE HIGHLIGHTS

Research background

Stent insertion has been widely used as an effective alternative to improve the quality of life of patients with benign esophageal strictures (BES). However, the metallic stents implantation is associated with some severe complications, such as migration, tissue ingrowth, and instent restenosis. Stents made from biodegradable alloy or polymer are able to provide enough force to tear the benign stricture of the esophagus, as well as reduce complications caused by stents.

Research motivation

Magnesium alloy stents are a good candidate because of biological safety, but they show a poor corrosion resistance and a quick loss of mechanical support *in vivo*. Silicone coating could prolong degradation time of magnesium alloy and enhance the support force of magnesium alloy stents.

Research objectives

The aim of the present study was to evaluate the technique feasibility and therapeutic effect of and tissue response to silicone-covered bio-degradable magnesium stent insertion into the benign esophageal stricture in rabbits.

Research methods

The silicone-covered magnesium stent was made of the magnesium alloy wires through cross-linked mesh, and was fabricated by covering with a silicone membrane. The mechanical testing demonstrated that silicone-covered magnesium stent possessed good flexibility and elasticity, and could provide adequate support *in vivo*. Fifteen rabbits underwent silicone-covered biodegradable magnesium stent insertion into the benign esophageal stricture under fluoroscopic guidance (stent group). The wall reconstruction and tissue reaction of stenotic esophagus were compared with those of six stenosis esophagus models (control group). Esophagography was performed at 1, 2, and 3 wk after stent insertion.

Research results

Histological examination revealed that the inflammation scores at 4 wk in the BES rabbits with stent implantation (stent group) were similar to those in the control rabbits (control group). Both the epithelial and smooth muscle cell layers were significantly thinner in the stent group than in the control group. The smooth muscle actin layer in the muscle layer was thinner in the stent group than in the control group. Without causing severe injury or collagen deposition, implantation of silicone-covered magnesium stent provided reliable support for at least 2 wk in rabbits.

Research conclusions

The present study demonstrated that insertion of silicone-covered magnesium esophageal stent is a promising approach for treating benign esophageal stricture without causing severe injury or tissue reaction.

Research perspectives

The silicone-covered magnesium stent can provide reliable support for at least 1 wk, however, reliable support of the silicone-covered biodegradable magnesium for 2 wk is not enough and associated with high migration rates. There are still some limitations in this study. Further study is needed to reduce the biodegradation rate and prolong the support time. Longer follow-up study is required for determining the efficacy, optimal insertion time, and tissue responses.

REFERENCES

1 **Ravich WJ**. Endoscopic Management of Benign Esophageal Strictures. *Curr Gastroenterol Rep* 2017; **19**: 50 [PMID: 28840483 DOI: 10.1007/s11894-017-0591-8]

2 **Poincloux L**, Rouquette O, Abergel A. Endoscopic treatment of benign esophageal strictures: a literature review. *Expert Rev Gastroenterol Hepatol* 2017; **11**: 53-64 [PMID: 27835929 DOI: 10.1080/17474124.2017.1260002]

3 **Samanta J**, Dhaka N, Sinha SK, Kochhar R. Endoscopic incisional therapy for benign esophageal strictures: Technique and results. *World J Gastrointest Endosc* 2015; **7**: 1318-1326 [PMID: 26722613 DOI: 10.4253/wjge.v7.i19.1318]

4 **Kim JH**, Song HY, Choi EK, Kim KR, Shin JH, Lim JO. Temporary metallic stent placement in the treatment of refractory benign esophageal strictures: results and factors associated with outcome in 55 patients. *Eur Radiol* 2009; **19**: 384-390 [PMID: 18726598 DOI: 10.1007/s00330-008-1151-2]

5 **Gambardella C**, Allaria A, Siciliano G, Mauriello C, Patrone R, Avenia N, Polistena A, Sanguinetti A, Napolitano S, Conzo G. Recurrent esophageal stricture from previous caustic ingestion treated with 40-year self-dilation: case report and review of literature. *BMC Gastroenterol* 2018; **18**: 68 [PMID: 29788901 DOI: 10.1186/s12876-018-0801-3]

6 **Vermeulen BD**, Siersema PD. Esophageal Stenting in Clinical Practice: an Overview. *Curr Treat Options Gastroenterol* 2018; **16**: 260-273 [PMID: 29557070 DOI: 10.1007/s11938-018-0181-3]

7 **Zhang YW**, Wei FX, Qi XP, Liu Z, Xu XD, Zhang YC. Efficacy and Safety of Endoscopic Intralesional Triamcinolone Injection for Benign Esophageal Strictures. *Gastroenterol Res Pract* 2018; **2018**: 7619298 [PMID: 30158968 DOI: 10.1155/2018/7619298]

8 **Yang K**, Ling C, Yuan T, Zhu Y, Cheng Y, Cui W. Polymeric Biodegradable Stent Insertion in the

Esophagus. *Polymers (Basel)* 2016; **8** [PMID: 30979258 DOI: 10.3390/polym8050158]
9 **Zhu Y**, Edmonds L, Zhao X, Chen X, Hu C, Cheng Y, Cui W. In vitro and in vivo evaluation of Rapamycin-eluting nanofibers coated on cardia stents. *RSC Adv* 2014; **4**: 34405-34411 [DOI: 10.1039/C4RA04771K]
10 **Yuan T**, Zheng R, Yu J, Edmonds L, Wu W, Cao J, Gao F, Zhu Y, Cheng Y, Cui W. Fabrication and evaluation of polymer-based esophageal stents for benign esophagus stricture insertion. *RSC Adv* 2016; **6**: 16891-16898 [DOI: 10.1039/C5RA23763G]
11 **Zhu YQ**, Cheng YS, Tang GY, Li MH, Zhao JG, Li F. Comparison of temporary stent insertion with pneumatic dilation of the same diameter in the treatment of achalasia patients: a retrospective study. *J Gastroenterol Hepatol* 2010; **25**: 499-505 [PMID: 20074159 DOI: 10.1111/j.1440-1746.2009.06107.x]
12 **Hindy P**, Hong J, Lam-Tsai Y, Gress F. A comprehensive review of esophageal stents. *Gastroenterol Hepatol (NY)* 2012; **8**: 526-534 [PMID: 23293566]
13 **Zhu Y**, Yang K, Cheng R, Xiang Y, Yuan T, Cheng Y, Sarmento B, Cui W. The current status of biodegradable stent to treat benign luminal disease. *Materials Today* 2017; **20**: 516-529 [DOI: 10.1016/j.mattod.2017.05.002]
14 **Sigounas DE**, Siddhi S, Plevris JN. Biodegradable esophageal stents in benign and malignant strictures - a single center experience. *Endosc Int Open* 2016; **4**: E618-E623 [PMID: 27556067 DOI: 10.1055/s-0042-105433]
15 **Repici A**, Vleggaar FP, Hassan C, van Boeckel PG, Romeo F, Pagano N, Malesci A, Siersema PD. Efficacy and safety of biodegradable stents for refractory benign esophageal strictures: the BEST (Biodegradable Esophageal Stent) study. *Gastrointest Endosc* 2010; **72**: 927-934 [PMID: 21034894 DOI: 10.1016/j.gie.2010.07.031]
16 **Tanaka T**, Takahashi M, Nitta N, Furukawa A, Andoh A, Saito Y, Fujiyama Y, Murata K. Newly developed biodegradable stents for benign gastrointestinal tract stenoses: a preliminary clinical trial. *Digestion* 2006; **74**: 199-205 [PMID: 17341853 DOI: 10.1159/000100504]
17 **Sabharwal T**, Gulati MS, Fotiadis N, Dourado R, Botha A, Mason R, Adam A. Randomised comparison of the FerX Ella antireflux stent and the ultraflex stent: proton pump inhibitor combination for prevention of post-stent reflux in patients with esophageal carcinoma involving the esophago-gastric junction. *J Gastroenterol Hepatol* 2008; **23**: 723-728 [PMID: 18410607 DOI: 10.1111/j.1440-1746.2008.05396.x]
18 **Yuan T**, Yu J, Cao J, Gao F, Zhu Y, Cheng Y, Cui W. Fabrication of a Delaying Biodegradable Magnesium Alloy-Based Esophageal Stent via Coating Elastic Polymer. *Materials (Basel)* 2016; **9** [PMID: 28773505 DOI: 10.3390/ma9050384]
19 Improving the biocompatibility of silicone: Surface Engineering. Materials Today 2003;6:19. [DOI: 10.1016/S1369-7021(03)01119-2]
20 **Esmaily M**, Svensson JE, Fajardo S, Birbilis N, Frankel GS, Virtanen S, Arrabal R, Thomas S, Johansson LG. Fundamentals and advances in magnesium alloy corrosion. *Pro Mater Sci* 2017; **89**: 92-193 [DOI: 10.1016/j.pmatsci.2017.04.011]
21 **Zhu YQ**, Yang K, Edmonds L, Wei LM, Zheng R, Cheng RY, Cui WG, Cheng YS. Silicone-covered biodegradable magnesium-stent insertion in the esophagus: a comparison with plastic stents. *Therap Adv Gastroenterol* 2017; **10**: 11-19 [PMID: 28286555 DOI: 10.1177/1756283X16671670]
22 **Stepanenko AA**, Dmitrenko VV. Pitfalls of the MTT assay: Direct and off-target effects of inhibitors can result in over/underestimation of cell viability. *Gene* 2015; **574**: 193-203 [PMID: 26260013 DOI: 10.1016/j.gene.2015.08.009]
23 **Hwang JC**, Jin B, Kim JH, Lim SG, Yang MJ, Kim SS, Shin SJ, Lee KM, Kim JH. Esophageal stricture induced by an ultraslim upper endoscope in a novel rabbit model of corrosive injury. *Scand J Gastroenterol* 2014; **49**: 30-34 [PMID: 24164403 DOI: 10.3109/00365521.2013.848229]
24 **Hair CS**, Devonshire DA. Severe hyperplastic tissue stenosis of a novel biodegradable esophageal stent and subsequent successful management with high-pressure balloon dilation. *Endoscopy* 2010; **42** Suppl 2: E132-E133 [PMID: 20405380 DOI: 10.1055/s-0029-1244011]
25 **Hirdes MM**, Siersema PD, van Boeckel PG, Vleggaar FP. Single and sequential biodegradable stent placement for refractory benign esophageal strictures: a prospective follow-up study. *Endoscopy* 2012; **44**: 649-654 [PMID: 22723182 DOI: 10.1055/s-0032-1309818]
26 **Gu X**, Zheng Y, Cheng Y, Zhong S, Xi T. In vitro corrosion and biocompatibility of binary magnesium alloys. *Biomaterials* 2009; **30**: 484-498 [PMID: 19000636 DOI: 10.1016/j.biomaterials.2008.10.021]
27 **Li X**, Chu CL, Liu L, Liu XK, Bai J, Guo C, Xue F, Lin PH, Chu PK. Biodegradable poly-lactic acid based-composite reinforced unidirectionally with high-strength magnesium alloy wires. *Biomaterials* 2015; **49**: 135-144 [PMID: 25725562 DOI: 10.1016/j.biomaterials.2015.01.060]
28 **Wong HM**, Yeung KW, Lam KO, Tam V, Chu PK, Luk KD, Cheung KM. A biodegradable polymer-based coating to control the performance of magnesium alloy orthopaedic implants. *Biomaterials* 2010; **31**: 2084-2096 [PMID: 20031201 DOI: 10.1016/j.biomaterials.2009.11.111]
29 **Wu Q**, Zhu S, Wang L, Liu Q, Yue G, Wang J, Guan S. The microstructure and properties of cyclic extrusion compression treated Mg-Zn-Y-Nd alloy for vascular stent application. *J Mech Behav Biomed Mater* 2012; **8**: 1-7 [PMID: 22402149 DOI: 10.1016/j.jmbbm.2011.12.011]
30 **Zhu YQ**, Cheng YS, Li F, Li MH, Zhao JG, Chen NW. Application of the newly developed stents in the treatment of benign cardia stricture: an experimental comparative study. *Gastrointest Endosc* 2011; **73**: 329-337 [PMID: 21111414 DOI: 10.1016/j.gie.2010.09.044]
31 **Zhu YQ**, Cui WG, Cheng YS, Chang J, Chen NW, Yan L. Evaluation of biodegradable paclitaxel-eluting nanofibre-covered metal stents for the treatment of benign cardia stricture in an experimental model. *Br J Surg* 2013; **100**: 784-793 [PMID: 23553755 DOI: 10.1002/bjs.9106]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3218-3230

DOI: 10.3748/wjg.v25.i25.3218

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

ORIGINAL ARTICLE

Basic Study

Nuclear magnetic resonance-based metabolomics and metabolic pathway networks from patient-matched esophageal carcinoma, adjacent noncancerous tissues and urine

Jia-Hao Liang, Yan Lin, Ting Ouyang, Wan Tang, Yao Huang, Wei Ye, Jia-Yun Zhao, Zhe-Ning Wang, Chang-Chun Ma

ORCID number: Jia-Hao Liang (0000-0002-6288-6288); Yan Lin (0000-0002-8284-9029); Ting Ouyang (0000-0003-4710-3069); Wan Tang (0000-0003-0404-1494); Yao Huang (0000-0001-5616-7828); Wei Ye (0000-0003-1459-0155); Jia-Yun Zhao (0000-0003-2446-2288); Zhe-Ning Wang (0000-0003-3754-0943); Chang-Chun Ma (0000-0002-5389-4250).

Author contributions: Lin Y conceived and designed the experiments; Liang JH and Lin Y contributed to NMR data acquisition; Liang JH, Ouyang T, Tang W and Huang Y analyzed the data; Lin Y wrote the paper; Ye W, Zhao JY and Wang ZN contributed to sample preparation; all authors approved the final version of the manuscript for publication.

Supported by the National Natural Science Foundation of China, No. 81471729 and No. 81101102; the Science and Technology and Planning Project of Guangdong Province, No. 2016A020216025; the Research Award Fund for Outstanding Young Teachers in Higher Education Institutions, Guangdong Province, No. YQ2015245; the National Natural Science Foundation of Guangdong Province, No. S2011010004973; the Department of Education of Guangdong Province, No. 2017KTSCX071.

Institutional review board statement: This study was

Jia-Hao Liang, Yan Lin, Ting Ouyang, Wan Tang, Yao Huang, Wei Ye, Jia-Yun Zhao, Zhe-Ning Wang, Department of Radiology, Second Affiliated Hospital, Shantou University Medical College, Shantou 515041, Guangdong Province, China

Chang-Chun Ma, Department of Radiation Oncology, Affiliated Tumor Hospital, Shantou University Medical College, Shantou 515041, Guangdong Province, China

Corresponding author: Yan Lin, PhD, Chief Doctor, Department of Radiology, Second Affiliated Hospital, Shantou University Medical College, No. 69. Dongshabei Road, Shantou 515041, Guangdong Province, China. 994809889@qq.com
Telephone: +86-13502958156

Abstract

BACKGROUND

Several studies have demonstrated a correlation between esophageal cancer (EC) and perturbed urinary metabolomic profiles, but none has described the correlation between urine metabolite profiles and those of the tumor and adjacent esophageal mucosa in the same patient.

AIM

To investigate how urinary metabolic phenotypes were linked to the changes in the biochemical landscape of esophageal tumors.

METHODS

Nuclear magnetic resonance-based metabolomics were applied to esophageal tumor tissues and adjacent normal mucosal tissues alongside patient-matched urine samples.

RESULTS

Analysis revealed that specific metabolite changes overlapped across both metrics, including glucose, glutamate, citrate, glycine, creatinine and taurine, indicating that the networks for metabolic pathway perturbations in EC, potentially involved in but not limited to disruption of fatty acid metabolism, glucose and glycolytic metabolism, tricarboxylic acid cycle and glutaminolysis. Additionally, changes in most urinary biomarkers correlated with changes in biomarker candidates in EC tissues, implying enhanced energy production for rapid cell proliferation.

reviewed and approved by the Second Affiliated Hospital, Shantou University Medical College Review Board (2018-44).

Informed consent statement: Informed consent was obtained from each subject prior to participation in this study.

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

Manuscript source: Unsolicited manuscript

Received: January 25, 2019
Peer-review started: January 25, 2019
First decision: February 26, 2019
Revised: May 13, 2019
Accepted: May 31, 2019
Article in press: June 1, 2019
Published online: July 7, 2019

P-Reviewer: Hashimoto N, Luyer MDP
S-Editor: Cui LJ
L-Editor: Filipodia
E-Editor: Ma YJ

CONCLUSION

Overall, these associations provide evidence for distinct metabolic signatures and pathway disturbances between the tumor tissues and urine of EC patients, and changes in urinary metabolic signature could reflect reprogramming of the aforementioned metabolic pathways in EC tissues. Further investigation is needed to validate these initial findings using larger samples and to establish the underlying mechanism of EC progression.

Key words: Esophageal cancer; Metabolites; Metabolic pathways; Nuclear magnetic resonance-based metabolomics; Tumor tissue; Urine

Core tip: Our research provides evidence for distinct metabolic signatures and metabolic pathway disturbances between the tumor tissues and urine of esophageal cancer patients, and changes in the urinary metabolic signature could reflect reprogramming of aforementioned metabolic pathways in esophageal tumor tissues.

Citation: Liang JH, Lin Y, Ouyang T, Tang W, Huang Y, Ye W, Zhao JY, Wang ZN, Ma CC. Nuclear magnetic resonance-based metabolomics and metabolic pathway networks from patient-matched esophageal carcinoma, adjacent noncancerous tissues and urine. *World J Gastroenterol* 2019; 25(25): 3218-3230
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3218.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3218

INTRODUCTION

Esophageal cancer (EC) is the eighth most common type of malignant tumor and the sixth leading cause of cancer-related death worldwide[1]. Identifying cancer-related biomarkers of EC is essential for its diagnosis and therapeutic intervention in the early stage, which in turn will significantly increase patient survival. While there are a few diagnostic/screening techniques, such as upper gastrointestinal endoscopy, endoscopy-based balloon cytology, and serum carcinoembryonic antigen (commonly known as CEA) test, high-throughput and sensitive molecular tools are required to elucidate specific disease biomarkers for optimal disease management. Metabolomics, in which the global changes of small molecular weight metabolites in a given biological specimen are investigated, has considerable potential to identify useful biomarkers, thereby stratifying subjects into disease or nondiseased categories[2,3]. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is a well-established, robust, noninvasive and reproducible method for quantifying metabolic profiles[4], and it has several advantages over other analytical techniques, such as liquid chromatography mass spectroscopy and gas chromatography mass spectroscopy, including nondestructive analysis of samples, minimal sample preparation, and the ability to detect multiple metabolites within a single experiment[5-8]. NMR data combined with multivariate statistical analysis, such as orthogonal partial least squares discriminant analysis (OPLS-DA), which can be utilized for disease cla-ssification (through the use of score plots) and biomarker detection (through the use of loading plots), allow for the identification of potential biomarkers in biological specimens and improve the ability to identify specific metabolic pathways and networks associated with the disease process.

Urine is a biological fluid commonly used by NMR-based metabolomics[9,10] because it is easily collected in large volumes with noninvasive procedures and may provide substantial diagnostic information for many cancer types[7,11-13]. Biomarkers in urine may be derived from cell apoptosis, glomerular filtration of blood plasma, cell sloughing, epithelial cell secretion of exosomes, and other processes[14]. Several NMR spectroscopy-based metabolomic studies have reported that metabolite compositions of urine samples from EC patients differ from those of healthy controls (HCs)[15,16]. However, little is known about the systemic mechanistic link between esophageal tissues and urine, owing to the invasiveness of tissue sampling and sensitivity of the urinary metabolome to factors such as environment, food and genetic composition.

The aim of this research was to profile parallel metabolites of EC tissues and ad-

jacent noncancerous tissues alongside urine samples from the same patients, to investigate how urinary metabolomic phenotypes associate with tumor tissue, and to identify specific disturbed metabolic pathways in esophageal tissues and urine. Such information would be vital to bridge the gap between the systemic metabolic characteristics of EC tissues and the corresponding samples of urine and may help advance the utility of urinary-based metabolites as relevant indicators of EC tissue microenvironment.

MATERIALS AND METHODS

Patient recruitment and sample collection

This study was approved by the Ethics Committee of Shantou University Medical College. Written informed consent was obtained from each subject prior to participation. Early-morning midstream urine samples were collected preoperatively from 41 EC patients and 40 HCs between August 2015 and October 2016 at the Second Affiliated Hospital of Shantou University Medical College. EC patients were diagnosed by microscopy, biopsy, or surgical resection, and the disease stage was determined according to the American Joint Committee on Cancer (AJCC) staging system for esophageal tumors: Stage I/II, 15 patients; stage III, 11 patients; stage IV, 15 patients. Each urine sample was mixed with 50 μL sodium azide preservative and stored at −80 °C until further analysis. Patient-matched EC tissues and their corresponding adjacent noncancerous tissues (~5 cm away from the tumor margin) were collected from 20 EC patients and immediately stored at −80 °C until NMR analysis. Exclusion criteria for all participants included use of antibiotics, nonsteroidal anti-inflammatory drugs, statins, or probiotics within 2 mo of study participation. Additional exclusion criteria for EC patients included chemotherapy or radiotherapy prior to surgery. The demographic and clinical characteristics of the EC patients and con-trols are summarized in Table 1.

Urine sample preparation

Frozen urine samples were thawed at room temperature and mixed to suspend any settled precipitate. After adding 300 μL PBS/D_2O buffer (0.1 M, pH 7.4) to 600 μL of each urine sample, the mixture was homogenized by vortexing for 60 s and then centrifuged at 8000 rpm for 10 min. Subsequently, a volume of 500 μL of the supernatant was transferred into an Eppendorf vial, to which 50 μL of a stock solution of sodium (3-trimethylsilyl)-2, 2, 3, 3-tetradeuteriopropionate (TSP)/D_2O was added, using a chemical shift reference (0.0 ppm) for spectral alignment. Finally, the resulting mixture was centrifuged at 10000 rpm for 5 min, and 500 μL of the prepared sample was transferred into a 5-mm high-resolution NMR tube for ^{1}H-NMR analysis.

Tissue sample preparation

The frozen tissue samples weighed ~300 mg and were thawed and cut into small pieces at room temperature. After adding 1.8 mL mixture containing 0.6 mL distilled water (2 mL/g tissue) and 1.2 mL methanol (4 mL/g tissue), the resulting mixture was homogenized at 16000 rpm for 80 s. After homogenization, the mixture was added to chloroform (4 mL/g tissue) and distilled water (4 mL/g tissue) and vortexed for 60 s. The suspension was then left on ice for 15 min and centrifuged at 2000 rpm for 5 min to facilitate separation of the liquid layers. The resulting supernatant was evaporated under a stream of nitrogen and then dried under vacuum for a minimum of 18 h. Subsequently, the lyophilized powder was redissolved with 550 μL PBS/D_2O buffer (0.1 mol/L, pH 7.4), to which 50 μL of a stock solution of TSP/D_2O was added. After homogenization and centrifugation at 10000 rpm for 5 min, 500 μL supernatant was transferred into a 5-mm high-resolution NMR tube for ^{1}H-NMR analysis.

^{1}H-NMR analysis

All samples were detected by a Bruker AVII 400 MHz NMR spectrometer (Bruker Biospin, Germany) operating at a ^{1}H frequency of 400.13 MHz. Magnetic field homogeneity was optimized by gradient or manual shimming prior to acquisition. The temperature was maintained at 298 K and lock performed on the D_2O signal. ^{1}H NMR spectra of urine samples were obtained using a 1D nuclear Overhauser enhancement spectroscopy pulse sequence [RD-90°-t*1*-90°-t*m*-90°- ACQ], with the following acquisition parameters: Recycle delay (RD) 1.5 s; t*1* 3 μs; mixing time, t*m* 100 ms; 90° pulse width 7.3 μs; number of scans (NS) 256; number of points, TD 32768; spectral width (SW) 8012.82 Hz; acquisition time (AQ) 2.04 s. Water suppression was achieved by irradiation of the water peak during RD and t*m*. Esophageal tissue ^{1}H NMR spectra were recorded using a standard (1D) Carr–Purcell–Meiboom–Gill pulse sequence with the following acquisition parameters: number of dummy scans 4; RD

Table 1 Summary of clinical and demographic features for study subjects and tumor characteristics

	EC	HC	χ^2	*P* value
Subjects, *n*	41	40		
Age (median, range), yr	60, 39-77	59, 28-78		
Sex			6.77	0.12
Male	31	19		
Female	10	21		
Cancer stage				
Stage I/II	15	-		
Stage III	11	-		
Stage IV	15	-		
CEA, ng/mL				
Positive	2	-		
Negative	31	-		
Not measured	8	-		
CA 19-9, U/mL				
Positive	2	-		
Negative	18	-		
Not measured	21	-		
Location				
Cervical	1	-		
Upper thoracic	5	-		
Middle thoracic	24	-		
Lower thoracic	11	-		
Symptoms				
Dysphagia	40	-		
Gastroesophageal reflux	27	-		

EC: Esophageal cancer; HC: Healthy control; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen.

70 ms; 90° pulse width 10 μs; NS 64; TD 65536; SW 8012 Hz; AQ 4.09 s.

^{1}H NMR spectral data processing

All free induction decays from 1D ^{1}H-NMR of the tissues and urine samples were multiplied by a 0.3 Hz exponential line broadening prior to Fourier transformation. ^{1}H-NMR spectra were then corrected for phase and baseline distortion and calibrated to TSP at 0.0 ppm by using MestReNova software (version 8.1.0, Mestrelab Research, Santiagode Compostella, Spain). To reduce the complexity of the NMR data, the spectral range from 0.8 to 9.0 ppm was segmented into buckets with the equal width of 0.004 ppm. The region of 5.5–4.5 ppm was discarded to eliminate imperfect water suppression. Each bucket was internally normalized to the total integral of the spectrum prior to pattern recognition analysis, to eliminate the dilution or bulk mass differences among samples due to the different sample weight.

Pattern recognition analysis and cross validation

To maximize class discrimination between EC patient and HC urine samples, as well as between EC tissues and their corresponding noncancerous tissues, multivariate data analysis was applied to the ^{1}H-NMR spectral data according to previously published and accepted methods[6,7]. The normalized NMR spectral data sets were unit variance scaled and analyzed using the SIMCA-P+ program (version 14.1, Umetrics AB; Umea, Sweden). OPLS-DA was applied to the analysis of ^{1}H-NMR spectral data to optimize the separation between experimental groups. The model quality was evaluated with the R^2Y and Q^2 values, reflecting the explained fraction of variance and the model predictability. R^2Y scores ranged between 0 and 1 and Q^2 scores between negative and 1, where a R^2Y score of 1 demonstrated that the model explained 100% of variance, and a Q^2 score closer to 1 indicated higher reliability of the prediction in the cross-validation procedure. Validation of the OPLS-DA model was also performed by means of a permutation test (400 times). The R^2Y in the permutated plot described

how well the data fitted with the derived model, whereas Q^2 described the predictive ability of the derived model ($Q^2 > 0.5$ considered as good and $Q^2 > 0.9$ as excellent). To further evaluate the predictive power of the robust OPLS-DA model, 80% of samples were applied to construct a model, which was used to predict the remaining 20% of samples. The variable importance in the projection (VIP) values of all peaks from OPLS-DA models was taken as a coefficient for peak selection. The VIP value was represented by a unitless number, where higher values suggested greater discriminatory power of the metabolite. Those variables with VIP > 1 were considered potential biomarker candidates for group discrimination.

Statistical analysis

The relative concentrations of those metabolites with VIP > 1 were calculated by integrating the signals in the spectra. Statistical significance was assessed using the Mann–Whitney *U* test, and $P < 0.05$ was considered statistically significant. The relative concentrations of those metabolites with VIP > 1 were calculated by integrating the signals in the spectra, and data are presented as the mean fold difference in EC metabolite abundance compared to controls. To further evaluate the diagnostic power of the potential biomarkers whose levels significantly differed between experimental groups, receiver operating characteristic (ROC) analysis in SPSS version 16.0 was performed, and the optimal area under the ROC curve (AUROC), specificity and sensitivity of the metabolites were calculated, where AUROC > 0.8 indicated excellent diagnostic ability. Pearson correlation analysis was used to assess the association of biomarker candidates between urine and tumor tissues of EC patients with an OPLS-DA model using a correlation coefficient cut-off of $|r|$ and correlation significance of $P < 0.05$. Correlation coefficients ranged from 1.0 (maximum positive correlation) to −1.0 (maximum anticorrelation), with a value of 0 representing no correlation. Correspondingly, $|r| > 0.44$ (calculated for the sample size of 20) was considered to be a statistically significant relationship between the two metabolites.

RESULTS

Metabolic profiles of esophageal tissues and urine samples

Representative 1D ^{1}H-NMR spectra of urine specimens acquired from EC patients, HCs and patient-matched esophageal tissue extracts are shown in Figure 1A and B. The standard 1D spectrum gave an overview of all metabolites. The major metabolites in the spectra were identified according to previous studies[17,18] and the Human Metabolome Database (http://www.hmdb.ca/). In all urine and esophageal tissue spectra, the aliphatic region at 0.8–4.2 ppm included numerous signals from the following water-soluble metabolites: Glutamate, glutamine, acetoacetate, citrate, cisaconitate, choline, creatine, creatinine and glycine, which are known to be involved in many biochemical processes, especially in energy metabolism.

Good discrimination between EC patients and HCs was achieved by an OPLS-DA scores plot generated from ^{1}H-NMR spectra of urine specimens (Figure 2A-a). The predictive ability of the model was calculated by internal validation ($R^2Y = 0.902$, $Q^2 = 0.682$, CV-ANONA $P < 0.01$), suggesting that the model possessed a satisfactory fit with good predictive power. In order to evaluate the robustness of the OPLS-DA model described above, a random permutation test was performed 400 times to further evaluate the robustness of this model, as exhibited by the steep R^2 and Q^2 regression lines and difference between R^2 and Q^2 ($R^2Y = 0.886$ and $Q^2 = 0.660$), indicating that this was an excellent model suitable for data analysis (Figure 2A-b). To further assess the predictive ability of the model for unknown samples, we randomly selected 80% of urine samples ("training set", 33 EC patients and 32 HCs) to construct an OPLS-DA model, which was then used to predict the remaining 20% of samples ("testing set", 8 EC patients and 8 HCs). As can be seen in Figure 2A-c, the testing sets of EC patients and HCs were correctly located in their corresponding region of the training sets. Urine metabolites that met the following conditions were considered potential metabolite biomarkers for EC detection: Levels of metabolites with VIP > 1 and the presence of a significant difference ($P < 0.01$) between metabolite levels of EC patients and HCs according to the Mann–Whitney *U* test. A total of ten urine metabolites were found to be significantly changed in EC patients compared to HCs (Table 2), including higher levels of acetoacetate, cis-aconitate, citrate and glutamate, together with lower amounts of glycine, taurine, creatinine, ethanolamine, glucose and hippurate.

Tissue profiles from EC tumor tissues and their corresponding adjacent noncancerous tissues were clearly separated using the OPLS-DA scores plot (Figure

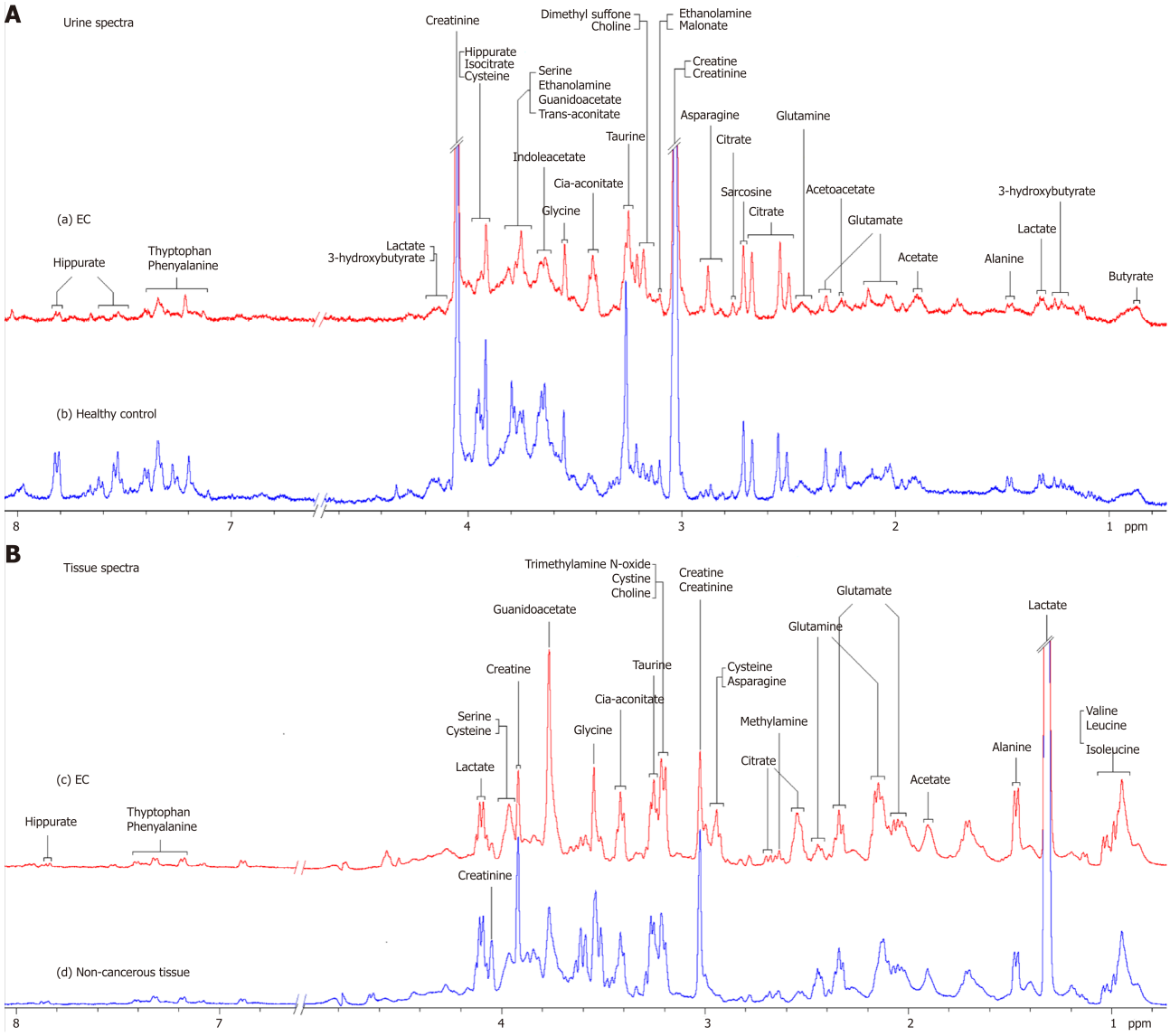


Figure 1 Proton nuclear magnetic resonance spectra. A: 400 MHz representative urine proton nuclear magnetic resonance (^{1}H-NMR) spectra obtained from esophageal cancer (EC) patient (a) and healthy control (b); B: Tissue ^{1}H-NMR spectra obtained from EC tissue (c) and adjacent noncancerous tissue (d) referenced to tetradeuteriopropionate (0.0 ppm).

2B-a). Model parameters of the 400 times permutation test generated $R^2Y = 0.908$ and $Q^2 = 0.842$ (Figure 2B-b). Moreover, the OPLS-DA model showed good performance for predicting the unknown samples (Figure 2B-c). Using these criteria (VIP > 1 and $P < 0.05$), the 15 metabolites listed in Table 3 were found to be significantly changed in EC tissues compared to their corresponding noncancerous tissues, including elevated levels of valine, leucine, alanine, acetate, citrate, succinate, choline and glutamate, as well as depleted levels of creatinine and creatine, glycine, threonine, taurine, glucose, and glutamine.

Metabolic changes that overlap across urine and EC tissues

Our parallel investigations revealed a few distinct and overlapping discriminatory metabolites between cancer tissues and urine of EC patients, including decreased levels of glucose, glycine, creatinine and taurine, together with increased levels of glutamate and citrate, as compared to their respective controls. Metabolic profiling associations between potential urine and tissue biomarkers were further analyzed, plotted as correlation heat maps color-coded by the strength of Spearman correlation coefficients (Figure 3). Changes in most potential urinary biomarkers (except for glutamate, citrate and glucose) were found to be correlated with changes in most biomarker candidates in EC tissues (except for glycine and acetate) ($|r| > 0.44$, $P < 0.05$).

Comparison of diagnostic performance of potential urinary biomarkers for

Table 2 Potential urine biomarkers for discriminating esophageal cancer patients from healthy controls

Metabolite	Chemicalshift, ppm	Fold difference		AUC (95%CI)	Related metabolic pathways
		EC / HC	*P* value		
Acetoacetate	2.25–2.28	1.55 ↑	< 0.001	0.745 (0.636–0.836)	Fatty acid metabolism, TCA cycle
Glutamate	2.04–2.06	1.22 ↑	0.002	0.702 (0.590–0.798)	Glutaminolysis, TCA cycle
Cis-aconitate	3.40–3.46	1.30 ↑	0.001	0.719 (0.608–0.813)	TCA cycle, glyoxylate and dicarboxylate metabolism
Citrate	2.48–2.54 2.64–2.66	1.27 ↑	0.002	0.643 (0.528–0.746)	TCA cycle
Hippurate	7.51–7.56 7.60–7.65 7.78–7.84	0.45 ↓	0.002	0.702 (0.591–0.799)	Gut microflora metabolism
Ethanola-mine	3.08–3.16	0.86 ↓	0.001	0.723 (0.613–0.817)	Fatty acid metabolism
Glycine	3.54–3.55	0.85 ↓	0.003	0.633 (0.518–0.737)	Amino acid metabolism
Creatinine	3.03–3.06 4.05–4.10	0.95 ↓	< 0.001	0.790 (0.685–0.872)	Urea metabolism, Creatinine metabolism
Taurine	3.26–3.28	0.53 ↓	< 0.001	0.763 (0.655–0.850)	Amino acid metabolism
Glucose	3.73–3.80	0.89 ↓	0.007	0.694 (0.581–0.792)	Glycolysis; TCA cycle

EC: Esophageal cancer; HC: Healthy control; AUC: Area under the curve; TCA: Tricarboxylic acid.

distinguishing EC

Given that urinary glutamate, citrate and glucose in EC patients did not show correlation with esophageal tissue biomarkers, a panel of urinary metabolite markers composed of acetoacetate, cis-aconitate, hippurate, ethanolamine, glycine, creatinine and taurine was selected to compare their diagnostic performance for distinguishing EC patients from HCs. ROC analysis of the comparison of single urinary biomarkers and their combination showed that combined metabolites of the aforementioned metabolites had better diagnostic performance than any single metabolite alone in discriminating EC patients from HCs, with sensitivity, specificity and area under the curve values of 92.68%, 92.50% and 0.971, respectively (Figure 4).

DISCUSSION

The current study performed parallel investigations of EC tissues and adjacent noncancerous mucosal tissues alongside patient-matched urine samples to investigate how changes of the urine metabolome were linked to the changes of EC tissues metabolic phenotypes. Our study showed significant metabolic alterations in both urine and tumor tissues of EC patients compared to their respective HCs. Correlative analysis of the altered metabolites across both matrices revealed a few distinct and overlapping discriminatory metabolites, such as glucose, glycine, creatinine and taurine (decreased levels) together with glutamate and citrate (increased amounts), suggesting that EC is associated with the following dysregulated metabolic pathway perturbations, including but not limited to fatty acid metabolism, glucose and glycolytic activity, tricarboxylic acid (TCA) cycle and glutaminolysis. Metabolic profiling correlations between esophageal tissues and urine showed that most potential urine biomarkers were correlated with most of the discriminating metabolites in EC tissues, indicating that changes in urine metabolic signature could reflect reprogramming of metabolic pathways in tumor tissues, highlighting the significance of the distinct urinary metabolic profiles as potential novel and nonin-vasive indicators for EC detection.

Our NMR-based metabolomic findings identified distinct disturbances occurring in both tissues and urine of EC patients compared to their respective controls (Figure 5). Tumor-microenvironment cooperation may occur in cancer cells, which exhibit a high rate of anabolic metabolism, by which they take up large amounts of nutrients to fuel the TCA cycle and oxidative phosphorylation. Therefore, in order to meet the increased demands of proliferation, tumor cells display changes in energy metabolism and nutrient uptake pathways[19]. In general, tumor tissue has depleted glucose and increased citrate and succinate, the TCA intermediates, reflecting high TCA cycle activity to maintain tumor promotion[8,20]. Reduced glucose and increased citrate levels were evident in EC patient urine, further indicating enhanced glycolysis under hypoxic conditions required for rapid cancer cell proliferation[21,22]. Acetate, a source for lipid and myelin synthesis[23], is derived from acetyl-CoA *via* the deacetylation of N-

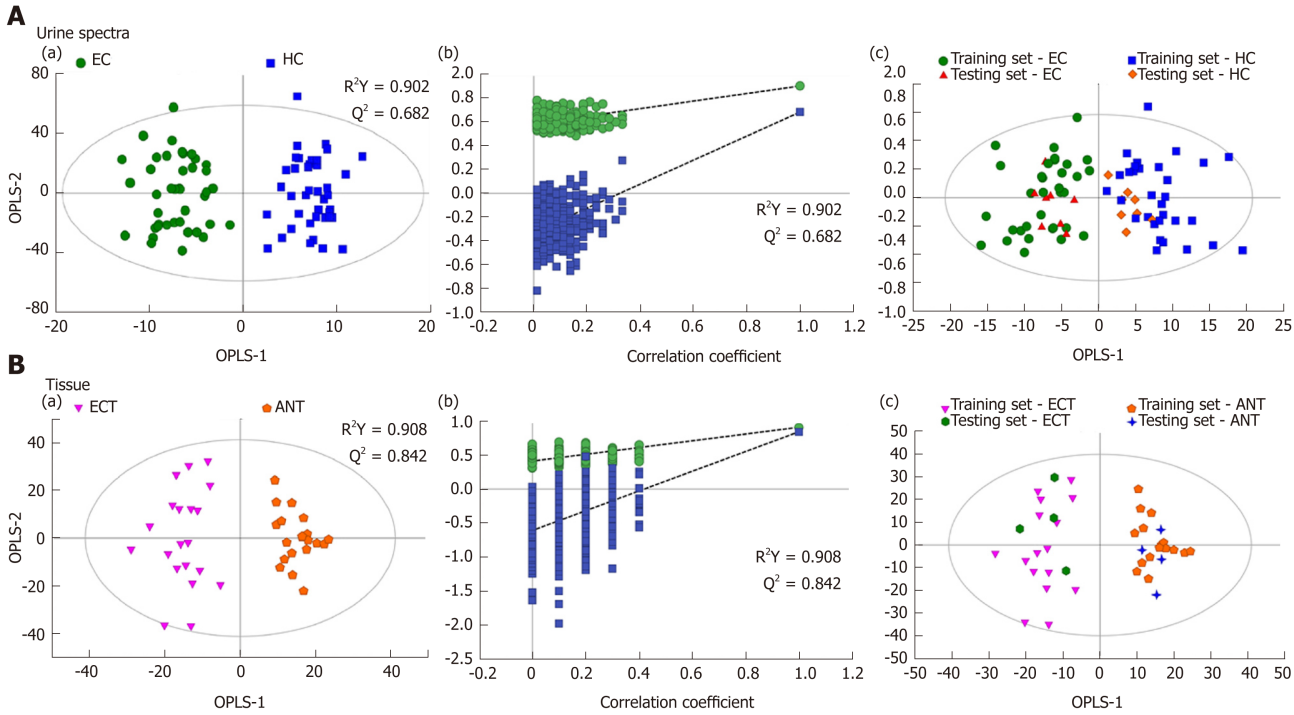


Figure 2 Pattern recognitions. A: Pattern recognition of urine metabolomic profiles analyzed by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. (a) orthogonal partial least squares discriminant analysis (OPLS-DA) scatter plot of urine samples based on esophageal cancer (EC) patients (green dots) and healthy controls (HCs) (blue squares); (b) Statistical validation of the corresponding OPLS-DA model by permutation analysis (400 times); (c) Score plots of OPLS-DA prediction model. 80% of samples were applied to construct the model, which was used to predict the remaining 20% of samples [testing set, 8 HCs (gold diamonds); 8 EC patients (red triangles)]; B: Pattern recognition of tissue metabolomic profiles analyzed by ^{1}H-NMR spectroscopy. (a) OPLS-DA scatter plot of EC tissue samples obtained (purple inverted triangles) and adjacent noncancerous tissue (orange pentagons); (b) Statistical validation of the corresponding OPLS-DA model by permutation analysis (400 times); (c) Score plots of OPLS-DA prediction model, with 80% of the samples applied to construct the model, which was used to predict the remaining 20% of samples [testing set, 4 EC tissues (green hexagons); 4 adjacent non-cancerous tissues (blue crosses)].

acetylaspartate. The observed elevation of acetate in EC tissues might result from an increase in fatty acid metabolism, and this observation is supported by elevation of alanine derived from metabolism of pyruvate, indicating activation of glycolysis to provide higher energy needs[24]. Glutamine is also regarded as important for energy production in proliferating cells, and it donates nitrogen for nucleotide synthesis, resulting in the formation of glutamate (glutaminolysis). The latter can be converted to α-ketoglutarate to increase transit through the TCA cycle, providing sustainable energy required for rapid cell proliferation[25]. Therefore, increased glutamate along with depleted glutamine in EC tissue could suggest changes in glutaminolytic activity for EC development. The increased glutamate in EC urine observed in this study could also suggest a high energy demand in proliferating cells due to augmented glutaminolysis[26]. Increased leucine and valine in tissues and increased acetoacetate in urine were in agreement with the ingested nutrients that fuel the TCA cycle to support cell proliferation. Similarly, cell growth and proliferation need amino acids to generate proteins required for cancer cell synthesis[27], therefore leading to decreased levels of glycine and threonine in EC pa-tients. Depleted creatine/creatinine levels in tumor tissues have been related to altered energy transfer processes and may reflect increased activity of creatine kinase, which has been previously reported to be lower in lung tumors compared to normal adjacent tissues[28]. Besides, creatinine levels in EC patient urine samples were significantly decreased compared to HCs, which has also been reported to be lower in urine samples from colorectal cancer patients[7]. The observed depletion of taurine in both tissues and urine of EC patients suggest a disruption in taurine metabolism and diffusion of gut microbes associated with EC tumors[29]. Ethanolamine is an important fatty acid for cellular membranes[30], and its decreased levels in EC urine could suggest increased consumption for biosynthesis of cellular membranes and indicate activation of fatty acid metabolism. The observed depletion of hippurate in EC urine was likely the result of gut microbiome perturbation associated with EC tumorigenesis. Hippurate is metabolized from benzoic acid, which is metabolized from the dietary polyphenol 3-hydroxyphenyl propionic acid by the gut microflora[31]. Choline is one of the major cell membrane phospholipids[32] and is overexpressed and highly active in tumor tissues and cell lines. We observed increased choline levels in EC tissues, which was consistent with previous

Table 3 Potential tissue biomarkers for discriminating esophageal cancer tissue from adjacent noncancerous tissue

Metabolite	Chemicalshift, ppm	Fold difference		AUC (95%CI)	Related metabolic pathways
		ECT / ANT	*P* value		
Valine	0.96–0.99 1.02–1.04	1.63 ↑	< 0.001	0.988 (0.889-1.000)	Amino acid metabolism
Leucine	0.93–0.95	1.32 ↑	< 0.001	0.852 (0.705-0.944)	Amino acid metabolism
Glutamate	1.97–2.08 2.32–2.39	1.30 ↑	< 0.001	0.930 (0.803-0.986)	Glutaminolysis, TCA cycle
Acetate	1.88–1.93	1.33 ↑	0.001	0.790 (0.632-0.902)	SCFA metabolism
Alanine	1.43–1.48	1.44 ↑	< 0.001	0.920 (0.789-0.982)	Amino acid metabolism, Gluconeogenesis
Choline	3.19–3.22	1.59 ↑	< 0.001	0.980 (0.876-1.000)	Choline metabolism, Lipid metabolism
Succinate	2.39–2.40	2.12 ↑	0.009	0.738 (0.575-0.864)	TCA cycle
Citrate	2.68–2.71	1.42 ↑	< 0.001	0.942 (0.820-0.991)	TCA cycle
Glucose	3.37–3.44 3.50–3.52	0.72 ↓	< 0.001	0.928 (0.800-0.985)	Glycolysis, TCA cycle
Creatinine	3.02–3.03 4.04–4.06	0.64 ↓	< 0.001	0.963 (0.849-0.997)	Urea metabolism, Creatine metabolism
Glycine	3.52–3.55	0.75 ↓	< 0.001	0.850 (0.702-0.943)	Amino acid metabolism
Threonine	3.58–3.62	0.60 ↓	< 0.001	0.933 (0.806-0.987)	Amino acid metabolism
Creatine	3.90–3.94	0.78 ↓	< 0.001	0.917 (0.786-0.981)	Urea metabolism, Creatine metabolism
Glutamine	2.42–2.48	0.77 ↓	< 0.001	0.895 (0.757-0.969)	Glutaminolysis, TCA cycle
Taurine	3.24–3.28 3.33–3.34	0.78 ↓	< 0.001	0.878 (0.735-0.960)	Amino acid metabolism

ANT: Adjacent noncancerous tissue; ECT: Esophageal cancer tissue; AUC: Area under the curve; TCA: Tricarboxylic acid.

reports[7], indicating that choline could be a viable tissue biomarker as-sociated with tumor promotion. However, this biomarker of EC tumor tissue was not completely detectable at the end of metabolism of urine biomarkers in this study.

In addition to specific metabolite differences between tumor tissues and urine in EC patients and HCs, we also evaluated the relationships between the metabolic networks in both tissues and urine. Decreased metabolite levels of glucose, glycine, creatinine and taurine, as well as increased citrate and glutamate in EC tissues, were also detectable in the urine of EC patients. These distinct and overlapping metabolites may reflect tumor cell shedding and represent metabolic pathway aberrations across both matrices. This potentially reveals linkages to disturbances of fatty acid metabolism, glucose and glycolytic activity, TCA cycle and glutaminolysis associated with tumor proliferation. Correlative analysis of metabolic profiling between EC tissues and urine showed that changes in most potential urinary biomarkers were correlated with changes in most biomarker candidates in EC tissues, implying enhanced energy production required for rapid cell proliferation. Creatinine was found to be the most sensitive predictor of EC in urine metabolite, with an AUC of 0.790. Overall, these associations provide evidence of distinct metabolic signatures and pathway disturbances across both matrices, and changes in urinary metabolic signature could reflect the EC tissue microenvironment.

In conclusion, our parallel investigations of EC patients through ^{1}H-NMR metabolomics revealed a great number of altered metabolites and metabolic pathway networks in EC patient urine and tumor tissues compared with HCs. We identified a few overlapping discriminatory metabolites across both matrices, derived from fatty acid metabolism (taurine and glycine), as well as metabolites (*e.g.*, glucose, glutamate, citrate and creatinine) involved in glucose and glycolytic metabolism, the TCA cycle and glutaminolysis. Correlative analysis of metabolic profiling across tumor tissues and urine in EC patients showed that changes in most potential urinary biomarkers were correlated with changes in most candidate biomarkers in EC tissues, implying enhanced energy production required for rapid cell proliferation. In summary, these associations provide clear evidence of different metabolic signatures and metabolic pathway disturbances between EC tissues and urine, and changes in urinary metabolic signatures could reflect the EC tissue microenvironment. Our study highlighted the significance of the distinct urinary metabolic profile as a potential noninvasive indicator of EC detection. Further investigation is needed to validate these initial findings using larger samples and to establish the mechanism underlying EC progression.

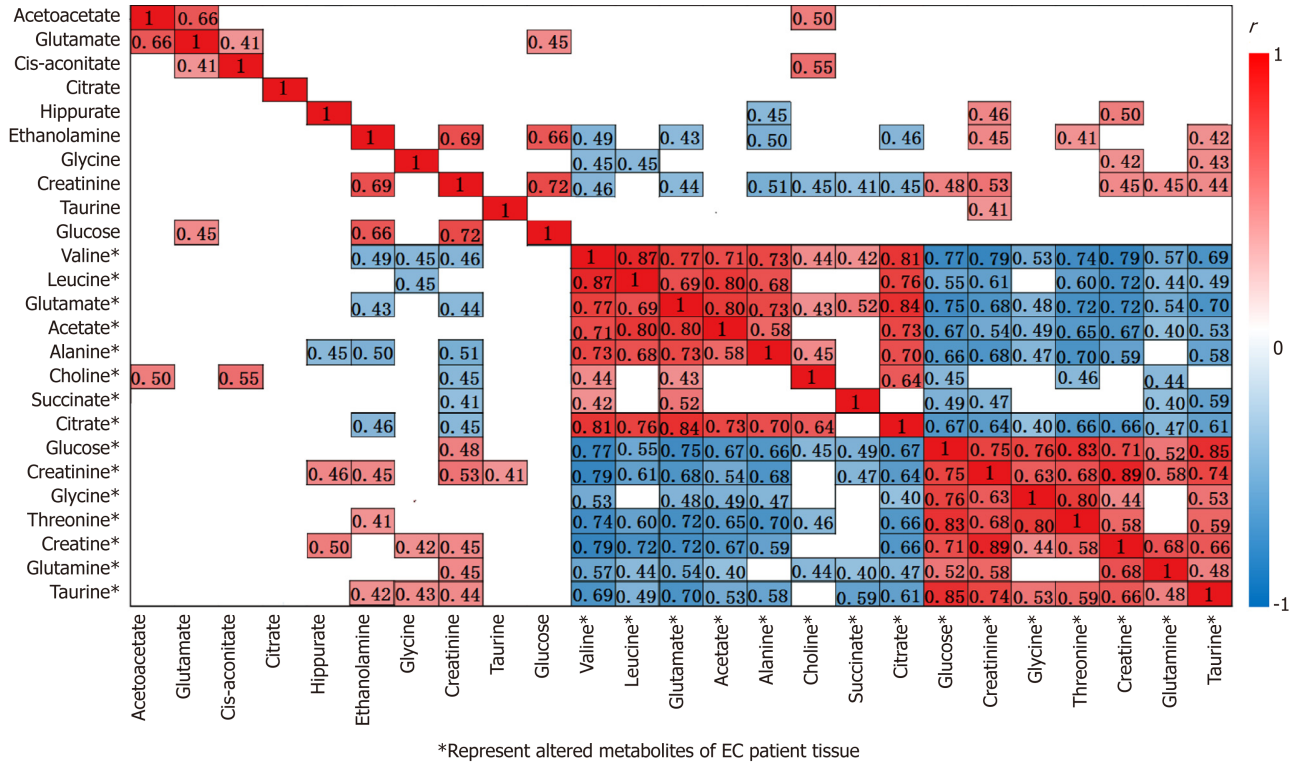


Figure 3 Correlation heat map color-coded by the strength of Spearman correlation coefficients (r) between metabolites found to be important in tumor versus control discrimination. Cut-off values of |r| > 0.4 and $P < 0.05$ have been used. The metabolites used are those listed in Tables 2 and 3 (metabolites labeled with "*" noted as tissue biomarkers). Red boxes indicate positive associations, and blue boxes indicate negative associations.

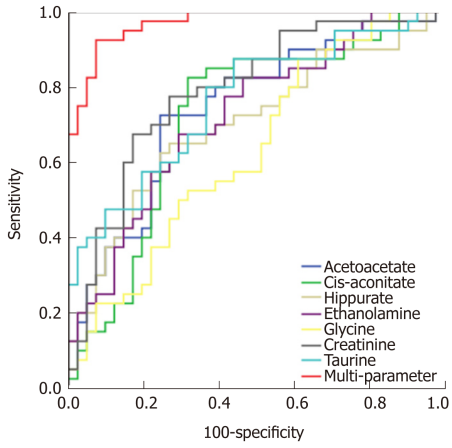


Figure 4 Comparison of single and combined metabolites receiver operating characteristic curves for distinguishing esophageal cancer patients from healthy controls.

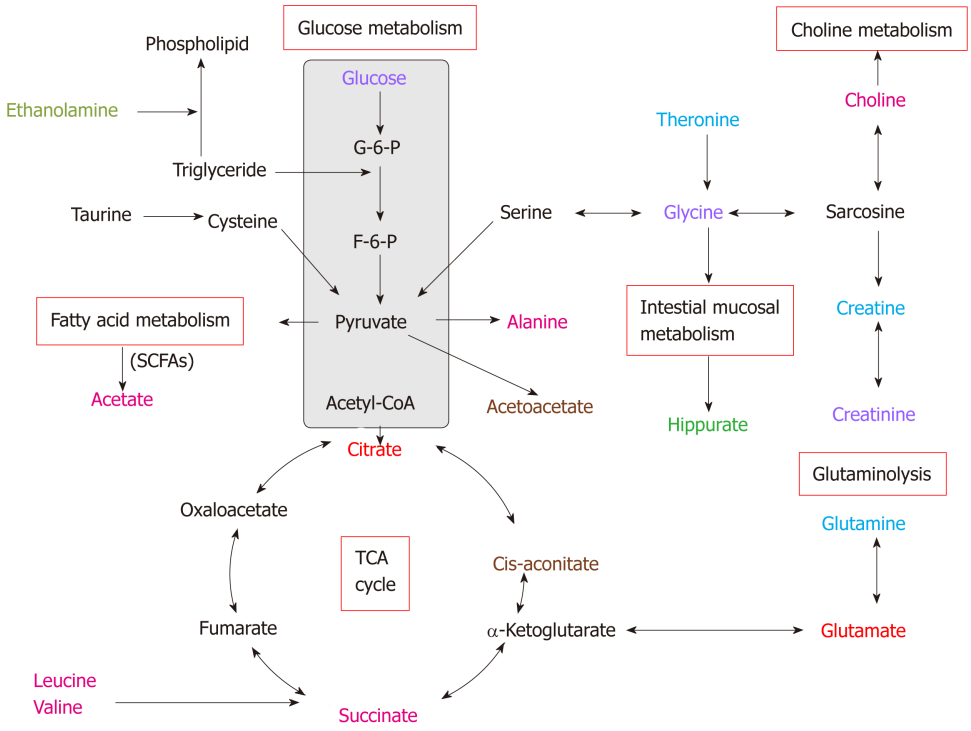


Figure 5 Altered metabolic pathways for the most relevant distinguishing metabolites (potential biomarkers) in urine and tissue samples between esophageal cancer patients and healthy controls. Red text: Increased with respect to control in both esophageal cancer (EC) patient tissue and urine; pink text: Increased with respect to control in EC tissue; orange text: Increased with respect to control in EC patient urine; purple text: Decreased with respect to control in both EC patient tissue and urine; blue text: Decreased with respect to control in EC tissue; green text: Decreased with respect to control in EC patient urine.

ARTICLE HIGHLIGHTS

Research background

A large number of studies have revealed changes of urinary metabolites between esophageal cancer (EC) and healthy controls (HCs), and some studies have demonstrated a correlation between EC and perturbed urinary metabolomic profiles.

Research motivation

However, none of the previous studies has described the correlation between urine metabolite profiles and those of the tumor and adjacent colonic mucosa in the same patient. Our study revealed a significant number of altered metabolites and metabolic pathway networks in EC patient urine and tumor tissues compared with HCs.

Research objectives

Our work is the first parallel investigation of esophageal tumor tissues and adjacent normal mucosal tissues alongside patient-matched urine samples to investigate how urinary metabolic phenotypes were linked to changes in the biochemical landscape of esophageal tumors.

Research methods

All samples were detected by a Bruker AVII 400 MHz nuclear magnetic resonance spectrometer, and all spectral data were applied to pattern recognition analysis and cross-validation by SIMCA-P software. Then, statistical significance was assessed using the Mann-Whitney *U* test and receiver operating characteristic analysis to calculate biomarker metabolites. Finally, we employed Pearson Correlation Analysis to assess the associations of biomarker candidates between urine and tumor tissues of EC patients.

Research results

Our study revealed metabolite changes that overlapped across both metrics, including glucose, glutamate, citrate, glycine, creatinine and taurine, indicating the networks for metabolic pathway perturbations in EC. Additionally, changes in most urinary biomarkers were correlated with changes in biomarker candidates in EC tissues.

Research conclusions

Our research is the first parallel investigation to investigate how urinary metabolic phenotypes were linked to the changes in the biochemical landscape of esophageal tumors. Our study showed significant metabolic alterations in both urine and tumor tissues of EC patients compared to their respective HCs. Our research revealed a few distinct and overlapping discri-

minatory metabolites, suggesting that EC is associated with the following dysregulated metabolic pathway perturbations. Furthermore, the metabolic profiling correlations between esophageal tissues and urine showed that most urine potential biomarkers were correlated with most of the discriminating metabolites in EC tissues, indicating that changes in the urine metabolic signature could reflect reprogramming of metabolic pathways in tumor tissue, highlighting the significance of the distinct urinary metabolic profiles as potential novel and noninvasive indicators for EC detection.

Research perspectives

With experiences in our study, we realized that many metabolites have associations in samples of cancer patients. In our same group, we are now investigating the serum samples of EC patients to see whether the same pattern of serum levels of amino acids can be found in EC patients.

ACKNOWLEDGEMENTS

The authors thank Ju-Rong Yang for kindly providing us with the NMR experimental setting and Dr. Hong-Jun Luo for handling of tissue samples.

REFERENCES

1 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]
2 **Lindon JC**, Nicholson JK, Holmes E, Everett JR. Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. *Magnetic Resonance An Ed J* 2015; **12**: 289-320 [DOI: 10.1002/1099-0534(2000)12:5<289::AID-CMR3>3.0.CO;2-W]
3 **Griffin JL**, Atherton H, Shockcor J, Atzori L. Metabolomics as a tool for cardiac research. *Nat Rev Cardiol* 2011; **8**: 630-643 [PMID: 21931361 DOI: 10.1038/nrcardio.2011.138]
4 **Lin Y**, Stephenson MC, Xin L, Napolitano A, Morris PG. Investigating the metabolic changes due to visual stimulation using functional proton magnetic resonance spectroscopy at 7 T. *J Cereb Blood Flow Metab* 2012; **32**: 1484-1495 [PMID: 22434070 DOI: 10.1038/jcbfm.2012.33]
5 **Duarte IF**, Gil AM. Metabolic signatures of cancer unveiled by NMR spectroscopy of human biofluids. *Prog Nucl Magn Reson Spectrosc* 2012; **62**: 51-74 [PMID: 22364616 DOI: 10.1016/j.pnmrs.2011.11.002]
6 **Lin Y**, Ma C, Liu C, Wang Z, Yang J, Liu X, Shen Z, Wu R. NMR-based fecal metabolomics fingerprinting as predictors of earlier diagnosis in patients with colorectal cancer. *Oncotarget* 2016; **7**: 29454-29464 [PMID: 27107423 DOI: 10.18632/oncotarget.8762]
7 **Wang Z**, Lin Y, Liang J, Huang Y, Ma C, Liu X, Yang J. NMR-based metabolomic techniques identify potential urinary biomarkers for early colorectal cancer detection. *Oncotarget* 2017; **8**: 105819-105831 [PMID: 29285295 DOI: 10.18632/oncotarget.22402]
8 **Lin Y**, Ma C, Bezabeh T, Wang Z, Liang J, Huang Y, Zhao J, Liu X, Ye W, Tang W, Ouyang T, Wu R. 1 H NMR-based metabolomics reveal overlapping discriminatory metabolites and metabolic pathway disturbances between colorectal tumor tissues and fecal samples. *Int J Cancer* 2019 [PMID: 30720869 DOI: 10.1002/ijc.32190]
9 **Nicholson JK**, Lindon JC. Systems biology: Metabonomics. *Nature* 2008; **455**: 1054-1056 [PMID: 18948945 DOI: 10.1038/4551054a]
10 **Rocha CM**, Carrola J, Barros AS, Gil AM, Goodfellow BJ, Carreira IM, Bernardo J, Gomes A, Sousa V, Carvalho L, Duarte IF. Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of blood plasma. *J Proteome Res* 2011; **10**: 4314-4324 [PMID: 21744875 DOI: 10.1021/pr200550p]
11 **Theodorescu D**, Wittke S, Ross MM, Walden M, Conaway M, Just I, Mischak H, Frierson HF. Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis. *Lancet Oncol* 2006; **7**: 230-240 [PMID: 16510332 DOI: 10.1016/S1470-2045(06)70584-8]
12 **M'Koma AE**, Blum DL, Norris JL, Koyama T, Billheimer D, Motley S, Ghiassi M, Ferdowsi N, Bhowmick I, Chang SS, Fowke JH, Caprioli RM, Bhowmick NA. Detection of pre-neoplastic and neoplastic prostate disease by MALDI profiling of urine. *Biochem Biophys Res Commun* 2007; **353**: 829-834 [PMID: 17194448 DOI: 10.1016/j.bbrc.2006.12.111]
13 **Theodorescu D**, Schiffer E, Bauer HW, Douwes F, Eichhorn F, Polley R, Schmidt T, Schöfer W, Zürbig P, Good DM, Coon JJ, Mischak H. Discovery and validation of urinary biomarkers for prostate cancer. *Proteomics Clin Appl* 2008; **2**: 556-570 [PMID: 19759844 DOI: 10.1002/prca.200780082]
14 **Pisitkun T**, Johnstone R, Knepper MA. Discovery of urinary biomarkers. *Mol Cell Proteomics* 2006; **5**: 1760-1771 [PMID: 16837576 DOI: 10.1074/mcp.R600004-MCP200]
15 **Hasim A**, Ma H, Mamtimin B, Abudula A, Niyaz M, Zhang LW, Anwer J, Sheyhidin I. Revealing the metabonomic variation of EC using ^{1}H-NMR spectroscopy and its association with the clinicopathological characteristics. *Mol Biol Rep* 2012; **39**: 8955-8964 [PMID: 22736106 DOI: 10.1007/s11033-012-1764-z]
16 **Davis VW**, Schiller DE, Eurich D, Sawyer MB. Urinary metabolomic signature of esophageal cancer and Barrett's esophagus. *World J Surg Oncol* 2012; **10**: 271 [PMID: 23241138 DOI: 10.1186/1477-7819-10-271]
17 **Wishart DS**, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorndahl T, Perez-Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A. HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res* 2013; **41**: D801-D807 [PMID: 23161693 DOI: 10.1093/nar/gks1065]
18 **Bouatra S**, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, Bjorndahl TC, Krishnamurthy R, Saleem F, Liu P, Dame ZT, Poelzer J, Huynh J, Yallou FS, Psychogios N, Dong E, Bogumil R, Roehring C, Wishart DS. The human urine metabolome. *PLoS One* 2013; **8**: e73076 [PMID: 24023812 DOI: 10.1371/journal.pone.0073076]

19 **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]
20 **Selak MA**, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell* 2005; **7**: 77-85 [PMID: 15652751 DOI: 10.1016/j.ccr.2004.11.022]
21 **Vander Heiden MG**, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324**: 1029-1033 [PMID: 19460998 DOI: 10.1126/science.1160809]
22 **Echeverry G**, Hortin GL, Rai AJ. Introduction to urinalysis: historical perspectives and clinical application. *Methods Mol Biol* 2010; **641**: 1-12 [PMID: 20407938 DOI: 10.1007/978-1-60761-711-2_1]
23 **Phelps TJ**, Suflita JM, Little B. Carbon dioxide corrosion and acetate: a hypothesis on the influence of microorganisms. *Corrosion Houston Tx* 2012; **64**: 854-859 [DOI: 10.5006/1.3279919]
24 **Chen JQ**, Russo J. Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. *Biochim Biophys Acta* 2012; **1826**: 370-384 [PMID: 22750268 DOI: 10.1016/j.bbcan.2012.06.004]
25 **Curthoys NP**, Watford M. Regulation of glutaminase activity and glutamine metabolism. *Annu Rev Nutr* 1995; **15**: 133-159 [PMID: 8527215 DOI: 10.1146/annurev.nu.15.070195.001025]
26 **Cairns RA**, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nature Reviews Cancer* 2011; **11**: 85
27 **Locasale JW**. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat Rev Cancer* 2013; **13**: 572-583 [PMID: 23822983 DOI: 10.1038/nrc3557]
28 **Joseph J**, Cardesa A, Carreras J. Creatine kinase activity and isoenzymes in lung, colon and liver carcinomas. *Br J Cancer* 1997; **76**: 600-605 [PMID: 9303358]
29 **van Stijn MF**, Vermeulen MA, Siroen MP, Wong LN, van den Tol MP, Ligthart-Melis GC, Houdijk AP, van Leeuwen PA. Human taurine metabolism: fluxes and fractional extraction rates of the gut, liver, and kidneys. *Metabolism* 2012; **61**: 1036-1040 [PMID: 22304837 DOI: 10.1016/j.metabol.2011.12.005]
30 **Amelio I**, Cutruzzolá F, Antonov A, Agostini M, Melino G. Serine and glycine metabolism in cancer. *Trends Biochem Sci* 2014; **39**: 191-198 [PMID: 24657017 DOI: 10.1016/j.tibs.2014.02.004]
31 **Nicholson JK**, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. *Nat Rev Microbiol* 2005; **3**: 431-438 [PMID: 15821725 DOI: 10.1038/nrmicro1152]
32 **Pun WK**, Chow SP, Fang D, Cheng CL, Leong JC, Ng C. Post-traumatic oedema of the foot after tibial fracture. *Injury* 1989; **20**: 232-235 [PMID: 2592102 DOI: 10.1586/14737159.2015.1039515]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3231-3241

DOI: 10.3748/wjg.v25.i25.3231

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

ORIGINAL ARTICLE

Retrospective Study

Prevalence and risk factors for Barrett's esophagus in Taiwan

Yan-Hua Chen, Hsien-Chung Yu, Kung-Hung Lin, Huey-Shyan Lin, Ping-I Hsu

ORCID number: Yan-Hua Chen (0000-0002-5241-258X); Hsien-Chung Yu (0000-0002-0261-0940); Kung-Hung Lin (0000-0003-2020-3756); Huey-Shyan Lin (0000-0003-4017-8359); Ping-I Hsu (0000-0003-3905-4674).

Author contributions: All authors helped to perform the research; Chen YH, Yu HC and Hsu PI designed the study and drafted the manuscript; Chen YH and Lin KH collected the data; Chen YH and Lin HS performed statistical analyses; Lin HS and Hsu PI revised the manuscript critically for important intellectual content.

Supported by the In-Hospital Research Project Funding of Kaohsiung Veterans General Hospital, No. VGHKS107-040.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of Kaohsiung Veterans General Hospital (VGHKS17-CT7-07).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.

Yan-Hua Chen, Hsien-Chung Yu, Kung-Hung Lin, Ping-I Hsu, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan

Yan-Hua Chen, Hsien-Chung Yu, Kung-Hung Lin, Health Evaluation Center, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan

Yan-Hua Chen, Kung-Hung Lin, Department of Nursing, Meiho University, Neipu Township, Pingtung County 91202, Taiwan

Hsien-Chung Yu, Institute of Health Care Management, Department of Business Management, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan

Huey-Shyan Lin, Department of Health-Business Administration, Fooyin University, Kaohsiung 83102, Taiwan

Ping-I Hsu, National Yang Ming University, Taipei 12221, Taiwan

Corresponding author: Ping-I Hsu, MD, Attending Doctor, Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, No. 386, Ta-Chung 1st Rd, Zuoying Dist., Kaohsiung 81362, Taiwan.
pihsu@vghks.gov.tw
Telephone: +886-7-3422121
Fax: +886-7-3468237

Abstract

BACKGROUND

Barrett's esophagus (BE) is a pre-malignant condition associated with the development of esophageal adenocarcinoma. The prevalence of BE in the general populations of Asian countries ranges from 0.06% to 1%. However, with lifestyle changes in Asian countries and adoption of western customs, the prevalence of BE might have increased.

AIM

To determine the current prevalence of BE in Taiwan, and to investigate risk factors predicting the presence of BE.

METHODS

This retrospective study was conducted at the Health Evaluation Center of Kaohsiung Veterans General Hospital in Taiwan. Between January 2015 and December 2015, 3385 subjects undergoing routine esophagogastroduodenoscopy examinations as part of a health check-up at the Health Evaluation Center were included. Patient characteristics and endoscopic findings were carefully reviewed. Lesions with endoscopic findings consistent with BE awaiting

Manuscript source: Unsolicited manuscript

Received: January 8, 2019
Peer-review started: January 8, 2019
First decision: January 30, 2019
Revised: February 24, 2019
Accepted: March 1, 2019
Article in press: March 2, 2019
Published online: July 7, 2019

P-Reviewer: Friedel D, Mastracci L, Zhao J
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

histological evaluation were judged as endoscopically suspected esophageal metaplasia (ESEM). BE was defined based on extension of the columnar epithelium ≥ 1 cm above the gastroesophageal junction and was confirmed based on the presence of specialized intestinal metaplasia (IM) in the metaplastic esophageal epithelium. Clinical factors of subjects with BE and subjects without BE were compared, and the risk factors predicting BE were analyzed.

RESULTS

A total of 3385 subjects (mean age, 51.29 ± 11.42 years; 57.1% male) were included in the study, and 89 among them were confirmed to have IM and presence of goblet cells *via* biopsy examination. The majority of these individuals were classified as short segment BE (n = 85). The overall prevalence of BE was 2.6%. Multivariate analysis disclosed that old age [odds ratio (OR) = 1.033; 95% confidence interval (CI): 1.012-1.055; P = 0.002], male gender (OR = 2.106; 95%CI: 1.145-3.872; P = 0.017), ingestion of tea (OR = 1.695; 95%CI: 1.043-2.754; P = 0.033), and presence of hiatal hernia (OR = 3.037; 95%CI: 1.765-5.225; P < 0.001) were significant risk factors predicting BE. The independent risk factor for the presence of IM in ESEM lesions was old age alone (OR = 1.029; 95%CI: 1.006-1.053; P = 0.014).

CONCLUSION

Current prevalence of BE among the general population in Taiwan is 2.6%. Old age, male gender, ingestion of tea and hiatal hernia are significant risk factors for BE.

Key words: Barrett's esophagus; Prevalence; Risk factors; Intestinal metaplasia; Taiwan

Core tip: The current prevalence of Barrett's esophagus (BE), based on the diagnostic criteria of the American College of Gastroenterology, is 2.6% among the general population in Taiwan. Its prevalence in Taiwan is the highest among the general population in Asian countries. Significant risk factors for BE include old age, male gender, ingestion of tea and the presence of hiatal hernia. In clinical practice, more attention should be paid when endoscopically suspected esophageal metaplasia is observed in older individuals, as these lesions have a higher likelihood of bearing intestinal metaplasia.

Citation: Chen YH, Yu HC, Lin KH, Lin HS, Hsu PI. Prevalence and risk factors for Barrett's esophagus in Taiwan. *World J Gastroenterol* 2019; 25(25): 3231-3241
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3231.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3231

INTRODUCTION

Barrett's esophagus (BE) is generally recognized as a pre-malignant condition and is associated with esophageal adenocarcinoma[1]. The American Gastroenterological Association defines BE as any extent of metaplastic columnar epithelium replacing the stratified squamous epithelium that normally lines the distal esophagus. Because intestinal metaplasia (IM) is the only type of esophageal columnar epithelium that clearly predisposes individuals to cancer development, its presence is a requirement for diagnosis[2]. However, the clinical guidelines of the American College of Gastroenterology (ACG) recommend that BE should be diagnosed only when the salmon-colored mucosa extend ≥ 1 cm into the tubular esophagus proximal to the gastroesophageal junction because of high inter-observer variability and the low risk for esophageal adenocarcinoma in cases of segments < 1 cm[3]. Periodic endoscopic surveillance for dysplastic or cancerous lesions is suggested for patients diagnosed with BE, although disagreement exists regarding the long-term survival benefit of such surveillance[4].

Risk factors for BE have been extensively evaluated. White male individuals with gastroesophageal reflux disease (GERD), hiatal hernia, obesity, cigarette smoking, low

birth weight and obstructive sleep apnea are more likely to develop BE[5-11]. Data concerning the role of alcohol intake in the development of BE are inconsistent. Additionally, some previous studies have found that wine consumption is inversely correlated with BE[8,12]. An inverse association between the presence of *Helicobacter pylori* (*H. pylori*) infection and BE has also been reported[13,14]

The prevalence of BE in western countries is between 0.5% and 2% of unselected individuals; in individuals with acid reflux symptoms, the prevalence is higher ranging from 5% to 15%[15]. In Asia, the previously reported prevalence of BE is lower than that in western countries. Tseng *et al*[16] reported that the prevalences of endoscopically suspected esophageal metaplasia (ESEM) and BE between 2003 and 2006 in Taiwan were 0.28% and 0.06%, respectively. Chang *et al*[17] showed that the prevalence of BE among subjects undergoing screening endoscopy in 2007 was 0.35%. However, with lifestyle changes in Asian countries such as increased western food consumption and adoption of western customs, the prevalence of BE might have increased.

The present study was conducted to (1) assess the current prevalence of BE among the general population in Taiwan, and (2) investigate independent risk factors predicting the development of BE in Taiwan.

MATERIALS AND METHODS

Subjects

Between January 2015 and December 2015, all consecutive outpatients who underwent routine esophagogastroduodenoscopy (EGD) examinations as part of a health check-up at their own expense at the Health Evaluation Center of the Kaohsiung Veterans General Hospital, Taiwan, were recruited into the present study. Exclusion criteria were (1) age less than 20 years, (2) refusal to undergo biopsy of suspicious gastrointestinal tract lesions, and (3) history of severe concomitant illness, including decompensated cirrhosis, uremia, and congestive heart failure.

Questionnaire

As a routine practice at the Health Evaluation Center, every subject was instructed to fill out a questionnaire detailing personal history, demographic data including age, gender, medical history, history of smoking, alcohol drinking, and coffee and tea consumption. Self-reported gastrointestinal discomforts including acid reflux symptoms or common upper gastrointestinal symptoms including epigastric pain or dyspepsia were also recorded.

Body mass index and body fat percentage

Personal data including body height, body weight, and body composition were readily accessible during routine physical examinations. They were recorded and transformed into body mass index (BMI) and body fat percentage (BFP) measurements, which were clinically important parameters for describing individuals as obese or overweight. BMI was calculated as weight/height^2 (kg/m^2), while BFP was determined with the bioelectrical impedance analysis method using the "X-Scan Plus II body composition analyzer (Jawon Medical Co., Ltd, Kyoungsan, South Korea)". The criteria of the Health Promotion Administration, Ministry of Health and Welfare of Taiwan, define obesity as (1) A percentage of body fat of ≥ 25 in males or ≥ 30 in females, or (2) BMI ≥ 27. Overweight was defined as a BMI of ≥ 24 and < 27. The participants were then classified into a normal or an obesity group based on BFP, and as normal, overweight, or obese based on BMI.

Study design

Clinical data including personal information from questionnaires, laboratory data, body weight, BMI, BFP, endoscopy reports and pathology report were collected through retrospective chart review. The endoscopes used for examination between January 2015 and August 2015 were GIF-XP260N, GIF-XQ260, GIF-Q260, and GIF-H260Z (Tokyo, Japan). New-generation endoscopes including GIF-H290Z and GIF-HQ290 had been introduced to our department for endoscopic examination since September 2015. All endoscopic examinations during this period were performed by seven experienced endoscopists. Most of the endoscopic procedures were performed under conscious sedation with the administration of sedative agents *via* the intravenous route by anesthesiologists. Among those not receiving conscious sedation, the attributed reasons were to old age, high risk in anesthesia due to underlying medical illness, or personal reasons. If more than one episode of endo-scopic examination was performed in the same individual during the study period, the result of the first endoscopy was used as the index data. Lesions with endoscopic findings consistent with BE awaiting histological evaluation were judged as ESEM[18]. The

presence and extent of ESEM were diagnosed according to the Prague C & M Criteria. The length of ESEM was measured using the circumferential extent (C value) and the maximum extent (M value) above the anatomic gastroesophageal junction (GEJ) in centimeters[19]. The endoscopic landmark for the GEJ was defined as the proximal margin of the gastric folds. When the value of "M" in the Prague C & M criteria was ≥ 3 cm, the lesion was defined as long-segment BE (LSBE); if the value of "M" was < 3 cm, the lesion was classified as short-segment BE (SSBE). It was common practice for us to perform biopsies in all patients with LSBE in a random manner from four quadrants of the lesions, 2 cm-apart, throughout the columnar-lined esophagus per the Seattle protocol. Target biopsy was used for individuals with small tongues of columnar mucosa and for all patients with any suspicious IM and dysplastic lesions under NBI evaluation. All specimens acquired were embedded in paraffin, stained with hematoxylin and eosin and then reviewed by eight experienced general pathologists. BE was defined based on extension of the columnar epithelium ≥ 1 cm above the GEJ and was histologically confirmed by the presence of IM epithelium within the columnar-lined esophagus which contains goblet cells[20].

The present study was approved by the Institutional Review Board of Kaohsiung Veterans General Hospital (VGHKS17-CT7-07).

Statistical analysis

The primary endpoint of the study was the prevalence of BE. To determine the risk factors for BE, clinical and endoscopic parameters were examined using univariate analysis. These parameters included age, gender, history of smoking, history of alcohol consumption, ingestion of coffee, ingestion of tea, coexistence of an underlying disease, presence of diabetes, hypertension, cardiovascular disease, *H. pylori* infection status, BFP, BMI, and endoscopic findings (including hiatal hernia, reflux esophagitis, peptic ulcer, and gastritis). The variables found to be statistically significant in univariate analysis were subsequently assessed using multivariate analysis to identify independent factors predicting BE. Categorical data were compared using the $\chi 2$ test or Fisher's exact test, as appropriate. The Student's *t*-test was used for the comparison of continuous data. SPSS (version 12.0 for Microsoft Windows) was used for all statistical analyses. A P value less than 0.05 was considered statistically significant. The statistical methods of this study were reviewed by Huey-Shyan Lin, the consultant of Research and Development, Department of Health, Kaohsiung City Government, and research consultants of several hospitals, Taiwan.

RESULTS

Characteristics of participating subjects and endoscopic findings

During the study period, a total of 3387 subjects were recruited. The majority of these individuals (68.5%, n = 2321) were physically robust and underwent their health check-up to rule out physical disorders, particularly malignancy. The remaining individuals were either employees (21.9%, n = 741) who were undergoing a regular physical check-up arranged by their employers or those suffering from physical discomforts (9.6%, n = 325). Of these, two who were aged below 20 years were excluded from the study. Thus, 3385 individuals (mean age, 51.29 ± 11.42 years; 57.1% male) were included in further analyses.

A total of 639 subjects were found to have reflux esophagitis, with a prevalence of 18.8%. Among them, males were predominant (n = 519, 81.2%). ESEM was found in 423 (12.5%) individuals, and 89 among them were confirmed to have IM and presence of goblets cells *via* biopsy examination. Therefore, the overall prevalence of BE was 2.6%. The majority of these individuals were classified as SSBE cases (n = 85) whereas the remaining four patients were considered to be LSBE cases. No dysplasia or adenocarcinoma was detected in any of the patients. Concomitant reflux esophagitis was identified in 31 of the 89 BE patients (34.8%).

Risk factors for BE

The baseline characteristics of BE subjects and of individuals without BE were compared and shown in Table 1. The mean age was significantly higher in individuals with BE than in those without BE. Male gender, alcohol consumption, betel nut consumption, cigarette smoking, ingestion of tea, hypertension, history of cardiovascular disease, abnormal waist circumference and high BMI were more common among BE subjects. Endoscopic findings such as hiatal hernia and reflux esophagitis were discovered more frequently in the BE group. Multivariate analysis revealed that old age [odds ratio (OR) = 1.033; 95%confidence interval (CI): 1.012-1.055; P = 0.002],

male gender (OR = 2.106; 95%CI: 1.145-3.872; P = 0.017), ingestion of tea (OR = 1.695; 95%CI: 1.043-2.754; P = 0.033), and presence of hiatal hernia (OR = 3.037; 95%CI: 1.765-5.225; P < 0.001) were significant risk factors predicting BE (Table 2).

Risk factors predicting the presence of IM in the ESEM lesions

Of the 423 individuals with ESEM, IM was detected using histology examination in 89 subjects. The ESEM subjects were further divided into two groups based on the presence of IM and their baseline characteristics were compared (Table 3). Univariate analysis revealed that subjects with IM were more likely to be older in age, of male gender, have abnormal waist circumference, and have history of hypertension or cardiovascular disease. Multivariate analysis showed that old age (OR = 1.029; 95%CI: 1.006-1.053; P = 0.014) was the only significant risk factor predicting the presence of IM (Table 4).

DISCUSSION

The current study showed that the prevalence of reflux esophagitis and BE in subjects undergoing routine health check-up in Taiwan was 18.8% and 2.6%, respectively. The data indicate that the prevalence of BE among the general population in Taiwan is comparable with that in the western countries, ranging from 0.5% to 2%. Our study also demonstrated that old age, male gender, ingestion of tea, and hiatal hernia were the independent risk factors predicting the presence of BE. In the subjects with ESEM, old age was the only independent risk factor associated with the presence of specialized IM.

The Guidelines of the ACG define BE as any change in length of distal esophageal epithelium that can be recognized as columnar type mucosa in endoscopy and confirmed to have intestinal metaplasia *via* biopsy of the tubular esophagus. The updated ACG guideline recommends that biopsy is crucial to confirm the presence of IM, because esophageal or gastric cardia cancer risk in subjects with columnar lined epithelium of the esophagus was significantly elevated in those with IM over those without IM in a population-based cohort study (0.38% per year *vs* 0.07% per year, respectively)[21]. Based on the definition, the prevalence of BE among the general population in Asia has been reported to range from 0.06% to 1%[16,17,22,23]. A previous study in a medical center of Taiwan reported that the prevalence of BE among individuals undergoing routine health check-up was 0.06%[17]. Park *et al*[22] conducted a nationwide study in South Korea and found a 0.84% prevalence of histology-proven BE in individuals undergoing routine health check-up. Peng *et al*[23] reported a 1% prevalence of histology-proven BE among the general population in China. The current study defined BE as ESEM ≥ 1 cm with the presence of biopsy-proven IM, and demonstrated that the prevalence of BE among the general population was 2.6% in Taiwan, indicating that BE is not an uncommon disease in Taiwan currently.

Previously well-discussed risk factors for BE have included older age, male gender and hiatal hernia, consistent with our findings[11,23,24]. Individuals of old age and male gender might have a predisposition for the development of BE based on epidemiological data, but the underlying mechanisms accounting for the associations between the development of BE and the two risk factors need further investigations. Phenomena such as impaired esophageal motility or gastric emptying and decreased lower esophageal sphincter (LES) tone have been observed in many elderly individuals, and the risk of acid-related esophageal mucosal injury might increase subsequently[25]. Further, gender-related differences in physiology and pathophysiology of the alimentary tract might contribute to the preponderance of BE in males. Estrogen has been found to have anti-inflammatory activity, contributing to tissue resistance in females in animal models[26,27]. Recently, Masaka *et al*[26] explored the role of estrogen (E2) in protecting esophageal damage in a chronic rat reflux esophagitis model. In addition, significant male-predominance in esophageal tissue damage due to exogenous nitric oxide (NO) has been found[26]. However, the detailed mechanism of estrogen action in controlling pathogenesis of the GERD spectrum remains unclear.

In the current study, tea ingestion was significantly associated with the development of BE. Such a finding has been rarely reported in previous studies. However, it undoubtedly poses a great impact on our daily clinical practice and care of the patient with BE, especially in Asian countries where the prevalence of tea ingestion is high. Several studies have shown that caffeine from coffee and tea induced or aggravated acid reflux by decreasing LES pressure (LESP)[28,29]. Gudjonsson *et al*[28] conducted a blinded crossover study of 12 healthy subjects to evaluate the effect of coffee and tea upon LES function. LESP was significantly lower after intra-

Table 1 Demographic data and endoscopic features of study groups, *n* (%)

Characteristics	Barrett's esophagus		*P* value
	Yes (*n* = 89)	No (*n* = 3296)	
Age (yr) (mean ± SD)	55.63 ± 10.49	51.18 ± 11.43	< 0.001[a]
Male gender	73 (82)	1859 (56.4)	< 0.001[a]
Smoking	23 (25.8)	576 (17.5)	0.041[a]
Consumption of alcohol	43 (48.3)	1080 (32.8)	0.002[a]
Consumption of betel nuts	5 (5.6)	52 (1.6)	0.016[a]
Ingestion of coffee	23 (25.8)	658 (20)	0.172
Ingestion of tea	26 (29.2)	624 (18.9)	0.015[a]
Presence of hypertension	31 (34.8)	619 (18.8)	< 0.001[a]
Presence of cardiovascular disease	33 (37.1)	742 (22.5)	0.001[a]
Presence of pulmonary disease	3 (3.4)	100 (3.0)	0.752
Presence of diabetes	8 (9)	224 (6.8)	0.419
Reflux symptoms	3 (3.4)	163 (4.9)	0.801
Waist			< 0.001[a]
Normal (< 90 cm for male, < 80 cm for female)	52 (58.4)	2566 (77.9)	
Obese (≥ 90 cm for male, ≥ 80 cm for female)	37 (41.6)	730 (22.1)	
Body fat percentage			0.072
Normal (< 25 cm for male, < 30 cm for female)	50 (56.8)	2163 (66)	
Obese (≥ 25 cm for male, ≥ 30 cm for female)	38 (43.2)	1113 (34)	
Body mass index			0.002[a]
Normal (BMI < 24)	33 (37.1)	1818 (55.2)	
Overweight (24 ≤ BMI < 27)	34 (38.2)	960 (29.1)	
Obese (27 ≤ BMI)	22 (24.7)	518 (15.7)	
H. pylori infection	14 (15.7)	603 (18.3)	0.536
Endoscopic findings			
Reflux esophagitis	31 (34.8)	608 (18.4)	< 0.001[a]
Hiatal hernia	71 (79.8)	1739 (52.8)	< 0.001[a]
Gastritis	68 (76.4)	2263 (68.7)	0.119
Gastric ulcer	45 (50.6)	1345 (40.8)	0.065
Duodenal ulcer	5 (5.6)	218 (6.6)	0.709
Gastric and duodenal ulcer	47 (52.8)	1421 (43.1)	0.069
Inlet patch	8 (9)	167 (5.1)	0.137

[a]*P* < 0.05. BMI: Body mass index; *H. pylori*: *Helicobacter pylori*; SD: Standard deviation.

gastric instillation of regular coffee and tea. The data for lower esophageal pH paralleled those for LESP[28]. Another single-blinded experimental study performed by Lohsiriwat *et al*[29] evaluated the effect of caffeine on LES and esophageal peristaltic contractions in healthy Thai adults. The result indicated that caffeine affected esophageal function, resulting in a decrease in basal LESP and distal esophageal contraction, which is known to promote esophageal reflux[29]. Additionally, tea consumption has been shown to increase gastric acid secretion[30]. Theophylline existing in black tea and green tea was also reported to induce esophageal acid reflux through inhibition of LESP[31]. It is therefore reasonable to expect that tea ingestion might be a risk factor for BE[32-34]. However, only a few studies have examined the relationship of coffee or tea with BE, and their data have been inconsistent. No association between risk of BE and consumption of coffee or tea was found by Sajja *et al*[35]. An Italian study conducted by Filiberti *et al*[36] revealed that tea intake reduced the risk of BE and reflux esophagitis. A double-blind study performed by Pehl *et al*[37] compared the impact of regular and decaffeinated coffee on esophageal acidity in terms of esophageal pH measurements, and reported that the fraction of time for which esophageal pH was less than 4 was reduced in the decaffeinated coffee-consuming group potentially *via* a reduction in esophageal reflux. Chang *et al*[38] have recently studied the effect of reflux-provoking diets on acid reflux in Taiwan, and found that frequent tea consumption increased the risk of asymptomatic erosive

Table 2 Multivariate analysis of risk factors predicting Barrett's esophagus

Clinical factor	Coefficient	Standard error	Odds ratio (95%CI)	*P* value
Age	0.033	0.011	1.033 (1.012-1.055)	0.002
Male gender	0.745	0.311	2.106 (1.145-3.872)	0.017
Tea consumption	0.528	0.248	1.695 (1.043-2.754)	0.033
Hiatal hernia	1.111	0.277	3.037 (1.765-5.225)	< 0.001

CI: Confidence interval.

esophagitis in Taiwanese men. Therefore, with increased acid exposure over the esophageal mucosa, probably by decreasing LES pressure, tea ingestion is still a reasonable risk factor for BE. In the results of the present study, the proportion of subjects with reflux esophagitis was indeed higher in the BE group than in the non-BE group (34.8% *vs* 13.4% respectively, $P < 0.001$, Table 1), although the association was not significant in multivariate analysis.

The importance of IM, which is diagnosed as identification of goblet cells in the columnar-lined esophagus, could be explained based on higher risk of developing adenocarcinoma in such cases compared with cases of columnar metaplasia without goblet cells, as previously reported[21,39]. Of the 423 subjects labeled as ESEM in this study, IM was detected in 89 individuals. The detection rate of IM in metaplastic epithelium was 21% only. Many factors may lead to false negative detection of IM in daily practice. For example, the number of endoscopic biopsies taken may directly affect the yield rate of IM. Harrison *et al*[40]. found that the diagnostic yield of IM was 34.7% when four biopsies were taken, which increased to 67.9% with eight biopsies, and would have reached 100% if more than 16 biopsies were taken[40]. Moreover, the distribution of IM over the columnar-lined esophagus is markedly heterogeneous, which could cause sampling error. Chandrasoma *et al*[41] demonstrated that the prevalence and density of goblet cells between the most proximal and most distal levels were markedly different, and the probability of finding IM was highest when the biopsies were focused in the most proximal area of the columnar-lined esophagus. In this study, we adopted the Seattle protocol with four quadrant biopsies, 2 cm-apart, throughout the columnar-lined esophagus. Additionally, target biopsy was used for individuals with small tongues of columnar mucosa and for all patients with any suspicious IM and dysplastic lesions under NBI evaluation. Although obtaining 4-quadrant biopsy specimens at interval of every 1 cm throughout the columnar-lined esophagus might increase the yield rate of IM, the procedure time, the dose of anesthetic agents and biopsy-related bleeding rate would increase. Our Health Evaluation Center therefore used the Seattle protocol with 4-quadrant biopsies at interval of every 2 cm for ESEM. Furthermore, the esophageal biopsy specimens were interpreted by eight pathologists. Mastracci *et al*[42] revealed that the overall agreement rate of the diagnostic category of "BE with IM" between pathologists is moderate, with a K value of 0.599. This phenomenon might also be one of the confounding factors responsible for the different detection rates between ESEM and BE.

The results of the present study demonstrated that old age significantly increased the likelihood of discovering IM, with a 1.029-fold increase in odds ratio per year of age increase. There are some postulated reasons which might explain this phenomenon. First, the density and surface area of IM might increase over time, due to prolonged gastric acid stimulation[43]. Second, the prevalence of hiatus hernia, which is a risk factor for acid reflux, increases with age[44]. Third, many older individuals, as a result of underlying medical illness and medication, may experience decreases in salivary flow, esophageal motility, gastric emptying, and LES tone[25]. In clinical practice, more attention should be paid when ESEM is observed in older individuals, as these lesions have a higher likelihood of bearing IM.

The present study also had some limitations. First, it was conducted using a retrospective observational method, and was subject to confounding due to other unmeasured variables. Second, in real-world clinical practice, there may exist conditions affecting the detection rate such as poor compliance with standard biopsy protocol or insufficient observation over the E-C junction area.

In conclusion, the current prevalence of BE among the general population in Taiwan is 2.6%. Its prevalence in Taiwan is the highest in Asian countries, and is comparable with that in western countries. Old age, male gender, ingestion of tea and the presence of hiatal hernia are significant risk factors for the development of BE in Taiwan.

Table 3 Univariate analysis of risk factors in relation to presence of intestinal metaplasia in the subjects with columnar lined epithelium of the esophagus, *n* (%)

Characteristics	ESEM		*P* value
	With specialized IM (BE) (*n* = 89)	No specialized IM (*n* = 334)	
Age (yr) (mean ± SD)	55.63 ± 10.49	51.36 ± 11.27	0.001[a]
Male gender	73 (82)	226 (67.6)	0.008[a]
Smoking	23 (25.8)	66 (19.8)	0.211
Consumption of alcohol	43 (48.3)	124 (37.1)	0.055
Consumption of betel nuts	5 (5.6)	6 (1.8)	0.059
Ingestion of coffee	23 (25.8)	90 (26.9)	0.834
Ingestion of tea	26 (29.2)	90 (26.9)	0.67
Presence of hypertension	31 (34.8)	72 (21.6)	0.010[a]
Presence of cardiovascular disease	33 (37.1)	80 (24)	0.013[a]
Presence of pulmonary disease	3 (3.4)	10 (3.0)	0.741
Presence of diabetes	8 (9)	26 (7.8)	0.710
Reflux symptoms	3 (3.4)	20 (6.0)	0.437
Waist			0.007[a]
Normal (< 90 cm for male, < 80 cm for female)	52 (58.4)	244 (73.1)	
Obese (≥ 90 cm for male, ≥ 80 cm for female)	37 (41.6)	90 (26.9)	
Body fat percentage			0.275
Normal (< 25 cm for male, < 30 cm for female)	50 (56.8)	211 (63.2)	
Obese (≥ 25 cm for male, ≥ 30 cm for female)	38 (43.2)	123 (36.8)	
Body mass index			0.121
Normal (BMI < 24)	33 (37.1)	157 (47)	
Overweight (24 ≤ BMI < 27)	34 (38.2)	122 (36.5)	
Obese (27 ≤ BMI)	22 (24.7)	55 (16.5)	
H. pylori infection	14 (15.7)	68 (20.4)	0.326
Endoscopic findings			
Reflux esophagitis	31 (34.8)	112 (33.5)	0.818
Hiatal hernia	71 (79.8)	289 (86.5)	0.112
Gastritis	68 (76.4)	245 (73.4)	0.56
Gastric ulcer	45 (50.6)	143 (42.8)	0.191
Duodenal ulcer	5 (5.6)	25 (7.5)	0.542
Gastric and duodenal ulcer	47 (52.8)	152 (45.5)	0.22
Inlet patch	8 (9)	30 (9)	0.998
Length of ESEM (cm)	1.42 ± 0.84	1.31 ± 0.48	0.243

[a]*P* < 0.05. BE: Barrett's esophagus; BMI: Body mass index; ESEM: Endoscopically suspected esophageal metaplasia; *H. pylori*: *Helicobacter pylori*; IM: Intestinal metaplasia; SD: Standard deviation.

Table 4 Multivariate analysis of risk factors in relation to presence of specialized intestinal metaplasia

Clinical factor	Coefficient	Standard error	Odds ratio (95%CI)	*P* value
Age	0.029	0.012	1.029 (1.006-1.053)	0.014 [a]

[a]*P* < 0.05. CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Barrett's esophagus (BE) is generally recognized as a pre-malignant condition and is associated with the development of esophageal adenocarcinoma. The presence of intestinal metaplasia (IM) is generally required for diagnosis because it is the only type of esophageal columnar epithelium that clearly predisposes individuals to cancer development. The updated guidelines of the

American College of Gastroenterology recommend that BE should be diagnosed when there is extension of salmon-colored mucosa into the tubular esophagus extending ≥ 1 cm proximal to the gastroesophageal junction with biopsy confirmation of IM. The prevalence of BE in the general populations of Asian countries ranges from 0.06% to 1%, which is lower than that in western countries. However, with adoption of western customs and lifestyle changes in Asian countries, the prevalence of BE might have increased.

Research motivation

Currently, there is a lack of universal diagnostic criteria for BE because the definition varies among different countries and is updated as time goes by. Nevertheless, the most updated guidelines from the American College of Gastroenterology provide a pragmatic framework for our daily clinical practice. We wished to update the current prevalence of BE in Taiwan based on these criteria strictly.

Research objectives

To determine the current prevalence of BE in Taiwan, and to investigate risk factors predicting the presence of BE.

Research methods

Subjects undergoing routine esophagogastroduodenoscopy examinations as part of a health check-up at the Health Evaluation Center of Kaohsiung Veterans General Hospital in Taiwan were included. Subjects aged below 20 years or refused biopsy examination were excluded. Endoscopic findings consistent with BE awaiting histological evaluation were judged as endoscopically suspected esophageal metaplasia (ESEM). The diagnosis of BE requires an extension of the columnar epithelium ≥ 1 cm above the gastroesophageal junction and the presence of specialized IM in the metaplastic esophageal epithelium. To determine the risk factors for BE, clinical and endoscopic parameters were examined using univariate analysis. The variables found to be statistically significant in univariate analysis were subsequently assessed using multivariate analysis to identify independent factors predicting BE. Categorical data were compared using the $\chi 2$ test or Fisher's exact test, as appropriate. The Student's *t*-test was used for the comparison of continuous data. A P value less than 0.05 was considered statistically significant.

Research results

A total of 3387 subjects were recruited in the study. Of these, two who were aged below 20 years were excluded from the study. Thus, 3385 individuals (mean age, 51.29 ± 11.42 years; 57.1% male) were included in further analyses. ESEM was found in 423 individuals, and 89 among them were confirmed to have IM and presence of goblet cells *via* biopsy examination. Therefore, the overall prevalence of BE was 2.6%. Factors that were significantly associated with a higher risk for BE *via* multivariate analysis included old age [odds ratio (OR) = 1.033; 95% confidence interval (CI): 1.012-1.055; P = 0.002], male gender (OR = 2.106; 95%CI: 1.145-3.872; P = 0.017), ingestion of tea (OR = 1.695; 95%CI: 1.043-2.754; P = 0.033), and presence of hiatal hernia (OR = 3.037; 95%CI: 1.765-5.225; P < 0.001). Old age alone was the only independent risk factor for the presence of IM in ESEM lesions (OR = 1.029; 95%CI: 1.006-1.053; P = 0.014).

Research conclusions

The current prevalence of BE among the general population in Taiwan is 2.6%. Its prevalence in Taiwan is not only the highest in Asian countries but also comparable with that in western countries. Adoption in western customs and foods might have contributed to this phenomenon substantially. From this study, we confirmed that old age, male gender, and presence of hiatal hernia were solid risk factors for BE. Besides, ingestion of tea, a common habit of Asian people, is also significantly associated with the development of BE in Taiwan. Such a finding has been rarely reported in previous studies. The results of the present study demonstrated that old age significantly increased the likelihood of discovering IM in ESEM lesions, with a 1.029-fold increase in odds ratio per year of age increase. From this point, more attention should be paid when ESEM is observed in older individuals in clinical practice, as these lesions have a higher likelihood of bearing IM.

Research perspectives

As this is a retrospective observational study and was subject to confounding due to other unmeasured variables, the true prevalence of BE might have been underestimated. Well-designed prospective clinical trials are needed to reveal the real prevalence of BE in the future. The exact mechanism responsible for the impact of tea ingestion on the development of BE is not clear. Further studies focusing on this topic are required.

REFERENCES

1 **Sharma P**. Clinical practice. Barrett's esophagus. *N Engl J Med* 2009; **361**: 2548-2556 [PMID: 20032324 DOI: 10.1056/NEJMcp0902173]
2 **American Gastroenterological Association**. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. American Gastroenterological Association medical position statement on the management of Barrett's esophagus. *Gastroenterology* 2011; **140**: 1084-1091 [PMID: 21376940 DOI: 10.1053/j.gastro.2011.01.030]
3 **Shaheen NJ**, Falk GW, Iyer PG, Gerson LB; American College of Gastroenterology. ACG Clinical

Guideline: Diagnosis and Management of Barrett's Esophagus. *Am J Gastroenterol* 2016; **111**: 30-50; quiz 51 [PMID: 26526079 DOI: 10.1038/ajg.2015.322]
4 **ASGE Standards of Practice Committee**. Evans JA, Early DS, Fukami N, Ben-Menachem T, Chandrasekhara V, Chathadi KV, Decker GA, Fanelli RD, Fisher DA, Foley KQ, Hwang JH, Jain R, Jue TL, Khan KM, Lightdale J, Malpas PM, Maple JT, Pasha SF, Saltzman JR, Sharaf RN, Shergill A, Dominitz JA, Cash BD. Standards of Practice Committee of the American Society for Gastrointestinal Endoscopy. The role of endoscopy in Barrett's esophagus and other premalignant conditions of the esophagus. *Gastrointest Endosc* 2012; **76**: 1087-1094 [PMID: 23164510 DOI: 10.1016/j.gie.2012.08.004]
5 **Johansson J**, Håkansson HO, Mellblom L, Kempas A, Johansson KE, Granath F, Nyrén O. Risk factors for Barrett's oesophagus: a population-based approach. *Scand J Gastroenterol* 2007; **42**: 148-156 [PMID: 17327933 DOI: 10.1080/00365520600881037]
6 **Abrams JA**, Fields S, Lightdale CJ, Neugut AI. Racial and ethnic disparities in the prevalence of Barrett's esophagus among patients who undergo upper endoscopy. *Clin Gastroenterol Hepatol* 2008; **6**: 30-34 [PMID: 18063419 DOI: 10.1016/j.cgh.2007.10.006]
7 **Kubo A**, Cook MB, Shaheen NJ, Vaughan TL, Whiteman DC, Murray L, Corley DA. Sex-specific associations between body mass index, waist circumference and the risk of Barrett's oesophagus: a pooled analysis from the international BEACON consortium. *Gut* 2013; **62**: 1684-1691 [PMID: 23355549 DOI: 10.1136/gutjnl-2012-303753]
8 **Steevens J**, Schouten LJ, Driessen AL, Huysentruyt CJ, Keulemans YC, Goldbohm RA, van den Brandt PA. A prospective cohort study on overweight, smoking, alcohol consumption, and risk of Barrett's esophagus. *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 345-358 [PMID: 21173169 DOI: 10.1158/1055-9965.EPI-10-0636]
9 **Forssell L**, Cnattingius S, Bottai M, Edstedt Bonamy AK, Lagergren J, Agréus L, Akre O. Increased risk of Barrett's esophagus among individuals born preterm or small for gestational age. *Clin Gastroenterol Hepatol* 2013; **11**: 790-794 [PMID: 23376800 DOI: 10.1016/j.cgh.2013.01.024]
10 **Leggett CL**, Gorospe EC, Calvin AD, Harmsen WS, Zinsmeister AR, Caples S, Somers VK, Dunagan K, Lutzke L, Wang KK, Iyer PG. Obstructive sleep apnea is a risk factor for Barrett's esophagus. *Clin Gastroenterol Hepatol* 2014; **12**: 583-8.e1 [PMID: 24035775 DOI: 10.1016/j.cgh.2013.08.043]
11 **Shiota S**, Singh S, Anshasi A, El-Serag HB. Prevalence of Barrett's Esophagus in Asian Countries: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* 2015; **13**: 1907-1918 [PMID: 26260107 DOI: 10.1016/j.cgh.2015.07.050]
12 **Kubo A**, Levin TR, Block G, Rumore GJ, Quesenberry CP, Buffler P, Corley DA. Alcohol types and sociodemographic characteristics as risk factors for Barrett's esophagus. *Gastroenterology* 2009; **136**: 806-815 [PMID: 19111726 DOI: 10.1053/j.gastro.2008.11.042]
13 **Corley DA**, Kubo A, Levin TR, Block G, Habel L, Zhao W, Leighton P, Rumore G, Quesenberry C, Buffler P, Parsonnet J. Helicobacter pylori infection and the risk of Barrett's oesophagus: a community-based study. *Gut* 2008; **57**: 727-733 [PMID: 17895354 DOI: 10.1136/gut.2007.132068]
14 **Wang C**, Yuan Y, Hunt RH. Helicobacter pylori infection and Barrett's esophagus: a systematic review and meta-analysis. *Am J Gastroenterol* 2009; **104**: 492-500; quiz 491, 501 [PMID: 19174811 DOI: 10.1038/ajg.2008.37]
15 **Runge TM**, Abrams JA, Shaheen NJ. Epidemiology of Barrett's Esophagus and Esophageal Adenocarcinoma. *Gastroenterol Clin North Am* 2015; **44**: 203-231 [PMID: 26021191 DOI: 10.1016/j.gtc.2015.02.001]
16 **Tseng PH**, Lee YC, Chiu HM, Huang SP, Liao WC, Chen CC, Wang HP, Wu MS, Lin JT. Prevalence and clinical characteristics of Barrett's esophagus in a Chinese general population. *J Clin Gastroenterol* 2008; **42**: 1074-1079 [PMID: 18360296 DOI: 10.1097/MCG.0b013e31809e7126]
17 **Chang CY**, Lee YC, Lee CT, Tu CH, Hwang JC, Chiang H, Tai CM, Chiang TH, Wu MS, Lin JT. The application of Prague C and M criteria in the diagnosis of Barrett's esophagus in an ethnic Chinese population. *Am J Gastroenterol* 2009; **104**: 13-20 [PMID: 19098843 DOI: 10.1038/ajg.2008.43]
18 **Vakil N**, van Zanten SV, Kahrilas P, Dent J, Jones R; Global Consensus Group. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol* 2006; **101**: 1900-1920; quiz 1943 [PMID: 16928254 DOI: 10.1111/j.1572-0241.2006.00630.x]
19 **Sharma P**, Dent J, Armstrong D, Bergman JJ, Gossner L, Hoshihara Y, Jankowski JA, Junghard O, Lundell L, Tytgat GN, Vieth M. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. *Gastroenterology* 2006; **131**: 1392-1399 [PMID: 17101315 DOI: 10.1053/j.gastro.2006.08.032]
20 **Haggitt RC**, Reid BJ, Rabinovitch PS, Rubin CE. Barrett's esophagus. Correlation between mucin histochemistry, flow cytometry, and histologic diagnosis for predicting increased cancer risk. *Am J Pathol* 1988; **131**: 53-61 [PMID: 3354644]
21 **Bhat S**, Coleman HG, Yousef F, Johnston BT, McManus DT, Gavin AT, Murray LJ. Risk of malignant progression in Barrett's esophagus patients: results from a large population-based study. *J Natl Cancer Inst* 2011; **103**: 1049-1057 [PMID: 21680910 DOI: 10.1093/jnci/djr203]
22 **Park JJ**, Kim JW, Kim HJ, Chung MG, Park SM, Baik GH, Nah BK, Nam SY, Seo KS, Ko BS, Jang JY, Kim BG, Kim JW, Choi YS, Joo MK, Kim JI, Cho MY, Kim N, Park SH, Jung HC, Chung IS; H. pylori and GERD Study Group of Korean College of Helicobacter and Upper Gastrointestinal Research. The prevalence of and risk factors for Barrett's esophagus in a Korean population: A nationwide multicenter prospective study. *J Clin Gastroenterol* 2009; **43**: 907-914 [PMID: 19417682 DOI: 10.1097/MCG.0b013e318196bd11]
23 **Peng S**, Cui Y, Xiao YL, Xiong LS, Hu PJ, Li CJ, Chen MH. Prevalence of erosive esophagitis and Barrett's esophagus in the adult Chinese population. *Endoscopy* 2009; **41**: 1011-1017 [PMID: 19967617 DOI: 10.1055/s-0029-1215291]
24 **Kuo CJ**, Lin CH, Liu NJ, Wu RC, Tang JH, Cheng CL. Frequency and risk factors for Barrett's esophagus in Taiwanese patients: a prospective study in a tertiary referral center. *Dig Dis Sci* 2010; **55**: 1337-1343 [PMID: 19557516 DOI: 10.1007/s10620-009-0872-7]
25 **Tack J**, Vantrappen G. The aging oesophagus. *Gut* 1997; **41**: 422-424 [PMID: 9391234 DOI: 10.1136/gut.41.4.422]
26 **Masaka T**, Iijima K, Endo H, Asanuma K, Ara N, Ishiyama F, Asano N, Koike T, Imatani A, Shimosegawa T. Gender differences in oesophageal mucosal injury in a reflux oesophagitis model of rats. *Gut* 2013; **62**: 6-14 [PMID: 22287598 DOI: 10.1136/gutjnl-2011-301389]
27 **Velders M**, Schleipen B, Fritzemeier KH, Zierau O, Diel P. Selective estrogen receptor-β activation

stimulates skeletal muscle growth and regeneration. *FASEB J* 2012; **26**: 1909-1920 [PMID: 22278942 DOI: 10.1096/fj.11-194779]
28 **Gudjonsson H**, McAuliffe TL, Kaye MD. [The effect of coffee and tea upon lower esophageal sphincteric function.]. *Laeknabladid* 1995; **81**: 484-488 [PMID: 20065484]
29 **Lohsiriwat S**, Puengna N, Leelakusolvong S. Effect of caffeine on lower esophageal sphincter pressure in Thai healthy volunteers. *Dis Esophagus* 2006; **19**: 183-188 [PMID: 16722996 DOI: 10.1111/j.1442-2050.2006.00562.x]
30 **Ruggiero P**, Rossi G, Tombola F, Pancotto L, Lauretti L, Del Giudice G, Zoratti M. Red wine and green tea reduce H pylori- or VacA-induced gastritis in a mouse model. *World J Gastroenterol* 2007; **13**: 349-354 [PMID: 17230601 DOI: 10.3748/wjg.v13.i3.349]
31 **Berquist WE**, Rachelefsky GS, Kadden M, Siegel SC, Katz RM, Mickey MR, Ament ME. Effect of theophylline on gastroesophageal reflux in normal adults. *J Allergy Clin Immunol* 1981; **67**: 407-411 [PMID: 7229228 DOI: 10.1016/0091-6749(81)90087-7]
32 **Bhatia SJ**, Reddy DN, Ghoshal UC, Jayanthi V, Abraham P, Choudhuri G, Broor SL, Ahuja V, Augustine P, Balakrishnan V, Bhasin DK, Bhat N, Chacko A, Dadhich S, Dhali GK, Dhawan PS, Dwivedi M, Goenka MK, Koshy A, Kumar A, Misra SP, Mukewar S, Raju EP, Shenoy KT, Singh SP, Sood A, Srinivasan R. Epidemiology and symptom profile of gastroesophageal reflux in the Indian population: report of the Indian Society of Gastroenterology Task Force. *Indian J Gastroenterol* 2011; **30**: 118-127 [PMID: 21792655 DOI: 10.1007/s12664-011-0112-x]
33 **Winters C**, Spurling TJ, Chobanian SJ, Curtis DJ, Esposito RL, Hacker JF, Johnson DA, Cruess DF, Cotelingam JD, Gurney MS. Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. *Gastroenterology* 1987; **92**: 118-124 [PMID: 3781178 DOI: 10.1016/0016-5085(87)90847-X]
34 **Burgess JN**, Payne WS, Andersen HA, Weiland LH, Carlson HC. Barrett esophagus: the columnar-epithelial-lined lower esophagus. *Mayo Clin Proc* 1971; **46**: 728-734 [PMID: 5128394]
35 **Sajja KC**, El-Serag HB, Thrift AP. Coffee or Tea, Hot or Cold, Are Not Associated With Risk of Barrett's Esophagus. *Clin Gastroenterol Hepatol* 2016; **14**: 769-772 [PMID: 26681488 DOI: 10.1016/j.cgh.2015.12.007]
36 **Filiberti RA**, Fontana V, De Ceglie A, Blanchi S, Grossi E, Della Casa D, Lacchin T, De Matthaeis M, Ignomirelli O, Cappiello R, Rosa A, Foti M, Laterza F, D'Onofrio V, Iaquinto G, Conio M. Association between coffee or tea drinking and Barrett's esophagus or esophagitis: an Italian study. *Eur J Clin Nutr* 2017; **71**: 980-986 [PMID: 28488688 DOI: 10.1038/ejcn.2017.64]
37 **Pehl C**, Pfeiffer A, Wendl B, Kaess H. The effect of decaffeination of coffee on gastro-oesophageal reflux in patients with reflux disease. *Aliment Pharmacol Ther* 1997; **11**: 483-486 [PMID: 9218070 DOI: 10.1046/j.1365-2036.1997.00161.x]
38 **Chang CH**, Wu CP, Wang JD, Lee SW, Chang CS, Yeh HZ, Ko CW, Lien HC. Alcohol and tea consumption are associated with asymptomatic erosive esophagitis in Taiwanese men. *PLoS One* 2017; **12**: e0173230 [PMID: 28264069 DOI: 10.1371/journal.pone.0173230]
39 **Bandla S**, Peters JH, Ruff D, Chen SM, Li CY, Song K, Thoms K, Litle VR, Watson T, Chapurin N, Lada M, Pennathur A, Luketich JD, Peterson D, Dulak A, Lin L, Bass A, Beer DG, Godfrey TE, Zhou Z. Comparison of cancer-associated genetic abnormalities in columnar-lined esophagus tissues with and without goblet cells. *Ann Surg* 2014; **260**: 72-80 [PMID: 24509200 DOI: 10.1097/SLA.0000000000000424]
40 **Harrison R**, Perry I, Haddadin W, McDonald S, Bryan R, Abrams K, Sampliner R, Talley NJ, Moayyedi P, Jankowski JA. Detection of intestinal metaplasia in Barrett's esophagus: an observational comparator study suggests the need for a minimum of eight biopsies. *Am J Gastroenterol* 2007; **102**: 1154-1161 [PMID: 17433019 DOI: 10.1111/j.1572-0241.2007.01230.x]
41 **Chandrasoma PT**, Der R, Dalton P, Kobayashi G, Ma Y, Peters J, Demeester T. Distribution and significance of epithelial types in columnar-lined esophagus. *Am J Surg Pathol* 2001; **25**: 1188-1193 [PMID: 11688579 DOI: 10.1097/00000478-200109000-00010]
42 **Mastracci L**, Piol N, Molinaro L, Pitto F, Tinelli C, De Silvestri A, Fiocca R, Grillo F; ABRAM Study Group. Interobserver reproducibility in pathologist interpretation of columnar-lined esophagus. *Virchows Arch* 2016; **468**: 159-167 [PMID: 26563401 DOI: 10.1007/s00428-015-1878-5]
43 **Smout AJ**, Breedijk M, van der Zouw C, Akkermans LM. Physiological gastroesophageal reflux and esophageal motor activity studied with a new system for 24-hour recording and automated analysis. *Dig Dis Sci* 1989; **34**: 372-378 [PMID: 2920643 DOI: 10.1007/bf01536258]
44 **Roman S**, Kahrilas PJ. The diagnosis and management of hiatus hernia. *BMJ* 2014; **349**: g6154 [PMID: 25341679 DOI: 10.1136/bmj.g6154]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3242-3255

DOI: 10.3748/wjg.v25.i25.3242

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

ORIGINAL ARTICLE

Retrospective Study

Gut microbiota contributes to the distinction between two traditional Chinese medicine syndromes of ulcerative colitis

Ya-Li Zhang, Li-Ting Cai, Jun-Yi Qi, Yun-Zheng Lin, Yan-Cheng Dai, Na Jiao, You-Lan Chen, Lie Zheng, Bei-Bei Wang, Li-Xin Zhu, Zhi-Peng Tang, Rui-Xin Zhu

ORCID number: Ya-Li Zhang (0000-0002-3538-9832); Li-Ting Cai (0000-0001-6173-7143); Jun-Yi Qi (0000-0001-8294-9268); Yun-Zheng Lin (0000-0002-0378-503X); Dai-Yan Cheng (0000-0001-9919-4033); Na Jiao (0000-0003-3976-6313); You-Lan Chen (0000-0002-4304-5693); Lie Zheng (0000-0002-8243-3736); Bei-Bei Wang (0000-0002-8864-5174); Li-Xin Zhu (0000-0001-7904-1769); Zhi-Peng Tang (0000-0001-5695-8072); Rui-Xin Zhu (0000-0002-5070-6453).

Author contributions: Zhang YL and Cai LT contributed equally to this work, and both performed the majority of research; Qi JY, Lin YZ, Dai YC, Jiao N, Chen YL, Zheng L, and Wang BB performed the research and analyzed the data; Zhu LX, Zhu RX, and Tang ZP designed and coordinated the research; Zhang YL and Cai LT wrote and revised the paper.

Supported by the National Natural Science Foundation of China, No. 81704009, No. 81873253, No. 81573892, and No. 81770571; and the Project of Shanghai Municipal Health and Family Planning Commission, No. 201640122.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of LongHua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and

Ya-Li Zhang, Jun-Yi Qi, You-Lan Chen, Zhi-Peng Tang, Institute of Digestive Diseases, LongHua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Li-Ting Cai, Yun-Zheng Lin, Na Jiao, Bei-Bei Wang, Rui-Xin Zhu, Department of Bioinformatics, School of Life Sciences and Technology, Tongji University, Shanghai 200092, China

Yan-Cheng Dai, Department of Gastroenterology, Shanghai Traditional Chinese Medicine-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200082, China

Lie Zheng, Department of Gastroenterology, Traditional Chinese Medicine Hospital of Shanxi Province, Xi'an 710000, Shanxi Province, China

Li-Xin Zhu, Genome, Environment and Microbiome Community of Excellence, the State University of New York at Buffalo, Buffalo, NY 14214, United States

Corresponding author: Zhi-Peng Tang, PhD, Doctor, Professor, Institute of Digestive Diseases, LongHua Hospital, Shanghai University of Traditional Chinese Medicine, 725 Wanping Road, Shanghai 200032, China. zhipengtang@sohu.com
Telephone: +86-21-6438-5700

Abstract

BACKGROUND

Ulcerative colitis (UC) is considered to be closely associated with alteration of intestinal microorganisms. According to the traditional Chinese medicine (TCM) theory, UC can be divided into two disease syndromes called Pi-Xu-Shi-Yun (PXSY) and Da-Chang-Shi-Re (DCSR). The relationships among gut microbiota, TCM syndromes, and UC pathogenesis have not been well investigated.

AIM

To investigate the role of gut microbiota in UC and the distinction of microbiota dysbiosis between PXSY and DCSR syndromes.

METHODS

From May 2015 to February 2016, UC patients presenting to LongHua Hospital who met the established inclusion and exclusion criteria were enrolled in this retrospective study. Fresh stool specimens of UC patients with PXSY or DCSR were collected. The feces of the control group came from the health examination population of Longhua Hospital. The composition of gut bacterial communities in stool samples was determined by the pyrosequencing of 16S ribosomal RNA.

medical data collection prior to study enrolment.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study

Manuscript source: Unsolicited manuscript

Received: April 12, 2019
Peer-review started: April 15, 2019
First decision: May 16, 2019
Revised: June 2, 2019
Accepted: June 8, 2019
Article in press: June 9, 2019
Published online: July 7, 2019

P-Reviewer: Day AS, Madnani MA, Serban ED, Tsujikawa T, Vasudevan A
S-Editor: Yan JP
L-Editor: Wang TQ
E-Editor: Ma YJ

The high-throughput sequencing reads were processed with QIIME, and biological functions were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

RESULTS

The composition of gut bacterial communities in 93 stool samples (30 healthy controls, 32 patients with PXSY syndrome, and 31 patients with DCSR syndrome) was determined by the pyrosequencing of 16S ribosomal RNA. Beta diversity showed that the composition of the microbiota was different among the three groups. At the family level, Porphyromonadaceae, Rikeneliaceae, and Lachnospiraceae significantly decreased while Enterococcus, Streptococcus, and other potential pathogens significantly increased in UC patients compared to healthy subjects. At the genus level, *Parabacteroides*, *Dorea*, and *Ruminococcus* decreased while *Faeca-libacterium* showed increased abundance in UC compared to healthy controls. Five differential taxa were identified between PXSY and DCSR syndromes. At the genus level, a significantly increased abundance of *Streptococcus* was observed in DCSR patients, while *Lachnoclostridium* increased in PXSY patients. The differential functional pathways of the gut microbiome between the PXSY and DCSR groups mainly included lipid metabolism, immunity, and the metabolism of polypeptides.

CONCLUSION

Our study suggests that the gut microbiota contributes to the distinction between the two TCM syndromes of UC.

Key words: Ulcerative colitis; Intestinal microbiota; Pi-Xu-Shi-Yun syndrome; Da-Chang-Shi-Re syndrome; Traditional Chinese medicine

Core tip: Ulcerative colitis (UC) is considered to be closely associated with alteration of intestinal microorganisms. According to the traditional Chinese medicine (TCM) theory, UC can be divided into Pi-Xu-Shi-Yun syndrome (syndrome of spleen deficiency and dampness, PXSY) and Da-Chang-Shi-Re syndrome (syndrome of dampness-heat in the large intestine, DCSR). This study showed that the gut microbiota was different between patients with PXSY syndrome and those with DCSR syndrome. The genus *Streptococcus* was significantly more abundant in DCSR patients than in PXSY patients, while *Lachnoclostridium* increased in PXSY patients. Our study suggests that the gut microbiota contributes to the distinction between the two TCM syndromes of UC.

Citation: Zhang YL, Cai LT, Qi JY, Lin YZ, Dai YC, Jiao N, Chen YL, Zheng L, Wang BB, Zhu LX, Tang ZP, Zhu RX. Gut microbiota contributes to the distinction between two traditional Chinese medicine syndromes of ulcerative colitis. *World J Gastroenterol* 2019; 25(25): 3242-3255
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3242.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3242

INTRODUCTION

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD). According to relevant epidemiological studies, the incidence of UC is high in Western developed countries, particularly in North America (19.2 per 100000 person-years) and Europe (24.3 per 100000 person-years)[1]. The incidence of UC has been increasing in Asia, especially in China, in recent years[2-4]. The high incidence of UC in China is particularly concentrated in coastal areas, such as Guangzhou (2.22 per 100000 person-years) and Hong Kong (1.66 per 100000 person-years)[5,6]. Notably, UC patients have a higher incidence of colorectal cancer than healthy individuals[7,8]. Moreover, UC is often difficult to treat and is accompanied by a high recurrence rate. Since di-fferent individuals have different manifestations of UC, more accurate diagnosis and treatment of UC for every patient have been emphasised, and precision medicine for

UC is needed.

According to the traditional Chinese medicine (TCM) theory, the same disease can be divided into different syndromes, and different treatments would be used clinically depending on the different syndromes. For instance, UC can be divided into Pi-Xu-Shi-Yun syndrome (syndrome of spleen deficiency and dampness, PXSY) and Da-Chang-Shi-Re syndrome (syndrome of dampness-heat in the large intestine, DCSR)[9]. Shi-Re syndrome is caused by dysfunction of the Pi ("spleen") and Wei ("stomach"), and Pi-Xu syndrome has been shown to be involved in dysfunction of the vegetative nervous system of the gastrointestinal tract and immune pathways[10]. PXSY syndrome is a deficiency syndrome, while the DCSR syndrome is a sthenia syndrome. Studies have proven that TCM works for treating UC patients[11-13] and that TCM drugs are closely associated with immunity[14,15], such as reducing pro-inflammatory cytokines[16]. Nevertheless, the essential molecular mechanisms of TCM remain unknown.

Although the pathogenesis of UC is not yet clear, most studies discuss that the dysbiosis of commensal bacterial populations is a major factor in disease pathogenesis, which interacts with genetic susceptibility and could lead to dysregulation of the immune response to bacterial antigens in IBD[17,18]. Round *et al*[19] suggested that dysregulation of the gut microbiota might be the cause of UC. The microbiota in the gut affects the health of the host by interacting with genes and the environment. Some studies[20-22] found that the biodiversity of intestinal bacteria in patients with UC is significantly reduced and that the increase in destructive microbiota in the gut leads to disorders in the intestinal environment. Guo *et al*[23] showed that the Chinese medicine "Red Ginseng" was effective in relieving symptoms of UC and could promote the growth of probiotic bacteria *in vitro*. However, the relationships among gut microbiota, TCM syndromes, and UC patho-genesis are unclear.

Because the majority of studies have focused on the relationship between intestinal microbiota and the onset of UC and the contribution of gut microbiota in these two distinct TCM syndromes is still unclarified, this study compared the difference in microbial composition and function between PXSY and DCSR syndromes to determine the molecular mechanism of TCM in UC by investigation of the gut microbiota.

MATERIALS AND METHODS

Patient recruitment

This study was approved by the LongHua Hospital, Shanghai University of Traditional Chinese Medicine. Three groups of adults were recruited in this study (PXSY patients, DCSR patients, and healthy controls). Volunteers under investigation in the hospital were approached if the doctors deemed that they had either UC or a normal colon at colonoscopy. Two types of TCM syndromes of UC, DCSR syndrome and PXSY syndrome, were identified according to the "dysentery" diagnosis standard of UC.

The main clinical manifestations of UC are diarrhoea, mucous pus, and blood accompanied by abdominal pain, tenesmus and back weight, and systemic symptoms of varying degrees. The course of disease is more than 4-6 wk. Mucous purulent blood is the most common symptom of UC. Currently, there is still a lack of the gold standard for UC diagnosis, which is mainly based on the combination of clinical manifestations and endoscopic, pathological, and histological findings as well as the exclusion of infectious and non-infectious colitis. We can distinguish these two syndromes according to patients' clinical manifestations, tongue coating, and pulse condition. Clinical manifestations of DCSR syndrome were abdominal pain, tenesmus, stools containing blood and white mucus, a burning sensation around the anus, and scanty, dark-yellow urine. The tongue coating was greasy and slightly yellow. Pulse was slippery and rapid. While clinical manifestations of PXSY syn-drome were recurrent loose stools and increased frequency of bowel movements, especially after eating greasy food, poor appetite, gastric or abdominal distension, a sallow complexion, and lassitude. The tongue coating was pale and thin. The pulse was weak and thready[24,25].

The exclusion criteria for UC patients were: (1) Below the age of 18 or above the age of 60 years; (2) Use of microecological preparations or antibiotics within the previous two weeks; (3) Previous gastrointestinal surgery; (4) Severe hypertension, diabetes, coronary heart disease, or other internal diseases; (5) Pregnancy, lactation, or planned pregnancy; (6) Uncooperativeness; and (7) Other autoimmune diseases.

DNA extraction and PCR amplification

Faecal samples were collected from volunteers and stored at -80 °C before DNA isolation. After the DNA was extracted, the integrity of the sampled bacterial genome was examined using agar gel electrophoresis. The V4 hyper-variable region was PCR-amplified with forward primer 515F (5′-GTGCCAGCCMGCCGCGG-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Unique barcodes were attached to the primers to allow multiplex deep sequencing. The 25-μL reaction system contained 2 μL DNA extract, 0.4 μL forward primer, 0.4 μL reverse primer, 0.2 μL Taq DNA, and 2.5 μL 10 × buffer. PCR cycling parameters were initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR product was electrophoresed on a 2.0% agarose gel for 30 min at 120 V, followed by staining with ethidium bromide (0.5 μg/mL) for 20 min, and the brightness of the image strip was observed.

Processing sequencing samples

Trimmomatic[26] (version 0.36) and QIIME[27] software (version 1.8.1) were applied for sequence processing and analysis. In this study, reads with a length less than 200 bp were excluded from further processing and analysis. All the joined sequences were identified as chimaeras with USEARCH[28], and chimaeric sequences were also filtered. Operational taxonomic units (OTUs) were grouped by a *de novo* strategy against the Greengenes database with an identity > 97%. Microbiota taxonomy was assigned by RDP classifier. In each group, OTUs with reads of more than half the sample size of that group were kept. Good's coverage index was used to detect the sequencing depth. The Chao1 index was calculated to evaluate the microbial diversity. Principal coordinate analysis (PCoA) was performed to determine the de-gree of dissimilarity among groups using the unweighted UniFrac method.

Prediction and differential analysis of functions

The microbial 16S rRNA sequencing data were used to make OTU tables *via* the closed reference OTU picking method. The microbial community metagenome prediction was done with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[29]. OTU tables were normalized by 16S rRNA copy number, followed by metagenome predictions against Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology and compilation into KEGG pathways. Finally, linear discriminant analysis effect size (LEfSe)[30] on galaxy online (http://huttenhower.sph.harvard.edu/ galaxy)[31] was carried out to detect inner function differentials among groups. Linear discriminant analysis scores larger than 2 and *P*-values from the Kruskal-Wallis test less than 0.05 were considered significant.

Statistical methods

Since the distribution of each clinical characteristic followed a normal distribution except of age, the *P*-value of each clinical characteristic was calculated by the chi-square test, while age was estimated by the rank sum test. A Kruskal-Wallis test with post hoc Dunn's test was performed to compare relative abundances of taxa and microbial diversity among the three groups. To examine significant differences between the UC and control groups, a Wilcoxon rank-sum test with two-tailed distribution was performed. Differential taxa were identified with adjusted *P*-values < 0.05 (adjusted by the Bonferroni correction) for multiple comparisons, and a false discovery rate < 0.05 was considered significantly different.

RESULTS

Human subjects

Clinical characteristics of the 93 human subjects are shown in Table 1. Of these subjects, 63 were diagnosed with UC based on colonoscopy at LongHua Hospital, Shanghai University of Traditional Chinese Medicine. Among these volunteers, 31 UC patients were diagnosed with DCSR syndrome, 32 UC patients were diagnosed with PXSY syndrome, and 30 individuals had no specific digestive system diseases and were recruited as healthy controls. All volunteers provided informed consent before participating in the experiment. Among all the individuals, there was no significant difference in any clinical factor, such as gender, age, or inflammation location (P > 0.05) (Table 1). PCoA plots in Appendix Figure suggests that gender and age had no obvious influence on sample clustering.

Characterization of microbiota ecological diversities

Table 1 Characteristics of patients and healthy subjects

	HC	DCSR	PXSY
Gender			
Male	15	15	17
Female	15	16	15
Age (mean ± SD), yr	43.13 ± 10.98	44.16 ± 11.49	41.94 ± 13.42
Duration of disease			
< 1 yr		4	4
1-3 yr		11	13
3-5 yr		12	11
> 5 yr		4	4
Inflammation location			
Rectum		5	5
Left colon		21	22
Whole colon		5	5
Disease activity			
Clinical remission		7	9
Mild activity		9	9
Moderate activity		7	7
Severe activity		8	7

HC: Healthy controls; DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome. There were no significant differences among the three groups in gender, age, duration of disease, inflammation location, or disease activity ($P > 0.05$).

Gut microbiomes of 93 samples (Table 1) were analysed by 16S rRNA pyrosequencing, and a total of 3494198 sequences were obtained after quality control. Good's coverage estimator was determined to measure the sequencing depth of each sample. The alpha rarefaction curve (Figure 1) of each sample reached at the platform extracted approximately 2700 sequences per sample, and Good's coverage estimator of each group approached 100% (>98.9%), indicating that the sequencing depth was sufficient to reflect the majority of bacterial sequences in all samples.

The beta diversities of the healthy control, PXSY syndrome, and DCSR syndrome cohorts were assessed by UniFrac analysis. The PCoA plot (Figure 2) showed that the majority of samples were clustered by health status but not by age or gender (Appendix Figure), reflecting that an association between health status and gut microbiomes existed. Moreover, the UC patients were clustered into two groups according to the two TMC syndromes, PXSY and DCSR, which indicated that the gut microbiota may contribute to distinguishing these two syndromes.

Differences in microbiomes between healthy controls and patients with UC

Non-parametric statistical testing methods were used to analyse the differential microbes between UC patients and healthy subjects. The microbes with an average abundance greater than 1% in any of the groups (Table 2) are as follows:

At the family level, the relative abundance of Paraprevotellaceae, Porphyromonadaceae, Rikenellaceae, and Lachnospiraceae decreased in UC patients compared to healthy individuals, while Enterococcaceae and Streptococcaceae were significantly abundant in UC patients. At the genus level, the abundance of *Parabacteroides*, *Alistipes*, *Ruminococcus*, *Phascolarctobacterium*, *Dorea*, *Sutterella*, and an uncultured genus belonging to the family Lachnospiraceae decreased in the UC group compared to healthy controls. On the other hand, the genera *Enterococcus*, *Streptococcus*, and *Faecalibacterium* were more significantly abundant in UC patients than in the healthy cohort. Notably, the families Porphyromonadaceae and Lachnospiraceae exhibited a similar depleted trend in UC patients, as some studies have previously reported[32-34]. It has also been reported that the genera *Enterococcus* and *Streptococcus* were differentially abundant in UC, while *Ruminococcus* was also significant in the healthy cohort[24,35].

Differential microbiome analysis of the two UC syndromes

Intestinal microbiomes that were significantly different between PXSY and DCSR as determined by the Kruskal-Wallis test are highlighted in Table 2. Bacteroides and

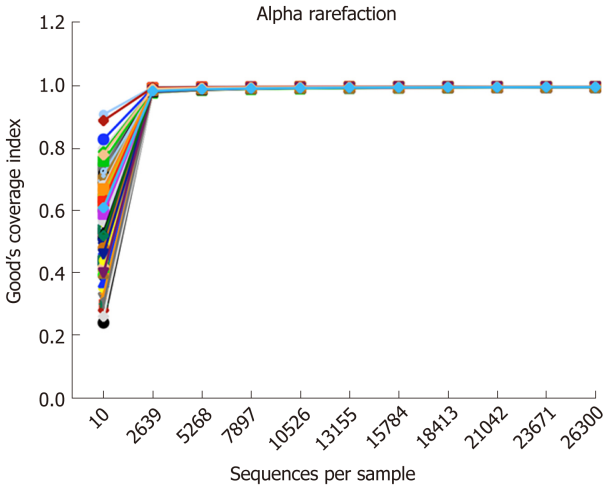


Figure 1 Rarefaction curves of all samples. The rarefaction curve of each sample reached a plateau when approximately 2700 sequences per sample were extracted, and Good's coverage estimator for each group approached 100% (> 98.9%).

Firmicutes were the dominant phyla in these samples, and differed significantly between the PXSY and DCSR groups. Firmicutes was significantly abundant in the DCSR cohort, while Bacteroides was differentially abundant in the PXSY cohort. The differential taxa at the family and genus levels are shown in Figure 3. Within the phylum Firmicutes, the family Streptococcaceae was significantly different between DCSR syndrome and PXSY syndrome patients. At the genus level, *Streptococcus* and *Lachnoclostridium* were significantly different between DCSR and PXSY patients. Known as a lactate producer, Streptococcus was more abundant in DCSR patients than in PXSY patients and healthy controls, indicating that DCSR patients might have more lactate in the gut than PXSY patients and controls. *Lachnoclostridium* is more abundant in PXSY patients than in DCSR patients and healthy controls. The species *Clostridium citroniae*, which belongs to the genus *Lachnoclostridium*, also exhibited the same tendency.

Lactate producers are abundant in DCSR syndrome patients

The lactate producer Streptococcus was increased in the DCSR group, which led us to explore the lactate-producing taxa in PXSY and DCSR syndromes. The relative abundance of the main lactate producers in the gut is shown in Figure 4. All of the main lactate producers showed a trend of increased relative abundance. Compared to healthy controls and PXSY patients, DCSR syndrome patients had significantly abundant lactic acid bacteria, suggesting that more lactic acid might accumulate in the guts of DCSR patients than in the guts of PXSY patients.

Differential functions between PXSY and DCSR

The gut microbiome composition of all groups was determined based on 16S rRNA gene sequencing data. KEGG pathways were categorized using PICRUSt, and ten differential functions were identified with LEfSe. The functional pathways of gut microbiomes between the PXSY and DCSR groups are shown in Figure 5. After differential function analysis, the functions related to liquid metabolism, such as synthesis and degradation of ketone bodies and fatty acid metabolism, were more significant in DCSR patients. Moreover, the RIG-I-like receptor (RLR) signalling pathway and benzoate degradation were also more abundant in DCSR patients. In the PXSY cohort, the genetic information processing functions of protein processing in the endoplasmic reticulum and the carbohydrate metabolism (amino sugar and nucleotide sugar metabolism) were abundant. In addition, nitrogen metabolism and prenyltransferase functions were abundant in the PXSY cohort.

DISCUSSION

The gut microbiota is closely associated with the host, and its dysregulation could lead to diseases and inflammation[36-39], such as IBD. Scaldaferri *et al*[40] found that the most severe inflammation was concentrated in the gut of IBD patients at the site with the largest number of microbes, suggesting that changes in the gut microbiota are

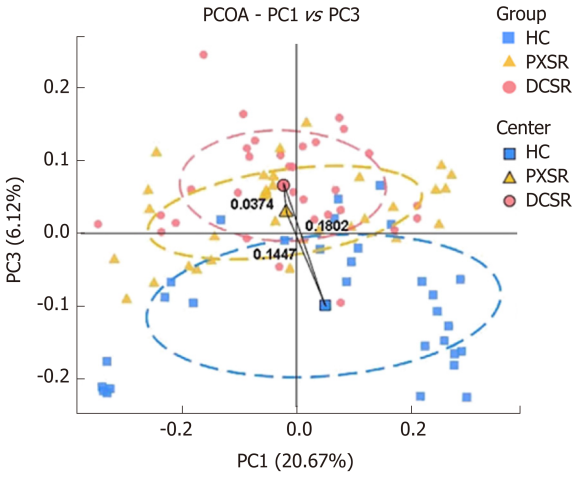


Figure 2 Principal coordinate analysis plot of the community structure in healthy controls, patients with Pi-Xu-Shi-Yun syndrome, and those with Da-Chang-Shi-Re syndrome. The majority of samples clustered by health status at the PC1 *vs* PC3 plot, indicating that health status was a major effect factor for the phylogenetic composition of these samples. Exceptions from all the three study groups were observed, reflecting the effect of other genetic and environmental factors on these microbiomes. PCOA: Principal coordinate analysis; HC: Healthy controls; DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome.

closely related to intestinal inflammation and that the microbiota affects the host through the immune system[41]. This study is pilot research about the contribution of the gut microbiota to the distinction between the two TCM syndromes of UC and the corresponding molecular mechanism.

According to the TCM theory, PXSY syndrome is a deficiency syndrome, while DCSR syndrome is a sthenia syndrome. Clinical manifestations of the DCSR syndrome focus on the empirical symptoms of intestinal inflammation and injury, while the clinical manifestations of PXSY syndrome focus on the deficiency symptoms of weakened digestion and absorption function. The main clinical symptoms of DCSR syndrome are abdominal pain, diarrhoea, mucous purulent stool, yellowish fur, and thready and slippery pulse. Some of these patients can also show symptoms such as burning pain in the anus, tenesmus, body heat, short red urine, dry or bitter mouth, and ozostomia. The main clinical manifestations in patients with PXSY syndrome include thin sloppy stool, stool mucus that is white rather than red, white frozen, pink tongue, tooth mark around the tongue, and white and greasy tongue coating. Some of these patients may have the symptoms such as dull abdominal pain, abdominal fullness and distention, poor appetite, tiredness, thready and weak pulse, and thready and slippery pulse.

The microbiota in the guts of the healthy cohort and UC patients was investigated in this study, which showed that the richness and diversity of the gut microbiota were different between patients and healthy controls. We found that the intestinal microflora was different between the PXSY and DCSR groups, which is essential for distinguishing the two TCM syndromes of UC and potentially contributes to personalized medicine.

Microbiota and the pathology of DCSR syndrome

Using comparisons among PXSY patients, DCSR patients, and healthy subjects, our study illustrated that the genus *Streptococcus* significantly increased in UC patients, especially in patients with DCSR syndrome. The excessive growth of *Streptococcus* in DCSR syndrome patients might indicate the pathological mechanisms of DCSR syndrome.

Increased Streptococcus in DCSR syndrome

Dysbiosis of gut microbiota stimulates the inflammatory response of the host and even induces serious complications[42-44]. The RLR signalling pathway is associated with inflammation[45-47]. Here, *Streptococcus*, together with other pathogens in the gut, could stimulate RLRs and produce pro-inflammatory cytokines such as TNF-α *via* the RLR signalling pathway to promote inflammation (Figure 6). However, the continuous stimulation of the RLR signalling pathway results in an excessive inflammatory response. This excessive inflammatory response leaves the intestinal barrier function impaired, and then more microorganisms in the intestinal lumen will pass through an already incomplete epithelial barrier. Finally, these microbiotas will cause

Table 2 Abundant taxa in healthy subjects and patients

	HC	UC	PXSY	DCSR	UC-H FDR	P-H P^1	D-H P^1	D-P P^1
Bacteroidetes	47.02	41.3	49.09	33.26	0.25	1.00	0.03	0.01
Paraprevotellaceae	1.04	0.18	0.35	0.00	0.02	0.07	0.02	1.00
Porphyromonadaceae	4.8	1.86	2.55	1.15	0.01	0.04	0.01	1.00
Parabacteroides	4.79	1.83	2.55	1.09	0.01	0.05	0.00	1.00
Rikenellaceae	1.66	0.71	1.15	0.25	0.04	0.11	0.04	1.00
Alistipes	1.53	0.59	0.94	0.24	0.03	0.06	0.03	1.00
Firmicutes	43.74	48.23	41.1	55.59	0.38	1.00	0.06	0.01
Enterococcaceae	0.03	0.68	0.31	1.05	0.01	0.02	0.03	1.00
Enterococcus	0.03	0.68	0.31	1.05	0.02	0.02	0.03	1.00
Streptococcaceae	0.7	2.57	1.25	3.95	0.01	0.79	0.00	0.01
Streptococcus	0.67	2.54	1.23	3.89	0.01	0.58	0.00	0.01
Lachnospiraceae	24.18	15.78	13.91	17.71	0.01	0.01	0.03	1.00
Uncultured	2.99	0.94	0.5	1.39	0.00	0.00	0.02	0.38
Dorea	8.87	2.83	2.51	3.17	0.01	0.00	0.02	1.00
*Lachnoclostridium**	1.16	1.04	1.65	0.41	0.2	0.01	1.00	0.01
Unknown	2.28	1.23	1.2	1.26	0.01	0.02	0.02	1.00
Ruminococcaceae	8.46	14.56	10.72	18.52	0.08	1.00	0.01	0.17
Faecalibacterium	4.24	10.75	6.48	15.15	0.00	0.11	0.00	0.09
Oscillospira	0.61	1.4	1.65	1.13	0.08	0.01	1.00	0.17
Ruminococcus	1.63	0.42	0.45	0.39	0.00	0.01	0.00	0.84
Veillonellaceae	6.4	8.44	10.24	6.58	0.37	0.6	1.00	1.00
Dialister	0.81	3.43	5.3	1.51	0.2	0.03	1.00	0.05
Phascolarctobacterium	1.13	0.68	0.5	0.86	0.04	0.01	0.26	0.61
Proteobacteria	7.1	8.6	7.61	9.63	0.63	1.00	1.00	1.00
Alcaligenaceae	1.77	1.29	0.95	1.64	0.04	0.02	0.19	1.00
Sutterella	1.77	1.29	0.95	1.64	0.05	0.02	0.19	1.00
Enterobacteriaceae	4.47	5.97	5.61	6.34	0.76	1.00	1.00	1.00
Unknown	0.14	0.62	0.22	1.03	0.06	0.38	0.02	0.65

The average abundance in gut microbiota in each study group is listed as a percentage. Differential taxa with an average abundance greater than 1% in any of the groups are listed. The significantly differential genera with *P*-values < 0.05 are highlighted in bold.
[1]Taxa that were renamed after blasting again in NCBI (The National Center for Biotechnology Information). P^1: *P*-value adjusted by the Bonferroni correction. HC: Healthy controls; DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome; UC: Ulcerative colitis; FDR: False discovery rate.

inflammation and damage to the intestinal epithelium layer.

Abundant lactate producers in DCSR syndrome

The majority of gut microbiota can ferment carbohydrates to produce short-chain fatty acids (SCFAs), lactate, and some inorganic compounds. Some of these products are beneficial to the health of the host, but some harm the intestinal tract, such as by causing damage to the intestinal epithelial barrier or promoting bowel inflammation.

Lactate can be produced by some microbes such as *Streptococcus* in the intestine. The lactate producers can ferment carbohydrates to produce lactic acid, and the accumulation of lactic acid can reduce the pH of the intestinal tract. It has been reported that the over-accumulation of lactate in the intestine of patients leads to a decrease in the pH[48-49]. In disease status, disorder of the gut microbiota appears, and blooms of lactate producers are significant in DCSR syndrome. *Streptococcus* in the presence of other lactate producers (*Enterococcus*, *Lactobacillus*, *Bifidobacterium* and so on)[50-54] can produce lactate. Nevertheless, the utilization rate of lactate by other microbes is decreased in disease status, which causes the over-accumulation of lactic acid in the gut so that the intestinal pH is reduced and the distribution and abundance of intestinal microbiota are changed. Additionally, the accumulation of lactate can stimulate the intestinal tract and aggravate inflammation.

Lachnoclostridium is raised in PXSY patients

This study showed that the abundance of *Lachnoclostridium* in the PXSY group was

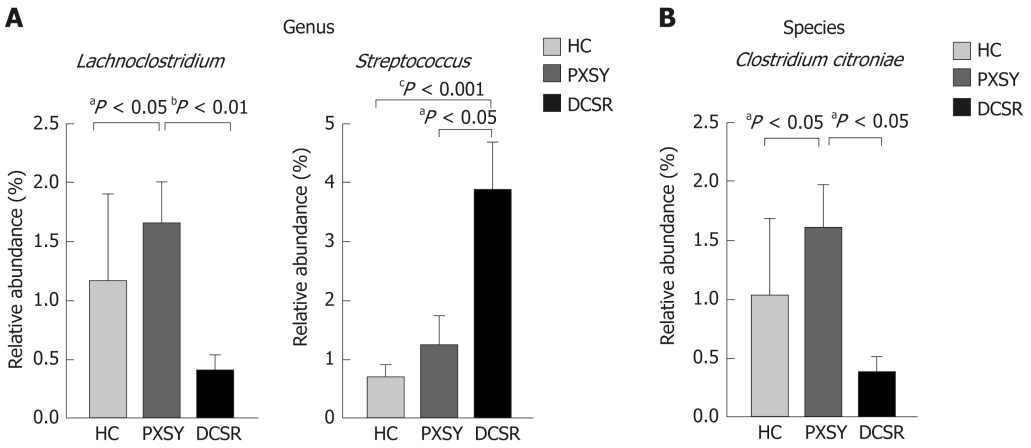


Figure 3 Differential taxa between Pi-Xu-Shi-Yun syndrome and Da-Chang-Shi-Re syndrome cohorts at the genus and species levels. A: Differential genera; B: Differential species. *Lachnoclostridium* is more abundant in Pi-Xu-Shi-Yun (PXSY) syndrome. *Lachnoclostridium* is more abundant in PXSY syndrome than in the shire group and healthy controls; the species of *Lachnoclostridium* named *Clostridium citroniae* also showed the same tendency. Streptococcus was more abundant in Da-Chang-Shi-Re patients than in PXSY and healthy controls. Relative abundance values are the mean ± SE (adjusted $^{a}P < 0.05$; adjusted $^{b}P < 0.01$; adjusted $^{c}P < 0.001$). HC: Healthy controls; DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome.

higher than that in the DCSR group. Members of *Lachnoclostridium* ferment some monosaccharides and disaccharides to acetate as the major SCFA products[55]. Within the genus *Lachnoclostridium*, the differential microbes between DCSR and PXSY patients, the main differential species is *Clostridium citroniae*, which also increased in DCSR patients. *Clostridium citroniae* contains D-cysteine desulfatase[56] and can ferment cysteine to sulfide, pyruvate, and ammonia as end products[57], and more *Clostridium citroniae* in the PXSY group suggested that more D-cysteine desulfatase might be present in the intestine of spleen-deficient PXSY patients than in DCSR patients.

The carbohydrate fermentation conducted by bacteria in the colon gives rise to the luminal SCFAs, including acetate, propionate, and butyrate. Among these SCFAs, but-yrate is considered to be the preferred source of nutrition and energy for colonic epithelial cells and is, to a major extent, metabolized by these cells[58]. In addition, butyrate is considered to have anti-inflammatory capacity, and the increase in sulfide may inhibit the oxidation of butyrate, which could damage the epithelial barrier and even result in more severe inflammation.

The gut microbiota could also activate a variety of pathogen-associated molecular patterns on the surface of intestinal epithelial cells and downstream signalling pathways, inducing epithelial cells to secrete anti-inflammatory substances[59,60]. However, the molecular and metabolic functions of most bacteria are still unknown[60].

Clostridium citroniae contains D-cysteine desulfhydrase, which can ferment cysteine to produce sulfides. Here, *Clostridium citroniae* was more abundant in PXSY patients than in DCSR patients and healthy subjects. While in patients with PXSY status, increased *Clostridium citroniae* results in increased D-cysteine desulfhydrase, which results in an increase in sulfides. Finally, sulfides inhibit the oxidization of butyric acid so that the epithelial cells cannot utilize the butyrate in the gut and cause epithelial cell apoptosis. Damage to colonic epithelial cells is reflected in the incomplete intestinal barrier, and gut microbes pass through the intestinal barrier to induce severe inflammation and ulceration. In brief, *Clostridium citroniae* might be detrimental to the health of the intestinal tract by inhibiting the utilization of butyrate.

PXSY syndrome reflects a kind of deficiency in immune activity, while DCSR syndrome is a kind of dysfunction in gut immunity. These differential trends in the microbiota between the two TCM syndromes of UC indicated that different effects were exerted on the mucosal immune activity and the epithelial barrier function by themselves. The genus *Streptococcus*, which could lead to inflammation, was significantly more abundant in DCSR patients. It is suggested that *Streptococcus* and other microbes in the gut might have a certain relationship with DCSR syndrome symptoms such as diarrhoea mixed with blood. These differential microbes are expected to be the markers and therapeutic targets for the clinical precision detection of UC.

There were several weaknesses in the study, like small sample size, non-blinding, and cross-sectional nature. In the following studies, the sample size needs to be further expanded to further strengthen and enrich the accuracy and stability of the research results. This study is a cross-sectional study, and it is difficult to determine the causal relationship between the results. In future studies, the intervention factors

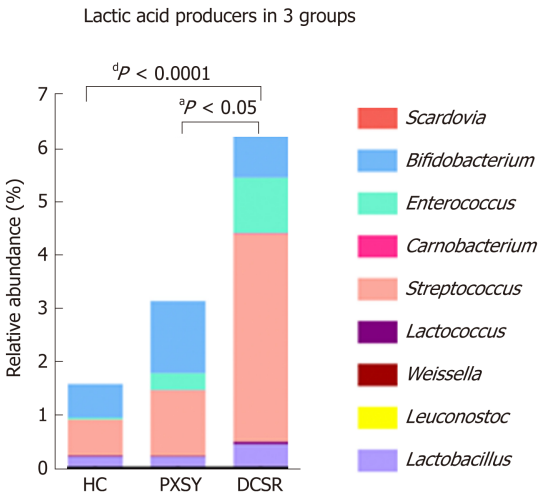


Figure 4 Lactic acid producers in the gut. Lactic acid bacteria were more abundant in ulcerative colitis patients, especially in the Da-Chang-Shi-Re (DCSR) group, than in healthy controls, suggesting that there might be more lactic acid in the guts of DCSR patients than in those of Pi-Xu-Shi-Yun patients. HC: Healthy controls; DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome.

can be considered and the dynamic observation before and after the intervention can be carried out.

In conclusion, the gut microbiota is different between patients with PXSY syndrome and those with DCSR syndrome. The genus *Streptococcus* is significantly more abundant in DCSR patients than in PXSY patients, while *Lachnoclostridium* increases in PXSY patients. Dysbiosis of the gut microbiota influences the immune responses of the host, which results in inflammation, and the microbial analysis of the two TCM syndromes essentially reflects different immune activities in the human body, but they all point to promotion of inflammation in the gut.

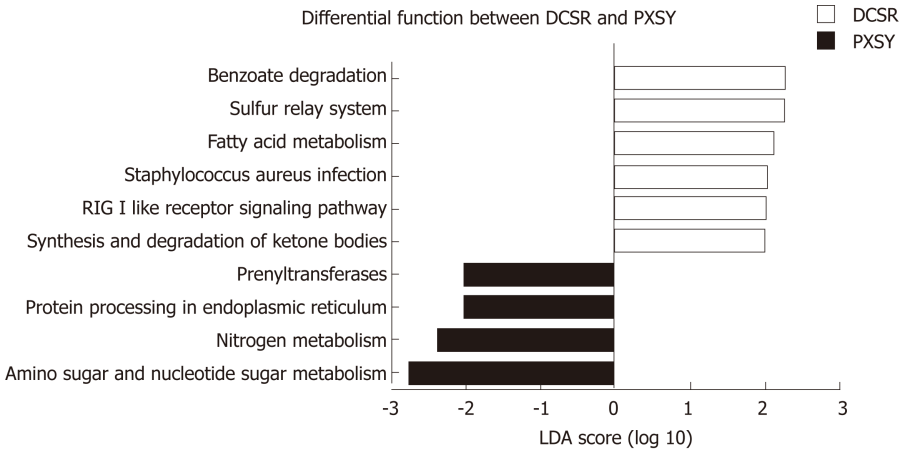


Figure 5 Functional pathways of gut microbiomes in the Pi-Xu-Shi-Yun and Da-Chang-Shi-Re groups. Microbial functions were predicted and categorized into KEGG pathways using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States. Linear discriminant analysis effect size was carried out to detect inner function differentials among groups. Significantly differential functions between Pi-Xu-Shi-Yun syndrome (yellow) and Da-Chang-Shi-Re syndrome (pink) are shown (LDA score > 2.0). DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome.

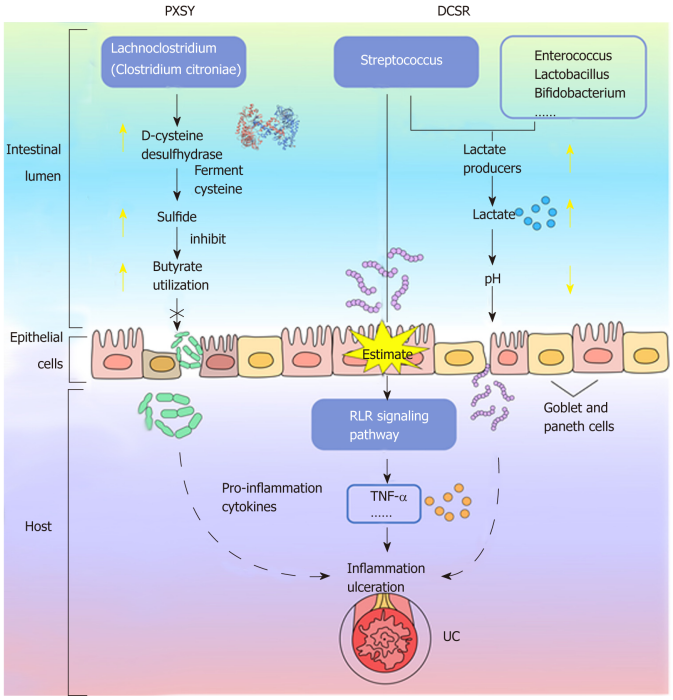


Figure 6 The probable contribution of the gut microbiota to the pathological mechanism of Da-Chang-Shi-Re and Pi-Xu-Shi-Yun syndromes. Da-Chang-Shi-Re and Pi-Xu-Shi-Yun syndromes might have different pathological mechanisms, which are based on the differential microbiota between these two syndromes. UC: Ulcerative colitis; DCSR: Da-Chang-Shi-Re syndrome; PXSY: Pi-Xu-Shi-Yun syndrome; RLR: RIG-I-like receptor.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is considered to be closely associated with alteration of intestinal microorganisms. According to the traditional Chinese medicine (TCM) theory, UC can be divided into Pi-Xu-Shi-Yun syndrome (syndrome of spleen deficiency and dampness, PXSY) and Da-Chang-Shi-Re syndrome (syndrome of dampness-heat in the large intestine, DCSR). PXSY syndrome is a deficiency syndrome, while the DCSR syndrome is a sthenia syndrome. However, the relationships among gut microbiota, TCM syndromes, and UC pathogenesis are unclear.

Research motivation

The majority of studies have focused on the relationship between intestinal microbiota and the onset of UC, and the contribution of gut microbiota in these two distinct TCM syndromes is still unclarified. This study aimed to compare the difference in microbial composition and function

between PXSY and DCSR syndromes to determine the molecular mechanism of TCM in UC by investigation of the gut microbiota.

Research objectives
The objective of this study was to investigate the role of gut microbiota in UC and the distinction of microbiota dysbiosis between PXSY and DCSR syndromes.

Research methods
We analysed gut microbiome composition of stool samples by 16S rRNA pyrosequencing. We assessed the beta diversity by UniFrac analysis. We also processed the high-throughput sequencing reads with QIIME, and further predicted biological functions using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

Research results
We determined the composition of gut bacterial communities in 93 stool samples (30 healthy controls, 32 patients with PXSY syndrome, and 31 patients with DCSR syndrome) by 16S rRNA pyrosequencing. Beta diversity showed that the composition of the microbiota was different among the three groups. We found that Porphyromonadaceae, Rikeneliaceae, and Lachnospiraceae significantly decreased while Enterococcus, Streptococcus, and other potential pathogens significantly increased in UC patients compared to healthy subjects at the family level. We further found that *Parabacteroides*, *Dorea*, and *Ruminococcus* decreased while *Faecalibacterium* showed increased abundance in UC compared to healthy controls at the genus level. Five differential taxa were identified between PXSY and DCSR syndromes. We observed a significantly increased abundance of *Streptococcus* in DCSR patients at the genus level, while *Lachnoclostridium* increased in PXSY patients. Additionally, we found that the differential functional pathways of the gut microbiome between the PXSY and DCSR groups mainly included lipid metabolism, immunity, and the metabolism of polypeptides.

Research conclusions
The present study identified that the gut microbiota is different between patients with PXSY syndrome and those with DCSR syndrome. The genus *Streptococcus* is significantly more abundant in DCSR patients than in PXSY patients, while *Lachnoclostridium* increases in PXSY patients. The microbial analysis of the two TCM syndromes essentially reflects different immune activities in the human body, but they all point to promotion of inflammation in the gut.

Research perspectives
The relationship between TCM syndromes and intestinal flora is an interesting and important research topic. Our study preliminarily explored the characteristics and differences of intestinal flora of patients with two different TCM syndrome. Further studies are required to confirm our findings and to clarify the precise mechanism of intestinal flora characteristics related to TCM deficiency and positive syndrome.

REFERENCES

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

2 **Ng SC**. Epidemiology of inflammatory bowel disease: Focus on Asia. *Best Pract Res Clin Gastroenterol* 2014; **28**: 363-372 [PMID: 24913377 DOI: 10.1016/j.bpg.2014.04.003]

3 **Manichanh C**, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 599-608 [PMID: 22907164 DOI: 10.1038/nrgastro.2012.152]

4 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: A systematic review of population-based studies. *Lancet* 2018; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

5 **Kaplan GG**. The global burden of IBD: From 2015 to 2025. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 720-727 [PMID: 26323879 DOI: 10.1038/nrgastro.2015.150]

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

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

8 **Desai D**, Shah S, Deshmukh A, Abraham P, Joshi A, Gupta T, Deshpande R, Khandagale V, George S. Colorectal cancers in ulcerative colitis from a low-prevalence area for colon cancer. *World J Gastroenterol* 2015; **21**: 3644-3649 [PMID: 25834332 DOI: 10.3748/wjg.v21.i12.3644]

9 **Lu YH**, Cong LL. [Study on the Chinese medical syndrome distribution of ulcerative colitis]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2012; **32**: 450-454 [PMID: 22803420]

10 **Chen Z**, Wang P. Clinical Distribution and Molecular Basis of Traditional Chinese Medicine ZHENG in Cancer. *Evid Based Complement Alternat Med* 2012; **2012**: 783923 [PMID: 22829858 DOI:

10.1155/2012/783923]
11 **Dai YC**, Zhang YL, Wang LJ, Guo Q, Yang K, Ye RH, Tang ZP. Clinical presentation and treatment strategies for ulcerative colitis: A retrospective study of 247 inpatients. *Chin J Integr Med* 2016; **22**: 811-816 [PMID: 26501692 DOI: 10.1007/s11655-015-2118-1]
12 **Dai YC**, Zheng L, Zhang YL, Chen X, Chen DL, Tang ZP. Effects of Jianpi Qingchang decoction on the quality of life of patients with ulcerative colitis: A randomized controlled trial. *Medicine (Baltimore)* 2017; **96**: e6651 [PMID: 28422869 DOI: 10.1097/MD.0000000000006651]
13 **Zheng L**, Zhang YL, Dai YC, Chen X, Chen DL, Dai YT, Tang ZP. Jianpi Qingchang decoction alleviates ulcerative colitis by inhibiting nuclear factor-κB activation. *World J Gastroenterol* 2017; **23**: 1180-1188 [PMID: 28275298 DOI: 10.3748/wjg.v23.i7.1180]
14 **Lin X**, Yi Z, Diao J, Shao M, Zhao L, Cai H, Fan Q, Yao X, Sun X. ShaoYao decoction ameliorates colitis-associated colorectal cancer by downregulating proinflammatory cytokines and promoting epithelial-mesenchymal transition. *J Transl Med* 2014; **12**: 105 [PMID: 24766737 DOI: 10.1186/1479-5876-12-105]
15 **Liu DY**, Guan YM, Zhao HM, Yan DM, Tong WT, Wan PT, Zhu WF, Liu HN, Liang XL. The protective and healing effects of Si Shen Wan in trinitrobenzene sulphonic acid-induced colitis. *J Ethnopharmacol* 2012; **143**: 435-440 [PMID: 22751005 DOI: 10.1016/j.jep.2012.05.060]
16 **Zou Y**, Lin J, Li W, Wu Z, He Z, Huang G, Wang J, Ye C, Cheng X, Ding C, Zheng X, Chi H. Huangqin-tang ameliorates dextran sodium sulphate-induced colitis by regulating intestinal epithelial cell homeostasis, inflammation and immune response. *Sci Rep* 2016; **6**: 39299 [PMID: 27982094 DOI: 10.1038/srep39299]
17 **Lepage P**, Häsler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, Ott S, Kupcinskas L, Doré J, Raedler A, Schreiber S. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* 2011; **141**: 227-236 [PMID: 21621540 DOI: 10.1053/j.gastro.2011.04.011]
18 **Becker C**, Neurath MF, Wirtz S. The Intestinal Microbiota in Inflammatory Bowel Disease. *ILAR J* 2015; **56**: 192-204 [PMID: 26323629 DOI: 10.1093/ilar/ilv030]
19 **Round JL**, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009; **9**: 313-323 [PMID: 19343057 DOI: 10.1038/nri2515]
20 **Wlodarska M**, Kostic AD, Xavier RJ. An integrative view of microbiome-host interactions in inflammatory bowel diseases. *Cell Host Microbe* 2015; **17**: 577-591 [PMID: 25974300 DOI: 10.1016/j.chom.2015.04.008]
21 **Stephani J**, Radulovic K, Niess JH. Gut microbiota, probiotics and inflammatory bowel disease. *Arch Immunol Ther Exp (Warsz)* 2011; **59**: 161-177 [PMID: 21445715 DOI: 10.1007/s00005-011-0122-5]
22 **Cucchiara S**, Stronati L, Aloi M. Interactions between intestinal microbiota and innate immune system in pediatric inflammatory bowel disease. *J Clin Gastroenterol* 2012; **46** Suppl: S64-S66 [PMID: 22955361 DOI: 10.1097/MCG.0b013e31826a857f]
23 **Guo M**, Ding S, Zhao C, Gu X, He X, Huang K, Luo Y, Liang Z, Tian H, Xu W. Red Ginseng and Semen Coicis can improve the structure of gut microbiota and relieve the symptoms of ulcerative colitis. *J Ethnopharmacol* 2015; **162**: 7-13 [PMID: 25554637 DOI: 10.1016/j.jep.2014.12.029]
24 **Nishikawa J**, Kudo T, Sakata S, Benno Y, Sugiyama T. Diversity of mucosa-associated microbiota in active and inactive ulcerative colitis. *Scand J Gastroenterol* 2009; **44**: 180-186 [PMID: 18825588 DOI: 10.1080/00365520802433231]
25 **Branch of Gastrointestinal Diseases**. China Association of Chinese Medicine. [Consensus on Chinese medical diagnosis and treatment of ulcerative colitis (2009)]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2010; **30**: 527-532 [PMID: 20681286]
26 **Bolger AM**, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; **30**: 2114-2120 [PMID: 24695404 DOI: 10.1093/bioinformatics/btu170]
27 **Caporaso JG**, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; **7**: 335-336 [PMID: 20383131 DOI: 10.1038/nmeth.f.303]
28 **Edgar RC**, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011; **27**: 2194-2200 [PMID: 21700674 DOI: 10.1093/bioinformatics/btr381]
29 **Langille MG**, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013; **31**: 814-821 [PMID: 23975157 DOI: 10.1038/nbt.2676]
30 **Segata N**, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011; **12**: R60 [PMID: 21702898 DOI: 10.1186/gb-2011-12-6-r60]
31 **Afgan E**, Baker D, van den Beek M, Blankenberg D, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Eberhard C, Grüning B, Guerler A, Hillman-Jackson J, Von Kuster G, Rasche E, Soranzo N, Turaga N, Taylor J, Nekrutenko A, Goecks J. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res* 2016; **44**: W3-W10 [PMID: 27137889 DOI: 10.1093/nar/gkw343]
32 **Lavelle A**, Lennon G, O'Sullivan O, Docherty N, Balfe A, Maguire A, Mulcahy HE, Doherty G, O'Donoghue D, Hyland J, Ross RP, Coffey JC, Sheahan K, Cotter PD, Shanahan F, Winter DC, O'Connell PR. Spatial variation of the colonic microbiota in patients with ulcerative colitis and control volunteers. *Gut* 2015; **64**: 1553-1561 [PMID: 25596182 DOI: 10.1136/gutjnl-2014-307873]
33 **Mar JS**, LaMere BJ, Lin DL, Levan S, Nazareth M, Mahadevan U, Lynch SV. Disease Severity and Immune Activity Relate to Distinct Interkingdom Gut Microbiome States in Ethnically Distinct Ulcerative Colitis Patients. *MBio* 2016; **7**: pii: e01072-16 [PMID: 27531910 DOI: 10.1128/mBio.01072-16]
34 **Frank DN**, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 2007; **104**: 13780-13785 [PMID: 17699621 DOI: 10.1073/pnas.0706625104]
35 **Ohkusa T**, Koido S. Intestinal microbiota and ulcerative colitis. *J Infect Chemother* 2015; **21**: 761-768 [PMID: 26346678 DOI: 10.1016/j.jiac.2015.07.010]
36 **Jiao N**, Baker SS, Nugent CA, Tsompana M, Cai L, Wang Y, Buck MJ, Genco RJ, Baker RD, Zhu R, Zhu

L. Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: A meta-analysis. *Physiol Genomics* 2018; **50**: 244-254 [PMID: 29373083 DOI: 10.1152/physiolgenomics.00114.2017]

37 **Jiao N**, Baker SS, Chapa-Rodriguez A, Liu W, Nugent CA, Tsompana M, Mastrandrea L, Buck MJ, Baker RD, Genco RJ, Zhu R, Zhu L. Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD. *Gut* 2018; **67**: 1881-1891 [PMID: 28774887 DOI: 10.1136/gutjnl-2017-314307]

38 **Zhu L**, Liu W, Alkhouri R, Baker RD, Bard JE, Quigley EM, Baker SS. Structural changes in the gut microbiome of constipated patients. *Physiol Genomics* 2014; **46**: 679-686 [PMID: 25073603 DOI: 10.1152/physiolgenomics.00082.2014]

39 **Zhu L**, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, Gill SR. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: A connection between endogenous alcohol and NASH. *Hepatology* 2013; **57**: 601-609 [PMID: 23055155 DOI: 10.1002/hep.26093]

40 **Scaldaferri F**, Gerardi V, Lopetuso LR, Del Zompo F, Mangiola F, Boškoski I, Bruno G, Petito V, Laterza L, Cammarota G, Gaetani E, Sgambato A, Gasbarrini A. Gut microbial flora, prebiotics, and probiotics in IBD: Their current usage and utility. *Biomed Res Int* 2013; **2013**: 435268 [PMID: 23991417 DOI: 10.1155/2013/435268]

41 **Hooper LV**, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; **336**: 1268-1273 [PMID: 22674334 DOI: 10.1126/science.1223490]

42 **Tahara T**, Arisawa T. Pathogenesis of CpG island methylator phenotype-positive colorectal cancers: Role of genetic alteration and colonic flora. *Epigenomics* 2014; **6**: 249-251 [PMID: 25111477 DOI: 10.2217/epi.14.25]

43 **Cui DJ**, Yang XL, Hu M, Yang LC. Effects of Preoperative Methotrexate on Complications After Surgery for Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2017; **23**: E2 [PMID: 27893545 DOI: 10.1097/MIB.0000000000000971]

44 **Van Der Sloot KW**, Joshi AD, Bellavance DR, Gilpin KK, Stewart KO, Lochhead P, Garber JJ, Giallourakis C, Yajnik V, Ananthakrishnan AN, Alizadeh BZ, Xavier RJ, Khalili H. Visceral Adiposity, Genetic Susceptibility, and Risk of Complications Among Individuals with Crohn's Disease. *Inflamm Bowel Dis* 2017; **23**: 82-88 [PMID: 27893544 DOI: 10.1097/MIB.0000000000000978]

45 **Chu H**, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nat Immunol* 2013; **14**: 668-675 [PMID: 23778794 DOI: 10.1038/ni.2635]

46 **Errett JS**, Gale M. Emerging complexity and new roles for the RIG-I-like receptors in innate antiviral immunity. *Virol Sin* 2015; **30**: 163-173 [PMID: 25997992 DOI: 10.1007/s12250-015-3604-5]

47 **Nakhaei P**, Genin P, Civas A, Hiscott J. RIG-I-like receptors: Sensing and responding to RNA virus infection. *Semin Immunol* 2009; **21**: 215-222 [PMID: 19539500 DOI: 10.1016/j.smim.2009.05.001]

48 **Vernia P**, Caprilli R, Latella G, Barbetti F, Magliocca FM, Cittadini M. Fecal lactate and ulcerative colitis. *Gastroenterology* 1988; **95**: 1564-1568 [PMID: 3181680 DOI: 10.1016/S0016-5085(88)80078-7]

49 **Hirschhaeuser F**, Sattler UG, Mueller-Klieser W. Lactate: A metabolic key player in cancer. *Cancer Res* 2011; **71**: 6921-6925 [PMID: 22084445 DOI: 10.1158/0008-5472.CAN-11-1457]

50 **Le Blay G**, Michel C, Blottière HM, Cherbut C. Prolonged intake of fructo-oligosaccharides induces a short-term elevation of lactic acid-producing bacteria and a persistent increase in cecal butyrate in rats. *J Nutr* 1999; **129**: 2231-2235 [PMID: 10573555 DOI: 10.1093/jn/129.12.2231]

51 **Belenguer A**, Duncan SH, Holtrop G, Anderson SE, Lobley GE, Flint HJ. Impact of pH on lactate formation and utilization by human fecal microbial communities. *Appl Environ Microbiol* 2007; **73**: 6526-6533 [PMID: 17766450 DOI: 10.1128/AEM.00508-07]

52 **de Matos BM**, Brighenti FL, Do T, Beighton D, Koga-Ito CY. Acidogenicity of dual-species biofilms of bifidobacteria and Streptococcus mutans. *Clin Oral Investig* 2017; **21**: 1769-1776 [PMID: 27660160 DOI: 10.1007/s00784-016-1958-1]

53 **Belenguer A**, Holtrop G, Duncan SH, Anderson SE, Calder AG, Flint HJ, Lobley GE. Rates of production and utilization of lactate by microbial communities from the human colon. *FEMS Microbiol Ecol* 2011; **77**: 107-119 [PMID: 21395623 DOI: 10.1111/j.1574-6941.2011.01086.x]

54 **Yutin N**, Galperin MY. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environ Microbiol* 2013; **15**: 2631-2641 [PMID: 23834245 DOI: 10.1111/1462-2920.12173]

55 **Feng Y**, Stams AJM, de Vos WM, Sánchez-Andrea I. Enrichment of sulfidogenic bacteria from the human intestinal tract. *FEMS Microbiol Lett* 2017; 364 [PMID: 28158432 DOI: 10.1093/femsle/fnx028]

56 **Nagasawa T**, Ishii T, Kumagai H, Yamada H. D-Cysteine desulfhydrase of Escherichia coli. Purification and characterization. *Eur J Biochem* 1985; **153**: 541-551 [PMID: 3908101 DOI: 10.1111/j.1432-1033.1985.tb09335.x]

57 **Tedelind S**, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: A study with relevance to inflammatory bowel disease. *World J Gastroenterol* 2007; **13**: 2826-2832 [PMID: 17569118 DOI: 10.1186/1471-230X-7-17]

58 **Cawich SO**, Harnarayan P, Budhooram S, Naraynsingh V. Axillary Artery Injury Accompanying Humeral Neck Fracture. *Int J Angiol* 2015; **24**: 296-299 [PMID: 26648673 DOI: 10.1055/s-0034-1372246]

59 **Gupta AK**, Harris JD, Erickson BJ, Abrams GD, Bruce B, McCormick F, Nicholson GP, Romeo AA. Surgical management of complex proximal humerus fractures-a systematic review of 92 studies including 4500 patients. *J Orthop Trauma* 2015; **29**: 54-59 [PMID: 25162974 DOI: 10.1097/BOT.0000000000000229]

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

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3256-3267

DOI: 10.3748/wjg.v25.i25.3256

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

ORIGINAL ARTICLE

Observational Study

Assessing significant fibrosis using imaging-based elastography in chronic hepatitis B patients: Pilot study

Hee Sun Park, Won Hyeok Choe, Hye Seung Han, Mi Hye Yu, Young Jun Kim, Sung Il Jung, Jeong Han Kim, So Young Kwon

ORCID number: Hee Sun Park (0000-0003-0869-269X); Won Hyeok Choe (0000-0002-8019-5412); Hye Seung Han (0000-0002-3591-9995); Mi Hye Yu (0000-0003-0519-7853); Young Jun Kim (0000-0003-2358-1170); Sung Il Jung (0000-0002-4588-209X); Jeong Han Kim (0000-0002-8383-8524); So Young Kwon (0000-0003-4290-1950).

Author contributions: Choe WH and Park HS contributed to study conception and design; Choe WH, Kim JH and Kwon SY contributed to collection of clinical data; Park HS, Han HS, Yu MH, Kim YJ, Jung SI and Kwon SY contributed to data acquisition, data analysis and interpretation; Park HS, Choe WH, Han HS, Yu MH, Kim YJ, Jung SI, Kim JH and Kwon SY contributed to writing of article, editing, reviewing and final approval of article.

Institutional review board statement: Based on the Declaration of Helsinki, the Institutional Review Board of Konkuk University Hospital approved the retrospective use of the clinical, biochemical, and radiographic data for the present study.

Informed consent statement: The requirements for informed consent were waived due to the retrospective design.

Conflict-of-interest statement: The authors declare they have no potential conflicts of interest.

Data sharing statement: No

Hee Sun Park, Mi Hye Yu, Young Jun Kim, Sung Il Jung, Department of Radiology, Konkuk University School of Medicine, Seoul 05030, South Korea

Won Hyeok Choe, Jeong Han Kim, So Young Kwon, Department of Internal Medicine, Konkuk University School of Medicine, Seoul 05030, South Korea

Hye Seung Han, Department of Pathology, Konkuk University School of Medicine, Seoul 05030, South Korea

Corresponding author: Won Hyeok Choe, MD, PhD, Professor, Department of Internal Medicine, Konkuk University Medical Center, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwangjin-gu, Seoul 05030, South Korea. 20050101@kuh.ac.kr
Telephone: +82-2-20305010
Fax: +82-2-20305029

Abstract

BACKGROUND

Accurate detection of significant fibrosis (fibrosis stage 2 or higher on the METAVIR scale) is important especially for chronic hepatitis B (CHB) patients with high viral loads but with normal or mildly elevated alanine aminotransferase (ALT) levels because the presence of significant fibrosis is accepted as the indication for antiviral treatment. Liver biopsy is the reference standard for diagnosing significant fibrosis, but it is an invasive procedure. Consequently, noninvasive imaging-based measurements, such as magnetic resonance elastography (MRE) or two-dimensional shear-wave elastography (2D-SWE), have been proposed for the quantitative assessment of liver fibrosis.

AIM

To explore MRE and 2D-SWE to identify fibrosis stage, and to compare their performance with that of serum-based indices.

METHODS

The study enrolled 63 treatment-naïve CHB patients with high viral loads but with normal or mildly elevated ALT levels who underwent liver biopsy before a decision was made to initiate antiviral therapy. MRE and 2D-SWE were performed, and serum-based indices, such as FIB-4 and aspartate transaminase to platelet ratio index (APRI), were calculated. The diagnostic performances of MRE, 2D-SWE, FIB-4, and APRI for assessing significant fibrosis (≥ F2) and cirrhosis (F4) were evaluated with liver histology as the reference standard, using

additional data are available.

STROBE statement: The authors have read and checked the STROBE checklist.

Manuscript source: Unsolicited manuscript

Received: April 19, 2019
Peer-review started: April 19, 2019
First decision: May 9, 2019
Revised: May 20, 2019
Accepted: June 8, 2019
Article in press: June 8, 2019
Published online: July 7, 2019

P-Reviewer: Cheungpasitporn W, Ierardi E, Mihaila RG, Tamori A, Yao D
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

receiver operating characteristic analyses.

RESULTS

The liver fibrosis stage was F0/F1 in 19, F2 in 14, F3 in 14, and F4 in 16 patients, respectively. MRE significantly discriminated F2 from F0/1 ($P = 0.022$), whereas 2D-SWE showed a broad overlap in distinguishing those stages. MRE showed a higher correlation coefficient value with fibrosis stage than 2D-SWE with fibrosis stage (0.869 *vs* 0.649, Spearman test; $P < 0.001$). Multivariate linear regression analyses showed that fibrosis stage was the only factor affecting the values of MRE ($P < 0.001$), whereas body mass index ($P = 0.042$) and fibrosis stage ($P < 0.001$) were independent factors affecting 2D-SWE values. MRE performance for diagnosing significant fibrosis was better [area under the curve (AUC) = 0.906, positive predictive value (PPV) 97.3%, negative predictive value (NPV) 69.2%] than that of FIB-4 (AUC = 0.697, $P = 0.002$) and APRI (AUC = 0.717, $P = 0.010$), whereas the performance of 2D-SWE (AUC = 0.843, PPV 86%, NPV 65%) was not significantly different from that of FIB-4 or APRI.

CONCLUSION

Compared to SWE, MRE might be more precise non-invasive assessment for depicting significant fibrosis and for making-decision to initiate antiviral-therapy in treatment-naïve CHB patients with normal or mildly-elevated ALT levels.

Key words: Antiviral therapy; Chronic hepatitis B; Liver fibrosis; Magnetic resonance elastography; Ultrasound elastography

Core tip: The present study investigated magnetic resonance elastography (MRE) and two-dimensional shear-wave elastography (2D-SWE) to identify significant fibrosis, and to compare their performance with that of serum-based indices in treatment-naïve chronic hepatitis B (CHB) patients with borderline-normal alanine aminotransferase levels, who should be considered for initiation of antiviral therapy depending on the presence of significant fibrosis. Our data demonstrated that MRE was a more accurate and noninvasive measurement for detecting significant fibrosis, compared to 2D-SWE as well as serum-based indices, and our results suggested that MRE could be used as a basis for anti-HBV treatment-decisions in treatment-naïve CHB patients.

Citation: Park HS, Choe WH, Han HS, Yu MH, Kim YJ, Jung SI, Kim JH, Kwon SY. Assessing significant fibrosis using imaging-based elastography in chronic hepatitis B patients: Pilot study. *World J Gastroenterol* 2019; 25(25): 3256-3267
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3256.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3256

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem, causing chronic liver disease. If left untreated, chronic HBV infection may potentially lead to complications such as cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC)[1]. Therefore, effective antiviral treatment in chronic hepatitis B (CHB) patients can reduce the disease progression towards HBV-related cirrhosis and the risk of HCC development[1,2].

Accurate staging of liver fibrosis in CHB patients is necessary not only for predicting the long-term clinical course but also for determining whether and when to begin antiviral therapy. Recent clinical guidelines have recommended that CHB patients with high serum HBV-DNA levels [hepatitis B e-antigen (HBeAg) positive patients with serum HBV-DNA levels > 20000 IU/mL or HBeAg-negative patients with serum HBV-DNA levels > 2000 IU/mL] and elevated alanine aminotransferase (ALT) levels of twice the upper limit of normal (ULN) or greater should be considered for antiviral treatment[3-5]. CHB patients with high viral loads and significant fibrosis (METAVIR scoring system ≥ F2) should also be considered for treatment even if the ALT level is normal or mildly elevated (less than 2 times) because long-term viral

suppression reduces liver-related complications, such as decompensated cirrhosis or HCC, in these patients[3-5].

Liver biopsy is still considered the "gold standard" for the evaluation of significant fibrosis in CHB patients[6]. However, its utilization is often restricted because its invasiveness can cause lifethreatening complications[7]. Moreover, tissue obtained *via* biopsy represents approximately only 1/50000 of the liver volume, which may result in a sampling error and is associated with considerable interobserver variability in the microscopic evaluation. Furthermore, repeating the liver biopsy to monitor changes in liver fibrotic burden is generally not feasible in clinical practice[7,8]. To overcome these limitations of liver biopsy, noninvasive serum- and imaging-based measurements for staging liver fibrosis have been developed[9,10].

To date, noninvasive methods incorporating serum-based indices or imaging-based tests using elastography have been increasingly used to assess liver fibrosis[11]. A variety of serum-based indices have been evaluated to predict the degree of liver fibrosis[10,12]. Among those, aspartate transaminase (AST)-to-platelet ratio index (APRI) and fibrosis index based on four factors (FIB-4) are commonly used for identifying liver fibrosis and cirrhosis in CHB patients because they are easily calculated with routine laboratory tests, and they have successfully predicted liver fibrosis in large cohorts[13]. However, their main disadvantage is their low accuracy in detecting mild to intermediate stages of fibrosis[10,11,13]. Imaging-based methods of elastography estimate liver stiffness that is associated with the severity of fibrosis by applying mechanical waves and by measuring their propagation speed through tissue using imaging[14-16]. Elastographic modalities can be either ultrasound (US)-based or magnetic resonance imaging (MRI)-based. US-based elastography techniques include strain-based imaging, transient elastography (TE), and shear wave elastography (SWE)[17,18]. MRI measures tissue stiffness with magnetic resonance elastography (MRE)[19,20]. These techniques have been proven superior to conventional cross-sectional imaging for the evaluation of fibrosis and cirrhosis, especially in the pre-cirrhotic stages[19-22]. Several studies comparing the diagnostic performance of serum-based indices and imaging-based elastographies have been published[17,23], but little is known regarding their diagnostic performances that can be used to inform the applicability of these modalities to whether and when to initiate antiviral therapy in treatment-naive CHB patients with high viral loads but with normal or mildly elevated ALT levels.

Therefore, the objective of this study was to evaluate the liver stiffness values of MRE and two-dimensional SWE (2D-SWE) to assess liver fibrosis and to compare their diagnostic performances with those of FIB-4 and APRI for the prediction of significant fibrosis, which is an indicator for initiating antiviral therapy in treatment-naïve CHB patients with high viral loads but with borderline-normal or mildly elevated ALT levels.

MATERIALS AND METHODS

Patients

Between March 2013 and February 2018, 67 treatment-naïve CHB patients with high viral loads but borderline-normal or mildly elevated ALT levels who underwent liver biopsy at Konkuk University Medical Center before a decision was made to initiate antiviral therapy were recruited. The following inclusion criteria were applied: (1) Hepatitis B surface antigen (HBsAg) positivity more than 6 months, HBeAg positive patients with > 20000 IU/mL, or HBeAg-negative patients with > 2000 IU/mL, normal ALT values (our laboratory reference value was 40 IU/L), or less than two times ULN; (2) Absence of any previous or concomitant anti-HBV therapy; (3) No liver comorbidity, including hepatitis C virus (HCV) coinfection, chronic ethanol consumption (more than 20 g of alcohol per day), HIV coinfection, or autoimmune hepatitis; (4) Availability of liver histologic assessment after liver biopsy, and time interval between liver biopsy and MRE/ 2D-SWE within 2 wk; and (5) Availability of both MRE and 2D-SWE, and time interval between MRE and 2D-SWE within 3 d. Patients who have clinical features or complications of liver cirrhosis, including ascites, medium/large gastroesophageal varices, or moderate to severe thrombocytopenia (platelet counts < 80000/μL), were excluded because they should be considered for antiviral treatment without requiring liver biopsy for confirmation of liver cirrhosis. Patients under 35 years of age were also excluded because they might stay in the immune-tolerant phase of chronic HBV infection. Our Institutional Review Board approved this study, waiving informed consent because of its retrospective nature.

MR elastography

All MR examinations were performed using a 3-T MR unit (Magnetom Skyra, Siemens Medical Solutions, Erlangen, Germany). Patients were asked to hold their breath at the end-expiratory period to obtain a consistent position of the liver for each phase offset. When the acquisition was completed, wave images were automatically processed by the MR scanner, and images depicting tissue stiffness (elastograms) were generated (Figure 1A-D). These quantitative images represented shear stiffness in units of kilopascals (kPa). In addition, the elastogram was reviewed automatically by the intrinsic software for artifacts, such as significant wave interference and oblique wave propagation. Elastograms with 95% confidence mapping were produced by excluding the artifact area. MRE technical failure was considered when the following occurred: (1) Wave images showed no wave propagation; (2) Anatomic images showed severe respiratory motion artifact along the z-axis; or (3) Substantial loss of signal in the liver parenchyma suggesting an iron overload was present[16].

The mean shear stiffness of the liver was calculated by placing a manually specified region of interest (ROI) into the stiffness map of MRE images. The stiffness value of the liver parenchyma was calculated as the mean value in four ROIs (mean area, 4044.8 ± 1715.8 mm^2) placed by one radiologist.

SWE technique

Measurements for 2D SWE were obtained by using an Aixplorer US system (SuperSonic Imagine, Aix-en-Provence, France) equipped with a broadband convex transducer (SC6-1). The operator was a single board-certified abdominal radiologist with more than 10 years of liver US experience and more than one year of clinical experience performing real-time elastography studies. SWE examinations were performed in the right lobe of the liver through the intercostal space. Liver stiffness measurements were obtained within an ROI of 10 mm^2 in diameter at the area where the elasticity image was most homogeneously displayed. SWE measurement failure was considered when little or no signal was obtained in the SWE box, and an appropriate color-coded elasticity map was not acquired. Five consecutive acquisitions were obtained in the same location of the liver for each patient. Each measurement was performed during a separate breath hold. The system calculated the mean, maximum, minimum, and standard deviation of the elasticity value of each measurement in kPa (Figure 1E). The mean value of five liver stiffness measurements was calculated.

FIB-4 and APRI formulae

The FIB-4 values were calculated automatically using the formula [age (years) × AST (U/L)]/{platelets (10^9/L) × [ALT (U/L)]$^{1/2}$}[24], in which the age of the patient was the age at the time of the liver biopsy. The APRI values were calculated using the formula (AST/upper limit of normal)/[platelet count (10^9/L)] × 100[25]. Our laboratory reference value of AST was 40 IU/L.

Histopathologic analysis

Biopsy specimens were fixed in formalin and embedded in paraffin. Thereafter, 4-mm-thick slices were cut and stained with hematoxylin-eosin. All specimens were analyzed by a pathologist who was blinded to the MRE results, SWE results, and the clinical data and who had 10 years of clinical experience interpreting liver pathologic examinations. The fibrosis stage and the degree of inflammation in the liver were assessed based on the METAVIR scoring system as shown below: F0, no fibrosis; F1, portal fibrosis; F2, periportal fibrosis; F3, septal fibrosis; and F4, cirrhosis[26]. In this study, a fibrosis stage of F2 or higher was considered to indicate significant fibrosis. Inflammatory activity was graded as A0 to A3: A0, no activity; A1, mild activity; A2, moderate activity; A3, severe activity.

Statistical analysis

Quantitative variables were expressed as the mean ± standard deviation (SD), which were analyzed with a *t*-test or a Mann-Whitney *U*-test, and categorical variables were demonstrated with numbers and percentages and compared using the Chi-squared method or Fisher's exact test, when appropriate. Correlations between noninvasive methods and liver histological fibrosis stages were assessed using the Spearman correlation test. The strength of the correlation coefficients was classified as follows: 0.0-0.2, very weak; 0.2-0.4, weak; 0.4-0.7, moderate; 0.7-0.9, strong; and 0.9-1.0, very strong correlation. The difference between two dependent correlations was calculated by the Steiger test. Factors affecting liver stiffness values of the MRE or 2D-SWE were first analyzed with univariate testing, and those with $P < 0.05$ were subsequently included in a multivariate linear regression analysis. The diagnostic performance of noninvasive methods was assessed using receiver-operating characteristic (ROC)

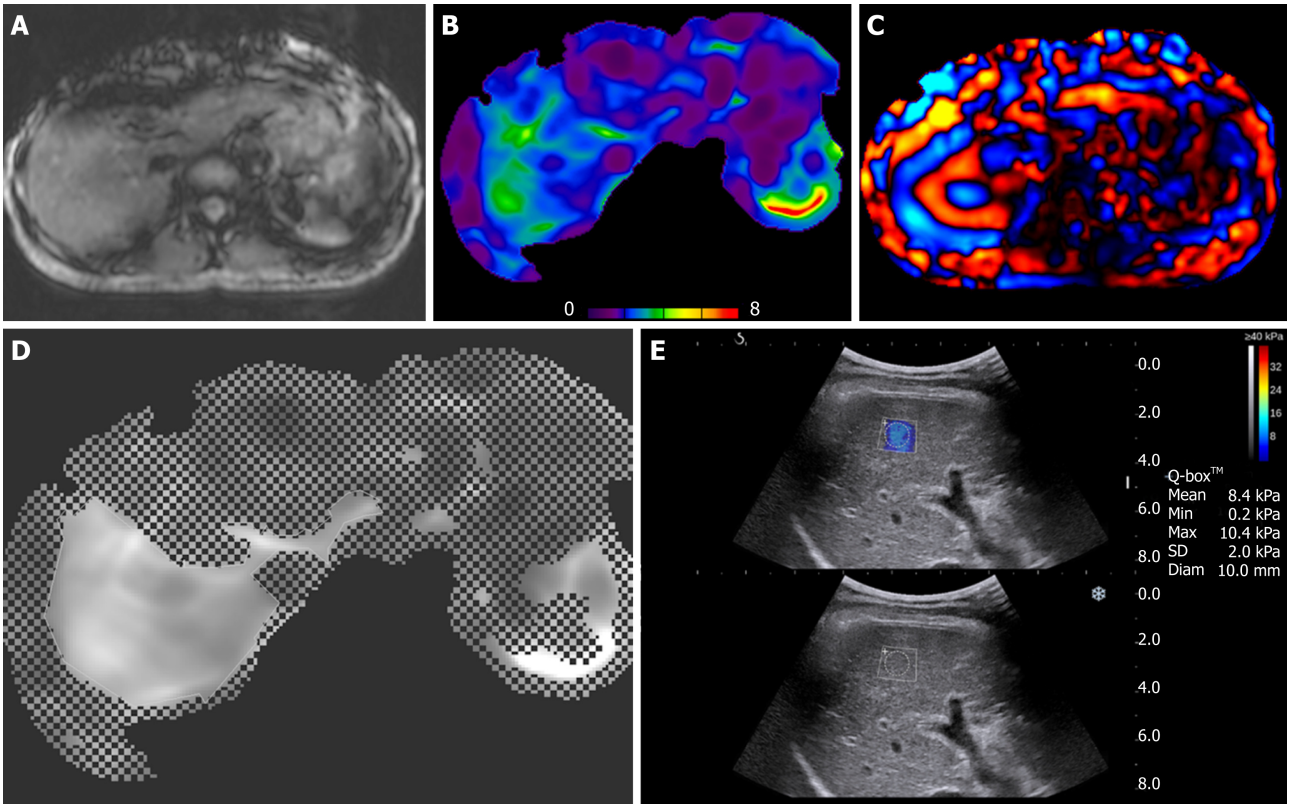


Figure 1 Images of magnetic resonance elastography (3A, 3B, 3C, 3D) and two-dimensional shear-wave elastography (3E) in 42-year old treatment-naïve chronic hepatitis B woman with fibrosis stage 3 on METAVIR score. A: anatomic image, B: elastography with color mapping, C: wave image, D: confidence map of an elastography in right lobe of the liver, and E: two-dimensional shear-wave elastography (2D-SWE) (top) and gray-scale (bottom) images of the right hepatic lobe. Her liver stiffness values of magnetic resonance elastography and 2D-SWE were 2.66 kPa and 8.4 kPa, respectively.

analysis; areas under the curve (AUCs) with 95% confidence intervals, sensitivity, specificity, and positive and negative predictive values were used for the classification of significant fibrosis (≥ F2) and cirrhosis (F4). AUCs were compared using the method of DeLong *et al*. A *P* value less than 0.05 was considered to indicate a significant difference. All statistical analyses were performed by using commercially available software programs (SPSS version 17, SPSS, Chicago, IL, United States; MedCalc, version 11.6, MedCalc Software, Mariakerke, Belgium).

RESULTS

Patient characteristics

Among 67 participants, MRE failed to provide liver stiffness values in one patient because there were no visible waves on MRE images due to overweight (BMI = 27.9) (technical failure rate, 1.5%). With regard to 2D-SWE, a proper elasticity map was not ade-quately displayed in three patients due to overweight (n = 2), or uncontrolled respiration (n = 1), yielding a 4.5% technical failure rate.

Finally, a total of 63 patients who could be successfully measured using both MRE and 2D-SWE were evaluated in this study. All 63 patients were treatment naïve and included 37 men and 26 women, with a median (range) age of 50 (30-68) years. The mean (± SD) levels of serum ALT were 44 ± 20.8 U/L. The median HBV-DNA levels of 35 HBeAg-positive CHB patients and 28 HBeAg-negative patients were 6.93 ± 1.25 $\log_{10}$ IU/mL and 4.35 ± 0.59 $\log_{10}$ IU/mL, respectively. Histopathologically, 3, 16, 14, 14, and 16 patients were diagnosed with fibrosis stage F0 to F4, respectively. The main characteristics of the patients are shown in Table 1.

Relationship between MRE, 2D-SWE, FIB-4, APRI and histological findings

The measurements of MRE, 2D-SWE, FIB-4 and APRI for different fibrosis stages are shown in Table 2. All measurements increased as the fibrosis score increased (MRE, F = 50.642, ^{a}P < 0.001; 2D-SWE, F = 16.063, ^{b}P < 0.001; FIB-4, F = 8.608, ^{c}P < 0.001; APRI, F = 4.165, ^{d}P = 0.010). Distributions of the liver stiffness values of MRE, 2D-SWE, and the FIB-4 and APRI scores in comparison with the different fibrosis stages using

Table 1 The baseline characteristics of the enrolled treatment-naïve chronic hepatitis B patients with normal or minimally raised alanine aminotransferase levels

Characteristics (n = 63)	
Sex, male/female	37/26
Age, mean (± SD) yr	50.8 (± 8.9)
Body mass index, mean (± SD) kg/m^2	23.4 (± 3.4)
AST, mean (± SD), IU/L (normal 4-40 IU/L)	43.5 (± 22.6)
ALT, mean (± SD), IU/L (normal 4-40 IU/L)	44.0 (± 20.8)
Platelet counts, mean (± SD), × 10^3/mm^3	163.5 (± 39.4)
Prothrombin time, mean (± SD), INR	1.05 (± 0.08)
Total bilirubin, mean (± SD), mg/dL	0.75 (± 0.47)
Albumin, mean (± SD), g/dL	3.97 (± 0.24)
γ-glutamyl transferase, mean (± SD), U/L	47.5 (± 31.0)
HBeAg status, positive/negative	35/28
HBV-DNA, mean (± SD), $\log_{10}$ IU/mL	5.78 (± 1.64)
Grade of inflammatory activity (0/1/ 2/3)	9/26/15/13
Fibrosis stage (0/1/2/3 /4)	3/16/14/ 14/16

ALT: Alanine transaminase; AST: Aspartate transaminase; INR: International normalized ratio; SD: Standard deviation.

METAVIR scores as the reference methods are shown in Figure 2. MRE revealed a statistical significance in distinguishing between F0/1 and F2 fibrosis stages (^eP = 0.022), whereas 2D-SWE showed a broad overlap for those stages. Compared to MRE and 2D-SWE, large overlaps existed even with F4 fibrosis stage in FIB-4 and APRI, and they showed a wide range of readings (large SDs).

MRE showed strong correlations with fibrosis stage (MRE, r = 0.869, ^fP < 0.001; Spearman correlation), whereas 2D-SWE, FIB-4 and APRI scores showed a moderate correlation with fibrosis stage (SWE, r = 0.649, ^gP < 0.001; FIB-4, r = 0.517, ^hP < 0.001; APRI, r = 0.431, ^iP < 0.001: Spearman correlation). Using the Steiger test, the correlation coefficient between the liver stiffness values of MRE and liver fibrosis stage is significantly higher than that between the liver stiffness values of 2D-SWE and fibrosis stage (^jP < 0.001). MRE and 2D-SWE measurements showed a moderate correlation with each other (MRE and 2D-SWE, r = 0.669, ^kP < 0.001), while there were moderate or weak correlations between radiology-based and serum-based measurements (MRE and FIB4, r = 0.465, ^lP < 0.001; MRE and APRI, r = 0.378, ^mP = 0.002; 2D-SWE and FIB4, r = 0.553, ^nP < 0.001; 2D-SWE and APRI, r = 0.396, ^oP = 0.001: Spearman correlation).

Analyses of clinical parameters associated with liver stiffness values measured by MRE or 2D-SWE

We investigated the factors that affect liver stiffness values by MRE and 2D-SWE. These parameters include sex, age, body mass index (BMI), platelet counts, total bilirubin, albumin, AST, ALT, γ-GT, prothrombin time, HBeAg status, HBV-DNA levels, inflammatory grade, and liver fibrosis stage (Table 3). Concerning MRE, a univariate analysis revealed correlations between liver stiffness values of MRE and platelet counts, inflammatory grade, and liver fibrosis stage, and a multivariate analysis showed that only the liver fibrosis stage was an independent factor affecting liver stiffness values of MRE. Concerning 2D-SWE, a univariate analysis revealed correlations between liver stiffness values of 2D-SWE and BMI, platelet counts, inflammatory grade, and liver fibrosis stage, and a multivariate analysis showed that not only the liver fibrosis stage but also BMI were independent factors affecting liver stiffness values of 2D-SWE.

Comparing liver stiffness values measured by MRE or 2D-SWE from FIB-4 or APRI scores for the diagnosis of significant fibrosis (≥ F2) and cirrhosis (F4)

The areas under ROC curve (AUCs), cut-off values, sensitivity, specificity, positive predictive values, and negative predictive values for the diagnosis of significant fibrosis (≥ F2) and cirrhosis (F4) using radiology-based or serum-based measurement indices are shown in Table 4. The AUCs for MRE, 2D-SWE, FIB-4, and APRI scores were 0.906, 0.843, 0.697, and 0.717, respectively, for the diagnosis of significant fibrosis, and 0.894, 0.816, 0.786, and 0.701, respectively, for the diagnosis of cirrhosis.

Table 2 Statistics of liver stiffness value measured by magnetic resonance elastography and two-dimensional shear wave elastography, fibrosis index based on four factors score, and aspartate transaminase-to-platelet ratio index score with the Spearman's coefficients according to fibrosis stages

	F0/F1 (*n* = 19)	F2 (*n* = 14)	F3 (*n* = 14)	F4 (*n* = 16)	*r*	*P* value
MRE	1.96 ± 0.43	2.46 ± 0.54	2.91 ± 0.45	3.91 ± 0.50	0.869	< 0.001
2D-SWE	6.09 ± 1.58	8.05 ± 2.23	8.16 ± 2.08	11.39 ± 3.01	0.649	< 0.001
FIB-4	1.75 ± 0.61	1.76 ± 0.60	2.48 ± 0.91	3.21 ± 1.45	0.517	< 0.001
APRI	0.58 ± 0.20	0.71 ± 0.46	0.95 ± 0.44	1.04 ± 0.58	0.431	< 0.001

MRE: Magnetic resonance elastography; 2D-SWE: Two-dimensional shear wave elastography; FIB-4: Fibrosis index based on four factors; APRI: Aspartate transaminase-to-platelet ratio index.

The AUCs of the MRE and 2D-SWE for the diagnosis of significant fibrosis were more than 0.80, with no statistically significant differences between indicators. The performance of MRE for the diagnosis of significant fibrosis was significantly better than that of serum-based measurements by pairwise comparison of the ROC curves (MRE *vs* FIB-4, $^{p}P = 0.002$; MRE *vs* APRI, $^{q}P = 0.010$, respectively). In addition, the performance of SWE was not significantly different compared to FIB-4 or APRI for the diagnosis of significant fibrosis (Figure 3A).

The AUCs of the radiology-based measurements for the diagnosis of cirrhosis were more than 0.80, and their performance was not significantly different from that of serum-based measurements for the identification of cirrhosis (F4) (Figure 3B).

DISCUSSION

The accurate diagnosis of significant fibrosis is of particular clinical value for treatment-naïve CHB patients with high viral loads but with normal or mildly elevated ALT levels because it is considered an indicator for antiviral treatment[3-5]. Among 63 patients analyzed in our study, 44 (69.8%) patients should need to initiate antiviral therapy because they were diagnosed with significant fibrosis. If they did not undergo liver biopsy, they did not fulfill the indications for antiviral therapy. Therefore, a main application of our research is intended to reduce the need for invasive liver biopsy by assessing and comparing noninvasive measurements for a precise diagnosis of significant fibrosis and, consequently, to assist in making antiviral treatment decisions. Our results showed that MRE was able to better discriminate significant fibrosis from normal or mild fibrosis than 2D-SWE. Furthermore, MRE showed a higher correlation coefficient value with fibrosis stage than that between 2D-SWE and fibrosis stage. Moreover, the performance of MRE for diagnosing significant fibrosis was better than that of FIB-4 and APRI, whereas the performance of SWE was not significantly different from that of FIB-4 or APRI. Furthermore, liver fibrosis stage was the only independent factor affecting the liver stiffness values of MRE, whereas BMI as well as liver fibrosis stage can affect the liver stiffness values of 2D-SWE. In addition, technical failure rate was lower in MRE ($n = 1$, 1.5%) than in 2D-SWE ($n = 3$, 4.5%). In our study, MRE could significantly discriminate between F0/1 and F2 fibrosis stage ($P = 0.022$), whereas 2D-SWE showed a broad overlap for those stages. The correlation coefficient between fibrosis stage and the liver stiffness values of MRE ($r = 0.859$) is higher than that between fibrosis stage and the values of 2D-SWE, FIB-4, and APRI ($r = 0.647$, $r = 0.498$, $r = 0.442$, respectively). These data suggest that MRE has a better diagnostic performance in the identification of significant fibrosis than 2D-SWE as well as FIB-4 and APRI, and this is similar to a previous study comparing MR-based and US-based elastographies[17,19]. The possible reason may be that MRE can measure a larger volume of liver, and therefore potentially assesses the stiffness of nearly the entire liver, whereas SWE is able to analyze a smaller volume of liver[27,28]. Thus, MRE is more representative of liver parenchyma with less sampling variability[29,30].

The AUCs in our study showed that MRE has excellent diagnostic accuracy in the assessment of significant fibrosis. The AUC of MRE was numerically higher than that of 2D-SWE but the difference was statistically insignificant (0.906 *vs* 0.843). The statistical insignificance might be explained by the homogeneity of the patients in our study, as our study selected only CHB patients with normal or mildly elevated ALT levels, who are borderline in terms of a decision to initiate antiviral treatment, whereas the previous studies, which showed MRE has statistically significant higher

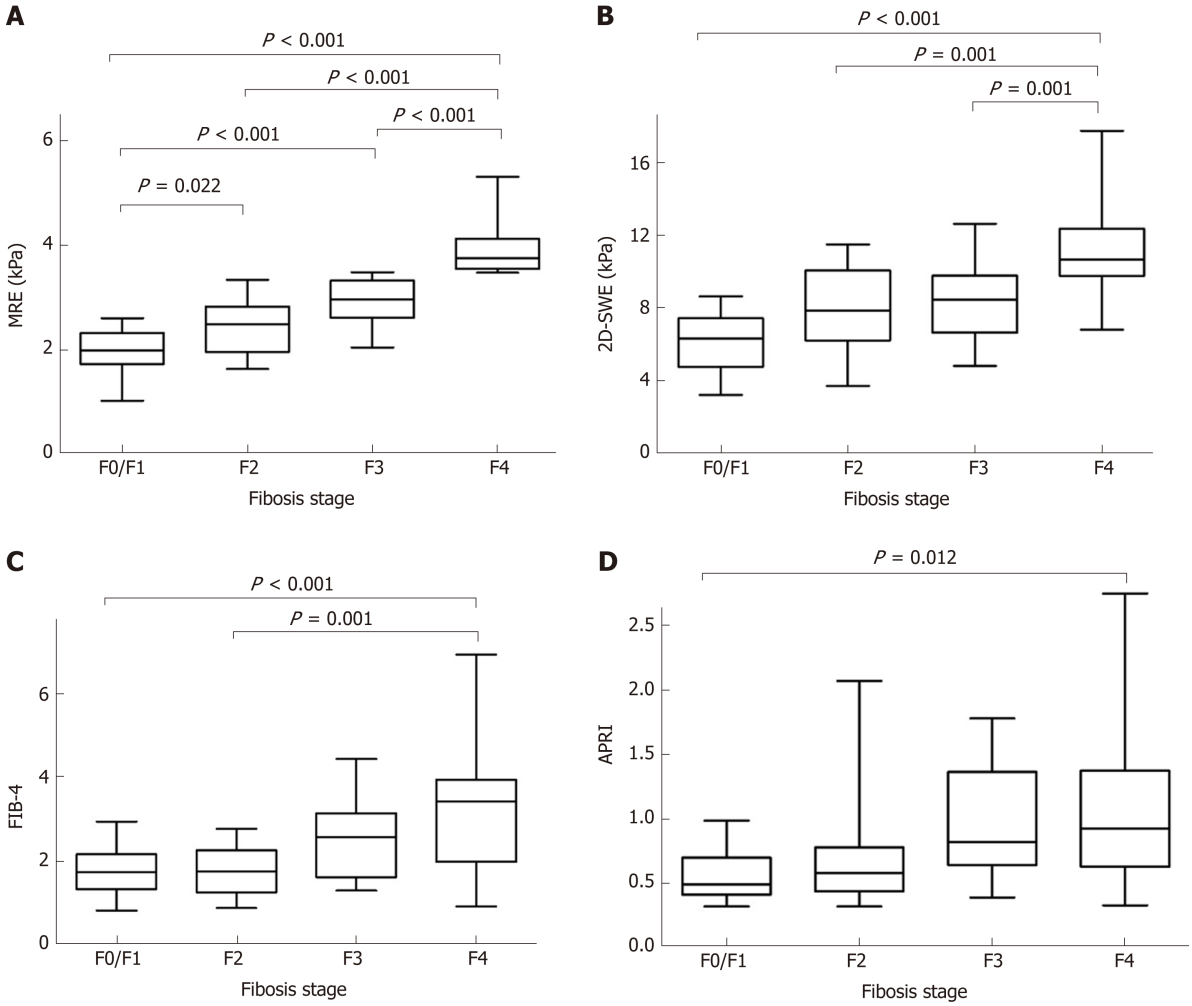


Figure 2 Box-and-whisker plots showing median and ranges for (A) magnetic resonance elastography, (B) two-dimensional shear-wave elastography, (C) fibrosis index based on four factors, (D) aspartate transaminase to platelet ratio index at different stages of liver fibrosis on METAVIR score. MRE: Magnetic resonance elastography; 2D-SWE: Two-dimensional shear-wave elastography; APRI: Aspartate transaminase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

accuracy than US-based elastography, enrolled participants with a wide range of ALT values[16,27,28,31,32]. Compared to serum-based indices, the diagnostic performance of MRE for diagnosing significant fibrosis is better than those of FIB-4 and APRI, whereas the performance of 2D-SWE is not significantly different from those of FIB-4 and APRI. These data suggested that among MRE and 2D-SWE, only MRE might help identify CHB patients who may benefit from treatment compared to serum based indices, such as FIB-4 or APRI.

We also investigated the confounding factors affecting liver stiffness values by MRE and 2D-SWE, including sex, age, BMI, platelet counts, total bilirubin, albumin, AST, ALT, γ-GT, prothrombin time, HBeAg status, HBV-DNA levels, inflammatory grade, and liver fibrosis stage. Except for liver fibrosis stage, the multivariate linear regression analysis revealed no associations between those factors and liver stiffness values of MRE. However, BMI and liver fibrosis stage were independent factors affecting liver stiffness values of 2D-SWE, and these data suggested that BMI might be a confounder that decreases liver stiffness values of 2D-SWE, potentially causing underestimation of the real liver fibrosis stage. The reason why BMI affect liver stiffness measurements of 2D-SWE is not clear. A possible explanation is that high BMI is the most common condition associated with hepatic steatosis, and several studies have shown that the liver stiffness value of US-based elastography is fundamentally influenced by hepatic liver fat content[33,34]. On the other hand, a few clinical studies revealed that hepatic steatosis did not affect liver stiffness values of MRE[35,36].

There are some limitations to the present study. First, the use of liver biopsy as the reference standard for assessing liver fibrosis has limitations associated with sampling errors, as well as intra- and interobserver variability, which are at least partly linked

Table 3 Factors associated with the values of liver stiffness measured by magnetic resonance elastography and two-dimensional shear wave elastography in univariate and multivariate linear regression analyses

Parameters	Factors associated with liver stiffness values by MRE				Factors associated with liver stiffness values by 2D-SWE			
	Univariate	*P* value	Multivariate	*P* value	Univariate	*P* value	Multivariate	*P* value
Sex, male/female	-0.054 (-0.508, 0.401)	0.815			0.685 (-0.840, 2.210)	0.372		
Age, yr	0.019 (-0.006, 0.044)	0.132			0.067 (-0.017, 0.151)	0.117		
BMI, kg/m^2	-0.048 (-0.114, 0.018)	0.149			-0.251 (-0.469, -0.034)	0.024	-0.186 (-0.366, -0.007)	0.042
AST, U/L	0.007 (-0.003, 0.017)	0.157			0.032 (-0.001, 0.065)	0.054		
ALT, U/L	0.002 (-0.009, 0.013)	0.674			0.000 (-0.037, 0.037)	0.995		
PLT counts, × 10^3/mm^3	-0.007 (-0.012, -0.001)	0.014	0.001 (-0.002, 0.005)	0.530	-0.027 (-0.045, -0.009)	0.004	-0.012 (-0.029, 0.004)	0.136
PT, INR	1.312 (-1.595, 4.220)	0.370			8.658 (-0.971, 18.288)	0.077		
Total bilirubin, mg/dL	0.331 (-0.140, 0.802)	0.165			0.098 (-1.517, 1.713)	0.904		
Albumin, g/dL	-0.540 (-1.510, 0.429)	0.270			-3.152 (-6.359, 0.055)	0.054		
γ-GT, U/L	0.004 (-0.003, 0.011)	0.302			0.012 (-0.012, 0.036)	0.327		
HBeAg status, +/-	0.166 (-0.282, 0.615)	0.461			1.052 (-0.445, 2.549)	0.165		
HBV-DNA, $\log_{10}$ IU/mL	-0.051 (-0.189, 0.086)	0.456			-0.076 (-0.541, 0.389)	0.745		
Inflammatory grade	0.363 (0.153, 0.573)	0.001	0.105 (-0.031, 0.241)	0.129	0.903 (0.163, 1.644)	0.018	0.220 (-0.411, 0.852)	0.487
Fibrosis stage	0.626 (0.520, 0.732)	< 0.001	0.609 (0.487, 0.731)	< 0.001	1.616 (1.116, 2.116)	< 0.001	1.276 (0.690, 1.863)	< 0.001

MRE: Magnetic resonance elastography; 2D-SWE: Two-dimensional shear wave elastography; ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Body mass index; INR: International normalized ratio; PLT: Platelet; PT: Prothrombin time; γ-GT: Gamma-GT.

to the size of the biopsy. Second, despite MRE has the best effectiveness, it is much more expensive than 2D-SWE and is available only in tertiary centers. Third, as the sample size of this study is relatively small, the present results need to be validated independently in further studies.

In conclusion, MRE might be a non-invasive and more precise measurement for the assessment of significant fibrosis compared to 2D-SWE as well as serum-based indices in treatment-naive CHB patients with high viral loads but with normal or mildly elevated ALT levels who should be considered for initiation of antiviral therapy depending on the presence of significant fibrosis.

Table 4 Diagnostic performance of magnetic resonance elastography and two-dimensional shear wave elastography, fibrosis index based on four factors score, and aspartate transaminase-to-platelet ratio index for evaluation of significant fibrosis (≥ F2) and cirrhosis (F4)

		AUC (95%CI)	*P* value	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MRE	≥ F2	0.906 (0.806, 0.965)	< 0.001	> 2.47 (kPa)	81.8	94.7	97.3	69.2
	F4	0.894 (0.791, 0.958)	< 0.001	> 3.46 (kPa)	88.9	97.8	94.1	95.6
2D-SWE	≥ F2	0.843 (0.730, 0.923)	< 0.001	> 6.73 (kPa)	84.1	68.4	86.0	65.0
	F4	0.816 (0.698, 0.902)	< 0.001	> 9.50 (kPa)	77.8	80.0	60.9	90.0
FIB-4	≥ F2	0.697 (0.568, 0.806)	0.003	> 1.80	70.5	63.2	81.6	48.0
	F4	0.786 (0.665, 0.880)	< 0.001	> 3.22	50.0	97.8	90.0	83.0
APRI	≥ F2	0.717 (0.590, 0.823)	0.001	> 0.49	84.1	52.6	80.4	58.8
	F4	0.701 (0.572, 0.810)	0.006	> 0.96	50.0	84.4	562	80.9

MRE: Magnetic resonance elastography; 2D-SWE: Two-dimensional shear wave elastography; FIB-4: Fibrosis index based on four factors; APRI: Aspartate transaminase-to-platelet ratio index; AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value.

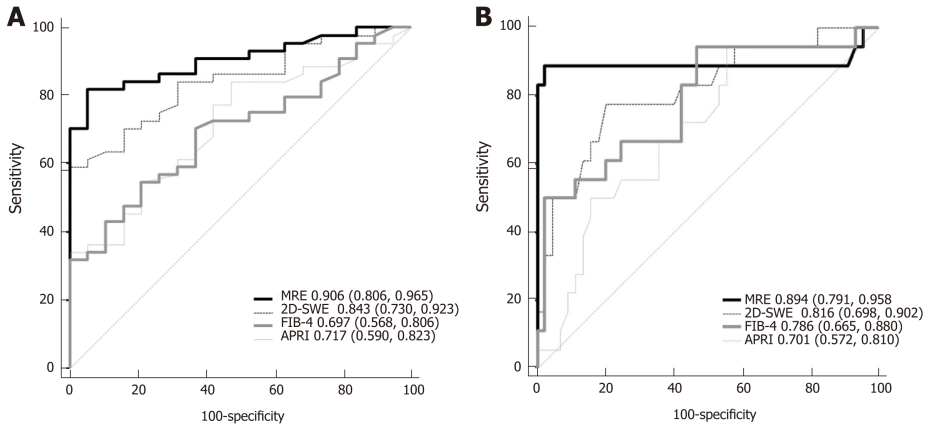


Figure 3 Graphs showing area under the receiver operating characteristic curves of magnetic resonance elastography, two-dimensional shear-wave elastography, fibrosis index based on four factors, and aspartate transaminase to platelet ratio index for prediction of significant fibrosis (A) and cirrhosis (B) in trea-tment-naive chronic hepatitis B patients with normal or mildly elevated alanine aminotransferase. MRE: Magnetic resonance elastography; 2D-SWE: Two-dimensional shear-wave elastography; APRI: Aspartate transaminase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

ARTICLE HIGHLIGHTS

Research background

Accurate detection of significant fibrosis (fibrosis stage 2 or higher on the METAVIR scale) is important especially for chronic hepatitis B (CHB) patients with high viral loads but with normal or mildly elevated alanine aminotransferase (ALT) levels because the presence of significant fibrosis is accepted as the indication for antiviral treatment. Liver biopsy is the reference standard for diagnosing significant fibrosis, but it is an invasive procedure. Consequently, non-invasive imaging-based measurements, such as magnetic resonance elastography (MRE) or two-dimensional shear-wave elastography (2D-SWE), have been proposed for the quantitative assessment of liver fibrosis.

Research motivation

Liver biopsy is still considered the "gold standard" for the evaluation of significant fibrosis in CHB patients. However, its utilization is often restricted because its invasiveness can cause life threatening complications. Moreover, tissue obtained *via* biopsy represents approximately only 1/50000 of the liver volume, which may result in a sampling error and is associated with considerable interobserver variability in the microscopic evaluation. Furthermore, repeating the liver biopsy to monitor changes in liver fibrotic burden is generally not feasible in clinical practice.

Research objectives

The objective of this study was to evaluate the liver stiffness values of MRE and two-dimensional SWE (2D-SWE) to assess liver fibrosis and to compare their diagnostic performances with those of FIB-4 and APRI for the prediction of significant fibrosis, which is an indicator for initiating antiviral therapy in treatment-naïve CHB patients with high viral loads but with borderline-

normal or mildly elevated ALT levels.

Research methods

The study enrolled 63 treatment-naïve CHB patients with high viral loads but with normal or mildly elevated ALT levels who underwent liver biopsy before a decision was made to initiate antiviral therapy. MRE and 2D-SWE were performed, and serum-based indices, such as FIB-4 and APRI, were calculated. The diagnostic performances of MRE, 2D-SWE, FIB-4, and APRI for assessing significant fibrosis (≥ F2) and cirrhosis (F4) were evaluated with liver histology as the reference standard, using receiver operating characteristic analyses.

Research results

The liver fibrosis stage was F0/F1 in 19, F2 in 14, F3 in 14, and F4 in 16 patients, respectively. MRE significantly discriminated F2 from F0/1 (P = 0.022), whereas 2D-SWE showed a broad overlap in distinguishing those stages. MRE showed a higher correlation coefficient value with fibrosis stage than 2D-SWE with fibrosis stage (0.859 *vs* 0.647, Spearman test; P < 0.001). Multiple-regression analyses showed that fibrosis stage was the only factor affecting the values of MRE (P < 0.001), whereas body mass index (P = 0.042) and fibrosis stage (P < 0.001) were independent factors affecting 2D-SWE values. The MRE performance for diagnosing significant fibrosis was better than FIB-4 (P = 0.002) and APRI (P = 0.010), whereas the performance of 2D-SWE was not significantly different from that of FIB-4 or APRI.

Research conclusions

MR elastography might be a non-invasive and more precise measurement for the assessment of significant fibrosis compared to 2D-SWE as well as serum-based indices in treatment-naïve CHB patients with high viral loads but with normal or mildly elevated ALT levels who should be considered for initiation of antiviral therapy depending on the presence of significant fibrosis.

Research perspectives

There are some limitations to the present study. First, the use of liver biopsy as the reference standard for assessing liver fibrosis has limitations associated with sampling errors, as well as intra and interobserver variability, which are at least partly linked to the size of the biopsy. Second, despite MRE has the best effectiveness, it is much more expensive than 2D-SWE and is available only in tertiary centers. Third, as the sample size of this study is relatively small, the pre-sent results need to be validated independently in further studies.

REFERENCES

1 **Seto WK**, Lo YR, Pawlotsky JM, Yuen MF. Chronic hepatitis B virus infection. *Lancet* 2018; **392**: 2313-2324 [PMID: 30496122 DOI: 10.1016/S0140-6736(18)31865-8]

2 **Cho EJ**, Kim SE, Suk KT, An J, Jeong SW, Chung WJ, Kim YJ. Current status and strategies for hepatitis B control in Korea. *Clin Mol Hepatol* 2017; **23**: 205-211 [PMID: 28942624 DOI: 10.3350/cmh.2017.0104]

3 **Terrault NA**, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH; American Association for the Study of Liver Diseases. AASLD guidelines for treatment of chronic hepatitis B. *Hepatology* 2016; **63**: 261-283 [PMID: 26566064 DOI: 10.1002/hep.28156]

4 **European Association for the Study of the Liver**. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]

5 **Sarin SK**, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int* 2016; **10**: 1-98 [PMID: 26563120 DOI: 10.1007/s12072-015-9675-4]

6 **Bravo AA**, Sheth SG, Chopra S. Liver biopsy. *N Engl J Med* 2001; **344**: 495-500 [PMID: 11172192 DOI: 10.1056/NEJM200102153440706]

7 **Bedossa P**, Carrat F. Liver biopsy: the best, not the gold standard. *J Hepatol* 2009; **50**: 1-3 [PMID: 19017551 DOI: 10.1016/j.jhep.2008.10.014]

8 **Rockey DC**, Caldwell SH, Goodman ZD, Nelson RC, Smith AD; American Association for the Study of Liver Diseases. Liver biopsy. *Hepatology* 2009; **49**: 1017-1044 [PMID: 19243014 DOI: 10.1002/hep.22742]

9 **Srinivasa Babu A**, Wells ML, Teytelboym OM, Mackey JE, Miller FH, Yeh BM, Ehman RL, Venkatesh SK. Elastography in Chronic Liver Disease: Modalities, Techniques, Limitations, and Future Directions. *Radiographics* 2016; **36**: 1987-2006 [PMID: 27689833 DOI: 10.1148/rg.2016160042]

10 **Martínez SM**, Crespo G, Navasa M, Forns X. Noninvasive assessment of liver fibrosis. *Hepatology* 2011; **53**: 325-335 [PMID: 21254180 DOI: 10.1002/hep.24013]

11 **Zeng DW**, Dong J, Liu YR, Jiang JJ, Zhu YY. Noninvasive models for assessment of liver fibrosis in patients with chronic hepatitis B virus infection. *World J Gastroenterol* 2016; **22**: 6663-6672 [PMID: 27547009 DOI: 10.3748/wjg.v22.i29.6663]

12 **Wu Z**, Dong X, Wang G, Zhao H; China HepB-Related Fibrosis Assessment Research Group. Clinical noninvasive markers for antiviral therapy decision in chronic hepatitis B with alanine aminotransferase less than two times upper limit of normal. *J Viral Hepat* 2019; **26**: 287-296 [PMID: 30380162 DOI: 10.1111/jvh.13030]

13 **Xiao G**, Yang J, Yan L. Comparison of diagnostic accuracy of aspartate aminotransferase to platelet ratio index and fibrosis-4 index for detecting liver fibrosis in adult patients with chronic hepatitis B virus infection: a systemic review and meta-analysis. *Hepatology* 2015; **61**: 292-302 [PMID: 25132233 DOI: 10.1002/hep.27382]

14 **Barr RG**, Ferraioli G, Palmeri ML, Goodman ZD, Garcia-Tsao G, Rubin J, Garra B, Myers RP, Wilson

SR, Rubens D, Levine D. Elastography Assessment of Liver Fibrosis: Society of Radiologists in Ultrasound Consensus Conference Statement. *Radiology* 2015; **276**: 845-861 [PMID: 26079489 DOI: 10.1148/radiol.2015150619]

15 **Kennedy P**, Wagner M, Castéra L, Hong CW, Johnson CL, Sirlin CB, Taouli B. Quantitative Elastography Methods in Liver Disease: Current Evidence and Future Directions. *Radiology* 2018; **286**: 738-763 [PMID: 29461949 DOI: 10.1148/radiol.2018170601]

16 **Yoon JH**, Lee JM, Joo I, Lee ES, Sohn JY, Jang SK, Lee KB, Han JK, Choi BI. Hepatic fibrosis: prospective comparison of MR elastography and US shear-wave elastography for evaluation. *Radiology* 2014; **273**: 772-782 [PMID: 25007047 DOI: 10.1148/radiol.14132000]

17 **Zhuang Y**, Ding H, Zhang Y, Sun H, Xu C, Wang W. Two-dimensional Shear-Wave Elastography Performance in the Noninvasive Evaluation of Liver Fibrosis in Patients with Chronic Hepatitis B: Comparison with Serum Fibrosis Indexes. *Radiology* 2017; **283**: 873-882 [PMID: 27982760 DOI: 10.1148/radiol.2016160131]

18 **Tsochatzis EA**, Gurusamy KS, Ntaoula S, Cholongitas E, Davidson BR, Burroughs AK. Elastography for the diagnosis of severity of fibrosis in chronic liver disease: a meta-analysis of diagnostic accuracy. *J Hepatol* 2011; **54**: 650-659 [PMID: 21146892 DOI: 10.1016/j.jhep.2010.07.033]

19 **Venkatesh SK**, Wang G, Lim SG, Wee A. Magnetic resonance elastography for the detection and staging of liver fibrosis in chronic hepatitis B. *Eur Radiol* 2014; **24**: 70-78 [PMID: 23928932 DOI: 10.1007/s00330-013-2978-8]

20 **Lee JE**, Lee JM, Lee KB, Yoon JH, Shin CI, Han JK, Choi BI. Noninvasive assessment of hepatic fibrosis in patients with chronic hepatitis B viral infection using magnetic resonance elastography. *Korean J Radiol* 2014; **15**: 210-217 [PMID: 24643284 DOI: 10.3348/kjr.2014.15.2.210]

21 **Venkatesh SK**, Ehman RL. Magnetic resonance elastography of liver. *Magn Reson Imaging Clin N Am* 2014; **22**: 433-446 [PMID: 25086938 DOI: 10.1016/j.mric.2014.05.001]

22 **Petitclerc L**, Sebastiani G, Gilbert G, Cloutier G, Tang A. Liver fibrosis: Review of current imaging and MRI quantification techniques. *J Magn Reson Imaging* 2017; **45**: 1276-1295 [PMID: 27981751 DOI: 10.1002/jmri.25550]

23 **Liu J**, Zhao J, Zhang Y, Ji Y, Lin S, Dun G, Guo S. Noninvasive Assessment of Liver Fibrosis Stage Using Ultrasound-Based Shear Wave Velocity Measurements and Serum Algorithms in Patients With Viral Hepatitis B: A Retrospective Cohort Study. *J Ultrasound Med* 2017; **36**: 285-293 [PMID: 28039877 DOI: 10.7863/ultra.16.01069]

24 **Sterling RK**, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, S Sulkowski M, Torriani FJ, Dieterich DT, Thomas DL, Messinger D, Nelson M; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317-1325 [PMID: 16729309 DOI: 10.1002/hep.21178]

25 **Wai CT**, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518-526 [PMID: 12883497 DOI: 10.1053/jhep.2003.50346]

26 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293 [PMID: 8690394 DOI: 10.1002/hep.510240201]

27 **Singh S**, Venkatesh SK, Wang Z, Miller FH, Motosugi U, Low RN, Hassanein T, Asbach P, Godfrey EM, Yin M, Chen J, Keaveny AP, Bridges M, Bohte A, Murad MH, Lomas DJ, Talwalkar JA, Ehman RL. Diagnostic performance of magnetic resonance elastography in staging liver fibrosis: a systematic review and meta-analysis of individual participant data. *Clin Gastroenterol Hepatol* 2015; **13**: 440-451.e6 [PMID: 25305349 DOI: 10.1016/j.cgh.2014.09.046]

28 **Xiao H**, Shi M, Xie Y, Chi X. Comparison of diagnostic accuracy of magnetic resonance elastography and Fibroscan for detecting liver fibrosis in chronic hepatitis B patients: A systematic review and meta-analysis. *PLoS One* 2017; **12**: e0186660 [PMID: 29107943 DOI: 10.1371/journal.pone.0186660]

29 **Lee Yj**. , Lee JM, Lee JE, Lee KB, Lee ES, Yoon JH, Yu MH, Baek JH, Shin CI, Han JK, Choi BI. MR elastography for noninvasive assessment of hepatic fibrosis: reproducibility of the examination and reproducibility and repeatability of the liver stiffness value measurement. *J Magn Reson Imaging* 2014; **39**: 326-331 [PMID: 23589232 DOI: 10.1002/jmri.24147]

30 **Huwart L**, Sempoux C, Vicaut E, Salameh N, Annet L, Danse E, Peeters F, ter Beek LC, Rahier J, Sinkus R, Horsmans Y, Van Beers BE. Magnetic resonance elastography for the noninvasive staging of liver fibrosis. *Gastroenterology* 2008; **135**: 32-40 [PMID: 18471441 DOI: 10.1053/j.gastro.2008.03.076]

31 **Cui J**, Heba E, Hernandez C, Haufe W, Hooker J, Andre MP, Valasek MA, Aryafar H, Sirlin CB, Loomba R. Magnetic resonance elastography is superior to acoustic radiation force impulse for the Diagnosis of fibrosis in patients with biopsy-proven nonalcoholic fatty liver disease: A prospective study. *Hepatology* 2016; **63**: 453-461 [PMID: 26560734 DOI: 10.1002/hep.28337]

32 **Ichikawa S**, Motosugi U, Morisaka H, Sano K, Ichikawa T, Tatsumi A, Enomoto N, Matsuda M, Fujii H, Onishi H. Comparison of the diagnostic accuracies of magnetic resonance elastography and transient elastography for hepatic fibrosis. *Magn Reson Imaging* 2015; **33**: 26-30 [PMID: 25308096 DOI: 10.1016/j.mri.2014.10.003]

33 **Yoneda M**, Suzuki K, Kato S, Fujita K, Nozaki Y, Hosono K, Saito S, Nakajima A. Nonalcoholic fatty liver disease: US-based acoustic radiation force impulse elastography. *Radiology* 2010; **256**: 640-647 [PMID: 20529989 DOI: 10.1148/radiol.10091662]

34 **Gaia S**, Carenzi S, Barilli AL, Bugianesi E, Smedile A, Brunello F, Marzano A, Rizzetto M. Reliability of transient elastography for the detection of fibrosis in non-alcoholic fatty liver disease and chronic viral hepatitis. *J Hepatol* 2011; **54**: 64-71 [PMID: 20932598 DOI: 10.1016/j.jhep.2010.06.022]

35 **Lee DH**, Lee JM, Han JK, Choi BI. MR elastography of healthy liver parenchyma: Normal value and reliability of the liver stiffness value measurement. *J Magn Reson Imaging* 2013; **38**: 1215-1223 [PMID: 23281116 DOI: 10.1002/jmri.23958]

36 **Venkatesh SK**, Wang G, Teo LL, Ang BW. Magnetic resonance elastography of liver in healthy Asians: normal liver stiffness quantification and reproducibility assessment. *J Magn Reson Imaging* 2014; **39**: 1-8 [PMID: 24123300 DOI: 10.1002/jmri.24084]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3268-3280

DOI: 10.3748/wjg.v25.i25.3268

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

META-ANALYSIS

Botulinum toxin injections after surgery for Hirschsprung disease: Systematic review and meta-analysis

Daniëlle Roorda, Zarah AM Abeln, Jaap Oosterlaan, Lodewijk WE van Heurn, Joep PM Derikx

ORCID number: Daniëlle Roorda (0000-0001-9740-4957); Zarah AM Abeln (0000-0001-7522-420X); Jaap Oosterlaan (0000-0002-0218-5630); Lodewijk WE van Heurn (0000-0002-8001-1222); Joep PM Derikx (0000-0003-0694-7679).

Author contributions: Roorda D, van Heurn LWE and Derikx JPM designed the research; Roorda D and Abeln ZAM performed the systematic review and data-extraction; Roorda D and Derikx JPM analysed the data; Roorda D, Abeln ZAM, Oosterlaan J, van Heurn LWE and Derikx JPM wrote and revised the paper.

Conflict-of-interest statement: All authors have no conflicts of interest to disclose.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Daniëlle Roorda, Zarah AM Abeln, Lodewijk WE van Heurn, Joep PM Derikx, Department of Pediatric Surgery, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam Reproduction and Development, Amsterdam 1105 AZ, Netherlands

Jaap Oosterlaan, Department of Pediatrics, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam, Emma Neuroscience Group, Amsterdam Reproduction and Development, Amsterdam 1105 AZ, Netherlands

Lodewijk WE van Heurn, Joep PM Derikx, Department of Pediatric Surgery, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam Gastroenterology and Metabolism, Amsterdam 1105 AZ, Netherlands

Corresponding author: Daniëlle Roorda, MD, BSc, MD, Research Associate, PhD Student, Department of Pediatric Surgery, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam Reproduction and Development, Meibergdreef 9, Amsterdam 1105 AZ, Netherlands. d.roorda@amc.uva.nl
Telephone: +31-20-5665693
Fax: +31-20-5669683

Abstract

BACKGROUND

A large proportion of patients with Hirschsprung disease experience persistent obstructive symptoms after corrective surgery. Persistent obstructive symptoms may result in faecal stasis that can develop into Hirschsprung-associated enterocolitis, a potential life-threatening condition. Important treatment to improve faecal passage is internal anal sphincter relaxation using botulinum toxin injections.

AIM

To give an overview of all empirical evidence on the effectiveness of botulinum toxin injections in patients with Hirschsprung disease.

METHODS

A systematic review and meta-analysis was done by searching PubMed, EMBASE and the Cochrane Library, using entry terms related to: (1) Hirschsprung disease; and (2) Botulinum toxin injections. 14 studies representing 278 patients met eligibility criteria. Data that were extracted were proportion of patients with improvement of obstructive symptoms or less enterocolitis after injection, proportion of patients with adverse effects and data on type botulinum toxin, mean dose, average age at first injection and patients with associated

Manuscript source: Invited manuscript

Received: March 14, 2019
Peer-review started: March 14, 2019
First decision: April 11, 2019
Revised: May 5, 2019
Accepted: May 31, 2019
Article in press: June 1, 2019
Published online: July 7, 2019

P-Reviewer: Tam PHK, Tang ST
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

syndromes. Random-effects meta-analysis was used to aggregate effects and random-effects meta-regression was used to test for possible confounding factors.

RESULTS

Botulinum toxin injections are effective in treating obstructive symptoms in on average 66% of patients [event rate (ER) = 0.66, P = 0.004, I^2 = 49.5, n = 278 patients]. Type of botulinum toxin, average dose, average age at first injections and proportion of patients with associated syndromes were not predictive for this effect. Mean 7 duration of improvement after one botulinum toxin injections was 6.4 mo and patients needed on average 2.6 procedures. There was a significant higher response rate within one month after botulinum toxin injections compared to more than one month after Botulinum toxin injections (ER = 0.79, *vs* ER = 0.46, Q = 19.37, P < 0.001). Botulinum toxin injections were not effective in treating enterocolitis (ER 0.58, P = 0.65, I^2 = 71.0, n = 52 patients). There were adverse effects in on average 17% of patients (ER = 0.17, P < 0.001, I^2 = 52.1, n = 187 patients), varying from temporary incontinence to mild anal pain.

CONCLUSION

Findings from this systematic review and meta-analysis indicate that botulinum toxin injections are effective in treating obstructive symptoms and that adverse effects were present, but mild and temporary.

Key words: Hirschsprung disease; Botulinum toxin; Internal anal sphincter; Obstructive symptoms; Enterocolitis; Adverse effects

Core tip: Patients with obstructive symptoms after surgery for Hirschsprung disease can benefit from intra-sphincteric botulinum toxin injections. This study indicates that Botulinum toxin injections are effective in treating obstructive symptoms after surgery for Hirschsprung disease and were associated with mild and temporary adverse effects. The proportion of patients that benefited from botulinum toxin injections was significantly higher within one month after administration (79%) compared to longer follow-up (46%). Beneficial effect was temporary and lasted on average 6 months, therefore requiring on average 2-3 injections per patient before satisfactory clinical improvement was achieved.

Citation: Roorda D, Abeln ZA, Oosterlaan J, van Heurn LW, Derikx JP. Botulinum toxin injections after surgery for Hirschsprung disease: Systematic review and meta-analysis. *World J Gastroenterol* 2019; 25(25): 3268-3280
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3268.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3268

INTRODUCTION

Hirschsprung disease is a congenital absence of ganglions of the distal gut, causing neonatal bowel obstruction. Treatment of Hirschsprung disease consists of surgical resection of the affected aganglionic bowel segment. Despite removal of the affected aganglionic bowel segment, about 8%-30% of patients experience persistent obstructive symptoms after corrective surgery, varying from mild constipation to ileus[1]. Causes of obstructive symptoms include: (1) Mechanical obstruction, such as anastomotic stricture or adhesions; (2) Residual aganglionosis; (3) Stool-holding behavior; (4) General motility disorders of the bowel; and (5) Anal outlet obstruction[1,2], caused by the absence of the recto-anal inhibition reflex or anal sphincter defects[1]. When treated inadequately, persistent obstructive symptoms may result in faecal stasis that can develop into Hirschsprung-associated enterocolitis, a potential life-threatening condition that occurs in 25% to 37% of patients after surgery[3]. Therefore, it is important to achieve adequate bowel passage in patients with Hirschsprung disease.

Many patients with Hirschsprung disease use dietary adaptations, laxatives or rectal irrigation to manage bowel passage after surgery. When these conservative

measures are not enough, a mechanical obstruction or residual aganglionosis needs to be ruled out, according to current consensus-based practice[1]. Current practice describes administration of intra-sphincteric botulinum toxin (BT) injections as a second step in treatment of obstructive symptoms after surgery, in order to obtain temporary relaxation of the internal anal sphincter, which subsequently improves faecal passage. We know from patients with childhood constipation and chronic anal fissures, that BT can be beneficial in treating constipation, regardless of sphincter dynamics[4], suggesting comparable beneficial effects for patients with Hirschsprung disease. Langer was the first to introduce treatment with BT injections for patients with Hirschsprung disease in 1997, as an alternative to myotomy of the anal sphincter and to use it as a predictive tool to assess necessity of sphincter myotomy[5].

Current consensus-based practice advises administration of 60-100 units BT diluted in 1.0 mL of saline with a maximum concentration of 100 IU/mL, given circumferentially at the level of the dentate line where the internal anal sphincter is located. The guideline also states that, BT injections need to be repeated every 3-6 mo as many times as necessary upon clinical improvement, as symptoms often will improve over time and BT injections generally become unnecessary at age older than five years. Alternatively, topical application of nitroglycerin or nifedipine cream or myotomy of the internal anal sphincter may be considered as treatment for post-operative obstructive symptoms. Non-operative management however is recommended, given the risk of faecal soiling after myotomy[1]. All these recommendations however, are consensus-based and are not substantiated by empirical evidence.

A meta-analysis on different treatment strategies for obstructive symptoms showed short-term improvement after BT injections in 77% of patients and decreased to 43% of patients in the long-term[6]. However, that meta-analysis did neither draw conclusions on effects on the prevalence of enterocolitis, nor on the complication rate and adverse events after BT injections. In addition, that meta-analyses did not assess potential predictors of effectiveness. Better knowledge is clearly needed and would benefit indication for treatment with BT injections and management of expectations of patients and their parents.

The current systematic review and meta-analysis aims to provide a comprehensive overview of all empirical evidence on: (1) Effects of treatment with BT injections on obstructive symptoms after surgery for Hirschsprung disease and factors moderating this effect (type of BT used, dose, age and the presence of associated syndromes); (2) Effects of treatment with BT injections on occurrence of post-operative Hirschsprung-associated enterocolitis; and (3) Complication rate and adverse effects after BT injections in patients after surgery for Hirschsprung disease.

MATERIALS AND METHODS

Search strategy

The search strategy combined two groups of search terms and their equivalents: "Hirschsprung disease" AND "Botulinum toxin injections". The search was performed in the electronic databases PubMed, EMBASE, Web of Science and the Cochrane Database using both simple search terms and hierarchical family forms (*e.g.*, Medical Subject Headings, Thesaurus, Emtree). The reference lists of eligible articles were also screened for additional articles. The last search was conducted in December 2018.

Study selection

A flow diagram of the study search and selection is provided in Figure 1. A total of 193 records were identified corresponding to 92 unique articles. Two authors (DR and ZAMA) independently assessed each article for eligibility. Conflicts in the selection process were solved by either consensus or by consulting a third reviewer (JD). Studies were included in this systematic review and meta-analysis if they: (1) Contained original patient data; (2) Described patients with Hirschsprung disease with post-operative obstructive symptoms or enterocolitis; (3) Described treatment of these patients with BT injections in the internal anal sphincter; (4) Described outcomes in terms of occurrence of obstructive symptoms and/or enterocolitis at follow-up; in (5) Were published in a peer-reviewed journal; and (6) Written in the English language. In case multiple articles reported on (partly) overlapping cohorts, we in-cluded the article that had the largest sample size to maximize generalizability of the sample and statistical power of our meta-analysis. In case articles reported patients with obstructive defecation problems that consisted of patients with Hir-schsprung disease and Internal Sphincter Achalasia and data about patients with Hirschsprung disease could not be extracted separately, the study was still included and the data were

extracted for the total sample. 14 studies were included in both the systematic review as in the meta-analysis[5,7-17].

Data extraction and synthesis

Primary outcome was effectiveness of BT injections in treating obstructive symptoms in patients after surgery for Hirschsprung disease, expressed as the proportion of patients with clinical improvement as reported in the various studies, the mean duration of improvement in months and the average number of BT needed to obtain satisfactory clinical improvement. Secondary outcomes were: (1) The proportion of patients that previously suffered from at least one episode of Hirschsprung-associated enterocolitis and were free of enterocolitis after treatment with BT injection; and (2) The proportion of patients with complications and/or adverse effects after BT injection. Primary and secondary outcome measures with the accompanying samples sizes were extracted from the included articles by two authors (DR and ZAMA) from the articles. In addition, possible confounding factors of effectiveness of BT injections in treating obstructive symptoms or Hirschsprung-associated enterocolitis (*e.g.*, type of BT, average dose, average age at first BT injection, proportion of patients with an associated syndrome and proportion of male patients) were extracted from the articles. In case when studies reported medians, these measures were considered as the best approximation of means.

Quality assessment

The quality of the included studies was evaluated using the Newcastle-Ottowa Quality Assessment Scale (NOS)[18]. This scale assesses study quality based on: (1) Selection procedure (4 points); (2) Comparability of controls (2 points); and (3) Outcome measurement (3 points). Thus, nine points can maximally be assigned to each study. In accordance with the Agency for Healthcare Research and Quality standards, quality of studies was considered "good" (Selection 3-4 AND Comparability 1-2 AND Outcome 2-3 points), "fair" (Selection 2 AND Comparability 1-2 AND Outcome 2-3 points) or "poor" (Selection 0-1 OR Comparability 0 OR Outcome 0 points). Quality assessment was conducted by two independent reviewers (ZA and DN) and in case of conflict, resolved with consensus.

Statistical analysis

Analyses were performed using Comprehensive Meta-Analysis Software (version 3.0, Biostat)[19]. For primary and secondary outcomes, the proportion of patients with clinical improvement on obstructive symptoms and enterocolitis, or with adverse effects was calculated for each study and expressed as the event rate (ER). The individual study's effect sizes were subsequently aggregated across studies into meta-analytic effect sizes using the random model to account for heterogeneity introduced by the included range of outcome definitions, differences in follow-up duration and methods for administering BT injections. Mean duration of improvement and mean number of BT injections needed were calculated by averaging these measurements from the studies. Post-hoc analysis tested differences between the meta-analytic findings pertaining to: Outcomes assessed within one month after administration *vs* outcomes assessed in follow-up longer than one month. If a meta-analytic effect size was built up by a minimum of 10 individual studies' effect sizes, we explored possible moderation effects on the outcomes of: (1) Type of BT; (2) Average dose; (3) Average age at first BT injection; (4) Proportion of patients with associated syndromes; and (5) Proportion of males. Only significant univariate moderators were further analyzed using multivariate meta-regression. Furthermore, post-hoc analysis were performed to test for possible differences between the different types of BT used for the injection, by aggregating effect sizes of observations for each type of BT. Sensitivity analyses were performed by repeating analyses after excluding studies that consisted of both patients with Hirschsprung disease and internal sphincter achalasia. ERs significantly higher than 0.50 are suggestive to be found not by chance and were arbitrarily considered to be clinically relevant. Heterogeneity was interpreted as small ($I^2 \leq 0.25$), medium (I^2 = 0.25-0.50) or strong ($I^2 \geq 0.50$), according to Higgins *et al*[20]. The possibility of publication bias was assessed by calculating Funnel plot asymmetry expressed as the Eggers regression intercept *t*[21], fail-safe *n* (fail-safe *n* values > 5 *k*+10 where considered robust)[22] and by calculating the moderating effect of samples sizes on effect sizes. A *P* value of 0.05 was considered statistically significant.

RESULTS

Characteristics of included studies

In this systematic review and meta-analysis, 14 studies (representing 278 patients) met

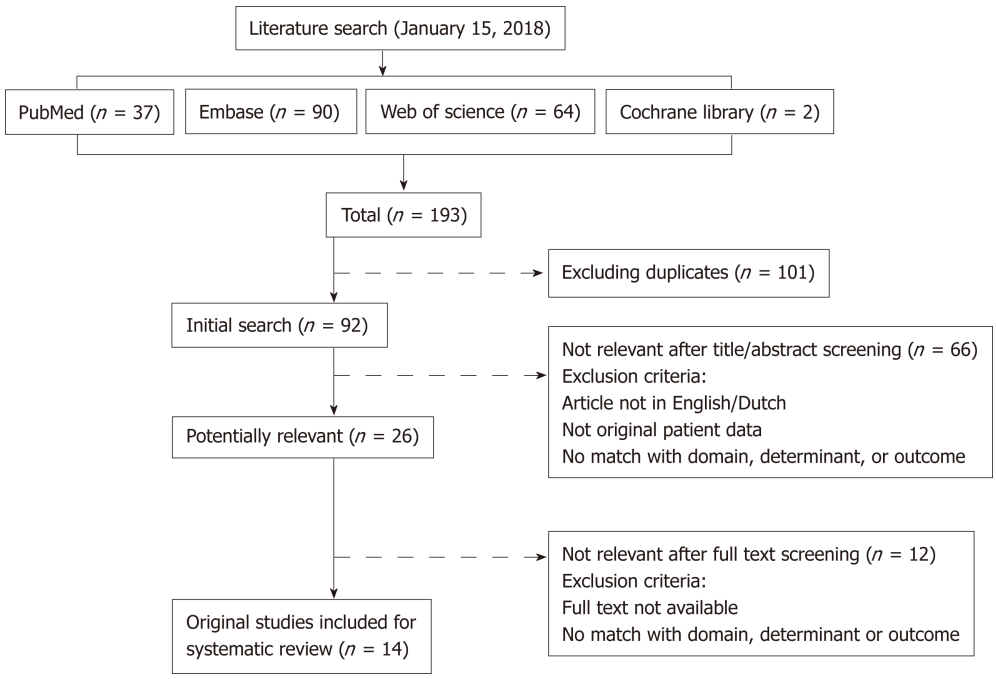


Figure 1 Flow chart illustrating details of the search strategy and the study selection process.

eligibility criteria and were included. Table 1 describes study characteristics of included studies. Length of follow-up after BT injections ranged from 6 to 126 mo. Dysport® (used in 4 of 14 studies) was administered with an average dose of 200 IU per procedure, whereas Botox® (used in 6 of 14 studies) was administered with an average mean dose of 95 IU per procedure, ranging from 60 to 120 IU per procedure. In the other four studies no details on the type of BT was provided. Ultrasonography was used to identify the internal anal sphincter in two studies, whereas in six studies palpation was used. The six other studies did not elaborate on their methods of identifying the internal anal sphincter. Mean age at administration of first BT injection was 4.5 years (SD 1.0 years). Proportion of patients with an associated syndrome was reported in seven studies and was on average 16% (ranging from 0-33%). The proportion of males in the included studies was on average 71% (SD 10%).

Improvement of obstructive symptoms

Primary outcome was reported in all 14 studies including 278 patients. Figure 2A shows a forest plot of the proportion of patients showing overall clinical improvement in each study and the aggregated ER of improvement of obstructive symptoms. Two of the 14 studies showed significant improvement of obstructive symptoms. The other 12 studies found no significant effect of treatment with BT injections. Meta-analytic aggregation of the effect sizes of all 14 studies showed significant effectiveness of BT injections, with improvement of obstructive symptoms in on average 66% of the patients [ER = 0.66, P = 0.004; 95% confidence interval (CI): 0.55-0.75, I^2 = 49.5%] (Table 2). There was a significant higher response rate within one month after BT injections (ER = 0.79, P < 0.001; 95%CI: 0.71-0.85, I^2 = 24.4%, n = 201 patients), compared to more than one month after BT injections (ER = 0.46, P = 0.50; 95%CI 0.34-0.58, I^2 = 61.8%, n = 241 patients) (Q = 19.37, P < 0.001). None of the tested moderators had a significant predictive value for the magnitude of studies' effect sizes in univariate analysis (*i.e.*, mean dose, n = 228, P = 0.28; mean age at first BT injections, n = 184, P = 0.81; proportion of patients with an associated syndrome, n = 160, P = 0.10; sex of patients, n = 201, P = 0.94). Subgroup comparison showed no significant differences in improvement of obstructive symptoms after administration of Botox® (ER = 0.72, 95%CI: 0.58-0.83, n = 8 studies) compared to Dysport® (ER = 0.57, 95%CI: 0.33-0.77, n = 4 studies) (Q = 0.46, P = 0.49, n = 242 patients). Mean duration of improvement after one BT injections was 6.4 mo, ranging from 1 to 60 mo (n = 97). Patients needed on average 2.6 procedures of BT injection (ranging from 1 to a maximum of 23 procedures per patient) before clinical improvement was obtained.

Improvement of enterocolitis

In the meta-analysis effectiveness of BT injections in treating enterocolitis three studies representing 52 patients were included. Figure 2B shows that none of the three

Table 1 Study characteristics of included studies

Study	Study design	Inclusion period	Sample size, n	Male (%)	Syndromal patients (%)	Total follow-up in months	Number of BT injections needed per patient	Age at first BT injection (yr)	Type BT	Mean dose (IU/injection)	Guidance at BT injection	Definition of outcomes	Improvement in obstructive symptoms < 1 mo	Prolonged improvement in obstructive symtpoms	Improvement of enterocolitis	Complications/ adverse effects	Decrease in mean resting pressure on anorectal manometry (mmHG)
Basson (2014)	Retrospective	2010-2014	11	67	NR	12-72	1pt: 1BTI 5pt: 2BTI 4pt: 3BTI 1pt: 4BTI	5	Dysport	200	Palpation	Successful-Improvement-Failed Favorable: Successful/improvement	10/11 (91%)	5/11 (45%)	NR	1 (transient soiling)	NR
Chumpitazi (2008)	Retrospective	1998-2007	30	80	10	41.2 ± 4.9	2.7 ± 0.2	5	Botox	NR	Palpation	Poor-Fair-Good-Excellent Favorable: Excellent/good	27/30 (90%)	11/30 (37%)	NR	8 (7 transients soiling, 1 anal pain)	NR
Chumpitazi (2011)	Retrospective	1998-2016	37	80	23	41.4 ± 4.5	2.8 ± 0.3	NR	Botox	100	NR	Poor-Fair-Good-Excellent Favorable: Excellent/good	33/37 (90%)	NA	NR	NR	NR
Church (2016)	Retrospective	2010-2015	30	NR	NR	20	87% in total: With US: 2 Without US: 1	3.1	NR	40	US-guided	Improvement of symptoms	NA	NR	3/4 (75%)	NR	NR
Han-Geurts (2014)	Retrospective	2002-2013	33	79	0	7.3 yr (1-24)	2 (1-5)	3.6	Dysport	200	NR	Poor-Fair-Good-Excellent Favorable: Excellent/good	25/33 (76%)	17/33 (52%)	7/19 (37%)	2	NR
Hemanshoo Thakkar (2017)	Retrospective	2002-2014	6	NR	NR	6 yr (1-12)		3	Dysport	200	US-guided	Short-term: Postoperative complications < 30 d Long-term: Rintala Bowel Function Score (BFS)	1/6 (17%)	NR	NR	NR	NR

Hosseini (2008)	Prospective	2002-2006	16	62	NR		8 pt: 1-3	NR	Dysport	NR	NR	Improvement in Constipation score (good/ recurrence/ non-responders)	14/16 (88%)	8/16 (50%)	NR	NR	NR
Jiang (2009)	Prospective	2000-2008	23	65	NR	12		NR	Botox	120	Palpation	Poor-Moderate-Excellent Favorable: Moderate/ Excellent	NR	21/23 (91%)	NR	9 (anal pain)	31-(8-30)
Koisuvalo (2009)	Retrospective	2005-2008	16 *	62	12.5	19 (3-43)	2 (1-4)	4	NR	100	NR	No effect-Little effect-Significant effect-Symptoms dissapeared Favorable: Significant effect/ symptoms dissapeared	10/16 (63%)	3/8 (38%)	1/4 (25%)	4 (increased soiling)	28-31 (2 w); 8-24 (8 m)
Langer (1997)	Prospective	NR	4	50	25		3 pt: 1, 1 pt 2	6	Botox	NR	Palpation	Improvement of obstructive symptoms, presence of incontinence	3/4 (75%)	1/4 (25%)	NR	1 (transient incontin -ence)	NR
Langer (2004)	Retrospective	NR	14	NR	NR	24	4 pt: 1, 9pt: 1-4	4	NR	150	NR	Improvement of obstructive symptoms, presence of incontinence	9/14 (64%)	4/14 (29%)	NR	NR	NR
Minkes (2000)	Prospective	NR	18	78	NR		8: 1; 10: 2-6	NR	Botox	60	Palpation	No response-Significant response	12/18 (67%)	5/18 (28%)	NR	4 (transient incontinence)	35-37
Patrus (2010)	Retrospective	1998-2008	22	78	5	5.0 ± 2.9 yr (0-10)	2 (1-23)	8.4	NR	120	NR	Improvement of obstructive symptoms, presence of incontinence	18/22 (81%)	6/22 (27%)	NR	0	NR
Wester (2015)	Retrospective	2007-2014	18	83	33	3, 8 yr (0, 1-8, 3)	3 (1-13)	2.4	Botox	100	Palpation	Good-Insufficient	NR	13/18 (72%)	NR	NR	NR

NR: Not reported; BT: Botulinum toxin.

Table 2 Main findings and risk of bias analysis

Effect	No. of studies	Event rate (95%CI)	Heterogeneity (I^2)	Significant predictors	Eggers intercept	Fail safe, *n*
Improvement of obstructive symptoms	14	ER = 0.66 95%CI: 0.55-0.75[a]	49.5%	None	-0.42	43
Decreasing enterocolitis incidence	3	ER = 0.58, 95%CI: 0.27-0.84[b]	71.0%	NA	3.27	0
Adverse effects	9	ER = 0.17, 95%CI: 0.10-0.29[c]	52.1%	NA	-2.78 [b]	101

[a]$P = 0.004$,
[b]$P < 0.01$,
[c]$P = 0.001$. ER: Event rate; NA: Not applicable; CI: Confidence interval.

studies showed significant effectiveness of BT injections. Meta-analytic aggregation of the effect sizes of all three studies showed non-significant effectiveness of BT injections, with improvement in on average 58% of the patients (ER = 0.58, $P = 0.65$; 95%CI: 0.27-0.84, $I^2 = 71.0\%$) (Table 2). The number of studies describing effects on enterocolitis did not allow for assessment of confounding factors.

Complications and adverse effects

In the meta-analysis on complications and adverse events after administration BT injections as shown in Figure 2C, nine studies representing 187 patients were included. Meta-analytic aggregation of the effect sizes of all nine studies showed significant occurrence of complications or adverse events in on average 17% of the patients (ER = 0.17, $P < 0.001$; 95%CI: 0.10-0.29, $I^2 = 52.1\%$) (Table 2). The number of studies describing adverse effects of treatment with BT injections did not allow for assessment of confounding factors. Adverse events that were described in the studies were mild and included: (1) Transient soiling or incontinence in a total of 17 patients; (2) Anal pain in nine patients; and (3) Muscle fatigue of the lower extremities in two patients. In two other patients adverse effects were present but not described in detail.

Quality of evidence and risk of publication bias

Overall judgement of quality of included studies, as well as scores on each domain of the NOS are presented in Table 3. All 14 studies were of poor quality, because of the observational uncontrolled study design. Funnel plot asymmetry as expressed by Eggers regression intercept was significant for findings on adverse effects ($P = 0.01$), but non-significant for other findings (P values ranged from 0.69 to 0.78). This indicates that there was a low risk op publication bias for findings on improvement of obstructive symptoms and enterocolitis. The latter observation was further supported by a significant positive correlation between sample size and ERs ($t = 0.07$, $P = 0.01$), suggesting there was a low risk of a publication bias. Fail safe n's ranged from 0 to 101, suggesting that only our findings on adverse effects were robust to the influence of publication bias. Results of the risk of bias analysis for every separate finding are presented in Table 2. Main effects were not significantly altered by excluding studies that included both patients with internal sphincter achalasia and Hirschsprung disease.

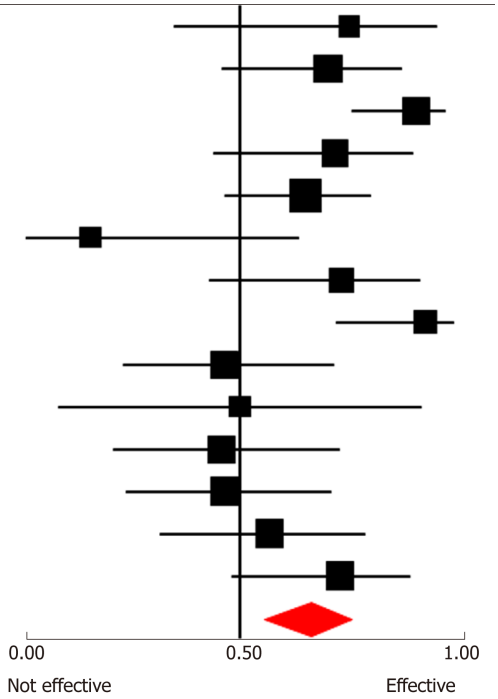
DISCUSSION

Current evidence

This systematic review and meta-analysis aimed to provide a comprehensive overview of all empirical evidence on: (1) Effectiveness of treatment with BT injections for obstructive symptoms; (2) Effectiveness of treatment with BT injections for enterocolitis; and (3) Complications and adverse event after BT injections in patients that underwent surgery for Hirschsprung disease. Our findings indicate that BT injections improve obstructive symptoms in most patients (66%), although the proportion of patients that benefits is significantly higher within one month after administration (79%) compared to the proportion that still benefits after one month of follow-up or longer (46%). This underlines the transient effect of BT injections and explains that most patients will need multiple injections before satisfactory clinical improvement of obstructive symptoms is achieved. Our results further show that

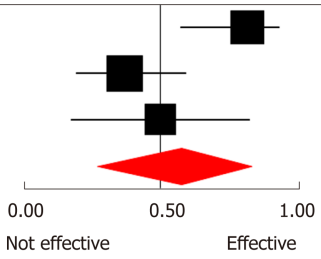
A

Study name	Event rate	Lower limit	Upper limit	*Z* value	*P* value
Basson, 2014	0.743	0.350	0.939	1.238	0.216
Chumpitazi, 2008	0.695	0.458	0.860	1.628	0.103
Chumpitazi, 2011	0.892	0.745	0.959	3.986	0.000
Church, 2016	0.712	0.439	0.887	1.540	0.124
Han-Geurts, 2014	0.646	0.465	0.793	1.586	0.113
Hemanshoo, 2017	0.167	0.023	0.631	-1.469	0.142
Hosseini, 2008	0.726	0.430	0.903	1.518	0.129
Jiang, 2009	0.913	0.711	0.978	3.177	0.001
Koisuvalo, 2009	0.465	0.236	0.710	-0.263	0.793
Langer, 1997	0.500	0.094	0.906	0.000	1.000
Langer, 2004	0.459	0.216	0.724	-0.286	0.775
Minkes, 2000	0.467	0.243	0.706	-0.256	0.798
Patrus, 2010	0.565	0.320	0.782	0.506	0.613
Wester, 2015	0.722	0.481	0.879	1.816	0.069
	0.655	0.550	0.747	2.860	0,004



B

Study name	Event rate	Lower limit	Upper limit	*Z* value	*P* value
Church, 2016	0.824	0.573	0.942	2.421	0.015
Han-Geurts, 2014	0.368	0.187	0.597	-1.133	0.257
Koisuvalo, 2009	0.500	0.168	0.832	0.000	1.000
	0.576	0.265	0.837	0.455	0.649



C

Study name	Event rate	Lower limit	Upper limit	*Z* value	*P* value
Basson, 2014	0.091	0.013	0.439	-2.195	0.028
Chumpitazi, 2008	0.267	0.139	0.450	-2.450	0.014
Church, 2016	0.033	0.005	0.202	-3.311	0.001
Han-Geurts, 2014	0.061	0.015	0.212	-3.757	0.000
Jiang, 2009	0.391	0.218	0.598	-1.034	0.301
Koisuvalo, 2009	0.250	0.097	0.508	-1.903	0.057
Langer, 1997	0.250	0.034	0.762	-0.951	0.341
Minkes, 2000	0.222	0.086	0.465	-2.210	0.027
Patrus, 2010	0.022	0.001	0.268	-2.662	0.008
	0.174	0.098	0.290	-4.627	0.000

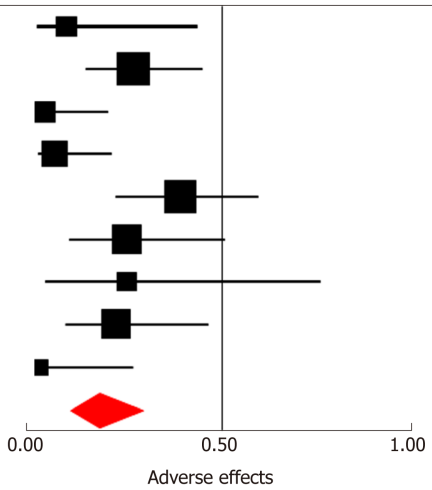


Figure 2 Event rates of effects of botulinum toxin injections in patients after surgery for Hirschsprung disease. A: Effectiveness of botulinum toxin injections in treating obstructive symptoms in patients with Hirschsprungs disease; B: Effectiveness of botulinum toxin injections in treating enterocolitis in patients with Hirschsprungs disease; C: Adverse effects of botulinum toxin injections in patients with Hirschsprungs disease.

Table 3 Quality of included studies

Study	Selection	Comparibility	Outcome	Total
Basson (2014)	2	0	3	Poor
Chumpitazi (2008)	3	0	3	Poor
Chumpitazi (2011)	3	0	3	Poor
Church (2016)	2	0	2	Poor
Han-Geurts (2014)	2	0	3	Poor
Hemanshoo Takkar (2017)	3	0	3	Poor
Hosseini (2008)	3	0	2	Poor
Jiang (2009)	3	0	3	Poor
Koisuvalo (2009)	3	0	3	Poor
Langer (1997)	3	0	3	Poor
Langer (2004)	3	0	2	Poor
Minkes (2000)	3	0	2	Poor
Patrus (2010)	3	0	3	Poor
Wester (2015)	3	0	3	Poor

current evidence on whether BT injections are effective in reducing Hirschsprung-associated enterocolitis is inconclusive. Our analysis lacked the power to make a very specific point estimate of effectiveness, as shown by the broad CI ranging from effectiveness of 27% to 84%. BT injections were associated with adverse effects in on average 17% of patients, with adverse effects varying from transient incontinence to anal pain and muscle fatigue.

Our results indicated that duration of improvement of obstructive symptoms was on average six months and that most patients need on average two to three injections. This in line with previous meta-analytic findings and with evidence on the effectiveness of BT injections in other patient groups, including chronic constipation, anal fissures and internal sphincter achalasia[4,6,23]. In addition, evidence from three studies (all included in our meta-analysis), indicated that short term response was predictive for long-term response, although studies used different cut-off points for defining short- and long term response[7-9].

Our analyses showed that differences between studies in the proportion of patients showing clinical improvement, could not be explained by differences in average dose and type of BT used or by patient characteristics. There was large heterogeneity between studies in the dosage administered, which suggests there is no current consensus on optimal dose. Dysport® was on average administered in higher dosages than Botox®. However, we could not test the unique contribution of dosage and type in multivariate analyses, because only ten studies described both type of BT as well as average dose used. We hypothesize that our findings do not reflect this difference in dosage, as neither type of BT nor average dose used correlated significantly to rates of clinical improvement in univariate analysis. Furthermore, our findings are in line with findings in patients with chronic anal fissures, in whom dose and type of BT were not predictive of clinical improvement[24,25].

With regard to age at first BT injection, our findings indicated the age at which the BT injection was administered was not correlated to the proportion of patients showing clinical improvement, suggesting that BT injections can be used at all ages. The proportion of patients with an associated syndrome was not correlated to the effectiveness of BT injection in the treatment of obstructive symptoms, suggesting that our results were not over- or underestimated by patients with an associated syndrome. Moreover, the average amount of patients with an associated syndrome in our study was comparable to what we know from the general Hirschsprung population[26].

Limitations of this study

Because of lack of power caused by the limited number of studies available, the small sample sizes and heterogeneity in outcome definitions, our meta-analysis could not assess the predictive value of a number of possible interesting predictors of treatment effectiveness, including length of aganglionosis (only six studies), type of reconstruction that was done, findings on anorectal manometry (three studies) and specific procedural aspects of BT injections. Individual studies included in this meta-analyses suggest that short-segment disease is associated with better responsiveness to BT

injections than long-segment disease[14,15]. Contrarily one study found no difference between short-segment and long-segment disease[16]. Three studies suggested that mean resting pressure decreases significantly after BT injections[11,12,14], but the degree of decrease in pressure was not predictive for clinical improvement[12]. One study by Church further suggests that US-guided BT injections decreases the amount of injections necessary compared to identifying the internal anal sphincter by palpation, although US-guided BT injections were not associated with higher response rates[17]. The study by Church also suggested that BT injection in the external anal sphincter is associated with higher response rates compared to injections in the internal anal sphincter[17]. In the majority of studies included in our systematic review (8/14 studies) residual aganglionosis or a mechanical obstruction was excluded as a cause of obstruction by barium enema and rectal biopsy before BT was administered. The other six studies did not exclude patients with these causes of obstruction from the study, but did not specifically report the cause of obstruction in individual patients prior to BT injections. Therefore, we could not compare differences in effectiveness of BT injections between different reasons of obstructive symptoms.

Another limitation of our study is the large heterogeneity between studies in outcome definitions and procedural aspects, including the position of the patients (lithotomy *vs* lateral decubitus position) during BT injection, the amount of injections administered and the number of sites in which BT was injected. This shows the lack of a standardized approach for BT injections.

Quality of evidence and risk of publication bias

Quality of evidence on the effects of BT injections was poor in all studies because of the lack of the use of randomized and controlled designs. Two studies assessed a combined sample of both patients with internal sphincter achalasia and Hirschsprung disease. This could account for a selection bias, resulting in an overestimation of effects, although sensitivity analyses in which these two studies were excluded showed no significant alteration of main effects. It may be hypothesized that the effects of BT injections are larger in patients with internal sphincter achalasia, as in these patients the absent rectoanal inhibitory reflex is the only explanatory factor for obstruction. The present systematic review and meta-analysis also carries the risk of assessment bias due to large variety of outcome definitions used in the included studies. There is only a risk of publication bias for our findings on complications and adverse effects, but our findings were robust to this influence. For other findings no evidence for risk of publication bias was found.

Conclusions and future implications

In this systematic review and meta-analysis we found evidence for improvement of obstructive symptoms after BT injections in patients that underwent surgery for Hirschsprung disease, although this effect was often transient and most patients needed multiple injections. Future studies using a standardized procedural approach and outcome definitions would be useful to determine dose-response effects and identify optimal dosages. Furthermore, better insight in predictors of clinical response would optimize treatment. Future studies should also assess factors that predict improvement of obstructive symptoms and enterocolitis incidence after BT injection, including length of aganglionosis and functional parameters such as mean resting pressures of the anal canal.

ACKNOWLEDGEMENTS

This research was not funded. The authors want to acknowledge the patient association of Hirschsprungs disease in the Netherlands for their support and cooperation in this research project.

ARTICLE HIGHLIGHTS

Research background

Patients with Hirschsprungs disease often suffer from persistent obstructive complaints after surgery. Improving faecal passage is important in these patients in order to prevent Hirschsprung-associated enterocolitis. Relaxation of the internal anal sphincter with botulinum toxin (BT) injections can be used to improve faecal passage.

Research motivation

BT injections are increasingly used to treat obstructive symptoms but an overview of the current evidence describing effectiveness of this treatment is lacking.

Research objectives
The objective of this study was to give a comprehensive overview of all evidence on effectiveness of intra-sphincteric BT injections to treat obstructive symptoms and enterocolitis in patients after surgery for Hirschsprungs disease, and to summarize evidence on its adverse effects.

Research methods
A systematic review and meta-analysis according to the PRISMA Guidelines was conducted, searching PubMed, EMBASE, Web of Science and Cochrane library using simple and hierarchical entry terms including "botulinum toxin injections" and "Hirschsprungs disease". Predefined predictors of effectiveness that were analysed were age at injection, sex, associated syndromes, dosage and type of BT used.

Research results
Data of 14 studies representing 278 patients were analysed. BT injections were effective in treating obstructive symptoms in 66% of patients, ranging from 79% in the first month of follow-up to 46% in follow-up longer than month. This was regardless of age at injection, sex, associated syndromes, dosage and type of BT used. Enterocolitis incidence was reduced in 57%, but the meta-analysis lacked power to draw conclusions. Mild adverse effects were present in 17%, which mainly consisted of temporary faecal incontinence or anal pain.

Research conclusions
Our systematic review and meta-analysis shows that BT injections effectively treat obstructive symptoms in patients after surgery for Hirschsprungs disease, regardless of age at injection, sex, associated syndromes, dosage and type of BT used. Furthermore, the data suggests that BT injections are associated with mild adverse effects. Evidence on effectiveness of BT injections in treating enterocolitis is limited and lacked power to draw conclusions. Our findings show that BT injections are a useful treatment modality in clinical practice.

Research perspectives
Future studies should further elucidate what factors predict good response to BT injections and subsequently if we can predict which patients can and cannot benefit from BT injections.

REFERENCES

1 **Langer JC**, Rollins MD, Levitt M, Gosain A, Torre L, Kapur RP, Cowles RA, Horton J, Rothstein DH, Goldstein AM; American Pediatric Surgical Association Hirschsprung Disease Interest Group. Guidelines for the management of postoperative obstructive symptoms in children with Hirschsprung disease. *Pediatr Surg Int* 2017; **33**: 523-526 [PMID: 28180937 DOI: 10.1007/s00383-017-4066-7]

2 **Bjørnland K**, Pakarinen MP, Stenstrøm P, Stensrud KJ, Neuvonen M, Granström AL, Graneli C, Pripp AH, Arnbjörnsson E, Emblem R, Wester T, Rintala RJ; Nordic Pediatric Surgery Study Consortium. A Nordic multicenter survey of long-term bowel function after transanal endorectal pull-through in 200 patients with rectosigmoid Hirschsprung disease. *J Pediatr Surg* 2017; **52**: 1458-1464 [PMID: 28094015 DOI: 10.1016/j.jpedsurg.2017.01.001]

3 **Gosain A**, Frykman PK, Cowles RA, Horton J, Levitt M, Rothstein DH, Langer JC, Goldstein AM; American Pediatric Surgical Association Hirschsprung Disease Interest Group. Guidelines for the diagnosis and management of Hirschsprung-associated enterocolitis. *Pediatr Surg Int* 2017; **33**: 517-521 [PMID: 28154902 DOI: 10.1007/s00383-017-4065-8]

4 **Zar-Kessler C**, Kuo B, Belkind-Gerson J. Botulinum toxin injection for childhood constipation is safe and can be effective regardless of anal sphincter dynamics. *J Pediatr Surg* 2018; **53**: 693-697 [PMID: 29395154 DOI: 10.1016/j.jpedsurg.2017.12.007]

5 **Langer JC**, Birnbaum E. Preliminary experience with intrasphincteric botulinum toxin for persistent constipation after pull-through for Hirschsprung's disease. *J Pediatr Surg* 1997; **32**: 1059-61; discussion 1061-2 [PMID: 9247234 DOI: 10.1016/S0022-3468(97)90399-7]

6 **Soh HJ**, Nataraja RM, Pacilli M. Prevention and management of recurrent postoperative Hirschsprung's disease obstructive symptoms and enterocolitis: Systematic review and meta-analysis. *J Pediatr Surg* 2018; **53**: 2423-2429 [PMID: 30236605 DOI: 10.1016/j.jpedsurg.2018.08.024]

7 **Basson S**, Charlesworth P, Healy C, Phelps S, Cleeve S. Botulinum toxin use in paediatric colorectal surgery. *Pediatr Surg Int* 2014; **30**: 833-838 [PMID: 24997611 DOI: 10.1007/s00383-014-3536-4]

8 **Chumpitazi BP**, Fishman SJ, Nurko S. Long-term clinical outcome after botulinum toxin injection in children with nonrelaxing internal anal sphincter. *Am J Gastroenterol* 2009; **104**: 976-983 [PMID: 19259081 DOI: 10.1038/ajg.2008.110]

9 **Han-Geurts IJ**, Hendrix VC, de Blaauw I, Wijnen MH, van Heurn EL. Outcome after anal intrasphincteric Botox injection in children with surgically treated Hirschsprung disease. *J Pediatr Gastroenterol Nutr* 2014; **59**: 604-607 [PMID: 25000353 DOI: 10.1097/MPG.0000000000000483]

10 **Thakkar HS**, Bassett C, Hsu A, Manuele R, Kufeji D, Richards CA, Agrawal M, Keshtgar AS. Functional outcomes in Hirschsprung disease: A single institution's 12-year experience. *J Pediatr Surg* 2017; **52**: 277-280 [PMID: 27912977 DOI: 10.1016/j.jpedsurg.2016.11.023]

11 **Hosseini SM**, Foroutan HR, Bahador A, Khosravi MB, Geramizadeh B, Sabet B, Zeraatian S, Razmi T, Banani SJ. Role of rectal biopsy in predicting response to intrasphincteric botulinum toxin injection for obstructive symptoms after a pullthrough operation. *Indian J Gastroenterol* 2008; **27**: 99-102 [PMID: 18787278]

12 **Jiang da P**, Xu CQ, Wu B, Li ZZ, Zhang YB, Han FY. Effects of botulinum toxin injection on anal achalasia after pull-through operations for Hirschsprung's disease: A 1-year follow-up study. *Int J Colorectal Dis* 2009; **24**: 597-598 [PMID: 18836730 DOI: 10.1007/s00384-008-0591-0]

13 **Langer JC**. Persistent obstructive symptoms after surgery for Hirschsprung's disease: Development of a diagnostic and therapeutic algorithm. *J Pediatr Surg* 2004; **39**: 1458-1462 [PMID: 15486887 DOI:

10.1016/j.jpedsurg.2004.06.008]
14 **Minkes RK**, Langer JC. A prospective study of botulinum toxin for internal anal sphincter hypertonicity in children with Hirschsprung's disease. *J Pediatr Surg* 2000; **35**: 1733-1736 [PMID: 11101725 DOI: 10.1053/jpsu.2000.19234]
15 **Patrus B**, Nasr A, Langer JC, Gerstle JT. Intrasphincteric botulinum toxin decreases the rate of hospitalization for postoperative obstructive symptoms in children with Hirschsprung disease. *J Pediatr Surg* 2011; **46**: 184-187 [PMID: 21238663 DOI: 10.1016/j.jpedsurg.2010.09.089]
16 **Wester T**, Granström AL. Botulinum toxin is efficient to treat obstructive symptoms in children with Hirschsprung disease. *Pediatr Surg Int* 2015; **31**: 255-259 [PMID: 25616563 DOI: 10.1007/s00383-015-3665-4]
17 **Church JT**, Gadepalli SK, Talishinsky T, Teitelbaum DH, Jarboe MD. Ultrasound-guided intrasphincteric botulinum toxin injection relieves obstructive defecation due to Hirschsprung's disease and internal anal sphincter achalasia. *J Pediatr Surg* 2017; **52**: 74-78 [PMID: 27836361 DOI: 10.1016/j.jpedsurg.2016.10.023]
18 **Wells GA**, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. 25 Apr. 2019; Available from: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp
19 **Borenstein M**, Hedges, L, Higgins, J, Rothstein, H. Comprehensive Meta-Analysis Version 3, Biostat, Englewood, NJ 2013. Available from: https://www.meta-analysis.com/
20 **Higgins JP**, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, Savovic J, Schulz KF, Weeks L, Sterne JA; Cochrane Bias Methods Group; Cochrane Statistical Methods Group. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* 2011; **343**: d5928 [PMID: 22008217 DOI: 10.1136/bmj.d5928]
21 **Egger M**, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997; **315**: 629-634 [PMID: 9310563 DOI: 10.1136/bmj.315.7109.629]
22 **Rosenthal R**. Writing meta-analytic reviews. *Psychol Bull* 1995; **118**: 183-192 [DOI: 10.1037/0033-2909.118.2.183]
23 **Friedenberg F**, Gollamudi S, Parkman HP. The use of botulinum toxin for the treatment of gastrointestinal motility disorders. *Dig Dis Sci* 2004; **49**: 165-175 [PMID: 15104353 DOI: 10.1023/B:DDAS.0000017434.53075.80]
24 **Bobkiewicz A**, Francuzik W, Krokowicz L, Studniarek A, Ledwosiński W, Paszkowski J, Drews M, Banasiewicz T. Botulinum Toxin Injection for Treatment of Chronic Anal Fissure: Is There Any Dose-Dependent Efficiency? A Meta-Analysis. *World J Surg* 2016; **40**: 3064-3072 [PMID: 27539490 DOI: 10.1007/s00268-016-3693-9]
25 **Gui D**, Rossi S, Runfola M, Magalini SC. Review article: Botulinum toxin in the therapy of gastrointestinal motility disorders. *Aliment Pharmacol Ther* 2003; **18**: 1-16 [PMID: 12848622 DOI: 10.1046/j.1365-2036.2003.01598.x]
26 **Moore SW**. Chromosomal and related Mendelian syndromes associated with Hirschsprung's disease. *Pediatr Surg Int* 2012; **28**: 1045-1058 [PMID: 23001136 DOI: 10.1007/s00383-012-3175-6]

World Journal of Gastroenterology

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

World J Gastroenterol 2019 July 7; 25(25): 3281-3282

DOI: 10.3748/wjg.v25.i25.3281

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

RETRACTION NOTE

Retraction Note: Construction of Gpm6a/ReelinGFPCreERT2 by BAC recombination using a specific gene in hepatic mesothelial or stellate cells

Hong-Bo Shi, Jin-Li Lou, Hong-Lin Shi, Feng Ren, Yu Chen, Zhong-Ping Duan

ORCID number: Hong-Bo Shi (0000-0002-3666-0196); Jin-Li Lou (0000-0001-9855-7904); Hong-Lin Shi (0000-0002-1203-4326); Feng Ren (0000-0002-8855-5400); Yu Chen (0000-0001-9936-547X); Zhong-Ping Duan (0000-0002-9397-6330).

Author contributions: Shi HB and Duan ZP wrote the paper; Lou JL, Shi HL, Ren F and Chen Y participated in the revision of the paper.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Manuscript source: Unsolicited manuscript

Received: January 10, 2019
Peer-review started: January 10, 2019
First decision: January 23, 2019
Revised: May 23, 2019
Accepted: May 31, 2019

Hong-Bo Shi, Hong-Lin Shi, Feng Ren, Zhong-Ping Duan, Beijing Institute of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China

Jin-Li Lou, Clinical Laboratory Center, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China

Yu Chen, Zhong-Ping Duan, Artificial Liver Center, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China

Corresponding author: Zhong-Ping Duan, MD, PhD, Professor, Beijing Institute of Hepatology, Beijing Youan Hospital, Capital Medical University, 8 Xitoutiao, Youwai Street, Fengtai District, Beijing 100069, China. duan2517@163.com
Telephone: +86-10-63291007
Fax: +86-10-63295258

Abstract

We have decided to retract the above article for further consideration due to some misunderstandings in communication.

Key words: Retraction note

Core tip: We have decided to retract the above article for further consideration due to some misunderstandings in communication.

Citation: Shi HB, Lou JL, Shi HL, Ren F, Chen Y, Duan ZP. Retraction Note: Construction of Gpm6a/ReelinGFPCreERT2 by BAC recombination using a specific gene in hepatic mesothelial or stellate cells. *World J Gastroenterol* 2019; 25(25): 3281-3282
URL: https://www.wjgnet.com/1007-9327/full/v25/i25/3281.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i25.3281

RETRACTION NOTE

Article in press: June 1, 2019
Published online: July 7, 2019

P-Reviewer: Chen YK, Tsoulfas G
S-Editor: Ma RY
L-Editor: Filipodia
E-Editor: Ma YJ

Retraction Note: Shi HB, Lou JL, Shi HL, Ren F, Chen Y, Duan ZP. Construction of Gpm6a/ReelinGFPCreERT2 by BAC recombination using a specific gene in hepatic mesothelial or stellate cells. *World J Gastroenterol* 2017; 23(2): 224-231. PMID: 28127196 DOI: 10.3748/wjg.v23.i2.224. The online version of the original article can be found at https://www.wjgnet.com/1007-9327/full/v23/i2/224.htm.

After discussion and agreement among all the authors, it was decided to retract the above article[1] for further consideration due to some misunderstandings in communication.

REFERENCES

1 **Shi HB**, Lou JL, Shi HL, Ren F, Chen Y, Duan ZP. Construction of Gpm6a/ReelinGFPCreERT2 by BAC recombination using a specific gene in hepatic mesothelial or stellate cells. *World J Gastroenterol* 2017; **23**: 224-231 [PMID: 28127196 DOI: 10.3748/wjg.v23.i2.224]

Published By Baishideng Publishing Group Inc
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, 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