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

World Journal of *Gastroenterology*

World J Gastroenterol 2015 November 28; 21(44): 12513-12734

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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

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2014-2017

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World Journal of Gastroenterology

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Weekly Volume 21 Number 44 November 28, 2015

CASE REPORT

World Journal of Gastroenterology
Volume 21 Number 44 November 28, 2015

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NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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Fax: +86-10-85381893
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PUBLISHER
Baishideng Publishing Group Inc
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Pleasanton, CA 94588, USA
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PUBLICATION DATE
November 28, 2015

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DOI: 10.3748/wjg.v21.i44.12513

World J Gastroenterol 2015 November 28; 21(44): 12513-12518
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Diagnosis and therapies for gastric non-invasive neoplasia

Motohiko Kato

Motohiko Kato, Department of Gastroenterology, National Hospital Organization Tokyo Medical Center, Tokyo 152-8902, Japan

Author contributions: Kato M solely contributed to this paper.

Conflict-of-interest statement: The author has no conflict of interest related to the manuscript.

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Correspondence to: Motohiko Kato, MD, PhD, Department of Gastroenterology, National Hospital Organization Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan. moto2hiko@icloud.com
Telephone: +81-3-34110111
Fax: +81-3-34129811

Received: April 28, 2015
Peer-review started: May 7, 2015
First decision: July 13, 2015
Revised: July 23, 2015
Accepted: September 28, 2015
Article in press: September 30, 2015
Published online: November 28, 2015

Abstract

There has been a great discrepancy of pathological diagnosis for gastric non-invasive neoplasia/dysplasia between Japanese and western pathologists. In Japan, lesions that most western pathologists diagnose as dysplasia are often considered adenocarcinoma based on nuclear and structural atypia regardless of the presence of invasion. In the Vienna classification, gastric non-invasive intraepithelial neoplasia (NIN) were divided into low grade and high grade (including intramucosal cancer of Japanese criteria). The diagnosis by both endoscopy and pathology of biopsy specimen is difficult. Recent advances of diagnostic modality such as magnified endoscopy and imaged enhanced endoscopy is expected to improve the diagnostic yield for NIN. There are two treatment strategies for NIN, observation and diagnostic therapy by endoscopic resection (ER). ER is acceptable because of its less invasiveness and high local control rate, on the other hand, cancer-developing rate of low-grade NIN is reported to be low. Therefore there is controversy for the treatment of gastric NIN. Prospective study based on unified pathological definition is required in the future.

Key words: Gastric; Non invasive intraepithelial neoplasia; Gastric; Adenoma; Adenocarcinoma; Diagnosis

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Core tip: The discrepancy of pathological diagnosis for gastric non-invasive neoplasia/dysplasia between Japanese and western pathologists was solved by Vienna classification. Although recent advances of diagnostic modality such as magnified endoscopy and imaged enhanced endoscopy is expected to improve the diagnostic yield for non-invasive intraepithelial neoplasia (NIN), precise prediction of histology is not easy by the findings of conventional white light endoscopy and pathologic findings of forceps biopsy. There is still a controversy regarding the treatment of NIN, observation and diagnostic therapy by endoscopic resection. Prospective study based on unified pathological definition is required in the future.

Kato M. Diagnosis and therapies for gastric non-invasive neoplasia. *World J Gastroenterol* 2015; 21(44): 12513-12518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12513.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12513

INTRODUCTION

Gastric cancer is one of the most common neoplasms worldwide, accounting for over 870000 new cases and more than 650000 deaths annually[1]. Early gastric cancer (EGC), which is defined as cancer of which the invasion depth remains mucosa or submucosa is known to have a good prognosis[2] and endoscopic resection (ER) is widely accepted as a local treatment for these lesions[3-7]. There is a benign non-invasive intraepithelial neoplasia (NIN), also called as gastric adenoma or dysplasia. On the contrary to colorectal adenoma, "adenoma-carcinoma sequence" in the stomach has not been proven, NIN is generally considered to be a premalignant lesion[8-10].

The diagnosis of NIN according to both endoscopic and histopathological findings is not always easy. Moreover there are some controversies concerning how to treat NIN[11]. In this editorial, we discuss clinical problems concerning diagnosis and treating NIN.

CHANGES IN CLASSIFICATION FOR GASTRIC INTRAEPITHELIAL NEOPLASIA

Vienna classification

It is known that there is a considerable difference in pathological diagnosis of gastric epithelial neoplasia by between western and Japanese pathologists. Western pathologists have used "dysplasia" for unequivocal neoplastic epithelium. Dysplasia was divided into high- and low-grade based on structural atypia and they seldom diagnosed as "adenocarcinoma" unless invasion was confirmed[12-14]. In Japan, lesions that most western pathologists diagnose as dysplasia are often considered adenocarcinoma based on nuclear and structural atypia regardless of the presence of invasion (Table 1).

To resolve some confusions caused by these diagnostic discrepancies between western and Japanese pathologists, in September 1998 approximately 30 pathologists from 12 countries met in Vienna and made a consensus on the terminology for gastrointestinal epithelial neoplasia, named the Vienna classification[11]. In this classification, gastric non-invasive neoplasia/dysplasia was divided into low grade (category 3) and high-grade (category 4). Category 4 includes high-grade adenoma/dysplasia", "non-invasive carcinoma (carcinoma *in situ*)" and "suspected invasive carcinoma" were clustered into a single category (category 4), termed "noninvasive high-grade neoplasia". In the Vienna classification revised in 2000[15], intra-mucosal carcinoma was added into category 4. The agreement on the diagnosis for category 4 among Japanese and western pathologists improved to 80% for gastric lesions[16].

Table 1 Vienna classification and Japanese classification of gastric cancer for diagnosis of gastric intraepithelial neoplasia

Vienna classification		Japanese	Western
Category 3	Low grade adenoma/ dysplasia (LGA)	Adenoma	Adenoma
Category 4.1	High grade adenoma/ dysplasia (HGA)	Adenoma/ cancer	Adenoma
Category 4.2	Non-invasive carcinoma (carcinoma *in situ*)	Cancer	Adenoma/cancer
Category 4.3	Suspicion of invasive carcinoma	Cancer	Adenoma/cancer
Category 5.1	Intramucosal carcinoma	Cancer	Adenoma/cancer
Category 5.2	Submucosal carcinoma or beyond	Cancer	Cancer

Difficulties of pathological diagnosis of specimen obtained by endoscopic forceps biopsy

Although pathological diagnosis established by endoscopic biopsy specimen is the gold standard for gastric epithelial neoplasia, discrepancy between final diagnoses established by endoscopically or surgically resected specimen would sometimes occur. The frequency of the discrepant diagnoses ranges widely in published reports. Recently we report that the diagnosis was changed in 44% of patients who were diagnosed as NIN proven by biopsy (95%CI: 39%-49%). Moreover, in that study, there were 2 lesions (0.42%) of adenocarcinoma with submucosal invasion of more than 500 μm, one of which involved the lymphatic duct[17]. The reasons for the difficulty in making an accurate diagnosis based on a biopsy specimen are as follows: (1) the structural atypia of both adenoma and well-differentiated adenocarcinoma is too subtle to detect in small biopsy specimens; and (2) cancer sometimes exists focally in the lesion and a sampling error might occur (Figure 1). Thus, pathologist might change the diagnosis from adenoma to carcinoma when they determine larger specimen.

ENDOSCOPIC DIAGNOSIS OF GASTRIC INTRAEPITHELIAL NEOPLASIA

Conventional white light endoscopy

Endoscopy has an advantage on the diagnosis of NIN because it is possible to assess the lesion as a whole. Some endoscopic findings have been reported to predict high-risk lesions for malignancy, lesion size, macroscopic type, color of the lesion, and surface pattern (Figure 2). Typical gastric low grade NIN reveals to have slightly elevated (Paris classification type 0-Ⅱa[18]) and whitish color with smooth surface. On the contrary, depressed macroscopic type, redness, and nodular surface are reported to reflect malignant histology. These endoscopic findings are

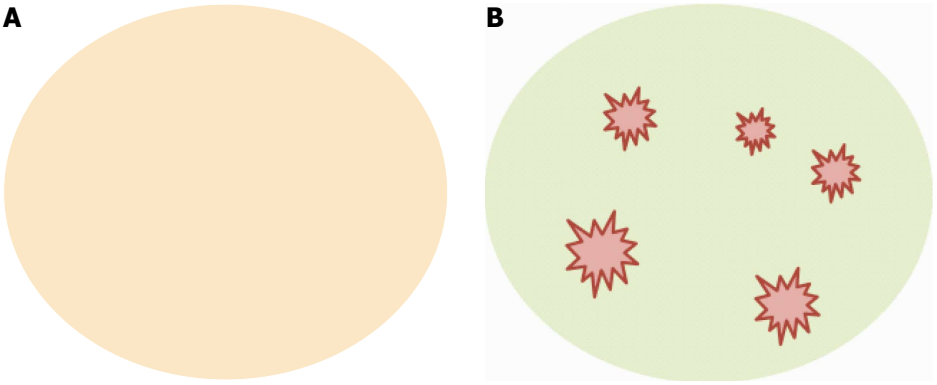

Figure 1 Pathological features of non-invasive neoplasia. A: If the structural atypia was weak, pathological diagnosis is difficult by small specimen obtained by endoscopic forceps biopsy; B: If cancer foci exist focally, sampling error may occur by endoscopic forceps biopsy.

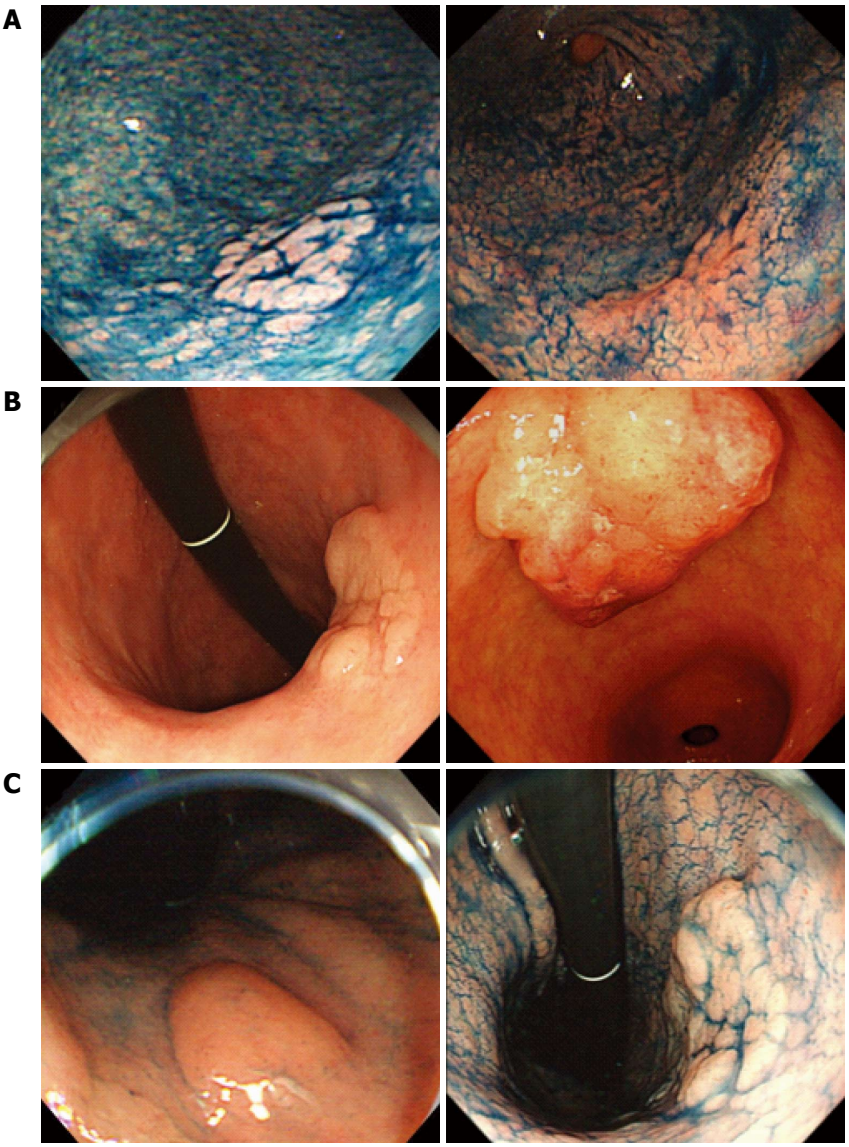

Figure 2 Endoscopic findings of non-invasive neoplasia. A: Macroscopic type:elevated (left), and depressed (right); B: Color:reddish (left), and discolored (right); C: Surface pattern: smooth (left) and nodular (right). List of abbreviations: ER: Endoscopic resection; QOL: Quality of life; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Table 2 Diagnosis yields for gastric non-invasive intraepithelial neoplasia of various endoscopic modality

Ref.	Modality	Sensitivity	Specificity	Accuracy
Cho *et al*[21], 2011	WLE	7.5%	99.4%	68.2%
Kato *et al*[17], 2011	WLE	42.0%	59.0%	56.0%
Kanesaka *et al*[28], 2014	NBI-ME	90.0%	87.8%	88.2%
Miwa *et al*[22], 2012	NBI-ME	82.4%	97.3%	NA
Yao *et al*[27], 2008	NBI-ME	94.0%	96.0%	98.7%
Wang *et al*[29], 2012	CLE	66.7%	92.3%	86.8%
Li *et al*[30], 2011	CLE	88.1%	98.6%	96.2%

WLE: White light endoscopy; CLE: Confocal laser endomicroscopy.

Table 3 Long-term follow-up outcomes of gastric non-invasive intraepithelial neoplasia

Ref.	Incrusion criteria	Duration	Regression	Accuracy
Suzuki *et al*[31], 2015	Vienna C3 and C4	NA	26.0%	0.0%
Yamada *et al*[32], 2004	Vienna C3	4.7Y	0.0%	2.7%
	Vienna C4	4.7Y	0.0%	10.0%
Saito *et al*[33], 2000	Adenoma	2Y	NA	6.3%
Kokkola *et al*[34], 1996	Mild dysplasia	NA	NA	0.0% (4% to moderate)
Bearzi *et al*[35], 1994	LGD	NA	49.4%	32.1%
Fertitta *et al*[36], 1993	Moderate and severe dysplasia	13M	NA	33.0%
Di Gregorio *et al*[37], 1993	Mild dysplasia	NA	74.0%	7.0%
Saraga *et al*[38], 1987	Mild and moderate dysplasia	42M	NA	1.6%
	Severe dysplasia	42M	NA	86.0%

NA: Not available.

useful because of convenience, however they are not satisfactory because the negative predictive value is not so high. We analysed the association between endoscopic findings[19-22] and final pathological findings in 468 NIN cases and lesion diameter larger than 20 mm and depressed macroscopic type were significantly more frequently seen cases who were diagnosed as adenocarcinoma after ER. However, the lesions were diagnosed as NIN based on smaller lesion size and elevated macroscopic type, the under-diagnosis rate was over 30%[17]. Therefore only conventional endoscopic diagnosis is not sufficient to make a precise pre- operative diagnosis.

Magnified endoscopy

Magnified endoscopy is reported to be useful for differentiation of gastric NIN. Tanaka analysed the diagnostic yield of magnified endoscopy with acetic acid spraying and they reported the diagnostic accuracy was over 95%. Moreover, Ohnita *et al*[23] reported the findings of magnified endoscopy with crystal violet dye correlates with the histological types of gastric epithelial neoplasia.

Recently novel diagnostic modalities, image enhanced endoscopy (IEE), are widely used. Narrow band imaging is a kind of IEE of which the usefulness have been reported for differential diagnosis or estimation of invasion depth for epithelial neoplasia arising from gastrointestinal tract[24-26]. Yao *et al*[27] reported that finding a white opaque substance on magnified endoscope with NBI could predict the final pathology of gastric NIN with a sensitivity of 94% and a specificity of 96%. Kanesaka *et al*[28] focused on crypt opening on magnified endoscopy with NBI and dense crypt opening pattern could predict malignant histology with a sensitivity of 90% and specificity of 87.8%.

Although the diagnostic yields of magnified endoscopy especially combined with IEE were excellent and seemed to be superior to conventional white light imaging (Table 2), most of the reports are single-centered retrospective study from high volume center, therefore expert bias is not negligible and the results should be confirmed by future prospective study (ideally randomized trial) to generalize the results.

Other modalities

There are a few reports concerning the diagnostic capability of confocal laser endomicroscopy (CLE) for identification of gastric superficial cancer/HGIN lesions[29,30]. Although these studies included only relatively small sample size of gastric NIN, the diagnostic yield of CLE is good and it might improve preoperative diagnosis (Table 2).

TREETMENT FOR GASTRIC NIN

There are two treatment strategies for gastric NIN, observation and aggressive endoscopic treatment as a diagnostic therapy.

Observation (including Helicobacter pylori eradication)

According to the studies focused on the long-term follow up outcomes for gastric NIN, the incidence of progression of histology widely ranged 0% to 86%[31-38] (Table 3). Histological grade is considered to caused this difference, in low grade NIN (Vienna category 3) the incidence rate of histological progression to high grade NIN or carcinoma remained less than 10%[31,32], moreover, some cases spontaneously regress during follow-up[31,35,37]. It is also reported that eradication therapy of *Helicobacter pylori* might reduce gastric cancer development[33] or it might accomplish regression of NIN from comparatively small studies[31,39]. These facts suggest that the malignant potential of low grade NIN is low and observation would be acceptable, whereas high grade NIN should be resected.

Endoscopic resection

The other strategy is diagnostic therapy. As mentioned

above, it is difficult to make an accurate pathological diagnosis with small biopsy specimens, ER might be used for the purpose of "total biopsy". It is reported that pathological diagnosis changes in 10% to 50% of the patients after ER[17,40-42]. ER is a less invasive treatment, however, complications such as perforation or bleeding sometimes occurs. In our comparatively large-scale retrospective study in which 468 NIN cases underwent ESD, R0 resection was accomplished in 97% of the patients and ESD-related bleeding and perforation rate was 5.4% and 4.7%. Most of the complications were managed conservatively and serious complication rate was 0.43%. These outcomes seem to be acceptable considering the high under-diagnosis rate of forceps biopsy[17]. The advantage of ER is to release the patients from physiological, psychological, and financial strains caused by repeated endoscopic examination with biopsies.

CONCLUSION

The discrepancy of pathological diagnosis for gastric non-invasive neoplasia/dysplasia between Japanese and western pathologists was solved by Vienna classification. Although recent advances of diagnostic modality such as magnified endoscopy and imaged enhanced endoscopy is expected to improve the diagnostic yield for NIN, precise prediction of histology is not easy by the findings of conventional white light endoscopy and pathologic findings of forceps biopsy. There is still a controversy regarding the treatment of NIN, observation and diagnostic therapy by ER. Prospective study based on unified pathological definition is required in the future.

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P- Reviewer: Vieth M **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Liu XM

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i44.12519

World J Gastroenterol 2015 November 28; 21(44): 12519-12543
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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FRONTIER

Update on pathogenesis and predictors of response of therapeutic strategies used in inflammatory bowel disease

Emilio G Quetglas, Zlatan Mujagic, Simone Wigge, Daniel Keszthelyi, Sebastian Wachten, Ad Masclee, Walter Reinisch

Emilio G Quetglas, Project Strategy and Science, Grünenthal GmbH, 52078 Aachen, Germany

Zlatan Mujagic, Daniel Keszthelyi, Ad Masclee, Internal Medicine, University of Maastricht Medical Center, 6229 Maastricht, The Netherlands

Simone Wigge, Translational Science, Grünenthal GmbH, 52078 Aachen, Germany

Sebastian Wachten, Molecular Pharmacology, Grünenthal GmbH, 52078 Aachen, Germany

Walter Reinisch, Internal Medicine. McMaster University, Hamilton 8LS 4L8, Canada

Author contributions: All authors made contributions to this manuscript.

Conflict-of-interest statement: Emilio G Quetglas, Simone Wigge and Sebastian Wachten are currently Grünenthal Employees. Zlatan Mujagic and Daniel Keszthelyi declare no conflict of interest. Ad Masclee has received research funding from Grünenthal, Ferring Pharmaceuticals, Falk Medical and DSM. Walter Reinisch Walter Reinisch has served as a speaker, consultant, and/or advisory board member for Abbott Laboratories, AbbVie, Aesca, Amgen, Astellas, Astra Zeneca, Biogen IDEC, Bristol-Myers Squibb, Cellerix, Chemocentryx, Celgene, Centocor, Danone Austria, Elan, Ferring, Galapagos, Genentech, Grünenthal, Johnson and Johnson, Kyowa Hakko Kirin Pharma, Lipid Therapeutics, Millenium, Mitsubishi Tanabe Pharma Corporation, MSD, Novartis, Ocera, Otsuka, PDL, Pharmacosmos, Pfizer, Procter and Gamble, Prometheus, Robarts Clinical Trial, Schering-Plough, Setpointmedical, Shire, Takeda, Therakos, Tigenix, UCB, Vifor, Yakult, Zyngenia, Austria, and 4SC.

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Correspondence to: Emilio G Quetglas, MD, PhD, Project Strategy and Science, Grünenthal GmbH, 52078 Aachen, Germany. emilio.quetglas@grunenthal.com
Telephone: +34-913-017809
Fax: +34-913-017809

Received: February 6, 2015
Peer-review started: February 7, 2015
First decision: May 18, 2015
Revised: August 5, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: November 28, 2015

Abstract

The search for biomarkers that characterize specific aspects of inflammatory bowel disease (IBD), has received substantial interest in the past years and is moving forward rapidly with the help of modern technologies. Nevertheless, there is a direct demand to identify adequate biomarkers for predicting and evaluating therapeutic response to different therapies. In this subset, pharmacogenetics deserves more attention as part of the endeavor to provide personalized medicine. The ultimate goal in this area is the adjustment of medication for a patient's specific genetic background and thereby to improve drug efficacy and safety rates. The aim of the following review is to utilize the latest knowledge on immunopathogenesis of IBD and update the findings on the field of Immunology and Genetics, to evaluate the response to the different therapies. In the present article, more than 400 publications were reviewed but finally 287 included based on design, reproducibility (or expectancy to be reproducible and translationable into humans) or already measured in humans. A few tests

have shown clinical applicability. Other, *i.e.*, genetic associations for the different therapies in IBD have not yet shown consistent or robust results. In the close future it is anticipated that this, cellular and genetic material, as well as the determination of biomarkers will be implemented in an integrated molecular diagnostic and prognostic approach to manage IBD patients.

Key words: Mucosal immunology; Biomarkers; Pharmacology; Mode of action; Therapeutic drug monitoring

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Core tip: The following article is an update on the latest findings on the pathogenesis of inflammatory bowel disease (IBD) and its correlation with genetic and non-genetic predictors of the efficacy of the different strategies of treatment. Although many therapies have been used for decades, this is a completely new approach that has become even more complicated with new therapies like biologics. While most of these strategies are still in a very early stage, and have not been validated in clinical practice, they have begun suggesting the direction in which physicians should start looking to establish the most adequate therapeutic strategy for each individual patient.

Quetglas EG, Mujagic Z, Wigge S, Keszthelyi D, Wachten S, Masclee A, Reinisch W. Update on pathogenesis and predictors of response of therapeutic strategies used in inflammatory bowel disease. *World J Gastroenterol* 2015; 21(44): 12519-12543 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12519.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12519

INTRODUCTION

The search for biomarkers that characterize specific aspects of inflammatory bowel disease (IBD), has received substantial interest in the past years and is moving forward rapidly with the help of modern technologies. Currently, biomarkers are more progressively used in routine clinical care of patients with IBD. Most biomarkers used are not disease specific, but in general reflect inflammation. The last decade has brought significant gains in insight to IBD genetics and pathogenesis. These insights have the potential to improve the utility of biomarkers currently in use in clinical practice or are under investigation in clinical trials[1].

Although some reviews have been recently published on biomarkers[1], the most lacking topic is possibly is to identify adequate biomarkers for predicting and evaluating therapeutic response to different therapies which is less developed. With the progress in genetics research in IBD, genetic markers are increasingly being proposed to improve stratification of patients. Nevertheless, none of the genetic variants associated with particular outcomes have shown sufficient sensitivity or specificity to be implemented in daily management, maybe with the exception on those related to thiopurine metabolism.

Along a same line of thinking, pharmacogenetics, the study of association between variability in drug response and genetic variation, has also received more attention as part of the endeavor for personalized medicine. The ultimate goal in this area of medicine is the adaptation of medication for a patient's specific genetic background and therefore to improve drug efficacy and safety.

The aim of the following review is to utilize the latest knowledge on immunopathogenesis of IBD and update the findings in the field of Immunology and Genetics, to evaluate the response to the different therapies with the intent to predict the outcome within the diverse therapeutic strategies.

IMMUNOPATHOGENESIS OF IBD

The exact cause of IBD is still unknown, but is thought to be due to a combination of a patient's microbiome, immune response, and the environment that result in an excessive and abnormal immune response against commensal flora in genetically susceptible individuals (Figure 1).

Epithelial cells are able to identify bacterial components *via* extracellular receptors like toll-like receptors (TLRs) on the cell surface or intracellular NOD-like receptors in the cytoplasm - NOD2 (nucleotide-binding oligomerization domain containing 2)/CARD15 (caspase-activating recruitment domain 15 receptor). NOD2 receptor, recognizes the muramyl dipeptide (MDP), the minimal bioactive peptidoglycan motif common to all bacteria[2]. MDP stimulation induces autophagy which controls bacterial replication and antigen presentation, and modulates both innate and adaptive immune responses[3-5]. Autophagy is involved in intracellular homeostasis, contributing to the degradation and recycling of cytosolic contents and organelles, as well as to the resistance against infection and removal of intracellular microbes[6-8]. In the innate immune arm, the association of IBD [specifically, Crohn's disease (CD)] with NOD2 mutations and the two-autophagy-related genes *ATG16L1* and *IRGM* suggests that alterations in the recognition and intracellular processing of bacterial components may have a role in the immunopathogenesis of the disease[9-11]. The unfolded protein response has been identified as a critical pathway in the maintenance of cellular homeostasis[12].

Barriers of protection

Upon penetration of luminal contents into underlying tissues due to leakage in the mucosal barrier, impaired clearance of foreign material from the lumen leads to a compensatory acquired immune response that

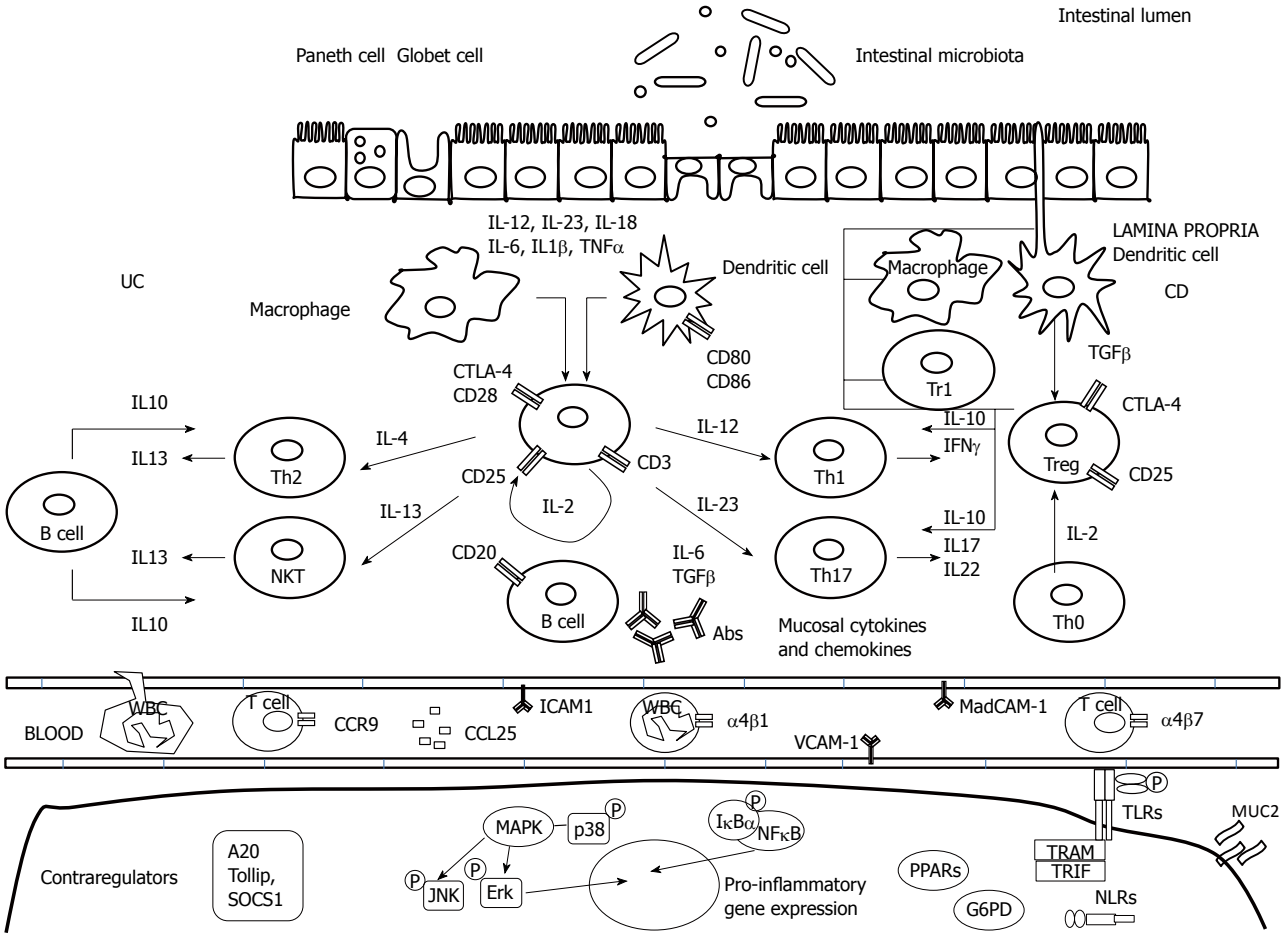

Figure 1 Inflammatory and regulatory pathways involved in inflammatory bowel disease pathogenesis. Crohn's disease (CD) is characterized by the generation of Th1- and Th17 T cell responses driven by the production of interleukin (IL)-12, IL-18, IL-23, IL-6 and tumor necrosis factor (TNF)-α by dendritic cells and macrophages. Th1-cells secrete IL-2, IL-17, interferon (IFN)-γ, and TNF-α. Ulcerative colitis (UC) is characterized by a Th2- T cell, and NKT response mediated by IL-5 and IL-13. T cell responses initiate an inflammatory cascade that involves endothelial activation, chemokine production, and white blood cell recruitment. Inappropriate triggering and maintenance of these pathogenic responses has been associated with innate immunity defects, *e.g.*, lack of efficient control by anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β. In the bottom of the figure, intracellular markers of activation are represented. Ab: antibody; CTLA-4: Cytotoxic T lymphocyte antigen-4; ICAM-1: Intercellular adhesion molecule-1; MAdCAM-1: Mucosal addressin cell adhesion molecule-1; VCAM-1: Vascular cell adhesion molecule-1; TLRs: Toll-like receptors; NLR: NOD-like receptor; NKT: Natural killer T.

can result in a chronic inflammatory state. Recently, a immunoregulatory dysfunction of hyperglycosylated mucin (MUC2) has been related to aggravation of IBD. Mucus does not seem to merely form a nonspecific physical barrier, but also constrains the immunogenicity of gut antigens by delivering tolerogenic signals[13].

Dendritic cells, as a part of the innate immune response, present antigens to naïve CD4+ helper T-cells and ensure tolerance to commensal flora by promoting their differentiation into regulatory T-cells. In response to over-activation of dendritic cells, there is a production of pro-inflammatory cytokines and a promotion of the differentiation of effector T-cells Th1, Th2 and Th17 (CD4+); moreover, over-activation induces a strong differentiation of CD8+ lymphocytes and other effector cells such as natural killer (NK) and NK T-cells while abolishing the production of regulatory cells[14].

Innate and adaptive immunity

Th1 cells, whose differentiation is induced by IL-12, produce a high amount of IFN-γ, TNF-α and IL-12, whereas Th2 cells release IL-4, IL-5 and IL-13[15]. An abnormal Th1 immune response is thought to predominate the intestinal inflammation in CD[16]. It has also been observed that in Ulcerative Colitis (UC), atypical NKT cells release higher amounts of the Th2 cytokine IL-13 than T cells from controls or CD patients[17,18]. However, recent data suggest that the CD-Th1 and UC-Th2 paradigms are not so straight forward[19,20].

The differentiation into Th17 cells, a subset of helper T-cells, is induced by IL-6 and TGF-β, acting in concert, and their expansion is promoted by IL-23. There is a delicate balance between Th17 and Treg. The absence of IL-6 drives Treg differentiation[21]. Mature Th17 cells are characterized by the secretion of copious amounts of IL-17A, IL-17F, IL-21, and IL-22[22-24]. The involvement of Th17 cells and, in particular, their signature cytokine IL-17A in intestinal inflammation has been extensively studied[25,26]. Only

when the Th17 cells are exposed to IL-23 they cease IL-10 production and attain their full pathogenic function[27].

TGF-β is produced by Treg cells and suppresses T-cell-mediated colitis in animal models[28]. TGF-β effects in IBD T cells are inhibited by the protein Smad7 and Smad7 is markedly overexpressed in IBD patients[29]. Inhibition of Smad7 *via* antisense DNA restored TGF-β sensitivity in IBD T cells has shown to be effective in murine models of experimental colitis[30,31]. Active IBD is dependent on the recruitment of mononuclear cells and leukocyte populations from the blood stream into the bowel wall. Recruitment is dependent on a series of steps known as rolling, tight binding/adhesion to endothelial cells, diapedesis, and migration of immune cells. This process is coordinated by selective adhesion molecules on the surface of immune cells and mucosal addressins on endothelial cells[32]. Selective adhesion molecules include cell-surface integrins that form heterodimers by various combinations of α- and β subunits. For gut homing of leukocytes, the interaction between α4/β7-integrins on T cells and the mucosal vascular addressing cell adhesion molecule 1 (MAdCAM-1) addressing on endothelial cells appears to be of crucial relevance.

Recent developments have classified NK cells as a subset of a new family of hematopoietic effector cells called innate lymphoid cells (ILCs). ILCs derive from an Id2 (inhibitors of DNA binding) expressing progenitor and the key cytokines secreted by ILCs tend to mirror those secreted by the T-helper cells of the adaptive immune system. Recent data has implicated ILCs, in particular group 3 ILCs in the development of IBD (ILCs IL-23 dependent with retinoid-related orphan receptor were found to be increased in the lamina propria of CD patients[33].

Based on this very recent knowledge, several of these molecules have been investigated as possible biomarkers/indicators of the immune response to therapies, however the results in sensitivity and specificity were moderate and validation was difficult. Here starts a review on the most promising ones[34].

PHARMACOTHERAPEUTIC OPTIONS

Besides nutritional and hygienic measures (smoking cessation), and the use of antibiotics to control symptoms there are several categories of medications used in the treatment of IBD: aminosalicylates (mesalazine), which are effective in treating mild-to-moderate episodes of UC and CD, as well as preventing relapses and maintaining remission[35-37], corticosteroids, recommended only for short-term use in order to achieve remission[38-40], thiopurines [azathioprine (AZA), mercaptopurine (MP)], effective at maintaining of clinical remission in steroid dependent IBD[41-43], methotrexate (MTX), positioned as an alternative immunosuppressive agent in patients with CD resistant or intolerant to AZA or MP[44-48], calcineurin inhibitors [cyclosporine (CsA), tacrolimus (Tac)], effective in the management of steroid refractory UC[49-51]; and, finally, the biologic therapies (adalimumab, certolizumab pegol, infliximab, golimumab, ustekinumab and vedolizumab) that interfere with the body's inflammatory response in IBD by targeting specific molecular players in the process such as cytokines and adhesion molecules[52-54].

Mesalazine

Mechanism of action: Mesalazine [(5-aminosalicylic acid (5-ASA)], also in the form of the pro-drug sulfasalazine, has been used for the treatment of UC for decades. It appears to act locally on colonic mucosa and reduces inflammation through a variety of anti-inflammatory processes (Figure 2). The current hypothesis is that 5-ASA activates a synthetic class of nuclear receptor. The anti-inflammatory actions of 5-ASA produce effects similar to activation of the γ-form peroxisome proliferator-activated receptors (PPAR-γ). PPAR-γ is a key receptor that mediates the effect of 5-ASA therapy in IBD by trans-repressing several key target genes such as nuclear factor B, signal transducers and activators of transcription: modulation of inflammatory cytokine production, modulation of RelA/p65 dephosphorylation, leading to decreased transcriptional activity of nuclear factor (NF)-κB, and reduced synthesis of prostaglandins and leukotrienes[55]. Activation of PPAR-γ also has anti-tumorigenic effects. PPAR-γ has a role in the regulation of intestinal inflammation and is highly expressed in the colon, where epithelial cells and macrophages are the main cellular sources of this nuclear receptor[56]. However, additional levels of activity at which the mechanism of action of mesalazine becomes apparent have been described. These include the inhibition of mediators of lipoxygenase and cyclooxygenase, IL-1, IL-2 and TNF-α. 5-ASA has also been recognized as a potent antioxidant and free-radical scavenger[55,57-61].

Measuring response to aminosalicylates in IBD: Heat shock proteins (Hsps) are a family of molecules that are typically involved in folding, refolding, translocation and degradation of intracellular proteins under normal and stress conditions[55,62]. Hsps can stimulate innate and adaptive immune responses and can also, by virtue of the sequence similarity between bacterial and human orthologs, become primary targets of autoimmunity due to a phenomenon known as molecular mimicry[63]. Thus, Hsps have been implicated in the pathogenesis of a number of chronic inflammatory and autoimmune diseases. Hsp60 and Hsp10 (Hsp60 co-chaperonin) are increased in the affected intestinal mucosa from patients with CD or UC[64]. Hsp60 and Hsp10 are increased in the cytoplasm of epithelial cells in CD and UC and also co-localised to epithelial cells of mucosal glands but not always in connective tissue cells of lamina propria, where only Hsp60 or, less often, Hsp10 is found[65].

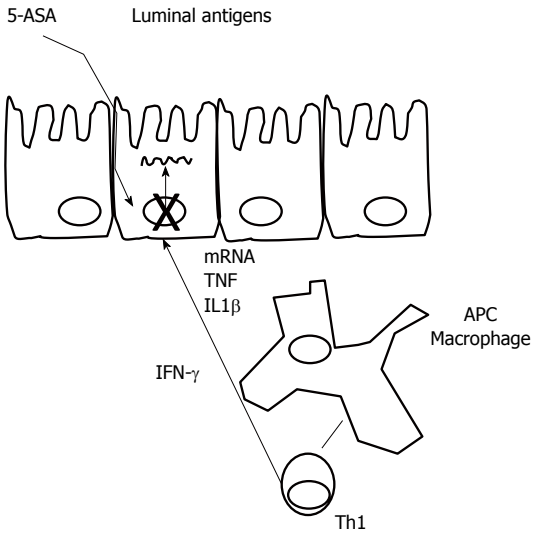

Figure 2 Mesalazine mode of action. Antigen presenting cells (APCs) recognize luminal antigens penetrating the colonic wall and through interaction with Th1 cells induce the production of interferon (IFN)-γ. IFN-γ activates epithelial cells. 5-aminosalicylic acid (5-ASA) is able to block transcription of inflammatory cytokines in colonic epithelial cells. IL: Interleukin; TNF: Tumor necrosis factor.

Tomasello *et al*[66], demonstrated that mucosal Hsp60 levels in UC patients decrease after therapy with either mesalazine alone or mesalazine plus probiotics, with the decrease in the latter being more pronounced. This same group has demonstrated that Hsp90 levels are high in UC mucosa, both in epithelium and lamina propria. Treatment with 5-ASA plus probiotics reduces Hsp90 levels in the lamina propria, while 5-ASA alone does not have any effect. However, Hsp90 levels within the epithelium were not affected by any of the treatment regimens. In fact, authors have found a linear correlation between Hsp90 and CD4 levels in lamina propria in both UC patients at diagnosis and 6 mo after 5-ASA alone therapy[67].

According to these and previously published results, it has been proposed that a synthetic Hsp90 inhibitor, able to block LPS-induced TLR4 signaling of CD4+ cells, could be applicable to treatment of autoimmune diseases involving inflammation and activation of the adaptive immune response[68]. The latest results show that Hsp10 levels in UC mucosa decrease after therapy. This decline is similar to what is previously described for Hsp60; however, in contrast to Hsp60, Hsp10 has been described as an anti-inflammatory agent. In conclusion, these results altogether indicate that determination of Hsp levels in intestinal mucosa as done in this study has a promising potential for monitoring response to treatment in UC.

Corticosteroids

Glucocorticoids (GC) are potent inhibitors of T cell activation and pro-inflammatory cytokines. However, failure to respond to glucocorticoid therapy is a risk factor for a progressive course of IBD[69,70]. In these patients reduced peripheral T lymphocyte GR binding affinity and abnormalities of glucocorticoid receptor activator protein (GR-AP)-1 interaction and increased expression of GRβ (a truncated splice variant of the normal isoform GRα that does not bind to glucocorticoid ligands) are observed[71].

Mechanism of action: Glucocorticoids mediate their anti-inflammatory responses by binding the intracellular glucocorticoid receptor (GR), a phosphorylated 92-kDa protein, which is a member of the nuclear receptor superfamily[72] (Figure 3). The unliganded receptor is sequestered in the cytoplasm, bound to heatshock proteins Hsp90 and Hsp70 and immunophilin FKBP59, a 59-kDa protein. Upon GC binding and dissociation from heterocomplex proteins, GR translocates into the nucleus; translocation is mediated by specific nuclear transport factors that belong to the importin β family of nuclear transporters, and in particular by importin 13[73]. The activated receptor then binds as homodimer to palindromic DNA-binding sites, the so-called glucocorticoid responsive elements (GREs), localized in the promoter region of target genes[74-76]. Although some GC anti-inflammatory effects are achieved through induction of anti-inflammatory genes, such as interleukin (IL)-10, annexin 1 and the inhibitor of NF-κB[77,78], transactivation enhances mainly the expression of genes involved in metabolic processes[79,80], and is therefore, responsible for the majority of unwanted side effects[81,82]. Indeed, the presence of GR on GRE might competitively prevent the binding of activator protein (AP)-1 and NF-κB on the same promoter regions or might trans-activate their inhibitor proteins. Furthermore, GRE-independent mechanisms of trans-repression also exist: the GR physically interacts with AP-1[83], NF-κB[84] and signal transducers and activators of transcription[85]. Trans-repression is believed to be responsible for the majority of the beneficial, anti-inflammatory effects of GCs[79,86-88].

Measuring response to corticosteroids in IBD: Research in impaired sensitivity to glucocorticoid inhibition in IBD has highlighted three potential molecular mechanisms: (1) decreased cytoplasmic glucocorticoid concentration secondary to increased P-glycoprotein-mediated efflux of glucocorticoid from target cells due to overexpression of the multidrug resistance gene (MDR1)[89-91]; (2) impaired glucocorticoid signaling because of dysfunction at the level of the glucocorticoid receptor[92,93]; and (3) constitutive epithelial activation of pro-inflammatory mediators, including NFκB, resulting in inhibition of glucocorticoid receptor transcriptional activity[94,95].

The multi-drug resistant (*MDR1*) gene codes for a drug efflux pump P-glycoprotein-170 (permeability-glycoprotein or Pgp), which is expressed on the apical surface of lymphocytes and intestinal epithelial cells and actively transports toxins and drugs out of

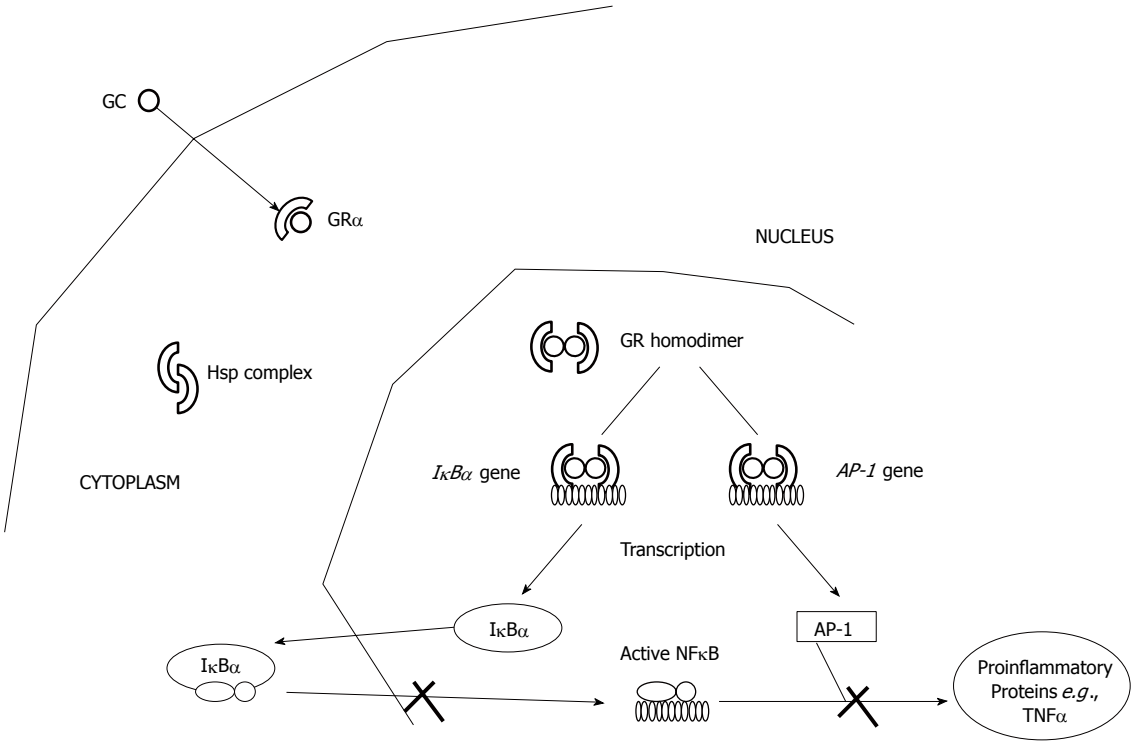

Figure 3 Glucocorticoids mode of action. AP: Activator protein; GC: Glucocorticoids; GR: Glucocorticoid receptor; TNF: Tumor necrosis factor; NF: Nuclear factor.

target cells, thereby removing toxic metabolites and xenobiotics from cells into urine, bile, and the intestinal lumen. This efflux pump also regulates the distribution and bioavailability of drugs, and in conclusion reduces their efficacy. To date, 15 MDR1 polymorphisms have been identified and a polymorphism in exon 26 (C3435T) of the MDR1 gene has been shown to be significantly correlated with levels of expression and function of P-gp-170 in healthy individuals. Healthy individuals are classified as: homozygous (C/C or"resistant" genotype and T/T or "responsive" genotype) or heterozygous (C/T). The C/C genotype is highly prevalent in West Africans (83%) and African Americans (61%) compared with 26% and 34% in caucasians and Japanese populations, respectively[90,91].

Pgp and MDR expression have been shown to be significantly higher in CD and UC patients requiring surgery due to failure of medical therapy[92]. In MDR1 knockout mice associations between C3435T and UC and G2677C/T and IBD have been described. Several other associations with SNPs in the TNF (tumor necrosis factor) gene and the macrophage MIF (migration inhibitory factor) gene and GC dependency or sensitivity have also been reported. According to these results a protective role for the MDR1 3435 C/C versus MDR1 3435 T/T genotype and C versus T allele for the progression of IBD is suggested[93-112].

Matrixmetalloproteinases (MMPs) make up a family of 24 human zinc-dependent endopeptidases and degrade practically all extracellular matrix components[102,103]. They are fundamental for tissue damage[103] and expressions correlate with the degree of inflammation in the gut[104,105]. MMP activity is inhibited by tissue inhibitors of MMPs (TIMPs), as well as nonspecific inhibitors such as α2-macroglobulin (α2M)[106]. TIMPs modulate the activity of soluble, matrix-bound, and cell-associated MMPs[106] and are upregulated in IBD[107,108]. α2M is a serum anti-proteinase, capable of almost universally inhibiting endoproteinases, and is thought to be the major plasma inhibitor of MMPs[106]. Pro-inflammatory cytokines, such as TNF-α, increase MMP production[109], and production of TNF-α correlates with both MMP and TIMP production in IBD[110]. Serum levels of MMP-7, -8, and -9, TIMP-1, and α2M, are elevated in active IBD. Both, GC and anti-TNF-α therapies reduce MMP-7 levels, but only in GC treated patients, the levels decline corresponding to levels of control patients. Interestingly, no significant changes in α2M are associated to GC treated group. MMP-7 and TIMP-1 seem promising in monitoring the effect of GC treatment. GCs inhibit MMP synthesis by controlling gene expression as well as by inducing the transcription of TIMPs[83]. While MMP-7/TIMP-2 ratio is associated with greater severity of UC[86], the decrease in MMP-7/TIMP-2 ratio in GC-treated patients is more likely a result of decrease in MMP-7 itself as TIMP-2 is not affected.

Several investigations have also identified GR abnormalities as potential mechanisms influencing response to glucocorticoid treatment in several inflammatory conditions as: (1) reduced peripheral T-lymphocyte GR binding affinity[91]; (2) abnormalities of GR-AP-1 binding in glucocorticoid resistant asthma,

suggesting a post-receptor mechanism[79]; and (3) increased expression of glucocorticoid receptor β (GRβ), a truncated splice variant of the normal isoform GRα that does not bind glucocorticoid ligands. GRβ is unable to transactivate glucocorticoid-responsive genes, and has therefore been suggested to act as a dominant-negative inhibitor of glucocorticoid action[92].

Honda *et al*[111] reported GRβ mRNA expression in 83% of the patients with steroid-resistant UC compared to only 9% in steroid-responsive patients, and 10% in healthy controls and chronic active CD patients. These results were confirmed in a recent study from Japan, where the authors looked at the frequency of GRα and β positive cells in colonic biopsies of GC-sensitive (n = 6) and GC-resistant (n = 8) UC patients[112]. They also found that there were significantly more GRβ-positive cells in the GC-resistant group than in the GC-sensitive and the control groups.

miRNAs are small (18-24 nucleotides) non-coding RNAs, which bind the 3'UTRs (mRNA that immediately follows the translation termination codon) and the coding exons of their target genes and inhibit gene expression. By affecting gene regulation, miRNAs are likely to be implicated in the control of diverse biological processes Moreover, miRNAs have important regulatory roles in the innate and adaptive immune system, and characteristic miRNA expression profiles have been demonstrated even in IBD[113]. A number of studies have shown that GCs can modify the expression profile of different miRNAs but to date it is not possible to recognize a specific miRNA pattern regulated by GCs. It has been demonstrated that activation of GR by GCs might induce or repress specific miRNAs in various target genes. The majority of studies have evaluated the effect of GCs on miRNA expression levels in tumor leukemic cells, during GC induced apoptosis[114]. Of interest, miRNA could target mRNAs encoded by genes involved in the importin pathway, or appear like potential regulators of components of the inflammasome pathway (key signalling platforms that detect pathogenic microorganisms and sterile stressors, and that activate the highly pro-inflammatory cytokines IL-1β and IL-18). Both importins and the inflammasome are involved in molecular mechanism of GC signaling: importin is a nuclear transport protein responsible for the translocation of the complex GR-GC into the nucleus[115], and variants of the inflammasome gene have been correlated with steroid resistance in pediatric IBD patients[116].

Conversely, NF-κB and GRα can mutually repress each other's transcriptional activity. Consequently, the debate as to whether inflammation drives glucocorticoid resistance or vice versa has refocused investigators' efforts into the critical role played by NF-κB[93]. Further investigations have shown that while the activation of AP-1 and the upstream kinases p38 and c-Jun N-terminal kinase (JNK) in steroid-sensitive patients with CD was mainly found in lamina propria macrophages, steroid-resistant patients revealed activation of all these mediators mostly in epithelial cells[94].

Gene expression profiling can be successfully used to stratify patients and identify transcriptional signatures associated with clinical parameters. Several predictor gene panels containing genes involved in immune mechanisms (PTN, OLFM4, LILRA2, CD36), autophagy or GC response (STS, MDM2) have been identified. This represents, the first biomarker discovery [predictor gene panels that contain genes involved in immune mechanisms (PTN, OLFM4, LILRA2, CD36), autophagy or GC response (STS, MDM2)] based on specifically designed analytical algorithms with potential value to predict GC response and need of surgery as well as with diagnostic value for IBD patients[117].

Thiopurines

Mechanism of action: *In vitro* studies have shown that AZA and 6MP exert their effect by controlling T cell apoptosis through modulation of Rac1 activation upon CD28 co-stimulation(118). Apoptosis induction required co-stimulation with CD28 and was mediated by specific blockade of Rac1 activation through binding of 6-thioguanine nucleotide (6-TGN) to Rac1 instead of guanine triphosphate (GTP). Activation of the Rac1 gene in turn leads to activation of mitogen-activated protein kinase (MAP kinase), NF-κB, bcl-x(L)(B cell lymphoma) and finally to a mitochondrial pathway of apoptosis. Thus AZA and 6MP convert a co-stimulatory signal into an apoptotic signal by modulating Rac1 activity. In Figure 4 the thiopurines biotransformation pathway is represented.

Measuring thiopurines derivatives: TPMT enzyme activity (measured by radioimmunoassay) is genetically determined and has been extensively reviewed[118]. In summary, TPMT enzyme activity can identify patients with high TPMT activity that metabolize 6-MP to 6-methyl-MP and therefore may be resistant to treatment with thiopurine drugs. It is estimated that TPMT deficiency is responsible for up to 30% of all adverse drug reactions (ADRs) experienced on AZA, but whilst TPMT deficiency strongly predicts the development of myelotoxicity, the most serious ADR of AZA therapy, it fails to account for over 70% of cases of myelotoxicity[119,120]. Another candidate enzyme for further study is xanthine oxidase /dehydrogenase (XDH)[121,122]. Blocking XDH activity using allopurinol (which, as recently described, also inhibits TPMT due to skewed drug metabolism)[123] is known to cause severe toxicity with conventional doses of AZA and safe co-prescription of allopurinol requires an AZA dose-reduction of approximately 80%[124]. A molybdenum cofactor[125] is essential for the action of three oxidases, XDH, aldehyde oxidase (AO) and sulphite oxidase. This molybdenum cofactor requires the action of molybdenum cofactor sulfurase (MOCOS). MOCOS

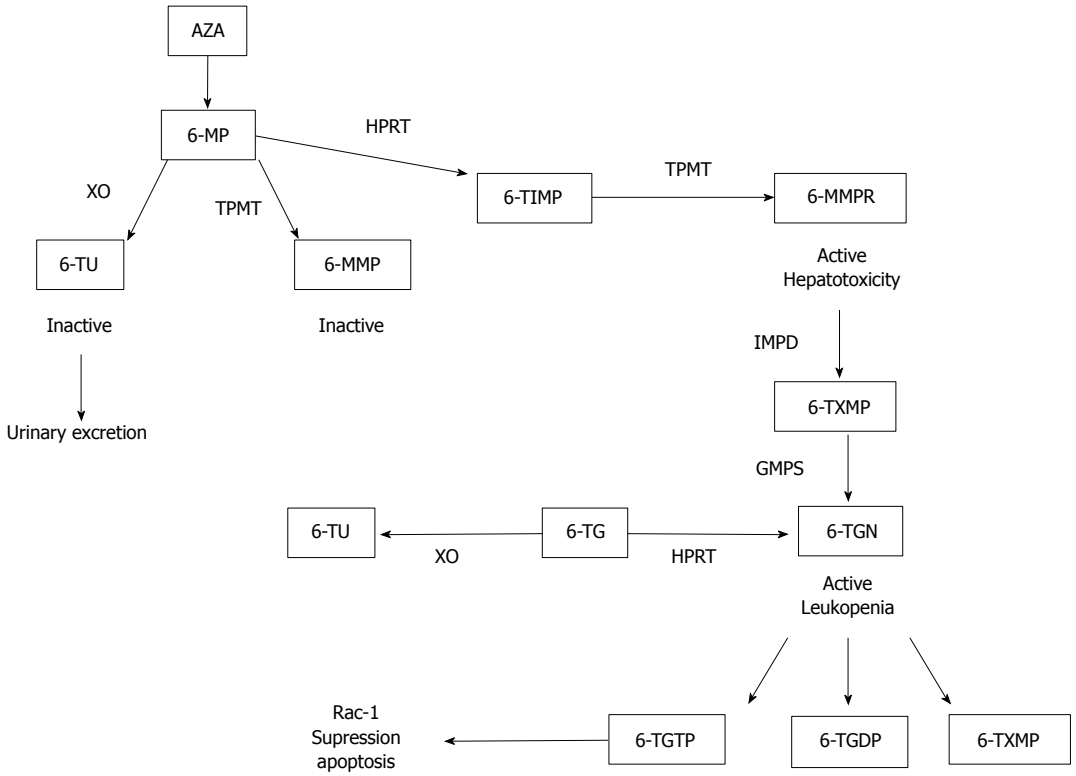

Figure 4 Thiopurines metabolic pathway. XO: Xanthin oxidase; 6-TU: 6-thiouracil; TPMT: Thiopurine methyltransferase; HPRT: Hypoxanthine phosporybosil transferase; 6-MMP: 6-methyl mercaptopurine; 6-TIMP: 6-thiosine 5' monophosphate; 6-MMPR: 6-methyl mercaptopurine ribonucleotide; IMPD: Inosine monophosphate dehydrogenase; 6-TXMP: 6-Thioxanthosine monophosphate; GMPS: Guanosine monophosphate synthetase; 6-TGN: 6-thioguanine nucleotide; 6-TG: 6-thioguanine; 6-TGDP: 6-thioguanine diphosphate; 6-TGTP: 6-thioguanine triphosphate.

deficiency (which results in the deficiencies of both XDH and AO, but not sulphite oxidase) is, in contrast, relatively benign, causing only a predisposition to renal stones (Type Ⅱ Xanthinuria)[126]. AO has been considered of minimal clinical significance and so it has not been carefully examined until recently.

Monitoring of 6-MP metabolites is a helpful, but not an indispensable tool in thiopurine non-responders to discriminate poor adherence and under-dosing from pharmacogenetic thiopurine resistance and thiopurine refractory disease. Several studies have reported that patients with IBD treated with AZA or 6-MP who respond to therapy have higher median concentrations of 6-TGN than patients who fail to respond to therapy[127-129]. One study in 93 patients with IBD reported that the median concentration of 6-TGN in responding patients was 312 pmol/8 × 10^8 RBCs compared with a median concentration of 199 in patients who failed to respond[129]. There was no difference in the median concentrations of 6-MMP between the 2 patient groups. The breakpoint between the lower two quartiles and the higher two quartiles of 6-TGN concentrations was 235 pmol/8 × 10^8 RBCs. Sixty-five percent of responding patients had an erythrocyte 6-TGN concentration > 235 as compared with only 27% of patients failing therapy. Thus, the authors suggested that clinicians should adjust AZA or 6MP doses to achieve 6-TGN concentrations > 235pmol/8 × 10^8 RBCs. The authors also reported that hepatotoxicity (defined as liver enzymes more than twice normal) occurred in 16 patients, and that the median 6-MMP concentrations were 5463 pmol/8 × 10^8 RBC's in patients with hepatotoxicity compared with only 2213 pmol/8 × 10^8 RBCs in patients without hepatotoxicity.

Unfortunately, dose escalation of thiopurines does not necessarily result in higher efficacy. Instead of increasing 6-TGN concentration, following increasing the thiopurine dose, some patients shift their metabolism towards the production of 6-MMP resulting in hepatotoxicity[130]. Another approach to bypass the influence of TPMT has been by direct administration of 6-TGN[131-133]. High concentrations of 6-TGN were achieved but the drug had to be stopped because of nodular regenerative hyperplasia in the liver[134].

Smith *et al*[135] have recently published the impact of introducing nucleotide monitoring into clinic. They obtained 608 TGN results from 189 patients with IBD. In non-responders, TGNs directed treatment change in 39/53 patients. When treatment was changed as directed by TGN, 18/20 (90%) improved *vs* 7/21 (33%) where the treatment decision was not TGN-directed ($P < 0.001$). Where treatment change was directed at optimization of thiopurine therapy, 14/20 achieved steroid-free remission at 6 mo *vs* 3/10 where the TGN was ignored ($P = 0.037$). Six per cent of patients were non-adherent, 25% under-dosed and 29% over-dosed by TGN. Twelve per cent of patients demonstrated

preferential thiopurine methylation; this group had low TGN levels and high risk of hepatotoxicity. In responders, adherence and dosing issues were identified and TGN-guided dose-reduction was possible without precipitating relapse. Mean cell volume (MCV), white blood cell count (WBC) and lymphocyte counts were not adequate surrogate markers. MCV/WBC ratio correlated with clinical response, but was less useful than TGN for guiding clinical decisions.

In a previous study, the same group identified the presence of the coding region SNP *AOX1* 3404G as a predictor of non-responsiveness to AZA therapy[136]. The authors suggested that those with a poor chance of responding to AZA (high TPMT activity and *AOX1*3404G variant) should be offered an alternative treatment as first-line therapy, which might include reduced dose azathioprine in combination with allopurinol, a combination which has been shown to circumvent the problem of hyper-methylation in some patients[137]. In patients with CD, the next immunomodulator considered for treatment would usually be MTX. It is possible that the same polymorphism *AOX1* c.3404A > G could also affect an individual's chance of response to MTX, as AO is known to metabolize MTX producing a 7-hydroxymetabolite, which is considered inactive.

Genotype variants, which have a functional impact, most commonly decrease the activity of the affected enzyme. If this is true for the *AOX1* 3404G variant, then the association with lack of clinical response would suggest that AO metabolites of AZA have immunosuppressive activity: 8-hydroxy-6MP did not slow the growth of rat sarcoma[138]; however, AO produces several other AZA metabolites on which no functional work has been carried out. Another possibility is that AO activity is increased in the presence of the *AOX1* 3404G variant. In this case, it is possible that overactive AO removes and inactivates a higher proportion of the ingested drug, resulting in decreased efficacy; but in this case, one would expect carriers of the *AOX1* sequence variant to have lower TGN levels.

Purinergic signaling and associated ectonucleotidases, such as CD39 and CD73, have been implicated in the pathogenesis of IBD. Adenosine generated by CD73 and CD39 components might play an important role in the resolution of inflammation and in the promotion of healing. The anti-inflammatory effects of AZA, have been ascribed to induce apoptosis of predominantly CD45RO+ memory T cells (within CD73+CD4+ T cells)[139].

Methotrexate

Mechanism of action: MTX, like folic acid, is a substrate for the enzyme folylpolyglutamate synthetase, which adds glutamic acid residues to these compounds. Parent MTX, polyglutamated MTX metabolites (MTXPG), and another major metabolite, 7-hydroxymethotrexate (7OH-MTX), are all folic acid analogues with inhibitory activity against many of the enzymes in the metabolic pathway of folic acid[140]. The principal cellular action of MTX, is competitive inhibition of the enzyme dihydrofolatereductase (DHFR)[141]. The metabolites of MTX have considerable importance as inhibitors of the folate-dependent enzymes distal to DHFR. MTXPG are preferentially retained intracellularly in a non-effluxable form in proportion to the length of the polyglutamate chain, and they account for more than 50% of intracellular drug 24 h after exposure[142]. 7-0H-MTX, which is the major circulating variant of MTX 24 h after a dose of the drug, undergoes polyglutamation 2.7-fold faster than does MTX, and it is also a 4.5-fold more potent inhibitor of 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase, and possibly other distal folate-dependent enzymes, than is MTX[143,144]. MTX is effective in the treatment of inflammatory diseases such as CD in low doses. The question is whether inhibition of T cell proliferation is a major mechanism of action in low-dose MTX treatment and if it is related to inhibition of DHFR or not. Some studies suggest that low-dose MTX is indeed able to inhibit lymphocyte proliferation through DNA synthesis inhibition[145]. However, anti-inflammatory properties of MTX were not always found to depend on lymphocyte proliferation inhibition. On the other hand, overt inhibition of cellular proliferation produced by inhibition of DHFR is not a requirement for efficacy but rather is a sign of toxicity of low-dose MTX therapy. The coadministration of folinic acid or leucovorin (fully reduced tetrahydrofolate), which bypasses blockage of DHFR, ameliorates many of the side effects of MTX. However, if given in excess quantities, it also retards the efficacy of the drug[146-148] (Figure 5).

Proliferation of T cells can also be inhibited by MTX through its inhibition of cytokines, such as IL-2, that promote proliferation. MTX generally inhibits Th1 cytokines and up-regulates or does not affect Th2 cytokines[149-151]. TNF-α, in particular, was found to be suppressed in both *ex vivo* stimulated T cells of patients with rheumatoid arthritis (RA) treated with MTX and *in vivo* and *in vitro* experiments in T cells and macrophages[152,153]. Also, the number of CD4+ T cells that secrete TNF-α was significantly lower in MTX-treated patients with RA than in untreated patients[154]. The inhibitory effect of MTX on TNF-α can be attributed to several mechanisms: high levels of adenosine[155]; inhibition by MTX of TNF-α promoter activity in lymphocytes[154]; inhibition of NF-κB activation, indirectly inhibiting subsequent TNF-α transcription[156]. IFN-γ, has also been shown to be inhibited by MTX.

In vivo, MTX enhances IL-10 production. Patients with RA treated with MTX showed increased numbers of IL-10-producing T cells, and *ex vivo* stimulated monocytes of patients with RA treated with MTX showed increased IL-10 production[154]. Interestingly, upregulation of IL-10-producing monocytes was observed only in patients who responded to therapy. In summary, pro-inflammatory cytokines and

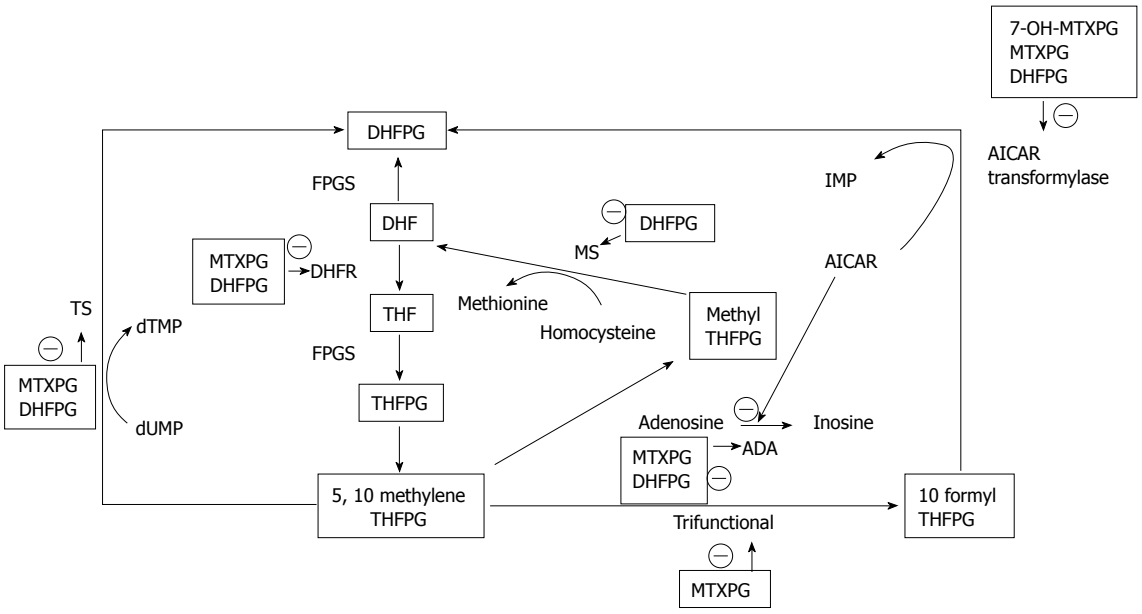

Figure 5 Methotrexate mode of action. DHF: Dihydrofolate; FPGS: Folil polyglutamate synthetase; DHFPG: Dihydrofolate polyglutamates; DHFR: Dihydrofolate reductase; THF: Tetrahydrofolate; THFPG: Tetrahydrofolate polyglutamates; TS: Thymidylate synthetase; MS: Methionine synthetase; ADA: Adenosine deaminase; IMP: Inosine monophosphate; AICAR: 5-aminoimidazole-4-carbox-amide ribonucleotide.

cytokines that promote proliferation are inhibited by MTX, whereas the anti-inflammatory cytokine IL-10 is upregulated. This may suggest that induction of IL-10 by MTX is one of its important anti-inflammatory mechanisms[157].

Genetic polymorphisms and treatment response: The uptake of MTX into cells is mainly controlled by the reduced folate carrier (RFC). A non-conservative polymorphism G80A (R27H) has been associated with higher plasma concentrations of MTX and a worse prognosis (event-free survival estimates) in children with acute lymphoblastic leukaemia treated with this drug[158].

The folypolyglutamase hydrolase, also known as c-glutamyl hydrolase (GGH), converts long-chain MTXPG into short-chain MTXPG and ultimately back to MTX resulting in higher efflux of the drug. Also, in acute lymphoblastic leukaemia patients, several polymorphisms in this gene have been identified including a putatively functional non-conservative SNP C452T (T127I) associated with low enzyme activity and higher accumulation of long-chain MTX-PG experiencing a better event-free survival[159,160].

Therapy with MTX results in a reduction of the reduced folate pool by inhibiting dehydrofolate reductase. Another enzyme, the methylenetetrahydrofolate reductase (MTHFR) is crucial for folate homeostasis by converting 5,10-methylene-tetrahydrofolate, the methyl-donor in dTMP synthesis, into 5-methyltetrahydrofolate, the carbon-donor required for methionine synthesis. Two common, non-synonymous polymorphisms in this gene have been found to influence MTX toxicity and efficacy. The SNP C677T results in a more thermolabile variant of the protein and has been associated with increased drug toxicity[161]. Interestingly, this SNP has also been associated with overall susceptibility to IBD in an Irish cohort of patients[162]. The second SNP, A1298C, also leads to a reduced activity of the MTHFR[163,164] and has been associated with increased efficacy in patients with RA[165].

There is also evidence from *in vitro* experiments that impaired folylpolyglutamasesynthase (VMcN1) activity may play a crucial role in MTX resistance[166,167].

A small clinical trial in 18 CD patients has de monstrated that individual RBC MTXGlu1-5 concentrations can be measured accurately and have low intra-patient variation at steady state. Unexpectedly, the study suggested that RBC MTXGlu4 and 5 concentrations correlated inversely with efficacy in patients with CD. In addition, high RBC MTXGlu4&5 concentrations were associated with an increased incidence of adverse effects. Although the findings were statistically significant, the number of subjects in this pilot study was small and, therefore, as stated by the authors, there is a high possibility of a type I statistical error[168].

Calcineurin inhibitors

Mechanism of action: Calcineurin inhibitors exert their cellular effects through binding to proteins called immunophilins[169]. Cyclophilins (CP) bind CsA and FK-binding proteins (FKBPs) bind Tac. Cyclophilin A is the most abundant cyclophilin in T lymphocytes, and the predominant Tac-binding immunophilin is the FKBP12. The CPs and FKBPs are structurally unrelated but both families have a cis-trans prolyl-peptidyl isomerase activity. The binding of CsA or Tac to its respective immunophilin enhances the immunophilin's affinity to calcineurin. Formation of such a complex results

in its binding to and inhibition of calcineurin[170]. In the process of T-cell activation calcineurin, which is a calmodulin-activated serine phosphatase, associates with and dephosphorylates inactive nuclear factor of activated T cells (NFAT). This leads to NFAT translocation to the nucleus and, in association with other transcription factors such as AP-1, initiation of downstream events involved in T-cell activation[171-173]. Within the members of the NFAT family, NFAT1, NFAT2, and NFAT4 participate in T lymphocytes cytokines transcriptional activation such as IL-2, IL-4, and CD40L[174]. So, in T cells NFAT proteins not only regulate activation but also are involved in the control of thymocyte development, T-cell differentiation and self-tolerance. The functional versatility of NFAT proteins is explained by their complex mechanism of regulation and their ability to integrate calcium signalling with other signalling pathways. The drug-immunophilin complex forms an inhibitory association with calcium-calmodulin-activated calcineurin, preventing its binding and activation of NFAT. CsA also inhibits mRNA transcription of IFN-γ by inhibiting NFAT translocation into the nucleus (Figure 6).

CsA and Tac, induce apoptosis of CD4+ T-lymphocytes[175]. CsA reduces the number of the anti-apoptotic Bcl-2-positive T cells[175]. The apoptotic activity of CsA is possibly mediated by the inhibition of cytokine release and the subsequent activation of ICE-like proteins (ICE is an acronym for IL-1β converting enzyme of caspase 1), known to play a chief role triggering the apoptotic cascade[176].

Apart from the above-mentioned effects on T lymphocytes, CsA inhibits antigen presenting cells activity and production of the B-lymphocyte activating factors[177]; attenuates adhesion interaction and trans-endothelial migration and infiltration of neutrophils by decreasing endothelial expression of cell adhesion molecules (E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1) and inhibits the anti-apoptotic NF-κB, a central transcription factor mediating inflammatory injury[178].

Influence of genetics on pharmacological behavior

Pharmacokinetics: CsA and Tac are among the most commonly used immunosuppressants in patients with organ transplantation or autoimmune diseases. However, both have a narrow therapeutic window and large inter-individual variability, resulting in therapeutic drug monitoring (TDM), necessary for adjusting the dose in order to reduce the toxicity and improve the efficacy.

To date, most transplant centers utilize whole-blood measurements of CsA trough levels as a means of TDM. However, it was demonstrated that the correlation of "therapeutic" trough levels with the actual drug exposure[179,180] or with clinical outcomes[181] was relatively poor. The determination of total AUC is the most accurate measure of drug exposure, and its values possibly correlate to some degree with the rate of successful outcomes. However, due to the cost and inconvenience of multiple blood measurements required for AUC determination, this method is impractical. A decade ago, prospective studies were underway examining the utility of a single measurement of 2-h (C2) CsA level, which showed to be associated with renal allograft rejection (blood concentrations of CsA during the early post dose period had been shown to correlate well with inhibition of calcineurin and IL-2 but still was, logistically difficult and plagued by a high intra-individual variability)[182,183].

Unlike the case of CsA, the trough levels of Tac correlate reasonably well with AUC and are the most common measure of Tac treatment monitoring[184]. Although TDM is widely recommended in clinical practice and has been conducted for approximately 30 years, this strategy for calcineurin inhibitors therapy is controversial according to recent reports[185]. In the past decade, the understanding of the pharmacogenomics of calcineurin inhibitors in transplantation has improved. Polymorphisms of genes coding for enzymes and transport proteins involved in the metabolism of these compounds have been thoroughly studied. CYP3A4 oxidizes CsA at multiple positions and is known to convert CsA into three major primary metabolites (AM1, AM9 and AM4N). CYP3A5 preferentially attacks at amino acid 9 and metabolizes CsA to only one primary metabolite (AM9). For Tac, the intrinsic clearance for CYP3A5 is approximately 2-fold higher than for CYP3A4. CYP3A5 catalyses the formation of four primary metabolites (M1, M2, M3 and M6). It is well established that the CYP3A5 A6986G (*3) SNP influences the pharmacokinetics of Tac in renal recipients[186]. Almost all studies have reported that recipients with the CYP3A5*3/*3 genotype (non-expressers) exhibit higher dose-adjusted Tac exposure (C0/dose, C2/dose or AUC/dose), and a lower dose requirement compared with the CYP3A5*1/*1 or*1/*3 carriers (expressers). With respect to CsA and the CYP3A5*3SNP, the results from clinical studies have not been able to reach a conclusion. ABCB1 (MDR1), encoding the transport protein P-glycoprotein, which pumps calcineurin inhibitors out of intestinal enterocytes, has had several of its SNPs investigated in renal transplant patients. The influence of these SNPs on the pharmacokinetics of CsA and Tac remains uncertain, as CYP3A4 also demonstrates inter-individual variation in a metabolic capacity and functional SNPs are few in the CYP3A4 gene and most studies found no association with pharmacokinetics of CsA, and controversial associations with Tac[186,187].

Pharmacodynamics: Enzymatic and immunological strategies are the two types of methods which can be used to assess the pharmacodynamics of these compounds. The former directly determines calcineurin activity, while the latter measures immune responsiveness at several levels[187,188]. Although these strategies are still in a very early stage, and

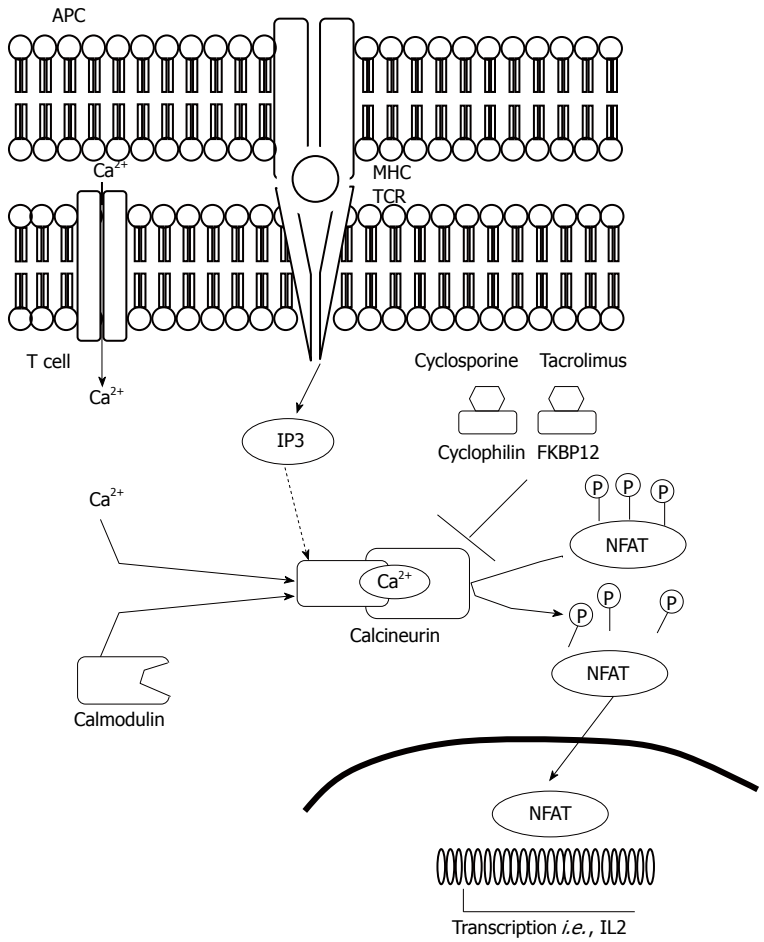

Figure 6 Mode of action of calcineurin inhibitors. APC: Antigen presenting cell; MHC: Major histocompatibility complex; NFAT: Nuclear factor of activated T cells; TCR: T cell receptor; IP3: Inositol triphosphate.

have not been validated in clinical practice, several clinical studies have reported associations of NFAT-regulated genes with biopsy-proven acute rejection and recurrent infections in renal recipients, making the expression of such genes a promising biomarker of pharmacodynamics[189,190]. In fact, functional polymorphisms in PPIA (coding for cyclophilin), FKBP1A (coding for FKBP12), PPP3CA/PPP3CB/PPP3R1 (coding for calcineurin), NFATC1/NFATC2/NFAT5 (coding for NFAT) and IL-2 (coding for IL-2) have been explored in other diseases[191-195].

Many important metabolic enzymes and transporters could also be modulated by orphan nuclear receptors, a large family of transcription factors regulating tissue gene expression, such as the pregnane X receptor (PXR), constitutive androstane receptor, the glucocorticoid receptor and more.

In recent years, epigenetics have been incorporated to the field of pharmacology referring to drug responses accounted for by epigenetic changes (DNA methylation, modification of histones in chromatin and RNA-mediated regulation of gene expression, as, miRNAs) instead of alterations in the DNA sequence. In contrast to SNPs, epigenetic characteristics can be altered by age, influenced by drugs and can interact with environments. Current evidence has revealed that the expression of CYP3A4 and CYP3A5 could be affected by a DNA methyltransferase inhibitor and miRNA-27b[196,197]. Besides the direct action on enzymes, miRNAs also regulate the expression of nuclear receptors, such as PXR[198].

Biologics

Mechanism of action - Anti TNF-α: TNF plays a central role as a pro-inflammatory cytokine that initiates the defense response to local injury. When present at low concentrations, it is believed to have beneficial effects, such as the augmentation of host defense mechanisms against infections. At high concentrations, TNF can lead to excess inflammation and organ injury. Both immune (macrophages, T cells, granulocytes, *etc.*) and non-immune cells (fibroblasts, neurons, smooth muscle cells) can produce TNF. It is initially produced as a cell surface-bound precursor (tmTNF), which can be enzymatically cleaved by TNF-α converting enzyme to form a soluble cytokine (sTNF). Both sTNF and tmTNF are biologically active and interact with either of 2 distinct receptors to exert their action: the p55 TNF receptor 1 (TNFR1) and the p75 TNFR2 that are present on a wide range of cell types. sTNF preferably binds to TNFR1 and generates the pro-inflammatory properties of

TNF: Activation of nuclear factor kappa-B1 (NF-κB1), which will result in the transcription of several inflammatory genes and the expression of other pro-inflammatory cytokines including IL-1 and IL-6 and enhancement of leukocyte migration by inducing expression of adhesion molecules by endothelial cells and leukocytes; caspase-8- and caspase-3-dependent apoptosis. tmTNF preferably binds to TNFR2 and initiates the immune-regulatory properties of TNF. This effect is attributed to the possibility of tmTNF serving as a receptor instead of a ligand for TNFR2 and inducing reverse signaling through this membrane-anchored ligand and triggering cell activation, cytokine suppression or apoptosis of the tmTNF bearing cell[199].

The four TNF antagonists available in the treatment of IBD can be divided into two categories based on their structure: the full-length monoclonal antibodies (mAbs) and those with only an antibody fragment. Infliximab, adalimumab and golimumab are the full-length IgG1 antibodies and their Fc region is capable of complement fixation and Fc-receptor mediated biologic activities. Certolizumab pegol lacks this Fc region and is therefore not able to perform these effector functions. All of these agents have TNF as target and are capable of binding sTNF and tmTNF with high affinity. However, differences in structure between antagonists cause different kinetic-binding parameters that can result in variable clinical efficacy. For example, etanercept, an anti-TNF approved for rheumatological diseases, is able to bind both forms of TNF but is not effective in CD, so other mechanisms must be responsible for the action of these agents[200]. These compounds exert a down-regulation of inflammatory cells in the inflamed bowel mucosa that is believed to be induced by apoptosis in tmTNF carrying cells. There is also *in vitro* evidence that infliximab induces cell lysis through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, both Fc-dependent[201]. One other possible mechanism of action that has been shuffled is the induction of regulatory macrophages, also an Fc-dependent mechanism. These macrophages have immunosuppressive capacities, play a crucial role in wound healing and have been shown to be up-regulated in patients responding to infliximab therapy[202] (Figure 7).

Anti IL12/23: IL-12 is the key inducer of Th1 cells while recent studies conducted on human cells suggest that a cocktail of cytokines, such as IL-23 and IL-1β, are critical for Th17 differentiation. Human Th17 cells are thought to produce several pro-inflammatory cytokines, including IL-17A and F, TNFα, IL-22, IL-26 and IFNγ[203]. Similar to IL-12, IL-23 can contribute to functional responses of several effector cell subtypes other than CD4+ T cells, including CD8+ T cells, NK, NKT, γδ T cells, and innate lymphoid cells[204-207]. There is increasing evidence of plasticity amongst certain Th subtypes, depending upon the cytokine microenviroment[208,209].

Ustekinumab is a human IgG1 kappa (κ) mAb that binds to the IL-12p40 subunit. This subunit of IL-12 was also found to associate with a p19 subunit to form IL-23[210]. Ustekinumab prevents human IL-12 and IL-23 from binding to the IL-12Rβ1 receptor chain of IL-12 (IL-12Rβ1/β2) and IL-23 (IL-12Rβ1/IL-23R) receptor complexes on the surface of NK and T cells but cannot bind to endogenous IL-12 or IL-23 that is already bound to receptor complexes. Thus, ustekinumab is unlikely to mediate Fc effector functions.

Mechanism of action - Anti-adhesion molecules: L-selectins are expressed on leukocytes, and P- and E-selectins are found on the endothelium. Although strong, these selectin bonds are short-lived and, consequently, the T cells roll over the endothelium from one selectin bond to the next[211]. This results in slowing of the lymphocytes and allows for transient interactions which enable the cell to encounter the cytokine-rich microenvironment that triggers subsequent firm adhesion and consequent migration through the blood vessel wall[212,213]. Secondary adhesion molecules, all members of the integrin family, function to stop the rolling lymphocytes and allow migration. Integrins are leucocyte cell-surface adhesion molecules that mediate both cell-cell and cell-extracellular matrix interactions[214] (Figure 8). The expression of integrins is activated by chemokines, which are released by T cells[215]. Integrins involved in the T-cell migration are as follows: leucocyte function-associated antigen 1 (LFA-1 or α2β2) and the two α4-integrins (α4β1 and α4β7). For the migration of leucocytes, these integrins bind to specific ligands at the endothelium called addressins or adhesion molecules. The α2β2 integrin, expressed on neutrophils, interacts with intercellular adhesion molecule-1 (ICAM-1) that is expressed on leucocytes, dendritic cells, fibroblasts, epithelial cells and endothelial cells[216,217]. The α4β1 integrin is expressed on most leucocytes, but not on neutrophils and binds to vascular cell adhesion molecule-1 (VCAM-1) and to components of the extracellular matrix such as fibronectin and thrombospondin[218]. The third family is the α4β7 integrin, which is expressed on the lymphocytes that colonise the gut and gut-associated lymphoid tissues and interacts with the MAdCAM-1 and this interaction activates the migration of lymphocytes to Peyer's patches[219,220]. Last, the αEβ7 integrin is another member of the β7 integrin family that it is expressed only in mucosal intraepithelial T lymphocytes and that binds selectively to E-cadherin on epithelial cells[221]. The expression of aEβ7 is elevated in UC and CD in the active phase of the disease[222,223], and the interaction of aEβ7/E-cadherin has been proposed to participate in the retention of T cells in the mucosal tissue[224]. Pro-inflammatory cytokines such as IL-1 and TNF[225-227] up-regulate the expression of ICAM-1 and MAdCAM-1. Treatment

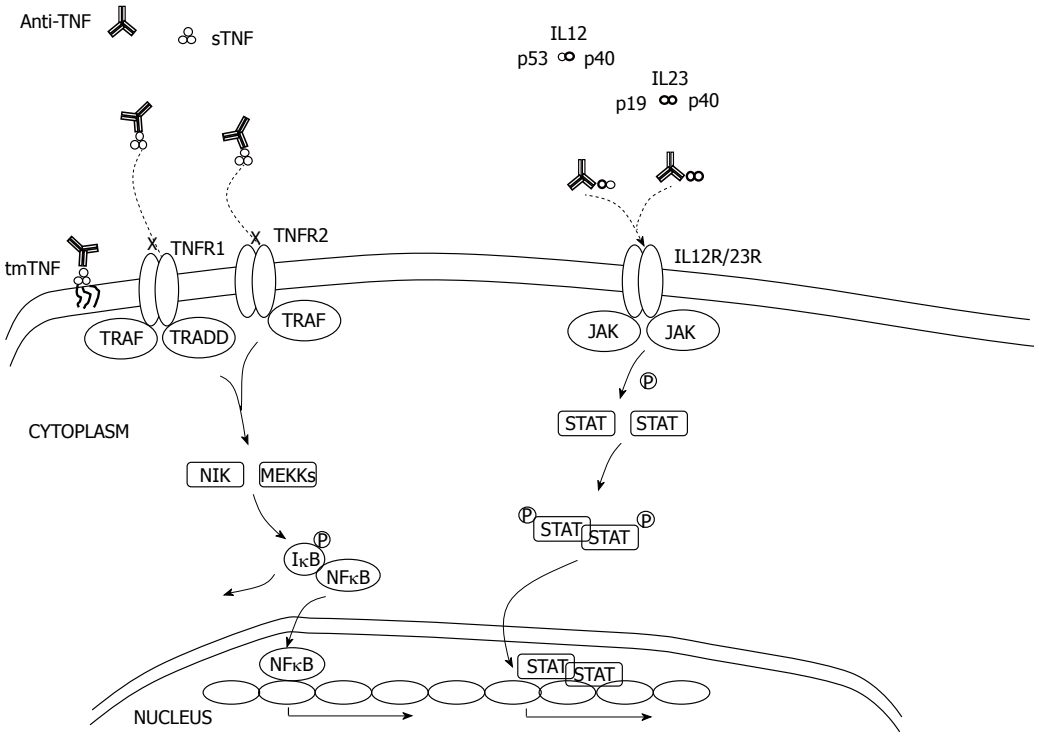

Figure 7 Mode of action of anti- tumor necrosis factor α and anti-IL-12/23. TNF: Tumor necrosis factor; tmTNF: Transmembrane TNF; sTNF: Soluble TNF; TRAF/TRADD: TLRs adaptors; JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

with IFX decreases the expression of ICAM-1[228]. Increased ICAM-1 expression in CD is present not only in the mucosa but also in the submucosa and muscular layers[229], which could be implied in the transmural nature of CD. Most anti-adhesion molecule therapies target the integrin family. The first drug developed was natalizumab, a mAb against α4 that is not gut-specific. However, its use in patients with CD has been limited by the development in some patients of progressive multifocal leukoencephalopathy (PML), an opportunistic brain infection that is caused by reactivation of latent JC polyomavirus. Newer monoclonal antibodies targeting the α4β7 integrin (vedolizumab, a humanized immunoglobulin G1 monoclonal antibody to α4β7 integrin, selectively blocks gut lymphocyte trafficking without interfering with trafficking to the central nervous system)[230,231] and β7 subunit of the heterodimeric integrins α4β7 and αEβ7 (etrolizumab)[232] are now in late clinical development.

Evaluation of clinical response: Lack of long experience managing these compounds, variability in response and the high economic cost which limits its use, makes it difficult to find studies which describe predictors of response within this therapeutic group. Generally, response can be classified in non-genetic and genetic predictors.

Anti-TNF-α

Histological changes: The effects of anti-TNF agents are mediated by multiple mechanisms including direct neutralization of soluble TNF and interaction with membrane-bound TNF. Anti-TNF agents act by reduction of pro-inflammatory cytokine levels, elimination or clearance of active inflammatory cells from inflamed tissue which can conceptually be achieved by a number of mechanisms including apoptosis induction, antibody and complement mediated cytotoxicity and inhibition of cell migration into the intestinal tissue. Effects of anti-TNF agents may vary according to their physical contact with TNF, which may also be influenced by structural differences in the non-TNF binding domain affecting the ability of each drug to interact with the immune system.

The binding avidity of infliximab, adalimumab and etanercept to sTNF and mTNF appears to be similar[233]. But the bond between the anti-TNF agent and TNF may be reversible and as such, the anti-TNF molecule may actually serve as a TNF reservoir. In support of this possibility, the concentrations of immunoreactive TNF were shown to rise in the circulation following infliximab[234] and adalimumab[235] administration in rheumatoid arthritis, probably due to drug-TNF complexes. *In vitro* studies demonstrated that the rate of dissociation of etanercept from TNF is higher than that of infliximab and that the released TNF was bioavailable[236].

Infliximab has been shown to inhibit the production of GM-CSF and IFNγ[237] *in vitro*[238]. Infliximab, adalimumab and certolizumab inhibit the production of IL-1β from LPS-activated macrophages in CD. Down regulation of mucosal chemokine molecules following treatment with Infliximab was also shown *in vivo* for

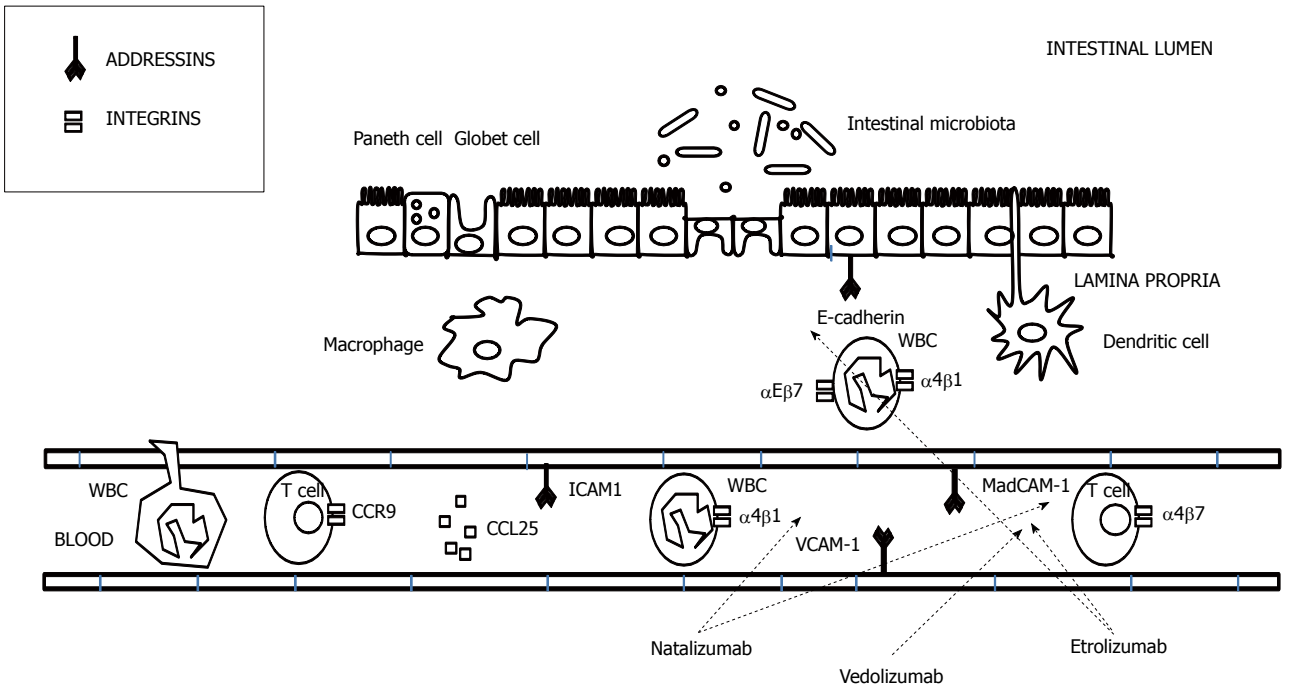

Figure 8 Mechanism of action of anti-adhesion drugs.

macrophage inflammatory protein (MIP)-1α, RANTES [Chemokine (C-C motif) ligand 5 (also CCL5)] and monocyte chemotactic protein (MCP)-1[239]. Another effect, less explored in CD and whose implications deserve further studies, is the change in tissue cellular populations following anti-TNF treatment. In one study, the population of FoxP3 positive cells was found to be reduced in the mucosa of active CD pediatric patients and infliximab treatment resulted in an increase on the mucosal number of these cells[240].

Ten Hove *et al*[241] observed apoptosis induction in mucosal CD3 positive T cells of CD patients treated with infliximab 24 h after drug administration. An additional study assessed mucosal biopsies from CD patients four weeks after treatment with standard infliximab induction doses and detected a significant increase in TUNEL-positive mucosal T cells[242]. Recently, another study used SPECT applied to infliximab-treated CD patients and detected positive up take in responders[243]. A similar profile of apoptosis induction was demonstrated *in vitro* for adalimumab[244]. However, no apoptosis induction was observed *in vitro* with certolizumab.

Another mechanism which is relevant for clearance of inflammatory cells is cytotoxicity. There is no study which definitively defines the relevance of these mechanisms *in vivo*. However, using Jurkat cells stably expressing uncleavable mTNF, Mitoma *et al*[246] demonstrated that infliximab and adalimumab exerted complement-dependent toxicity (CDC) equally, etanercept exerted CDC to a lesser extent and all three agents were capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC)[245]. Activation of p38 MAPK was demonstrated both *in vitro* and *in vivo* following exposure to infliximab. Differences between anti-TNF agents exist with respect to functional implications of reverse signaling. Kirchner *et al*[247] demonstrated that infliximab, but not etanercept inhibited LPS-induced cytokine secretion but both inhibited endothelial cell apoptotic factor. Similarly, infliximab and adalimumab inhibited secretion of IL-10 and IL-12 induced by LPS from monocytes, whereas etanercept did not[241]. In a different study, incubation of intestinal T cells derived from CD patients with infliximab and etanercept resulted in activation of the p38 MAPK pathway by infliximab only, although both reduced STAT3 activation[248].

Serological changes: Linked to the GCs chapter, anti-TNF-α therapies reduce MMP-7 levels but not to the levels of control patients as in GC therapy. α2M increase following treatment with infliximab. Anti-TNF-α therapy seems to induce α2M in serum, possibly to control disease activity. Anti-TNF-α therapy does not significantly alter serum TNF-α concentrations[249], and TNF-α can itself up-regulate MMP-7 expression[250]. This may explain why levels of MMP-7 remained elevated after anti-TNF-α treatment, compared with controls. Although TIMP-1 induction has been linked to the response to infliximab in CD in adults[251], unexpectedly weak TIMP-1 expression has been observed in pediatric IBD[252].

CD4+ T cells changes: infliximab appears to block up-regulation of CD73 on CD4+ T cells in response to TNF, but does not induce apoptosis in cells expressing high levels of CD73 *ab initio*. Doherty *et al*[243] speculated that the resolution of the inflammatory

response induced by infliximab in the lamina propria results in downstream reduction of the inflammatory mediators of CD73 expression.

Imaging changes: Recent progress concerning molecular imaging studies in animals and human patients implicates that this approach can be used to improve detection of mucosal lesions in wide-field imaging and for *in vivo* characterization of the mucosa with the ultimate goal of assessing the likelihood of response to targeted therapy with biological agents. In a recently published study, using confocal laser endomicroscopy with a fluorescent antibody *in vivo* demonstrated its potential to predict therapeutic response to subsequent biological treatment in human patients. As anti-TNF Abs suppress immune responses in CD by binding to membrane-bound TNF (mTNF) expressing mucosal cells, *in vivo* detection of such cells *via* fluorescent anti-TNF Abs was used to predict therapeutic efficacy in CD patients *via* molecular imaging[253,254].

Genetics and genomics: Genetic non-response to Infliximab appears to be stable in time implicating a genetic influence [patients homozygous for a TNF-α polymorphism (LTA NcoI-TNFc-aa13L-aa26 1-1-1-1 haplotype)][255]. Previous data have confirmed the association of the TNFα-1031C allele with CD and polymorphisms at the TNFα locus have been suggested as a tool to predict response to Infliximab[256]. Halavaty *et al*[257] reported that polymorphisms in FasL/Fas system and caspase-9 influenced the response to infliximab in luminal and fistulizing Crohn' s disease. In this study, Fas ligand-843 TT genotype was presented exhibiting the strongest association with the lack of response, while concomitant 6-MP/AZA therapy, however, was able to overcome the effect of unfavourable genotypes in luminal disease. Another study identified a 100% accurate predictive gene signature for (non)response to IFX in CD colitis (TNFAIP6, S100A8, IL11, G0S2, and S100A9), whereas no such a predictive gene set could be identified for CD ileitis[258]. In another interesting study, several parameters were investigated to determine early response to infliximab in patients with UC[255]. Homozygous carriers of IBD risk-increasing IL23R variants were more likely to respond to infliximab than were homozygous carriers of IBD risk decreasing IL23R variants. Similar conclusions were reached by Rismo *et al*[259] who found that high levels of Th17-defining cytokine IL-17A and Th1-defining cytokine IFN-γ can potentially predict a favorable outcome of infliximab therapy in patients with UC, whereas Th2 and T-reg related cytokines do not seem to be useful as predictive markers in relation to therapeutic outcome.

Anti IL-12/23 and anti-adhesion therapies

Although recently, IL-20, IL-21 and p40 have been proposed as potential biomarkers of treatment response to ustekinumab in psoriasis, no data have been published related to IBD until now[260].

CONCLUSION

In summary, great advances have been made in the last years for a better comprehension of the pathogenesis of IBD that undoubtedly will lead us to develop more accurate therapies and therapeutic strategies. Alterations in recognition and intracellular processing of bacterial components with involvement of ER stress and apoptotic cell death may have a meaningful role in the immunopathogenesis of the disease. The weakening of the mucosal defenses promoting excessive interactions between commensal microbiota and the mucosal immune system leads to a loss of tolerance and over-activation of dendritic cells starting the production of pro-inflammatory cytokines and a promotion of differentiation of effector T cells and CD8* lymphocytes. Also active IBD is dependent on the recruitment of mononuclear cells and leukocyte populations from the blood stream into the bowel wall. This recruitment is dependent on a series of steps coordinated by selective adhesion molecules on the surface of immune cells and mucosal addressins on endothelial cells.

SRecent years knowledge in the areas of Immunology and Genetics has allowed to start using some biomarkers to measure the responsiveness of CD and UC diseases to certain therapies: mucosal HSP60 and Hsp10 levels in UC treated with 5-ASA; serum MMP-7 in response to GC therapy; role of 6-TGN and 6-MMP in AZA's treated patients; inverse correlation between red blood cells MTXGlu1-5 and MTX efficacy in CD; through levels of Tac and 2-h CsA blood concentrations and lately, the likehood of response to targeted therapies with biological agents using immune cells imaging.

The goal of physicians treating patients with IBD, is to have to their disposal a molecular (serum, DNA, tissue-based) profile of patients which would allow them to choose the most appropriate management of the disease: prognosis of the disease outcome, most adequate therapy for an individual patient to reach success, most adequate therapy from a safety perspective, prediction on the intensity of follow-up, *etc.* At the moment, only TPMT testing prior to start of thiopurine analogues has shown clinical applicability, although it does not replace blood monitoring during treatment and some authors even defend the idea that the latest can substitute the former. Other genetic associations for the different therapeutic classes in IBD have not yet shown consistent or robust results but in the close future it is anticipated that this genetic marker's determination will be implemented in an integrated molecular diagnostic and prognostic approach to manage IBD patients.

ACKNOWLEDGMENTS

The authors would like to thank the unvaluable help of Katrin Seemayer (Grünenthal Information Management Services) for her contribution to the scientific information, criticism of the manuscript and subsequent corrections.

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P- Reviewer: Ledder OD **S- Editor**: Yu J **L- Editor**: A
E- Editor: Zhang DN

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DOI: 10.3748/wjg.v21.i44.12544

World J Gastroenterol 2015 November 28; 21(44): 12544-12557
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Hepatic applications of endoscopic ultrasound: Current status and future directions

Indu Srinivasan, Shou-Jiang Tang, Andreas S Vilmann, John Menachery, Peter Vilmann

Indu Srinivasan, Shou-Jiang Tang, Division of Digestive Diseases, Department of Medicine, University of Mississippi Medical Center, Jackson, MS 39216, United States

Andreas S Vilmann, Peter Vilmann, Department of Surgical Gastroenterology, Copenhagen University Hospital Herlev, 2730 Herlev, Denmark

John Menachery, Department of Gastroenterology, Rajagiri Hospital Aluva, Kerala 683112, India

Author contributions: All authors were involved in the planning the design and conduct of the review paper; Srinivasan I initial research was conducted; Srinivasan I and Tang SJ were involved in drafting the manuscript; all the authors then contributed equally in revising the manuscript and approving the final version.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

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Correspondence to: Peter Vilmann, MD, DSc, HC, FASGE, Department of Surgical Gastroenterology, Copenhagen University Hospital Herlev, Nørregade 10, Herlev 2730, Denmark. peter.vilmann@regionh.dk
Telephone: +45-38-682164

Received: May 8, 2015
Peer-review started: May 11, 2015
First decision: September 11, 2015
Revised: September 28, 2015
Accepted: October 12, 2015
Article in press: October 13, 2015
Published online: November 28, 2015

Abstract

The diagnosis and staging of various gastrointestinal malignancies have been made possible with the use of endoscopic ultrasound, which is a relatively safe procedure. The field of endoscopic ultrasound is fast expanding due to advancements in therapeutic endoscopic ultrasound. Though various studies have established its role in gastrointestinal malignancies and pancreatic conditions, its potential in the field of hepatic lesions still remains vastly untapped. In this paper the authors attempt to review important and landmark trials, case series and case studies involving hepatic applications of endoscopic ultrasound, thus not only providing an overview of utilization of endoscopic ultrasound in various liver conditions but also speculating its future role.

Key words: Endoscopic ultrasound; Drainage; Liver; Intrahepatic lesion

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Core tip: To review the available published trials, case series and case reports, discuss the implications and the future role of endoscopic ultrasound in the management of various liver conditions. Through this review paper we aim to provide a unified one stop educational experience as we have attempted to amalgamate all the published data.

Srinivasan I, Tang SJ, Vilmann AS, Menachery J, Vilmann P. Hepatic applications of endoscopic ultrasound: Current status and future directions. *World J Gastroenterol* 2015; 21(44): 12544-12557 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12544.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12544

INTRODUCTION

Since the initial description of endoscopic ultrasonography (EUS) in 1980's, EUS has become an indispensable method not only in diagnosing various gastrointestinal (GI) lesions but also in performing various therapeutic maneuvers[1-4]. It was the limitations imposed by standard ultrasonography that prompted the development of EUS. By placing the ultrasound transducer within the body, interposed air filled or bony structures could be avoided and simultaneously by reducing the distance it became feasible to adequately visualize deeper structures. The method is basically an endoscopy with ultrasound at the tip which helps to visualize not only the GI tract but also produce images of nearby structures such as the gall bladder and bile ducts, pancreas, liver, *etc.* Echoendoscopes are designed using either a radial or curvilinear array system[1]. Radial endosonography provides a high-resolution, 360 degrees circumferential imaging of the GI tract and its surrounding structures. However, it does not allow for fine needle aspiration (FNA), thus making way for development of the linear echoendoscope enabling FNA of lesions both within and adjacent to GI tract[2,3]. This ability is the cornerstone of interventional EUS and the method has been proven safe and reliable in diagnosing various benign and malignant conditions in the upper and lower GI tract as well as the mediastinum[3,4]. When compared with other techniques EUS-FNA has demonstrated improved accuracy and cost effectiveness. This was especially well demonstrated in pancreatic lesions and malignant tumors of the esophagus[3,4].

Till late 1990's the role of EUS in evaluating hepatic lesions was only described in a few case studies[5] but the case series by Nguyen *et al*[6] paved the way. They theorized that close proximity of the transducer to lesions coupled with clear visualizations and accessibility of the left lobe and central segments of the liver should facilitate the use of EUS in hepatic lesions. Since EUS-FNA was first reported in 1992[2], we have seen a tremendous growth of interventional EUS with various innovations such as EUS guided tru-cut biopsy, tumor ablative therapy, vascular interventions, and various transmural drainage procedures. However, though case series have been described limited trials advocating the use of EUS for liver lesions have been published and there are no dedicated guidelines establishing the role of EUS in hepatic applications at present. The aims of this review are to summarize the published reports on hepatic applications of EUS and to speculate on its future roles.

ANATOMICAL DESCRIPTION OF THE LIVER

The adjacent organs next to the liver include the stomach and duodenum (Figure 1). The liver has a dual blood supply with the portal vein (formed by the confluence of superior mesenteric vein and splenic vein) and the hepatic artery (branch of celiac artery). These vessels terminate at porta hepatis by dividing into right and left liver branches which undergo secondary and tertiary divisions to supply the various segments. Right, middle and left hepatic veins formed by the union of intersegmental collecting veins open into the inferior vena cava (IVC). The liver can subsequently be divided into 8 segments that are served independently by a secondary or tertiary branch of the portal triad. The caudate lobe is considered to be separate as it is supplied by both branches of the portal vein and drains independently into the IVC via hepatic veins. It is located posteriorly and considered to be segment one. The rest of the liver is divided by the main portal fissure hosting the middle hepatic vein which extends from the fundus of the gall bladder to the IVC. The left hepatic vein divides the left lobe into lateral (2, 3) and medial (4a, 4b) segments. The right hepatic vein divides the right lobe into anterior (5, 8) and posterior (6, 7) segments. The portal vein divides the liver into upper (2, 4a, 8, 7) and lower (3, 4b, 5, 6) segments. The segments are labeled in a clockwise manner. In a normal frontal view segments 1, 6 and 7 are not visible.

VISUALIZATION OF THE LIVER BY EUS

With currently available echoendoscopes, both mechanical and electronic transducers can scan over a range of frequencies ranging between 5 to 20 MHz. [1]Scanning at higher frequencies improves image resolution but limits the penetration of the ultrasound beam to 1 to 2 cm from the probe, whereas scanning with lower frequency provides images of structures up to 6 to 8 cm from the probe. In 1986, Rifkin *et al*[7] incorporated 9 MHz electronic transducer on to a traditional fiber optic gastro scope and concluded that it had a better resolution than 0.5 mm.

Both the left lobe and caudate lobe lie in close proximity to the stomach and duodenum, hence providing an easy access during EUS (Figures 2 and 3). The caudate lobe and gastrohepatic space can be accessed by EUS while these are anatomically more difficult to approach by trans-abdominal ultrasound. EUS is limited in its ability to access the portion of the right lobe adjacent to the dome of the diaphragm along with its lateral and inferior portions. In spite of this, most of the right is lobe is visible from the duodenum as well as the portal hilum with its structures.

PubMed SEARCH

On December 20th, 2014, the authors performed PubMed search using these key word sets: EUS in combination with hepatic lesion, hepatic application, hepatic intervention, hepatocellular carcinoma, and hepaticogastrostomy (HGS). The inclusion criteria are: (1) english language publications only; (2) EUS

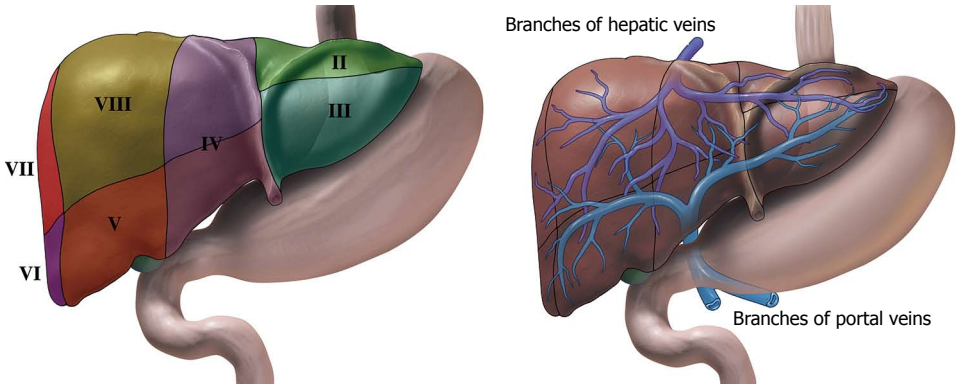

Figure 1 Illustrations of liver and its surrounding stomach and duodenum. A: The liver can subsequently be divided into 8 segments that is served independently by a secondary or tertiary branch of the portal triad. B: The left hepatic vein divides the left lobe into lateral (II2, III3) and medial (IV4a, IV4b) segments. The right hepatic vein divides the right lobe into anterior (V5, VIII8) and posterior (VI6, VII7) segments. The portal vein divides the liver into upper (II2, IV4a, VIII8, VII7) and lower (III3, IV4b, V5, VI6) segments. The segments are labeled in a clockwise manner. In a normal frontal view segments I1, VI6 and VII7 are not visible.

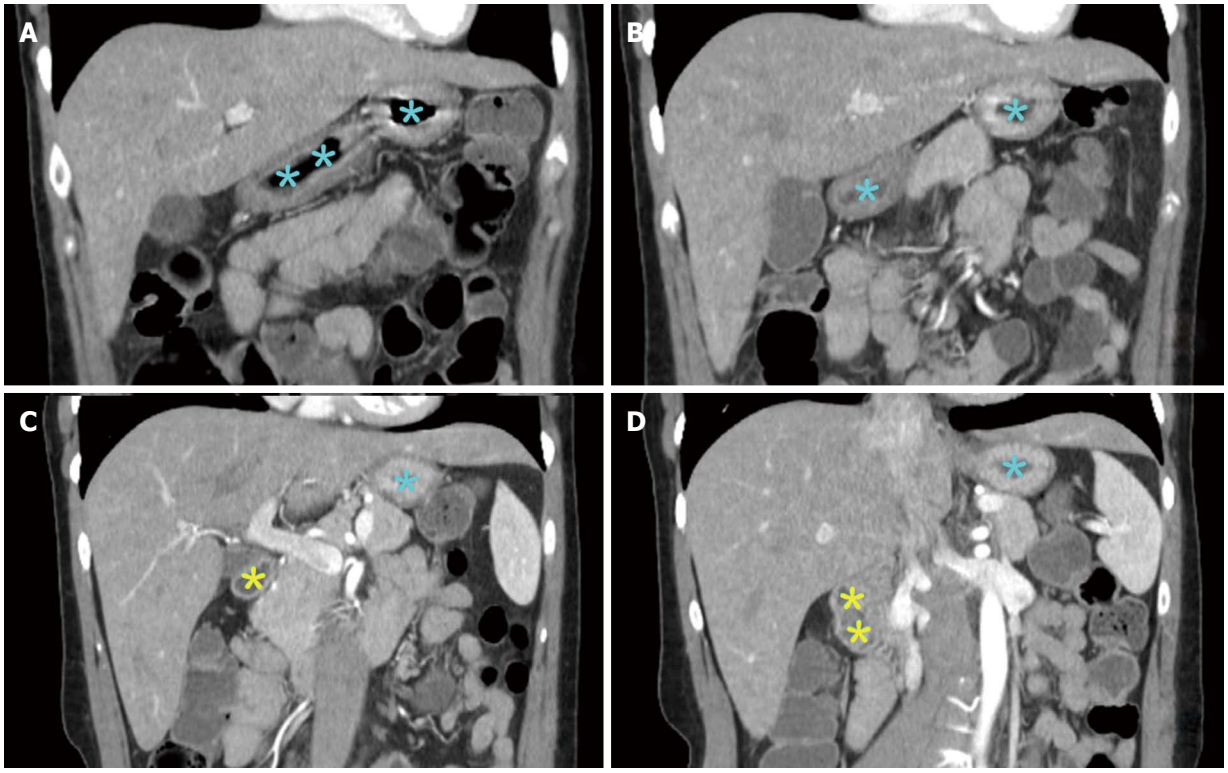

Figure 2 Selected computerized tomography scan images showing the liver and its surrounding stomach and duodenum. Both the left lobe and caudate lobe lie in close proximity to stomach (blue colored asterisk indicated gastric lumen) and duodenum (yellow colored asterisk indicated bulb and duodenal lumen), hence providing an easy access during endoscopic ultrasonography (EUS). The caudate lobe and gastrohepatic space can be accessed by EUS while are anatomically difficult to approached by trans-abdominal ultrasound. EUS is limited in its ability to access the portion of the right lobe adjacent to the dome of the diaphragm along with its lateral and inferior portions.

is utilized; and (3) case report, series, clinical studies, potential hepatic applications in animal models, reviews on this topic. The authors excluded reports about EUS utilization in extrahepatic bile duct, gallbladder, and other extrahepatic structures except in management and diagnosis of complications of portal hypertension. We also excluded intraductal EUS application. Each published paper was reviewed and only important information was extracted for this review. Initial search yielded 731 articles out of which 584 were discarded as they did not meet the inclusion criteria. Please refer to the attached PubMed result search flow sheet for further details (Figure 4).

ROLES OF EUS IN THE EVALUATION OF HEPATIC LESIONS

Focal liver lesions

Diagnostic EUS: Focal liver lesions include both benign (such as hepatic cysts, abscess, adenoma or hemangioma) and malignant (such as hepatocellular

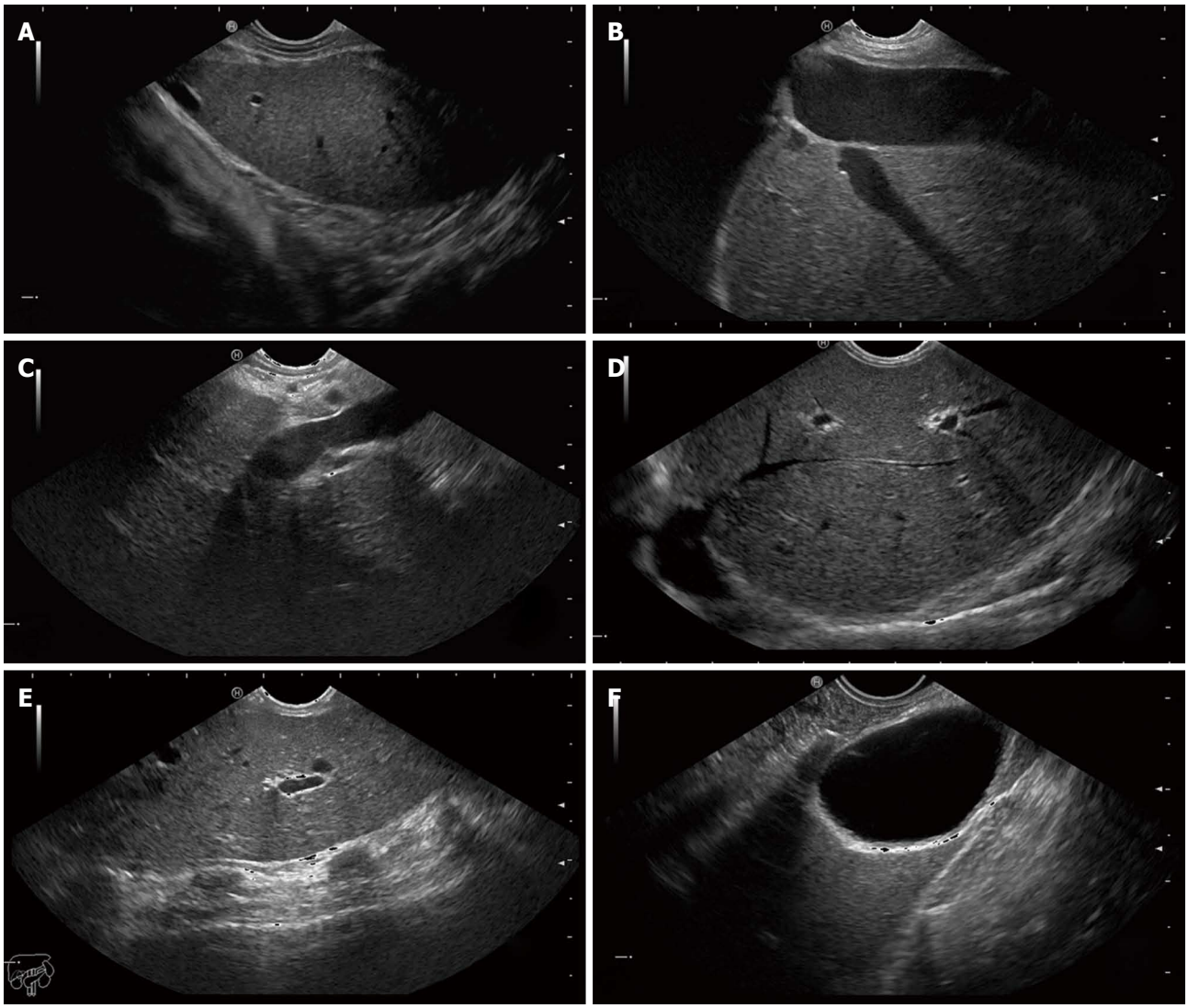

Figure 3 Endoscopic ultrasound images of the hepatic structures with the tip of the linear echoendoscope at different positions. A: Endoscopic ultrasound (EUS) image of the left liver lobe with the diaphragm. The image is obtained from the cardia region; B: EUS image of the left liver lobe with the inferior vena cava and a hepatic vein; C: EUS image of the liver at the portal ligament region showing from the transducer, the hepatic artery, the portal vein and a short segment of the common bile duct. The transducer is located in the stomach; D: EUS image of the liver looking over the hepatic dome; E: EUS image of the right hepatic lobe. Note the shadows from the ribs at the anterior abdominal wall; F: EUS image of the liver with the gall bladder. The transducer is located in the first part of the duodenum.

carcinoma, intrahepatic cholangiocarcinoma, biliary cystadenoma, and metastatic liver disease) lesions (Figure 5 and Table 1). These lesions were most often diagnosed by either abdominal imaging or by percutaneous tissue diagnosis. The last 15 to 20 years have seen rapid advancements in the applicability of EUS, especially combined with FNA cytology. Studies[8,9] have not only established its efficacy in evaluating intra-abdominal lesions but in also staging[10] of various GI tumors. However it was not till 1999[5] when EUS was used for clinical imaging of the liver. Studies[11,12] comparing intraoperative ultrasound to preoperative computed tomography (CT) scan and magnetic resonance imaging (MRI) had proven it to be superior owing to the proximity of the ultrasound probe to the liver parenchyma and the use of color flow Doppler - hence the idea of EUS for liver lesions was considered. Nguyen *et al*[6] evaluated the liver in 574 consecutive patients undergoing upper EUS examination for suspicion or history of GI or pulmonary tumor: 15 liver lesions were identified (5 were in the right lobe and 9 were in the left lobe) and underwent EUS-FNA, 14 of them were found to be malignant (one of the patients underwent FNA of two lesions as the first lesion revealed normal cytology). Surprisingly CT scan done prior to EUS identified only 3 of these liver lesions: 12 of the 15 lesions were less than 2 cm. Thus EUS became recognized as a modality to help detect small focal liver lesions. This was also demonstrated by a retrospective study reported by Prasad *et al*[13] where in liver lesions as small as 5 mm not seen by previously conducted noninvasive imaging were detected by EUS. The detection of metastatic disease is particularly important as it influences management of these patients. It is important to realize that EUS is a semi-invasive test

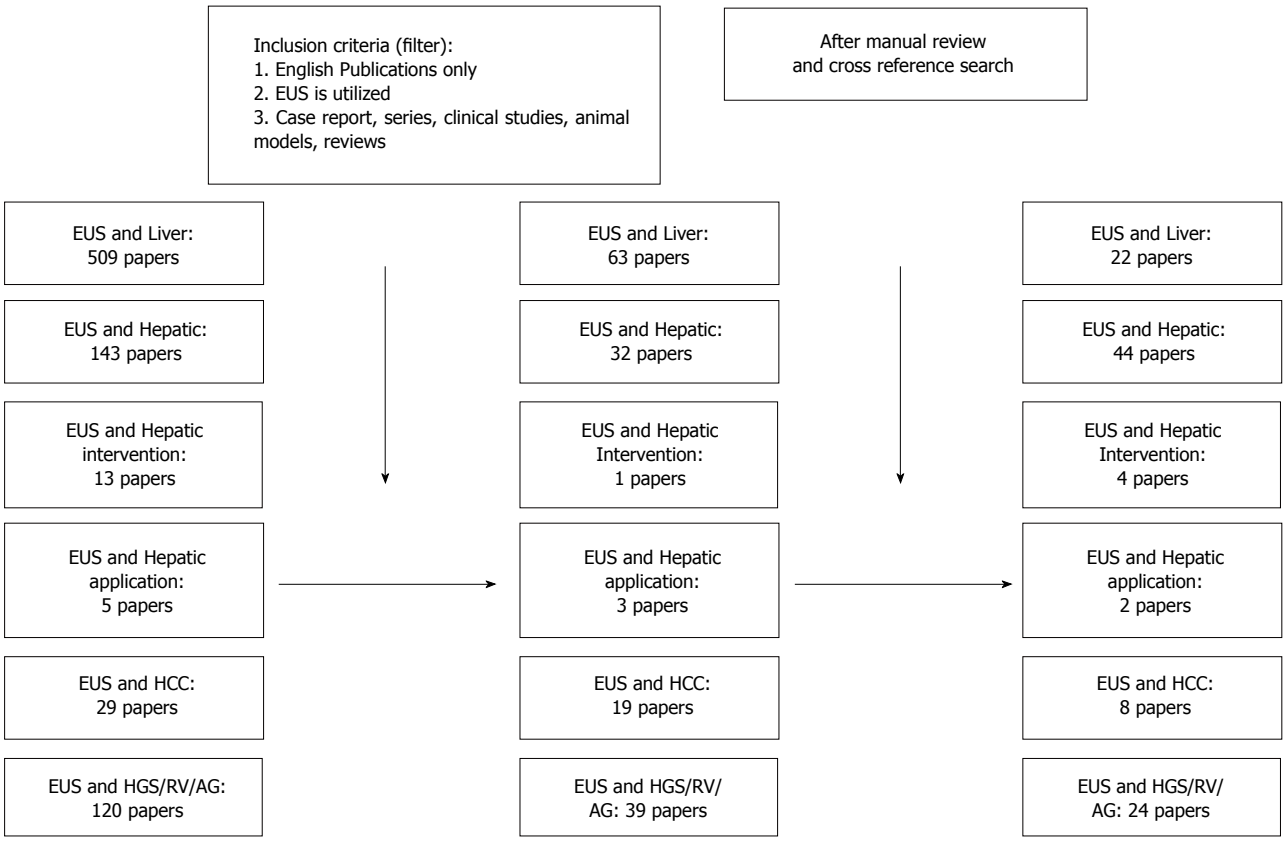

Figure 4 PubMed result search flow sheet. PubMed search was performed on December 20, 2014. EUS: Endoscopic ultrasound; HCC: Hepatocellular carcinoma; HGS: Hepaticogastrostomy.

with complications. Two patients in this study had duodenal injury. Hence, though the data appears promising more studies are needed to compare the risk benefit ratio and to establish a protocol for detection of liver masses. Awad *et al*[14] then evaluated 14 consecutive patients with a history of a known liver mass. They underwent both dynamic CT scan and EUS. EUS not only identified the lesions in all the 14 patients but it also recognized 4 new lesions smaller than 0.5 cm which had not been visualized by CT scan. In 2002 Tenberge *et al*[15] published a retrospective study wherein they sent a questionnaire to EUS-FNA centers around the world. Out of the 130 centers, 21 of them reported 167 cases of EUS-FNA of the liver with a complication rate of only 4%. Further, it helped to diagnose malignancy in cases of non-diagnostic FNA obtained under trans-abdominal ultrasound and localize primary tumor in cases where CT only reported liver metastasis. Thus the authors concluded that EUS-FNA of the liver was a safe procedure which should be considered in case the lesion is not accessible by trans-abdominal ultrasound or CT or when FNA is non diagnostic by these methods or when a liver lesion is detected during routine EUS. As described in the anatomy section EUS provides good visualization of the left hepatic lobe and EUS-FNA of liver lesions may provide important management information especially in case of detection of metastasis or hepatocellular carcinoma. Further it is hypothesized that in patients with cirrhosis percutaneous biopsy may be difficult owing to presence of ascites and coagulopathy. In such cases EUS may be a safer option as the transducer is only 1.5-3 cm away from the lesion and biopsy occurs under EUS guidance. A retrospective study[16] of 77 patients who underwent EUS FNA of various solid liver lesions helped to detect malignancy in 41% of patients who had previously had negative examination. This was the first study to include both malignant and benign lesions. They concluded that sensitivity of diagnosing malignancy ranged from 82% to 94% (7 patients from the nonmalignant group died without follow up imaging, biopsy or autopsy and hence could not be classified) and this group did not have any complications. The presence of two or more lesions with regular outer margins is more indicative of a malignant lesion. In a prospective study by Hollerbach *et al*[17] EUS-FNA provided appropriate biopsy specimen in 40/41 patients with an average of 1.4 needle passes. On combining both histological and cytological examination of the specimens they had a sensitivity of 94% and a specificity of 100% with a low 2.5% rate of minor complication. A Fritscher-Ravens *et al*[18] reported a case series of 10 patients with a biliary stricture at the hepatic hilum who underwent EUS-FNA. In 9/10 patients' adequate specimens were obtained and 8 of these lesions were found to be malignant

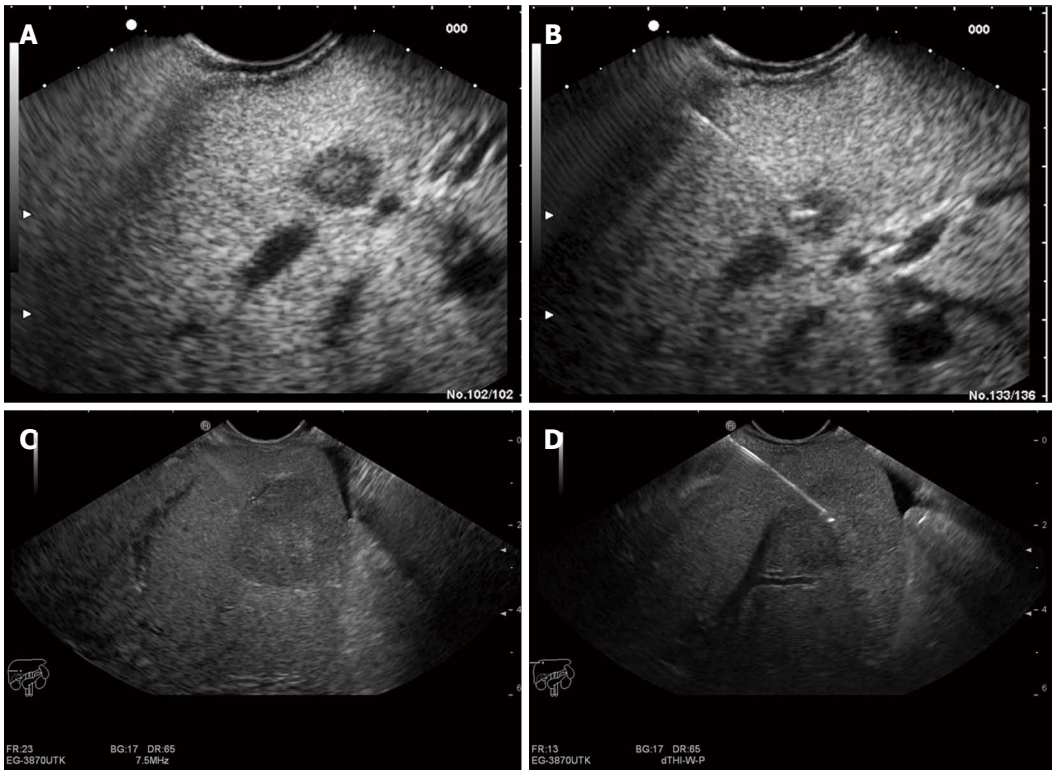

Figure 5 Endoscopic ultrasound image of lesion in the liver. A: Endoscopic ultrasound (EUS) image of an 8 mm metastatic lesion in the liver; B: Endoscopic ultrasound (guided biopsy from the same lesion; C: EUS image of a 25 mm lesion in the liver; D: EUS guided aspiration biopsy from the same lesion.

but one lesion was falsely identified as benign. This study highlights an important implication of EUS as various advances are being made in the management of cholangiocarcinoma, thus it is imperative that we have not only have accurate diagnosis but also stage the disease adequately. Studies so far have revealed that brush cytology from endoscopic retrograde cholangiopancreatography (ERCP) has variable sensitivity. Ryan[19] reported a sensitivity of only 44% while Glasbrenner *et al*[20] reported a sensitivity of 80%. Subsequently Crowe *et al*[21] in 2006 compared 34 percutaneous CT-FNA liver biopsies to 16 EUS-FNA liver biopsies and concluded that though they were comparable in terms of diagnostic utility for hepatic lesions, EUS was limited in its ability to access the portion of the right lobe adjacent to the dome of the diaphragm along with its lateral and inferior portions. Thus, EUS is an important tool to use in adjunction with other noninvasive imaging methods to not only detect occults metastasis but also to diagnose focal malignant lesions more so in the left hepatic lobe.

More recently, EUS elastography used for visualization of tissue elasticity during routine EUS[22] is an upcoming modality for diagnosing focal liver lesions. It is based on the same principle of trans-abdominal elastography but it offers an additional advantage of comparing the echoes over several seconds of normal breathing and blood circulation thus overcoming the need for applying manual pressure. This technique was utilized by Rustemovic *et al*[23] in diagnosis of focal liver masses Since the malignant tumor tissue is harder than benign tumors, efforts have been made to utilize this modality in differentiating benign from malignant tumors. This principle was already established in other tumors such as prostate[24], pancreas[25]and breast[26]. In 2008 Kato *et al*[27] established that intraoperative usage of real time elastrography could help distinguish between HCC and metastases by dividing the tumors into four different types based on elasticity. In 2012, Sandulesco *et al*[28] published a pilot study using real time sonography to differentiate focal liver lesions and concluded that the sensitivity, specificity, and accuracy of differentiation of benign and malignant masses were 92.5%, 88.8%, and 88.6%, respectively. Hara *et al*[29] described a novel approach to assess vascular invasion at the hepatic hilum by using the linear scanning which they postulated had a superior visualization of abdominal vessels than radial scanning.

Therapeutic EUS in focal liver lesions: More recently there are case reports pushing the frontiers of EUS from being just a diagnostic tool to one with therapeutic benefits. EUS guided ethanol injection in treatment of hepatic metastasis was first described in 2002[30]. Since then few more cases[31-33] have been reported but unfortunately there are no long term data or larger case series to draw any significant conclusions. In 2011 Di Matteo *et al*[34] described the use of Nd: YAG laser ablation of hepatocellular carcinoma in the caudate lobe. More recently fiducial placement for stereotactic body radiation under EUS guidance for hepatic and pancreatic malignancies was

Table 1 Reported diagnostic yields of endoscopic ultrasound guided fine needle aspiration of hepatic solitary lesions

Ref.	Patient number/lesion sampled	Diagnostic yields
Nguyen *et al*[6]	574/15	CT before EUS depicted liver lesions in 3 of the 14 patients (21%)
Awad *et al*[14]	14/9	EUS identified additional lesions in 28% of the patients and changed clinical management in 67% of the patients
TenBerge *et al*[15]	167/167	EUS-FNA diagnosed malignancy in 89% of cases after non diagnostic FNA under trans abdominal US guidance
DeWitt *et al*[16]	77/77	45 (58%) were diagnostic for malignancy, 25 (33%) were benign and seven (9%) were non diagnostic. EUS detected malignancy in 41% of patients with previously negative exam
HollerBach *et al*[17]	41/41	With combination of histological and cytological examination sensitivity and specificity for detecting malignancy was 94% and 100%
Prasad *et al*[13]	222/21	Diagnostic of malignancy in 15 (6.8%) 5 of whom (2.3%) had normal imaging prior
Crowe *et al*[21]	50/16	Diagnostic of malignancy in 56% of the cases, comparable to CT scan
McGrath *et al*[105]	98/5	The sensitivity of EUS-FNA for liver lesions was 80%. These lesions were not evident on prior noninvasive imaging
Singh *et al*[106]	132/26	The diagnostic accuracy of EUS/EUS-FNA and CT scan was 98% and 92% respectively

EUS: Endoscopic ultrasonography; FNA: Fine needle aspiration; CT: Computed tomography.

reported[35].

Hepatic cysts

With the increasing imaging using ultrasound and CT scan, simple hepatic cysts are now routinely detected in 2.5%-7% of the population[36]. In majority of the time they are asymptomatic and need no further treatment. Only 10%-16% of such cysts are producing symptoms such as abdominal pain, hepatomegaly, early satiety, bile duct compression necessitating the need for treatment. Traditionally a surgical approach (complete cyst excision or fenestration of cyst) or percutaneous aspiration were the modalities used to treat large symptomatic hepatic cysts. Percutaneous aspiration is associated with a recurrence rate of nearly 100% in 2 years[37]. In 1985 Bean and Rodan[38] described successful percutaneous aspiration of 6 hepatic cysts followed by sclerotherapy with alcohol. No recurrence was noted during a 6-18 mo follow up period. These authors concluded that alcohol caused cellular destruction followed by fibrotic obliteration of the cysts. Since then multiple studies have established the efficacy of percutaneous aspiration with sclerotherapy. Lee *et al*[39] conducted a retrospective study where he hypothesized that EUS guided aspiration and lavage therapy without a percutaneous drainage catheter, would enable it to be performed in a single step. A total of 17 patients with 19 hepatic cysts were enrolled with a median cysts volume of 368.9 mL. Ten cysts were drained by a percutaneous approach and 8 cysts underwent EUS guided aspiration and lavage treatment. In a 15-mo follow up the EUS guided group showed 100% reduction. The authors concluded that EUS guided drainage was a safe method for left sided hepatic cysts while percutaneous catheter drainage is preferred for large right sided cysts.

Hepatic abscesses

Hepatic abscesses are collections of infected materials in the parenchyma which usually develop directly from the biliary tree or from extension of intra-abdominal infection or hematogenously from bacteremia. Conventionally hepatic abscesses have been treated with either surgical or percutaneous drainage[40,41]. Owing to high morbidity and mortality (almost 32%) associated with surgical drainage[40,41]. percutaneous drainage with success rates of 80%-100% has emerged as the first line of therapy[42]. Unfortunately this is also associated with certain side effects[43] such as injury to surrounding vascular structures, intraperitoneal bleeding, hepato-venous fistula[44] and patient discomfort due to external drainage. EUS guided hepatic abscess drainage[45] was suggested as an alternative to overcome some of the complications. As described before, both the left lobe and caudate lobe lie in close proximity to the stomach and duodenum, hence providing an easy access during EUS. In 2005 Seewald *et al*[45] described a case report of a large hepatic abscess in the lateral segments of the left lobe that did not resolve with one week of intravenous antibiotic therapy. The authors performed EUS guided transgastric drainage of the hepatic abscess. At 6 mo follow up the patient still remained asymptomatic. In 2010 Noh *et al*[46] described a case series of three hepatic abscesses localized to the caudate lobe and the gastrohepatic space which were anatomically difficult to drain percutaneously. These patients underwent EUS guided drainage and had complete resolution on follow up. The EUS method was hypothesized to be better as it provided excellent visualization of the abscess cavity and the close approximation of the transducer to the cavity wall aided in direct passage of the needle into the cavity. Color Doppler prevented accidental puncturing of interposed vessels. A total of 7 cases have been reported with no single complication and a success rate of 100%[47]. Moving forward we need further studies to establish its efficacy and recommend as a standard therapy. Further, its use may be limited in right sided abscesses. As of now it can be offered to patients as an attractive alternative especially if percutaneous

drainage fails.

Hemobilia

With the advent of invasive hepatobiliary procedures[48] such as percutaneous biopsy, biliary drainage and transhepatic cholecystography the incidence of hemobilia has been on a rise. Most common causes include accidental or iatrogenic trauma. The bleeding typically starts within 4 wk[48] of the trauma though cases with longer time periods have been reported. They are most commonly diagnosed by hepatic angiography and often treated with embolization[49]. Cattan *et al*[50] described a case report where in hemobilia occurred nearly 4 mo after hepatic injury and was diagnosed successfully by EUS. The patient underwent upper endoscopy with both front viewing and side viewing endoscopy along with EUS which revealed the presence of mobile hyperechoic material with no acoustic shadow in the bile duct and gall bladder suggestive of hemobilia. Trakarnsanga *et al*[51] described another case where a patient presented with abdominal pain and jaundice and underwent EUS with Doppler which revealed a large cystic lesion with a detectable to and fro color flow arising from the common hepatic artery. Diagnosing hemobilia is always challenging, ultrasound and CT scan help by detecting the presence of hematoma or arteriovenous fistula. On occasions they may also detect blood clots in the bile ducts, however these findings are not always present[52]. Hence we postulate that in cases of unexplained GI bleeding especially after hepatic trauma, certain imaging studies such as EUS should be considered.

Portal hypertensive complications and hepatic cirrhosis

The past decade has seen increasing interest in using EUS for not only early diagnosis of portal hypertension but also for treatment of varices. EUS combined with the Doppler technique helps in the detailed evaluation of the distal esophagus. Though initial studies[53] were not encouraging, later studies[54] showed that EUS could adequately identify high risks of bleeding by determining the size of the varix. Careful examination of the gastroesophageal junction with upper endoscopy helps to identify large varices, however small varices and gastric varices may be missed. In a study by Choudhuri *et al*[55], gastric varices were detected more often with EUS than with endoscopy alone. In addition EUS can also help to identify deep venous plexus such as peri-esophageal and para-esophageal varices. In his study, Lee *et al*[56] compared cirrhotics and patients with dyspepsia to assess gastroesophageal varices and extra luminal venous abnormalities. EUS detected gastric varices in 30.8% when compared to 17.3% detected via upper endoscopy alone. Further with EUS extraluminal venous abnormality was noted in 92% of people with cirrhosis. These changes include early formation and engorgement of collateral vessels in the distal esophagus, proximal stomach and splenic vein. Thus there is a potential for possible early detection of cirrhosis as these changes cannot be seen by regular endoscopy. This was further demonstrated by a study done by Mckiernan *et al*[57] in Birmingham, United Kingdom where 16 children with intestinal failure underwent both endoscopy and EUS to assess for the need of combined intestinal and liver transplant if indicated by presence of intestinal failure associated liver disease. In 7 patients gastroesophageal varices was only detected by EUS and not by regular endoscopy thus resulting in fewer liver biopsies in this subset of patients. Various studies conducted over the last decade helped to establish that EUS can also be used to predict the recurrences of esophageal varices after therapy. Irisawa *et al*[58] in 2001 published a retrospective study of 38 patients who had undergone endoscopic injection sclerotherapy. Presence of severe type peri-esophageal collateral veins and large perforating veins were associated with increased recurrence of esophageal varices. In 2003[59] he studied 18 patients and concluded that para-esophageal collateral veins detected after the sclerotherapy sessions predicted recurrence. Sato *et al*[60] studied 306 patients whose varices had been treated with endoscopic injection sclerotherapy with endoscopic color Doppler ultrasonography and concluded that presence of patent inflowing perforating veins before and after sclerotherapy was predictive of early variceal recurrence. Presence of severe cardial sub mucosal veins and severe grade perforating veins[54] and presence of rapid hepatofugal flow velocity of 12 cm/s or more (The group tested a variety of cutoff points from 5 to 18 cm/s, and chose 12 cm/second as the cutoff point since it gave the maximal differences in prognoses between the low- and high-risk groups) in the left gastric vein[61] have also been reported to be associated with early recurrence of esophageal varices after treatment. Benefits of EUS extend not only to diagnosis but studies have shown therapeutic benefits as well. Especially in fundal varices which are not amenable to band ligation, EUS guided techniques such as injection of cyanoacrylate[62] and/or cyanoacrylate with coiling[63] eradicating of gastric varices have been useful. Romero-Castro *et al*[63] conducted a multicenter study comparing cyanoacrylate against EUS guided coil application. Though it was a small non randomized group, the EUS guided coil application group required fewer endoscopies and had fewer adverse effects. In a randomized control trial by de Paulo *et al*[64], EUS guided sclerotherapy was proven to be as effective as endoscopic sclerotherapy. However larger randomized controlled trials are needed to substantiate the claim.

Initially described in 1969[65], transjugular intra-hepatic portosystemic shunt has certainly come a long way to becoming an effective tool in the management of portal hypertension[66], especially in the management of refractory ascites and variceal bleeding

not responding to endoscopic therapy[67]. With the emerging role of EUS in diagnosis and management of various hepatobiliary conditions there have been studies conducted which push the boundaries a bit further. In 2004 Lai *et al*[68] demonstrated for the first time the feasibility of EUS guided extrahepatic portal vein puncture and portography in an animal model. Since then few studies[69,70] on porcine models have established the efficacy and feasibility of EUS guided portal vein catheterization in portal angiography and portal vein pressure measurements. Subsequently, in 2009 Buscaglia *et al*[71] reported the first successful endoscopic creation of intrahepatic portosystemic shunt (IPSS) in 10 porcine models without any complications. They concluded that their technique of EUS guided IPSS was technically feasible and a comparative alternative with few advantages such as avoiding the entrance through heart or IVC and decreasing the radiation exposure to both the patient and physician. Further studies are needed with large diameter covered stents[72], deployment of the proximal end of the stent into the hepatic vein-IVC confluence to promote stent patency[73] and in cirrhotics to test the safety of this technique prior to conducting human studies.

Detection of fibrosis of the liver has important management implications and although liver biopsy is still considered "a gold standard", studies have proven this technique to be less perfect owing to sampling errors, inter-observer variability[74] and complications[75]. Various serum markers and imaging tests[76] such as FibroScan (EchoSens, Paris, France)[77] have been developed and are being tested to assess their efficacy in staging the liver disease. In 2009, Rimbaş *et al*[78] postulated that mapping of the tissue elasticity distribution might prove to be useful in accurately determining stages of liver disease. He further commented that in comparison to FibroScan, real time EUS elastography not only allows for estimation of liver stiffness in all patients (irrespective of obesity) but it can also differentiate between steatosis and fibrosis thus giving it an edge. Further studies are needed to confirm these hypotheses.

Portal vein

In patients undergoing extensive hepatectomy preoperative embolization of portal vein branches causing atrophy of the segments to be removed with subsequent compensatory hypertrophy of the remaining segments[79] has proven to be safe and effective[79,80]. Matthes *et al*[81] reported the first successful EUS guided selective embolization of the portal vein with Enteryx (ethylene-vinyl alcohol copolymer) in a single swine model. The group thus concluded that EUS guidance appears to be feasible and a potential minimally invasive preoperative treatment option for patients undergoing extensive hepatectomy. Moving forward more studies need to be conducted to establish its clinical efficacy.

INTRAHEPATIC BILE DUCT

Currently ERCP is considered be a gold standard for relieving biliary obstruction and when this fails the only alternative is surgery or percutaneous approach both of which are unfortunately associated with higher mortality and morbidity[82]. With the advent of EUS and the easy access of left hepatic duct from the gastric wall paved the way for EUS guided transgastric approach to biliary system. This was initially described[83] in patients with biliary obstruction who had failed either endoscopic or percutaneous transhepatic drainage by Giovannini *et al*[83] in 2003. Following this few more case reports[84,85] were published expanding interventional EUS guided biliary drainage as an attractive alternative. Subsequently, Bories *et al*[86] published a pilot series of 11 patients who underwent EUS guided transgastric drainage of the left hepatic system. Out of 11 patients the procedure was a success in 10 of them. Panpimanmas *et al*[87] also reported successful EUS guided HGS in two patients with hilar cholangiocarcinoma who had failed ERCP. Currently there are three endoscopic drainage procedures[88,89] described in the literature which includes above mentioned EUS-guided transluminal biliary drainage including choledocoduodenostomy and hepaticogastrostomy (EUS-HGS), EUS-rendezvous technique (EUS-RV), and EUS-antegrade approach (EUS-AG) of which EUS-HGS is indicated in cases of surgically altered anatomy and duodenal obstruction with tumor invasion that precludes the passage of echo endoscope. There are certain limitations to this approach as were listed by Itoi *et al*[90] in his review such as nonapposed gastric wall and left liver lobe causing procedure failure, difficulty of puncture in cases of liver cirrhosis and risk of puncturing the portal vein. A multicenter retrospective study[91] was conducted across seven tertiary centers in Japan where 64 patients were enrolled. Out of these 20 of them underwent EUS-HGS. Technical success rate was 95% but the stent dysfunction rate and 3 mo dysfunction free patency rate was 32% and 51% respectively with 6/20 patients experiencing procedure related complications. The most common complications associated with this include bile leakage and stent misplacement. EUS guided ante grade stenting (EUS-AS) was developed as an alternative to reduce the complications. Ogura *et al*[92] described a pilot study in 12 patients where he combined the EUS-HGS with EUS-AS of the biliary obstruction using an uncovered metallic stent. At the time of follow up only one patient had experienced mild pancreatitis. Ogura *et al*[93] also described a novel method called locking stent method using the end-bare covered metallic stents to prevent stent dysfunction. Initially its scope was limited only to the left intrahepatic drainage but in 2014 Ogura *et al*[94]

described a successful biliary drainage of hepatic hilar obstruction further expanding its horizons. EUS-RV technique is indicated when the biliary cannulation fails or there is a biliary stricture that cannot be passed. It involves creation of temporary fistula using EUS and placement of guidewire via the biliary duct and ampulla into the duodenum. After this ERCP is re attempted to cannulate the bile duct using the guidewire. It can be divided into two types transhepatic and transduodenal. Transduodenal can be further divided into two types according to the endoscope position: long (push) and short (pull)[95]. In 2004 Mallery *et al*[96] described feasibility of rendezvous technique for biliary drainage in 6 cases. Few studies[97,98] with over 40 patients have been published which have reported technical success rate in 60's and 70's. A retrospective study by Dhir *et al*[99] comparing the precut papillotomy to Rendezvous technique in patients with failed cannulation revealing higher success rate with EUS-RV.

EUS guided antegrade techniques are suitable for patients with altered surgical anatomy[100] or upper intestinal obstruction which prohibits the scope from reaching the biliary orifice. It involves accessing the intrahepatic biliary duct by creation of a temporary fistula between the intestine and IHBD followed by dilation of the fistula with subsequent stent placement or balloon dilation for biliary obstruction.

Park *et al*[101] in 2012 successfully described EUS-guided transhepatic antegrade balloon dilation in a 45-year-old female with hepaticojejunostomy. She had presented with bilioenteric anastomotic stricture and had failed deep enteroscopy with a pediatric colonoscopy and double balloon enteroscope. Further in a prospective series by Park *et al*[102] 14 patients underwent antegrade technique out of which it was successful in 8 of them. A retrospective study by Shah *et al*[103] reported a success rate of 81%.

In 2013, Park *et al*[104] published a prospective series in which he not only described but also evaluated the technical feasibility and safety of EUS-guided hepaticoduodenostomy (EUS-HD) in cases of isolated right intrahepatic duct (IHD) obstruction. EUS guided cholangiography of the right IHD was successfully performed in all 6 patients and he described 3 kinds of approaches: (1) using a cholangiogram obtained by EUS-guided transduodenal puncture of the right hepatic duct as a "roadmap" to assist retrograde cannulation; (2) EUS-guided antegrade transanastomotic balloon dilation with or without stenting; and (3) EUS-BD with transluminal stenting between the right hepatic duct and the duodenal wall as an antegrade bypass stenting. Though it is an attractive alternative to PTBD in patients with isolated IHD more studies are needed to establish its efficacy.

CONCLUSION

Although diagnostic and therapeutic EUS is an established tool for upper GI and pancreatic applications, the authors believe that its indications and utilizations in hepatic pathologies are under-recognized. For example, EUS is able to depict and biopsy even small solid lesions in the liver that are either not visualized by other imaging modalities or visualized during routine staging procedures of GI or pulmonary cancers. However, its diagnostic role for hepatic applications is at present not fully defined. Comparative studies are needed. The therapeutic role of EUS in hepatic applications is increasing in particular regarding internal drainage procedures in patients with intrahepatic abscesses in the left lobe and in patients with biliary obstruction and altered anatomy of the GI tract. However close monitoring of the results is mandatory due to the risk of complications such as bile leakage.

EUS is definitely one of the most emerging technologies with significant clinical ramifications. There has also been a substantial interest in the future roles of EUS. The authors expect future development and expanded hepatic applications of EUS in two directions: improved diagnostic yield and evolved indications with new EUS imaging technologies and hard wares, and growth in therapeutic EUS with new EUS and other endoscopic devices. Since the left lobe and caudate lobe lie in close proximity to stomach and duodenum, providing an easy access during EUS, more EUS applications should be seen within left, caudate lobe, and gastrohepatic space. With current technology, EUS is limited in its ability to access the portion of the right lobe adjacent to the dome of the diaphragm along with its lateral and inferior portions, trans-abdominal ultrasound can complement EUS and achieve total hepatic coverage by ultrasound imaging. Interventional EUS and/or trans-abdominal ultrasound can be utilized to approach various pathologies associated with hepatic parenchyma, intrahepatic biliary system, and vascular structures. The authors expect to see more EUS guided injection ablative therapy for hepatic tumors or cysts with ethanol, sclerosant, chemotherapeutics, and biologics, and EUS guided fiducial placement for stereotactic body radiation.

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P- Reviewer: Kitano M, Mendez-Sanchez N, Picardi A
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12558

World J Gastroenterol 2015 November 28; 21(44): 12558-12575
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Hepatitis B virus therapy: What's the future holding for us?

Sobia Manzoor, Muhammad Saalim, Muhammad Imran, Saleha Resham, Javed Ashraf

Sobia Manzoor, Muhammad Saalim, Muhammad Imran, Saleha Resham, Atta-ur-Rahman School of Applied Bio-Sciences, Department of Healthcare Biotechnology, National University of Sciences and Technology, Islamabad 44000, Pakistan

Javed Ashraf, Islam Dental College, Sialkot 51310, Punjab, Pakistan

Author contributions: Manzoor S designed the research; Manzoor S and Saalim M searched the literature and drafted the manuscript; Manzoor S critically reviewed the manuscript; Imran M, Resham S, and Ashraf J helped in literature review and manuscript preparation.

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

Data sharing statement: All the data regarding the review is available from the corresponding author, Sobia Manzoor at: lcianunique@yahoo.com.

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Correspondence to: Sobia Manzoor, PhD, Assistant Professor, Atta-ur-Rahman School of Applied Bio-Sciences, Department of Healthcare Biotechnology, National University of Sciences and Technology, Islamabad 44000, Pakistan. lcianunique@yahoo.com
Telephone: +92-51-90856147
Fax: +92-51-90856102

Received: April 21, 2015
Peer-review started: April 22, 2015
First decision: June 25, 2015
Revised: July 24, 2015
Accepted: October 17, 2015

Article in press: October 20, 2015
Published online: November 28, 2015

Abstract

Hepatitis B is one of the leading causes of liver cancer worldwide and unfortunately the number of people affected with hepatitis B virus (HBV) infection is still on the rise. Although the HBV has been known to cause fatal illness since decades but the population effected by this lethal virus have still only a few options for its management. The major treatment strategies include interferons and nucleos(t)ide analogues. These agents have so far produced unsatisfactory results in terms of complete virus eradication. Interferons cannot be used for long term therapy because of their potential side effects. Prolong treatment with nucleos(t)ide analogues has also been reported to cause serious side effects besides the increasing resistance by the virus. The need for new innovative solutions for treatment of HBV has been realized by global research institutes and pharmaceutical industry. Present review focuses in detail on the new ideas that are being transformed into therapeutic tools for use as future therapies in HBV infection. Modern drug designing and screening methods have made the drug discovery process shorter and more reliable. HBV therapeutics will take a new turn in coming years owing to these intelligent drug designing and screening methods. Future therapy of HBV is aiming to include the use of vaccines (both prophylactic and therapeutic), immunomodulators such as antibodies, non-nucleoside antivirals such as RNAi and inhibitors of viral life cycle.

Key words: Hepatitis B virus treatment; Hepatitis B virus vaccines; Immunomodulators; Non-nucleoside antivirals; Nucleoside analogues

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Core tip: The need to develop new therapeutic agents for hepatitis B virus treatment is the motivation factor for many research groups and pharmaceutical industries worldwide. The therapies in development from immunomodulation agents to non-nucleoside viral inhibitors hope to replace the current treatments with the promise of increased efficacy, minimum side effects and shorter duration of cure.

Manzoor S, Saalim M, Imran M, Resham S, Ashraf J. Hepatitis B virus therapy: What's the future holding for us? *World J Gastroenterol* 2015; 21(44): 12558-12575 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12558.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12558

INTRODUCTION

Chronic hepatitis B virus infection (CHB) lies among chief public health threats globally and is basically a main reason for the development of liver cirrhosis and hepatocellular carcinoma with substantial disease burden; therefore representing the high unmet medical requirement[1]. Almost more than five hundred million people are projected to be persistently infected with the hepatitis B and C virus and they are at high risk of developing chronic liver disease (CLD), cirrhosis and hepatocellular carcinoma (HCC) ultimately. Hepatitis B virus (HBV) is 3.2 kb, partially double-stranded DNA enveloped virus capable of infecting hepatocytes. It belongs to the family *Hepadnaviridae*[2]. Currently there are eight HBV genotypes ranging from A-H[3,4]. Its virion particle consists of a core particle encapsulating the viral genome, polymerase, nucleocapsid protein, besides a lipoprotein envelope containing viral antigens. There are four major open reading frame sin viral DNA genome that include: (1) The pre-core/core gene, that codes for nucleocapsid protein and for the non-structural, secreted, pre-core protein, and hepatitis B e antigen (HBeAg); (2) polymerase gene that codes for enzyme, RNase H reverse transcriptase, and terminal protein domains; (3) PreS2/M-, PreS1/L-, Surface/S-gene that code for the 3 envelope proteins; besides (4) *X* gene, that codes for regulatory X-protein[5,6]. Molecular virology of HBV dictates that it is not directly cytopathic[7] and upon infection, it remains in latent state within the hepatocytes[8].

Increasing evidence showed that distinct geographic distributions of HBV genotypes may influence disease severity and response to treatment. It has been observed that HBV genome integration witint host chromosome is not vital for life cycle of HBV. The disease progression by HBV depends upon the clinical spectrum that is wide, ranging from a subclinical inactive carrier state, to advanced chronic hepatitis, cirrhosis that leads to decompensation, and ultimately culminating in hepatocellular carcinoma. The lifecycle of HBV within a cell is shown in Figure 1[6].

The dynamic natural history of CHB infection involves a complex interaction between the host immune system and the virus. During the course of chronic exposure to HBV, persistent inflammation process accompanies liver damage and cell death. These elements lead to chronic liver disease[7]. Carriers of HBV are susceptible to the development of[2] cirrhosis and decompensation within liver along with 100-fold high risk of development of hepatocellular carcinoma (HCC)[1,9-11]. Viral proteins play their roles through altering gene expression. These proteins augment oncogenesis, metastases and resistance to apoptosis and growth inhibition. HBV genome contains a gene coding for the HBx protein that has been studied to potentially contribute in inducing hepatocytes malignancy and transformation. However there are immense number of unanswered questions within the process of developing and progression of carcinogenesis by the virus as well as the perturbed signaling pathways within the liver. Virologists are following the trend of research that is focused on life cycle of the virus as well the cell signaling pathways that are disturbed during pathogenesis leading to the development of cancer. The most obvious and prominent reason for poor management of HBV infection is delayed detection/diagnosis or detection at the stage where the liver has reached to end stage liver disease. Therefore, timely diagnosis and CHB treatment is vital for the reduction of mortality and morbidity[1]. There are many key factors that impede adequate treatment like: apprehensions to initiate, end, financial cost and resistance of therapy[12]. However, obstacles HBV-related chronic liver disease may be compact by viral suppression. There are following goals of the therapy: to improve quality of life and promote survival by prevention of advancement to cirrhosis and decompensated cirrhosis, HCC and death through continuous inhibition of HBV replication.

Broadly, depending upon the treatment duration there are two different treatment options for patients with CHB infection: (1) Therapies that are of fixed duration including immunomodulators like standard/conventional or PEGylated interferon-α (IFN-α); and (2) Long-term treatment with nucleos(t)ide analogues lamivudine, adefovirdipivoxil, entecavir, tenofovir or telbivudine.

Current therapies aims at persistent suppression of viral replication that typically results in biochemical remission and reduced histological activity of chronic hepatitis. Consequently, the risk of progression to next stage *i.e.,* cirrhosis, also decreases. Therefore, reducing incidence of HCC in non-cirrhotic and some extent in cirrhotic patients[6,13,14]. However, many patients that undergo the currently available therapies do not show long-lasting control after the withdrawal of treatment. Specially, rates of hepatitis B surface

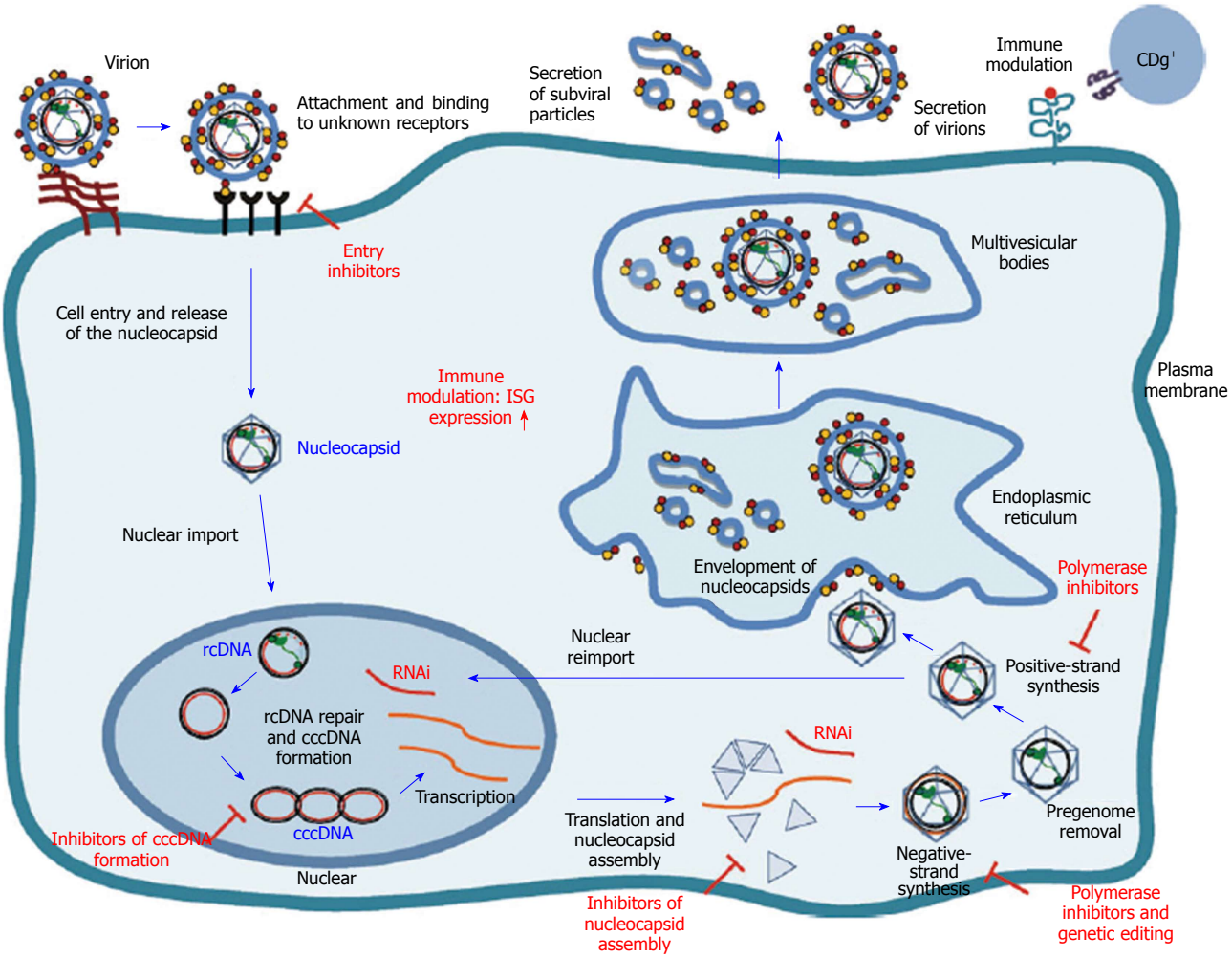

Figure 1 Hepatitis B virus life cycle along with inhibitors targeting the various stages of the hepatitis B virus lifecycle (Adapted from Grimm *et al*[6] 2011). Following attachment of virus to the receptors, cell entry and release of nucleocapsid, nuclear import of virus to nucleus, transcription and translation leads to the synthesis of covalently closed circular DNA (cccDNA), envelopment of nucleocapsid within endoplasmic reticulum, formation of multivesicular bodies and finally secretion of subviral and virion particles. Moreover red bar lines shows the inhibitors targeting various stages of the virus life cycle such as: entry inhibitors, inhibition of cccDNA formation, and inhibition of assembly, polymerase inhibition and genetic editing and immunomodulation targeting the cell surface receptors. ISG: Immune serum globulin.

antigen (HBsAg) loss and seroconversion to hepatitis B surface antibody (HBsAb) are very low[15,16].

The objective of this current review is to provide an update on the recent advances in HBV therapeutics addressing all the drugs available in the market and those in clinical trials to provide an update to those who are either working on HBV or in health care sector.

CURRENT THERAPIES FOR HBV

Currently, there are sevendrugs approved by Food and Drugs Administration (FDA) for the treatment of chronic hepatitis B infection. These drugs are intron A (IFN-α), Pegasys (Pegylated Interferon), Epivir HBV (Lamivudine), Hepsera (Adefovir), Baraclude (Entecavir), Tyzeka (Telbivudine), and Viread (Tenofovir). These seven drugs have been licensed by FDA but not a single drug provides a complete cure; but they do stop/slow down the progressive liver damage. There are limitations of each drug; Interferon therapy offers fix treatment duration but mostly associated with serious side effects that lead to the cessation of therapy. Oral nucleoside or nucleotides inhibitors induce drug resistance by prolong use. The treatment response rate of currently used drugs against HBV infection two years post-treatment is summarized in Table 1[17]. The treatment response rate is defined in terms of undetectable HBeAg, HBeAg seroconversion (absence of hepatitis B e antigen in serum and the presence of antibodies against it) and HBV DNA less than 300/400 copies/mL in HBeAg negative patients.

Pegasys (Pegylated Interferon)

IFN-α-2a/b was the first approved treatment option for CHB infection in most of the countries. However later on, the improved pharmacokinetics properties, efficacy and convenient dosing of Pegylated Interferon have caused the replacement of standard IFN-α-2b. Pegasys is given as a shot once a week as compare to

Table 1 A list of hepatitis B virus vaccines in development

Treatment	Undetectable HBV DNA in HBeAg positive patients	Anti-HBeAg serconversion in HBeAg positive patients	HBV DNA < 300/400 copies/mL in HBeAg negative patients
Pegylated interferon	25	30	63
Lamivudine	39	22	72
Adefovir	21	12	51
Entecavir	67	22	90
Telbivudine	60	26	88
Tenofovir disoproxil fumarate	74	21	91

HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

standard interferon that is given as a shot three times a week[18-20]. The half-life of interferon is increased by pegylation. The treatment success rate of Pegasys is 24% as compared to 12% of standard interferon[21]. Pegasys is a better treatment option than lamivudine in terms of HBV DNA suppression and seroconversion of HBsAg[1]. The treatment success rate of Pegasys and lamivudine was 32% and 19% respectively[22,23]. Pegasys is not recommended for those CHB who are victims of autoimmune hepatitis, liver cirrohosis, show serious side effects or having age below one year. Interferon blocks DNA synthesis of HBV. Pegasys treatment is the most favorable for those CHB patients possessing strong immune system with high level of liver enzymes, infected after childhood and the virus is replicating.

Epivir-HBV (Lamivudine)

Lamivudine is a cytidine nucleoside analogue that inhibits the reverse transcriptase enzyme of HBV. Lamivudine is an orally administered and well tolerated at a daily dosage of 100 mg for one year[24]. It profoundly suppresses serum HBV DNA and showed bio-availability for more than 80%[25]. The rate of HBeAg seroconversion (absence of hepatitis B e antigen in serum and the presence of antibodies against it) in CHB patients by Epivir-HBV or Lamivudine for the treatment duration of one year is 32%[26,27] and increases up to 65% by five years treatment[28]. Additionally the treatment response is not dependent on ethnicity[20]. Lamivudine therapy can be stopped after the seroconversion and sustained viroloigcal response is maintained in approximately 80% of cases. The treatment is associated with the development of viral resistance that occurs by mutations in the YMDD motif of HBV polymerase. The incidence of lamivudine resistant HBV mutants increases upto 70% by five years treatment. The viral breakthrough occurs at 6-9 mo of therapy and detectable HBV DNA at six month of therapy is linked with the emergence of higher resistance rate. Combinational therapy of lamivudine with other therapies such as PEG-IFN or adefovirhave no significant effect on the treatment response, however the rate of emergence of viral resistance is reduced[29,30].

Hepsera (Adefovir)

Hepsera is the tradename foradefovirdipivoxil. Adefovir is a nucleotide analogue which has shown activity against HBV. Hepsera is daily administered as 10 mg oral tablets. The most common serious side effects associated with hepsera are rash, swelling of throat, lips, tongue and face, difficulty in breathing and proximal kidney tubular dysfunction[31]. A recent clinical trial of seventy treatment naive HBV patients demonstrated that the treatment efficacy in terms of HBV reactivation was almost comparable for lamivudin and adefovir *i.e.,* 37.1% and 28.6% respectively. 61.5% patients on lamivudine developed drug resistance; however none of the patients on adefovir developed drug resistance. Thus, adefovir has the advantage over lamivudine in terms of developing lower drug resistance profile[32].

Baraclude (Entecavir)

Baraclude or entecavir is a potent inhibitor of HBV' s DNA polymerase enzyme[33]. Phase 3 clinical study of baraclude demonstrated significantly higher treatment efficacy than lamivudine in term of histologic, biochemical and virologic improvements[34]. Resistance to baraclude is not common; however it occurs in half number of patients who have used lamivudine. Baraclude is daily orally taken at a dosage of 0.5 mg for patients who are treatment naive for HBV treatment and at dosage of 1 mg for patients who demonstrated resistance for lamivudine[34].

Tyzeka (Telbivudine)

Tyzeka is orally taken at a daily dosage of 600 mg. It is more potent than lamivudine and adefovir. Like lamivudine and adefovir, it is commonly associated with the emergence of resistance. However, a recent meta-analysis study of telbivudine and lamivudine showed that telbivudine is a better treatment choice for HBV infection due to its lower rate of resistance[35].

Viread (Tenofovir disoproxil fumarate)

Tenofovir disoproxil fumarate is a nucleotide analogue that is also a potent inhibitor of HIV and HBV polymerase. It is orally taken at the dosage of 300 mg per day. It is more effective than lamivudine and adefovir. It is

Table 2 A Lists of hepatitis B virus vaccines in development

Name of vaccine	Type	Developed by	Phase
Sci-B-Vac	Prophylactic 3rd generation vaccine	SciVac Ltd, Israel	Approved in Israel, Asia and Africa
HEPLISAV-B	Prophylactic vaccine	Dynvax Technologies Corporation, United States	Phase Ⅲ
GS-4774	Therapeutic vaccine	Gilead Sciences, United States	Phase Ⅱ
Hepsyn-B	Therapeutic vaccine	Immune Targeting Systems Ltd, United Kingdom	Pre-clinical
VAXINE's HBV Vaccine	Therapeutic	Vaxine Pty, Ltd, Australia	Phase Ⅰ
VAXINE's HBV Vaccine	Prophylactic	Vaxine Pty, Ltd, Australia	Phase Ⅱ
HBV^+ DRibbles vaccine	Prophylactic and therapeutic	Southeast University, Nanjing, Jiangsu, China	Pre-clinical
ABX 203	Therapeutic	ABIVAX, France	Multicenter Phase Ⅱ/Ⅲ Trials
INO-1800	Therapeuti	Inovio Pharmaceuticals, United States and Roche	Phase Ⅰ (expected to start in 2015)
DV-601	Therapeutic	Dynvax Technologies Corporation, United States	Phase Ⅰb
Altravax Therapeutic Vaccine	Therapeutic	Altravax, Inc, United States	Pre-Clinical

HBV: Hepatitis B virus.

effective to telbivudine, lamivudine and entecavir resistant patients. However, it is less effective in adefovir resistant patients[36].

FUTURE THERAPIES FOR HBV

Vaccines

Currently used HBV Vaccine was developed initially in 1984[37]. The same vaccine that was developed from baker's yeast with a little modification is used to date. It was introduced in 1986 in United States. The HBsAg vaccine is 80%-100% efficacious in prevention of infection or hepatitis in those who receive the prescribed course of vaccine. However, the immunogenicity of vaccine is found to decline with age. After the age of 40 years, almost 90% of recipients respond positively to a three-dose series, and at the age of 60 years, merely 75% of vaccines are able to develop antibody titers that give protection[38]. Alum is the most widely used adjuvant in humans. It has an exceptional safety record and shows a strong bias toward Th2 type antibody responses while, a marginal Th1 cell-mediated response is observed *in vivo* and often requires multiple booster immunizations[39,40]. The need to develop a new vaccine that is able to ensure 100% efficacy with long lasting immune response *i.e.,* without booster doses has always been there. Another approach designated as therapeutic vaccination aims at designing immunotherapies that produce either specific or non-specific immune responses against HBV that could help to achieve sustained viral control by limited treatment[41,42]. The therapeutic vaccines are not only under trial for monotherapy but have also been evaluated as a combination therapy with existing anti-HBV. Chongqing Jiachen Biotechnology Ltd. Conducted a phase Ⅱ clinical trial of Synthesized Peptide εPA-44 with Entecavir in 2012[43]. Phase Ⅲ clinical trial of lamivudine and recombinant hepatitis B surface antigen was conducted by French National Agency for Research on AIDS and Viral Hepatitis in Dakar, Senegal in 2008[44]. Therapeutic vaccination has shown promising results in animal models by inducing T cell immune responses therefore, limiting viral infection[45]. Some of the vaccine products in development are discussed here. Table 2 summarizes various HBV vaccines currently in development.

Sci-B-Vac

Sci-B-Vac is a brainchild of two physicians from Israel at the Weizmann Institute of Science and is a product of Israel's SciGen. Sci-B-Vac is designated as the first commercially available third-generation vaccine effective against HBV and is already approved in Philippines, Hong Kong,Vietnam,India,Central Africaand Georgia. Sci-B-Vacsi not a single protein. It is a hollow "envelope" that contains three purified recombinant proteins which have been derived from a line of hamster cells - these cells are much closer to human cells instead of yeast. The vaccine has completed its clinical trial and the data shows that the SCI-B-VAC vaccine that contains the S-protein component of the HBV surface together with the PreS1 and Pre-S2, is considerably more immunogenic than a second- generation recombinant HBV vaccine[46,47]. The vaccine is equally efficient in all types of study populations including infants, adults and patients with end stage renal disease, non-responders to the already available HBV vaccines including obese and elderly[47-50]. It has also been shown that fewer number of doses *i.e.,* two are sufficient to provide adequate rapid seroprotection[51]. Recent studies have asserted that Sci-B-Vac is more efficient than Engerix-B (the most popular brand in market) due to its rapid and higher response[52]. It can be therefore said once FDA grants the approval Sci-B-Vac, it will capture the major share in market for HBV immunization.

GS-4744

GS-4774 is as a therapeutic vaccine that has been engineered to generate T cell immune responses against cells that contain HBV antigens along with the antiviral therapy with the aim of improving response rate in patients with CHB infection. GS-4774 is a heat-killed, recombinant, yeast-based immunotherapy designed to express antigens that are specific for HBV[53]. The GS-4774 Tarmogen expresses a fusion

protein utilizing sequences of the HBV contained in the four major HBV genotypes worldwide, in order to ensure applicability for this product across multiple markets[54]. Gilead initiated a phase 2 clinical trial in July 2014 investigating GS-4774 in individuals with chronic HBV infection who are not currently receiving any treatment[55]. According to latest data GS-4774 was found to be safe and well-tolerated in healthy subjects. The weekly and monthly regimens of GS-4774 both provided HBV-specific immune responses at all evaluated doses[56]. GS-4774 can therefore be regarded as a promising member of HBV Therapy Regimen providing physicians with a very reliable option for HBV treatment.

Heplisav-B or 1018 ISS adjuvant

Heplisav is a vaccine under development by Dynavax technologies and is currently in Phase-Ⅲ of clinical trials. It contains ISS *i.e.,* immunostimulatory sequences (1018 ISS) and HBsAg[57]. Phase Ⅲ clinical trials have that Heplisav-B a Toll-like receptor-9 (TLR-9) agonist produces a rapid and high titer leading to sustained seroprotection in healthy individuals as well as hyporesponders with lesser number of immunizations[57-59].

Heplisav is different from the vaccines already present in the market as it contains synthetic adjuvant called immunostimulatory sequences (ISS) instead of alum. ISS are cytosine phosphoguanosine (CpG) motifs that originate from bacterial DNA and are potent activators of immune system[60]. It has been shown that these CpG motifs use TLR-9 to stimulate the innate arm of immune system leading to the activation of a chain of immune responses intermediated by IL-18, IL-12 and IFN-γ derived from macrophages and natural killer (NK) cells. This chain of events promotes Th1 responses for both protein and vaccines derived from DNA[60]. Antibody production and B-cell proliferation are also significantly enhanced the presence of these CpG motifs[60,61].

Heplisav is clearly a vaccine of the future with an ability of producingseroprotection against HBV with lesser number of immunizations and in a rapid succession, especially amongst immunologically week populations and is therefore, ideal candidate to decrease the number of morbidities and mortalities of chronic HBV infections effectually.

Hepsyn-B

Hepsyn-B is a lead product of immune targeting systems (ITS). ITS has commenced lead optimization of Hepsyn B and has received a grant from the UK Biomedical Catalyst funding scheme for FP 02 development in Hepatitis B infection. Hepsyn-B is a fluoropeptide vaccine (Hepsyn-B), based on ITS's proprietary Depovaccine and Densigen technologies, to induce immune control over the disease, increase likelihood of clearance of infected liver cells and reducing the need for prolonged costly drug therapy[62,63]. Hepsyn-B™ is a DepoVaccine product that contains eight Densigens which are derived from highly conserved regions of the Hepatitis B virus. The product is intended to provide therapeutic benefit to patients who are chronically infected with Hepatitis B of any of the four major sub-types (A-D)"[63].

The vaccine is currently in pre-clinical development stages. The goal of current standard of care treatment is to reduce viral load and permit seroconversion so subjects progress to an inactive state where liver disease progression is halted. Antiviral T-cell immunity is a key driver of this outcome. It has been suggested that therapeutic vaccination with Hepsyn-B and other similar products will improve treatment outcomes and reduce reactivation rates by "actively reconstituting disease protective T-cell immune responses"[64].

Vaxine's HBV vaccine

The Australia based biotechnological company is in process of developing a HBV prophylactic as well as an HBV therapeutic vaccine. The prophylactic vaccine is based on combining the Advax™ adjuvant with a unique hepatitis B vaccine antigen. Vaxine's prophylactic HBV vaccine was ranked as one of the top 5 global products that entered the phase Ⅱ clinical testing in 2007 by Thomson Scientific. The company has received an approval to conduct the vaccine trial for HBV immunization of subjects with impaired immune systems because of diabetes, older age or kidney failure[65].

Vaxine's therapeutic HBV vaccine is actually a nasal vaccine that utilizes the ability of hepatitis B core antigen of stimulating a strong Th1 type response against hepatitis B surface antigen[65]. The vaccine is currently in phase 1 trial[66].

HBV$^+$ DRibbles vaccine

HBV$^+$ DRibbles vaccine is an autophagosome-based HBV vaccine from HBV-expressing hepatoma cells. This vaccine is a brainchild of scientists from Department of Microbiology and Immunology, Medical School of Southeast University and Cancer Research and Biotherapy Center, the Second Affiliated Hospital of Southeast University, Nanjing, Jiangsu, China and Laboratory of Cancer Immunobiology, Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR, United States. The vaccine has shown positive results by inducing polyvalent anti-HBV T-cell responses and therapeutic efficacy in mouse models that mimic acute and chronic HBV infection in human. The HBV$^+$ DRibbles produced from a HBV expressing cell line were effective as a prophylactic vaccine or a therapeutic vaccine to treat mice with established HBV replication. The study demonstrated that HBV$^+$ DRibbles mixed with α-Al_2O_3 nanoparticles could elicit an endogenous T-cell response that swiftly eliminatesan established "HBV infection".

The results suggested that HBV^+ DRibbles vaccine together with a potent adjuvant was capable to elicit therapeutic immune response and overcome HBV tolerance in the mouse model[67]. The efficiency of HBV^+ DRibbles vaccine can be attributed because to α-Al_2O_3 nanoparticles, in contrast to traditional Alum adjuvant, which are capable to deliver antigen for very efficient priming of cytotoxic lymphocytes and capable of boosting the anti-tumor efficacy of tumor-derived autophagosomes[68]. All these features make HBV^+ DRibbles vaccine a promising candidate as a future HBV therapeutic vaccine.

ABX 203

ABX 203 is the first collaborative result of ABIVAX France with CIGB Cuba. In order to shorten the current treatment period of HBV ABX 203 has been developed as a therapeutic vaccine candidate that is capable of delivering long-lasting control of the viral load. It is composed of both the surface and core antigens of the hepatitis B (HBs and Hbc antigens) and thus a mix of 2 viral proteins.

The Clinical trials of ABX203 have shown encouraging data that shows the efficacy of this vaccine in trial in healthy volunteers as well as in phase Ⅰ study and phase Ⅱ studies in patients with chronic HBV infection[69]. The vaccine has entered a multicenter phase Ⅱ/Ⅲ clinical trial that is estimated to be completed in 2017[70].

INO-1800

INO-1800 is a multi-antigen SynCon® DNA immuno-therapeutic vaccine that targets hepatitis B virus clades A and C surface antigens and HBV core antigens. Inovio pharmaceuticals has entered into agreement for collaboration with Roche to develop IN0-1800[71]. Preclinical trial data successfully reported generation of strong immune responses that include T cell and antibody activation by INO-1800 that leading to the elimination of targeted liver cells within mice. These results show the true potential of this DNA vaccine in treatment of HBV infection and prevention of progression into liver cancer. The company has planned to initiate a phase Ⅰ clinical trial of INO-1800 in 2015[72].

DV-601

DV-601 is a protein based therapeutic vaccine that was able to induce both cellular and humoral immune responses and was able to drive multi-functional and multi-specific T cell immune responses in the liver of chronically HBV infected mice. The vaccine employs the use of a novel protein based vaccine formulation that comprises HBsAg and hepatitis B core antigen along with ISCOMATRIX adjuvant. This particular formulation was able to successfully break the tolerance in this chronic viral disease[73]. In 2011 the drug successfully underwent Phase 1b clinical trials that showed all doses were generally well tolerated and safe in cohorts at all doses[74].

Altravax's therapeutic vaccine

Altravax is a privately held United States based bio-pharmaceutical company that bagged SBIR grant from NIH in January 2013[75,76]. Altravax has developed a therapeutic DNA vaccine product delivered by electroporation and expressing a mixture of HBsAg variants. A number of immunogenic HBsAg variants containing xenogeneic sequences with novel T epitopes have been identified using a directed molecular evolution approach. Mixing several variants has increased the immunotherapeutic potential of the combined vaccine by including many different xenogeneic epitopes. Beginning with seven individual variants, all possible 3-variant combinations are being screened using a tiered strategy to identify the most immunogenic mixtures in normal and HBsAg-transgenic mice[77]. Altravax's preclinical studies in animals indicated the effectiveness of the vaccine for therapeuticpurpose in chronic HBV patients. The product has now formally entered the preclinical development phase that will include manufacturing and safety studies that are prerequisite for submitting an investigational new drug (IND) application to FDA[77].

IMMUNOMODULATORS

There are many immunomodulators currently in development for HBV infection. A few of them are discussed here. These include recombinant interleukins, antibodies and agonists of pattern recognition receptors. Table 3 gives summary of immunomodulators currently in various stages of development for HBV infection.

CYT-107

Recombinant human interleukin-7 (CYT107) is an immune-modulator for immune T-cell retrieval and augmentation. IL-7 is produced as growth factor and cytokine by thymic or bone marrow stromal cells and other epithelia. IL-7 critically and non-redundantly stimulates the development of T lymphocyte by thymopoiesis and from the thymus downstream, on expansion of peripheral T-cells[78,79]. The repeated dose trials of first-generation of rhIL-7 were shown in pre-clinical and phase Ⅰ studies in oncology and HIV-infected patients that were well tolerated in recurrent dose trials, with long-term increases in both CD4 and CD8 T cells. The second-generation rhIL-7 is CYT107, a product made by Cytheris, a biopharmaceutical company in clinical stage. Trials conducted in clinical settings that recruited over 120 patients within Europe, Taiwan and North America have confirmed the ability of IL-7 to develop and protect CD4+ and CD8+ T-cells[79,80]. After initial safety evaluation CYT107 has now been successfully evaluated in phase Ⅰ/Ⅱa clinical

Table 3 Immunomodulators in pipeline for hepatitis B virus treatment

Immune enhancer	Function	Developer	Phase
CYT-107	Recombinant IL-7	Cytheris SA, France	Phase Ⅱ
TG-1050	Adenovirus based targeted immunotherapy	Transgene SA, France	Pre-clinical
GS-9620	TLR-7 agonist	Gilead Sciences, United States	Phase Ⅱ
GC1102	Monoclonal antibody Recombinant HBIg	Green Cross Corporation, Japan	Phase Ⅱ
CYT-003	TLR-9 agonist	Tekmira Pharmaceuticals Corporation, Canada	Pre-clinical (Phase Ⅱ for Asthma)
SB 9200	RIG-I and NOD2 activator	Spring Bank Pharmaceuticals, United States	Phase Ⅱ

HBV: Hepatitis B virus; HBIg: Hepatitis B Immunoglobulin; TLR: Toll-like receptor; RIG-I: Retinoic acid-inducible gene I; NOD2: Nucleotide-binding oligomerization domain containing 2; IL-7: Interleukin-7.

trial evaluating recombinant human Interleukin-7 (CYT107) in combination with the standardized antiviral treatment and vaccination in HBeAg-negative chronically infected HBV patients[81,82].

TG-1050

TG-1050 is a targeted immunotherapy candidate based on adenovirus-based for the treating chronic hepatitis B. The pre-clinical package for TG-1050 is capable of supporting TG-1050 to induce a vigorous, extensive, long-lasting T cells with features similar to those found in effected individuals who resolve infection along with antiviral activity. Significantly, TG-1050-educated T cells have the ability to recognize immune factors derived from all circulating strains of HBV viral genotypes, including genotypes B and C[64,83]. Additionally, a combined study conducted by Ruijin Hospital in Shanghai and Transgene Biopharmaceutical Technology (Shanghai) Co., Ltd. and Transgene SA, displayed that amount of antibodies produce against adenovirus are similar to those produced in chronically infected HBV patients and healthy individuals. The company plans to starta first-in-humans clinical evaluation in the next few months[84].

GS-9620

GS-9620 is an oral agonist of TLR-7. It is currently in phase Ⅱ clinical trial for treating individuals with CHB[85]. In previously conducted preclinical phase of the study, GS-9620 was able to induce a prolonged suppression of HBV antigens and serum levels of viral DNA within animal models of HBV induced hepatitis[86,87]. It has been established that the antiviral response mediated by GS-9620 in preclinical models of CHB is probably facilitated in part by the cytolytic activity of CD8+ T cells. Strong intrahepatic B cell response when initiated might have played a vital role in seroconversion of HBsAg antigen[88].

GC 1102

GC 1102 is a new recombinant hepatitis B immunoglobulin (HBIg) from Chinese Hamster Ovary (CHO) cells. It is a monoclonal antibody and has high affinity and avidity to hepatitis B surface antigen and several advantages compared to HBIg derived from blood plasma of human donors[89]. The antibody is currently in Phase Ⅱ Clinical Trials at Seoul Asan Medical Center Kore and is sponsored by Green Cross Corporation[90].

CYT-003

Tekmira's lead TLR-9 program, CYT-003, was in-licensed from Cytos Biotechnology Ltd., where it was evaluated clinically in allergic asthma and successfully completed Phase Ⅱ Clinical Trials for Asthma. The company is currently evaluating the utility of CYT-003 in HBV. TLR-9 agonists are a novel approach to the reactivation of immune system in patients chronically infected with HBV. Stimulation of cellular TLRs is expected to provoke a response mediated by innate arm of the immune system leading to the production of cellular proteins capable of targeting viral infections such as HBV[91].

SB 9200

SB 9200 a product of Spring Bank Pharmaceuticals that works by activation of pattern recognition receptors including retinoic acid-inducible gene Ⅰ and nucleotide-binding oligomerization domain containing 2, involved in the detection of viral RNA within cells. It up-regulates the host immune response selectively to viral infection by blocking replication of the virus and at the same time by produces endogenous interferon. It is potentially less likely to produce resistance because it targets the host proteins that are involved in the immune response in contrast to directly acting on the virus. The drug was found to cause a significant decline in preclinical animal models in viral load of HBV. The company is currently planning to proceed towards Phase Ⅱ clinical development of SB 9200 for HBV infected patients[92].

NON NUCLEOSIDE ANTIVIRALS

Non-nuceoside anti-virals (NNAVs) are the cornerstone of future therapies for HBV. These include phenyl-propenamides, hetero-aryl-di-hydro-pyrimidines, RNAis and Novel Protein Inhibitors. These approaches are being analyzed with great success in clinical trials

Table 4 Non-nucleoside anti-viral drugs in development for hepatitis B virus

Name	Mechanism	Developed By	Phase
CpAMs	Allosteric Modulation of HBCp	Assembly Biosciences, United States	Pre-clinical
AT61 and AT130	Assembly Activation (Inhibit Capsid Assembly)	Victorian Infectious Diseases Reference Laboratory, Australia	Pre-clinical
BAY41-4109	Assembly Activation (Inhibit Capsid Assembly)	AiCuris, Germany	Phase Ⅰ
NVR 3-778	HBV Cp Inhibition (cccDNA suppression)	NoviraTheraputics, United States	Phase Ⅰb
NVP018	Cyclophilin inhibition	Tekmira Pharmaceuticals Corporation, Canada	Phase Ⅰ
TKM-HBV$_{4G}$ and TKM-HBV$_{3G}$	RNAi	Tekmira Pharmaceuticals Corporation, Canada	Phase Ⅰ
STING agonists	PRR Activation	Tekmira Pharmaceuticals Corporation, Canada	Pre-clinical
ARC-520	RNAi	Arrowhead Research Corporation, United States	Phase Ⅱ
ALN-HBV	RNAi	Alnylam Pharmaceuticals, Inc. United States	Pre-clinical
GLS4	Assembly Activation (Inhibit Capsid Assembly)	HEC Pharm Group, China	Phase Ⅱ (China)
BSBI-25	cccDNA Inhibition	Baruch S. Blumberg Institute, United States	Pre- clinical
Birinapant	SMAC	TetraLogic Pharmaceuticals, United States	Phase I
CPI-431-32	Inhibition of cyclophilin A	Ciclofilin Pharmaceuticals, United States	Pre-clinical
Myrcludex B	HBV entry inhibition	Hepatera Ltd, Russia and MYR GmbH, Germany	Phase Ⅱ
Simvastatin	HMG CoA reductase inhibitor	University of Oklahoma and United States Veterans Administration	Phase I

RNAi: RNA inhibition; HBV Cp: Hepatitis B virus core protein; PRR: Pattern recognition receptors; SMAC: Synthetic small molecule and peptidomimetic of second mitochondrial-derived activator of caspases.

and most of them hold great promise in the future. The NNAVs in development for HBV are enlisted in Table 4.

Core protein allosteric modulators

Core protein allosteric modulators (CpAMs) belong to the category of anti-viral small molecules which are responsible for targeting the function of a virus DNA that is hidden in the infected livers of individuals suffering from hepatitis B. CpAMs have the ability to alter the activity of hepatitis B core protein that is vital for the virus's survival. Purified hepatitis B core protein can impulsively assemble, within seconds, to form complexes that are soccer-ball shaped and are identical to capsids in the infectious virus[93]. By careful examination of the mechanism of assembly, investigators at Indiana University in association with Assembly Biosciences have steered the discovery of a variety of families of small molecule CpAMs that are capable of reducing viral load and key antigens of virus selectively and effectively, which is considered to be the paramount marker of a functional cure[94]. In hepatitis B, CpAMs fight the virus by disturbing multiple aspects of the viral lifecycle, that include altering the function of a special viral DNA known as covalently closed circular DNA (cccDNA), that acts as the viral reservoir and hides in the infected liver cells nuclei. This cccDNA functions as a template for the assembly of viral proteins and extra copies of the viral genome, backing the persistence of the infection. Currently available HBV therapies do not affect cccDNA, because of which only 3 to 5 percent of hepatitis B patients become disease free, which requires most infected individuals to take these antivirals for the rest of their lives. HBV inhibition in this case has been achieved by causing mutations in capsid proteins that as a result affects the self-assembly, reverse transcription and packaging of HBV molecule[95,96]. The firm is currently working for selection of a first generation lead molecule[93].

Phenylpropenamides (AT-61 and AT-130)

AT-61 and AT-130 are phenylpropenamides that belong to non-nucleoside assembly activators (anti-capsid activity) for HBV treatment[97]. AT-130 acts by binding a hydrophobic pocket that is also capable of accommodating the previously characterized Hetero-aryl-di-hydro-pyrimidines or HAP compounds, but favors a distinctive quasi-equivalent site on the capsid surface. Therefore, this pocket acts as an uninhibited drug binding site and a potential target for different assembly effectors with a wide range of mechanisms of action[98,99]. Although both of these compounds have shown significant anti-viral activity against resistant strains of HBV but AT-130 has also shown greatest activity in cell culture[98,100]. AT-61 demonstrated antiviral specific activity in cell culture with minimal adverse effects[101]. It has been recently revealed that AT-130 enhances the rate of core protein (Cp) assembly and stabilizes favorably non-capsid polymers of Cp. Therefore, further strengthening the case of AT-130 as future non-nucleoside anti-HBV drug[101].

BAY 41-4109

BAY41-4109 belongs to HAPs or Hetero-aryl-di-hydropyrimidines which are non-nucleoside assembly activators that include potent anti virals such as HAP1, HAP12, BAY41-4109 acting as allosteric effectors that prompt an assembly-active state and, at high concentration, preferentially stabilize non-capsid polymers of Cp[102,103]. BAY 41-4109 has been shown to be used a valuable addition to current therapies for HBV in murine models, and it can also be tested as a therapeutic tool during the spread of resistant HBV strains that occurs during treatment with nucleoside

analogues[104]. These anti-capsid compounds show (CpAMs) significant impact on Cp nuclear functions at multiple levels: blocking of new cccDNA formation/ accumulation, reduction of an established cccDNA pool and inhibition of HBc occupancy and histone acetylation on the cccDNA that translate into a reduced pre-genomic RNA (pgRNA) transcription[101]. BAY 41-4109 is currently in Phase1 Clinical trial being developed by AiCuris, Germany[105]. Together these compounds represent an interesting avenue for HBV treatment that can provide great promise in the future.

NVR 3-778

Novira's lead core inhibitor candidate, NVR 3-778, disrupts the HBV lifecycle by inducing the assembly of defective capsids and is an effective drug inhibitor of HBV replication both *in vitro* cell culture models and a humanized liver mouse model of CHB. An excellent preclinical safety profile has been established for NVR 3-778 and a Phase 1 clinical trial is underway in New Zealand[106]. Phase Ia has been successfully completed and has revealed that the drug is safe and well tolerated in healthy volunteers[107]. Phase Ib of the Clinical Trials has been started and is expected to be completed in December 2015[106]. This novel mechanism of action promises to change the paradigm for CHB treatment. It can be administered and with the standard treatment for HBV *i.e.,* (nucleosides and interferon), Novira's core inhibitors are expected to provide greater and faster suppression of cccDNA and new virus production. Directly or indirectly, core inhibitors may also reduce HBsAg levels and release the block on immune response pathways to further reduce the time to cure. Higher and faster cure rates promoted by core inhibitors will in turn enable finite therapy so that many CHB patients will no longer require lifelong treatment[107].

NVP018 or OCB-030

NVP018 also known as OCB-030 is available in oral form and is a second-generation cyclophilin inhibitor based on sangamide with a well-differentiated preclinical profile in comparison with other cyclophilin inhibitors. Cyclophilins are a group of proteins that have been occupied in controlling innate immune responses subsequent to viral infection. Blocking of the of cyclophilins interaction with interferon signaling pathways is one of the mechanisms *via* which viruses take control of host innate immune response[108]. Data demonstrated at the International Liver Congress ™ (2014), the periodic meeting of the European Association for the Study of the Liver, showed that OCB-030 may also inhibit the HBV replication by two mechanisms *in vitro*. First, OCB-030 may directly inhibit several phases of viral replication in liver cells and second, it may indirectly act by augmenting the host immune response *via* Interefron regulators factors, including strong inhibition of an interaction that take splace between cyclophilin A and IRF9 (a key component of the JAK/STAT pathway). Results also showed that the possibility of developing resistance, a substantial clinical problem with current therapies for hepatitis B is very low with OCB-030[108,109]. Tekmira is planning to file an IND with the FDA, or and an equivalent agency in another country to launch the Phase Ⅰ clinical trials of the drug OCB-030 in 2015[110].

TKM-HBV

Tekmira's TKM-HBV database comprises two wet-lipid nanoparticle (LNP) interpretations TKM-HBV$_{4G}$ (4th generation LNP) and TKM-HBV$_{3G}$ (3rd generation LNP) interpretations. Both interpretations comprise a combination of three UsiRNA RNAi triggers that target highly conserved regions on the 4 HBV pgRNAs[111]. TKM-HBV also has shown diminution in HBsAg and cccDNA in the urokinase plasminogen activator-severe combined immunodeficiency (uPA/SCID) chimeric mouse model. Collectively data showed that TKM-HBV$_{3G}$ may facilitate at least a 1log$_{10}$ reduction in HBsAg in the treatment center, at safe therapeutic doses[112]. Preclinical trials of TKM-HBV$_{3G}$ data showed cccDNA reduction as a downstream result of inhibiting the assembly of all HBV proteins *via* the RNAi that includes preventing cccDNA episomal conservation and ultimate establishment by inhibiting the production of all HBV proteins. TKM-HBV's RNAi mechanism of action results in downstream inhibition of new HBV subviral particle and its formation. By vigorously inhibiting HBsAg and subviral particle construction, TKM-HBV may also ease the reactivation of immune system against HBV and cccDNA by activating humoral and T-cell facilitated immune mechanisms[112].

Second TKM-HBV formulation 4th gen LNP and preparation was publicized in November 2014 by Tekimira Pharmaceutical Corporation. TKM-HBV$_{4G}$ is basically times more effective than TKM-HBV$_{3G}$[113,114]. The study should evaluate the pharmacokinetics, acceptability and safety of the drug TKM-HBV$_{4G}$ and TKM-HBV$_{3G}$ in healthy subjects. TKM-HBV$_{3G/4G}$ has the potential to be a keystone drug in successfully treating CHB infection because of its ability to counter various elements of the HBV lifecycle[113].

Stimulators of interferon genes agonist

Stimulators of interferon genes (STING) agonists are activators of pattern recognition receptors (PRRs) activators present in the cytosol of immune cells. By stimulating genes responsible for interferon activation within body it may lead to the induction of the supplementary IFN-α and β, which exhibit the antiviral properties[114]. Tekmira in collaboration with Blumberg Institute and is attempting to investigate and identify a small orally active potent molecule that are the human STING agonists. The molecules shall possess the desired attributes which shall progress it to the human

clinical studies[115].

ARC-520

Arrowhead's ARC-520 is a product of RNAi (interference) technology, using short interfering RNA (siRNA) particles. These particles are injected in dynamic polyconjugate (DPC) nano particles bound to target ligands, which transport them to their target sites where viral infection has taken place. The DPCs are coated with a highly resistant polymer made of both polar and non-polar elements, which make them resistant to auto-immunity components in the bloodstream. On reaching the target sites, DPCs are taken up by endosomes containing the virus particles. The polymers coating the DPCs lyse the endosomes and the siRNAs interfere with the genetic machinery of the virus particles, knocking out virulence genes, hampering DNA replication, disturbing transcription and hence inhibiting protein synthesis. These mechanisms collectively prevent spread of virus particles further in the host[116]. ARC-520 aims to deliver a functional cure for the HBV infection and restore the adaptive immune system with the help of its RNAi mechanism. It has been tested successfully in mice and chimpanzee models, where it has shown to reduce the amount of viral DNA, HbsAg and HbeAg by as much as 90%-95%, lasting up to one month or more[117]. ARC-520 successfully completed its Phase Ⅰ Clinical Trials showing excellent safety and tolerability results in healthy volunteers[118,119]. The company is at present investigating the single dose Phase Ⅱa study in chronic HBV patients which so far have shown significant reduction in HBsAg in chronic HBV patients[120,121]. The company received an IND for Phase Ⅱ study of ARC-520 in January 2015[122].

ALN-HBV

ALN-HBV RNAi is a potent drug with an excellent therapeutic potential and its potent mechanism of action blocks all steps of the HBV life cycle including assembly, secretion of virus, Replication, and secretion of sub-viral antigens. The development of a candidate program targets to work with the Alnylam Pharmaceuticals Enhanced Stabilization Chemistry (ESC)-GalNAc-conjugate technology. The technology enables the subcutaneous dose administration with upgraded potency, safety, durability and wide therapeutic index of the drug. ESC-GalNAc conjugate can be developed into an excellent approach in the class of RNA therapeutics targeting HBV[123].

The pre-clinical studies with drug indicate substantial, multi-log reductions in HBV viral titers and surface antigen (HBsAg). The drug proved to support the evidence for an immune-mediated therapeutic effect in chronically infected chimpanzees. The results investigated another striking feature in achieving functional cure for HBV cure by the use of the RNAi therapeutics against the conserved regions of the HBV genome[124]. Alnylam Pharmaceuticals is expected launch the product name for its ALN-HBV Development Candidate by of 2015 and file an IND or IND equivalent around the end of 2015[125].

Morphothiadine or GLS4

Morphothiadine Mesilate (GLS4) is another HAP (heteroaryl-di-hydro-pyrimidines) compound. GLS4 is a pipeline product of HEC Pharm that can combine with the HBV core protein dimer, and interfere the assembly process and functions of HBV nucleocapsid, and thus it can effectively inhibit HBV replication through two active ways of inhibiting viral structure assembly and gene replication.

Compared to the traditional existing nucleoside analogues, MorphothiadineMesilate shows higher inhibitory activity in the HBV inhibition tests[125,126]. According to the company's website the Phase Ⅰ Clinical Trial application of GLS4 was submitted to China Food and Drug Administration (CFDA) in 2010, and received the approval letter in November 2011. The phase Ⅰ study was initiated early of 2012 and finished in Dec, 2012. Data in phase Ⅰ show that GLS4 has favorable safety and efficacy signals, and no confirmed drug-related adverse events are observed. It also shows favorable pharmacokinetic profile as a potential clinical candidate. Phase Ⅱa trial was initiated in early May. 2013 and results came out in February 2014. Filing for Phase Ⅱb trial has submitted to CFDA and the pharmaceutical is planning to launch it in China by 2016[127,128].

BSBI-25

BSBI-25 is a cccDNA inhibitor under development by Baruch S. Blumberg Institute. The compound is still in preclinical stage of development. The drug aims to work on the removal of cccDNA protecting hepatocytes and to prevent the replase of HBV due to rapid recover in serum HBV-DNA after termination of antiviral treatment. Encouraging evidence of cccDNA degradation in the absence of hepatotoxicity has been reported in which lymphotoxin-β and IFN-α receptor stimulation up-regulated the levels of the host factors APOBEC3A and APOBEC3B cytidine deaminases, respectively, the action of which mediated by the core protein in HBV-infected cells. The human liver needle biopsies and primary hepatocytes led to the cccDNA degradation and cytidine deamination, apurinic/apyrimidinic site formation that prohibited HBV recurrence[129,130].

Birinapant

Birinapant (TL32711) is a potent, bivalent SMAC (synthetic small molecule and peptidomimetic of second mitochondrial-derived activator of caspases) mimetic that binds with differential affinity to multiple members of the inhibitor of apoptosis proteins (IAP) family including cIAP1, cIAP2, XIAP, and ML-IAP[131,132]. Initially developed by Tetralogic Pharmaceuticals as

Table 5 Nucleoside analogs in development for hepatitis B virus

Name	MOA	Developed by	Phase
Clevudine	DNA polymerase inhibition	Bukwang Pharmaceutical, South Korea	Approved in South Korea and Philippines
Besifovir	DNA polymerase inhibition (pro-drug)	Ildong Pharmaceuticals and LG Life Sciences	Phase Ⅲ
Tenofoviralafenamide	DNA polymerase inhibition (pro-drug)	Gilead Sciences, United States	Phase Ⅲ
CMX157	DNA polymerase inhibition (pro-drug)	ContraVir Pharmaceuticals, United States	Phase Ⅲ
AGX-1009	DNA polymerase inhibition (pro-drug)	Agenix, Australia	Phase Ⅰ

HBV: Hepatitis B virus; MOA: Mechanism of action.

anticancer agent, this drug has proven its efficacy in infectious diseases as well. The use of the mouse model of HBV, birinapant showed excellent results it was well tolerated and exhibited activity in the clearance of cells infected with HBV. The drug is currently in Phase Ⅰ Clinical Trials undergoing safety and tolerability studies in CHB[132].

CPI-431-32

CPI-431-32, a novel agent developed by Ciclofilin Pharmaceuticals, targets and inhibits a host cellular enzyme known as cyclophilin A (CyPA). CyPA is responsible for activation of viral proteins critical for the life cycles of HCV, human immunodeficiency virus type 1 (HIV-1) and HBV. By understanding how cyclophilins mediate viral replication, the company has developed a host-targeting antiviral, CPI-431-32 which is used for the treatment of HCV, HBV, HIV-1, and infection with more than one of these viruses simultaneously (co-infection)[133,134].

Myrcludex B

Myrcludex B is a HBV entery inhibitor currently in Phase Ⅱ Clinical Trials. A joint venture of Hepatera Ltd and its development partner MYR GmbH, Myrcludex B. After positive preclinical and Phase 1 studies the drug has successfully completed phase Ⅱa of the clinical trials showing evidence that the inhibition of intrahepatic spread of HBV may become part of future curative regimes[135-137].

Simvastatin

Simvastatin is FDA approved 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA reductase inhibitors that are used to treat hypercholesterolemia. Besides exhibiting strong anti-HBV activity in cell culture models. Simvastatin has shown synergestic anti-HBV activity with nucleos(t)ide analogues including adefovir, entecavir, tenofovir and lamivudine[138]. The University of Oklahoma and the United States Veterans Administration successfully conducted a phase Ⅰ proof-of-concept study for the drug Simvastatin against the CHB patients that was completed in 2012[139].

NUCLEOSIDE/NUCLEOTIDE ANALOGS

Nucleoside analogs (NUCs) have so far served as the main weapon in the armory against hepatitis B along with the Interferons. However the development of resistance, biochemical irregularities, along with poor durability are the limitations of the NUCs[132]. The drug companies are therefore focusing on altering the approach of utilizing the NUCs as discussed under. Table 5 shows the nucleoside/nucleotide analogs development in for HBV.

Clevudine

Clevudine is a nucleoside analogue already approved in Phillipines and South Korea with incomplete licensing in Indonesia, Thailand, India, and Malaysia for the treatment of Hepatitis B. It has been found to inhibit HBV infection at the multiple steps of its replication cycle. The drug has an extended half-life which causes the significant reduction of cccDNA in animal models[140].

But despite of these potent antiviral activity Clevudine has shown to induce myopathy that is characterized by depletion of mitochondrial DNA on long term therapy[141,142]. This potent anti-viral needs to be administered with proper considerations of its ability to cause serious long-term side effects[143,144].

Besifovir/LB80380/ANA-380

LB80380 is an oral nucleotide pro-drug that incorporates into the viral DNA and prevents its replication. Phase Ⅱ Clinical Trials after Safety and Tolerance studies of the drug showed effective antiviral activity against viruses strains *i.e.,* wild-type and drug-resistant mutations[145,146]. Ildong Pharmaceuticals and LG Life Sciences are currently conducting Phase Ⅲ Clinical Trials of the drug to prove that besifovir is non-inferior to a control drug that are expected to be completed in July 2015 along with Phase Ⅰ Bioequivalence studies[147].

Tenofoviralafenamide

Tenofovir is the primary treatment for lamivudine-resistant HBV in the United States and Western Europe. There has been no Resistance to tenofovir characterized for three years after the drugs approvalfor HBV treatment[18]. Gilead Sciences is currently conducting Phase Ⅲ safety and efficacy studies of Tenofovir Alafenamide Versus Tenofovir Disoproxil Fumarate for Treatment of HBeAg-positive hepatitis B that is expected to be completed in November 2015[36,148]. Other than Tenofoviralafenamide

two more pro drugs of tenofovir namelyCMX157 (ContraVir Pharmaceuticals) and AGX-1009 (Agenix, Australia) are in Phase Ⅱ and Ⅰ of the clinical trials respectively[143].

In conclusion, HBV is a progressive liver disease and current therapies require continuous monitoring for serious side effects along with the development of resistance and therefore shorter durability. The increasing rates of resistance to antiviral therapy necessitates consideration of combination therapy and impels to look for novel treatment options. There is a deficiency of risk calculators including those similar to the Framingham risk score that evaluates the risk of coronary heart disease having a limited treatment of chronic HBV infection. The future therapies mentioned in this article are primarily focused on the designing of such therapies that require shorter duration of treatment, are more efficacious and have fewer side effects without the development of resistance. The idea to use therapeutic vaccines as monotherapy and along with the nucleoside/NUCs may prove to be of great success in designing future regimens for HBV therapy. The fact that pharmaceutical companies by realizing the increased demand of replacing the depleting army of NUCs are now focusing on Non-nucleoside antivirals including RNAi's and other novel protein inhibitors is a testimony that future therapy of HBV infection is non-NUCS.

ACKNOWLEDGMENTS

We are deeply thankful to Dr. Vijayakumar Velu and Dr. Chris Ibegbu for proof reading of the manuscript.

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P- Reviewer: Tai DI **S- Editor**: Gong ZM **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12576

World J Gastroenterol 2015 November 28; 21(44): 12576-12585
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Basic Study

Mucosal healing effect of nilotinib in indomethacin-induced enterocolitis: A rat model

Gozde Dervis Hakim, Mujde Soyturk, Mehtat Unlu, Pinar Ataca, Meral Karaman, Ozgul Sagol, Elif Borekci, Osman Yilmaz

Gozde Dervis Hakim, Mujde Soyturk, Department of Gastroenterology, School of Medicine, Dokuz Eylul University, Inciralti, Izmir 35340, Turkey

Mehtat Unlu, Ozgul Sagol, Department of Pathology, School of Medicine, Dokuz Eylul University, Inciralti, Izmir 35340, Turkey

Pinar Ataca, Elif Borekci, Department of Internal Medicine, School of Medicine, Dokuz Eylul University, Inciralti, Izmir 35340, Turkey

Meral Karaman, Osman Yilmaz, Department of Laboratory Animal Science, School of Medicine, Dokuz Eylul University, Inciralti, Izmir 35340, Turkey

Author contributions: Dervis Hakim G and Soyturk M designed the research; Dervis Hakim G and Ataca P performed the research; Sagol O and Karaman M contributed new reagents or analytic tools; Borekci E provided technical support; Unlu M And Sagol O contributed to the evaluations of the pathological data; Karaman M and Yilmaz O designed the rat conditions; Soyturk M analyzed the data; Dervis Hakim G wrote the paper; all authors read and approved the final manuscript.

Supported by Dokuz Eylul University Research Committee.

Institutional review board statement: The study was reviewed and approved by the Dokuz Eylul University Institutional Review Board.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Dokuz Eylul University [protocol number: B.30.2.DEÜ.0.01.00.00/15387].

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

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Correspondence to: Gozde Dervis Hakim, MD, Department of Gastroenterology, School of Medicine, Dokuz Eylul University, Inciralti, Mh., Mithatpaşa cad. No. 1606, İnciraltı yerleşkesi, Izmir 35340, Turkey. gozdedervis@gmail.com
Telephone: +90-505-2663138

Received: March 26, 2015
Peer-review started: March 28, 2015
First decision: May 18, 2015
Revised: June 15, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: November 28, 2015

Abstract

AIM: To investigate the effects of nilotinib in a rat model of indomethacin-induced enterocolitis.

METHODS: Twenty-one Wistar albino female rats obtained from Dokuz Eylul University Department of Laboratory Animal Science were divided into the following three groups: control (n = 7), indomethacin (n = 7) and nilotinib (n = 7). A volume of 0.25 mL of physiological serum placebo was administered to the control and indomethacin groups through an orogastric tube for 13 d. To induce enterocolitis, the indomethacin and nilotinib groups received 7.5 mL/kg indomethacin dissolved in 5% sodium bicarbonate and administered subcutaneously in a volume of 0.5 mL twice daily for three days. Nilotinib was administered 20 mg/kg/d in two divided doses to the nilotinib group of rats for 13 d through an orogastric tube, beginning on the same day as indomethacin administration. For 13 d, the rats were

fed a standard diet, and their weights were monitored daily. After the rats were sacrificed, the intestinal and colonic tissue samples were examined. The macroscopic and microscopic pathology scores were evaluated. The pathologist stained all tissue samples using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling method. Mucosal crypts and apoptotic cells were quantified. The platelet-derived growth factor receptor (PDGFR) α and β scores assessed by immunohistochemical staining method and tissue and serum tumor necrosis factor (TNF) α levels were determined by enzyme-linked immunosorbent assay.

RESULTS: Between days 1 and 13, the rats in the nilotinib and indomethacin groups lost significantly more weight than the controls (-11 g *vs* +14.14 g, *P* = 0.013; -30 g *vs* +14.14 g, *P* = 0.003). In the small intestinal and colonic tissues, the macroscopic scores were significantly lower in the nilotinib group than in the indomethacin group (1.14 ± 0.38 and 7.29 ± 2.98, *P* = 0.005; 1.14 ± 0.38 and 7.43 ± 2.64, *P* = 0.001, respectively), but the values of the nilotinib and indomethacin groups were similar to the control group. In the small intestinal and colonic tissues, the microscopic scores were significantly lower in the nilotinib group than in the indomethacin group (3.43 ± 2.99 and 7.67 ± 3.67, *P* = 0.043; 2.29 ± 0.76 and 8.80 ± 2.68, *P* = 0.003, respectively), but the values were similar to the control group. The PDGFR β scores in the small intestine and colon were significantly lower in the nilotinib group than in the indomethacin group (1.43 ± 0.79 and 2.43 ± 0.54, *P* = 0.021; 1.57 ± 0.54 and 3 ± 0, *P* =0.001), and the values were similar to controls. The colonic PDGFR α scores were significantly lower in the nilotinib group than in the indomethacin group (1.71 ± 0.49 and 3 ± 0, *P* = 0.001). The colonic apoptosis scores were significantly lower in the controls than in the nilotinib group (1.57 ± 1.13 and 4 ± 1.29, *P* = 0.007). Furthermore, the serum and tissue TNF-α levels were similar between the nilotinib and indomethacin groups.

CONCLUSION: In the indomethacin-induced enterocolitis rat model, nilotinib has a positive effect on the macroscopic and microscopic pathologic scores, ensuring considerable mucosal healing. Nilotinib decreases PDGFR α and β levels and increases the colonic apoptotic scores, but it has no significant effects on weight loss and the TNF-α levels.

Key words: Inflammatory bowel disease; Enterocolitis; Platelet-derived growth factor receptor; Tumor necrosis factor α; Tyrosine kinase inhibitor; Rats; Mucosal healing

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Core tip: The etiopathogenesis of inflammatory bowel diseases (IBDs) has not been clearly elucidated. Many management strategies that include targeting particular pathways involved in the development of IBD have been developed. In the present study, we aimed to investigate the effects of nilotinib in a rat model of indomethacin-induced enterocolitis. From our study, it appears that nilotinib had considerable effects on pathological scores, which indicated positive effects in mucosal healing. Nilotinib decreased platelet-derived growth factor receptor α and β and increased the colonic apoptotic scores, but it had no significant effects on weight loss or serum-tissue tumor necrosis factor α levels.

Dervis Hakim G, Soyturk M, Unlu M, Ataca P, Karaman M, Sagol O, Borekci E, Yilmaz O. Mucosal healing effect of nilotinib in indomethacin-induced enterocolitis: A rat model. *World J Gastroenterol* 2015; 21(44): 12576-12585 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12576.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12576

INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of idiopathic, chronic and relapsing inflammatory conditions of the gastrointestinal tract. The pathogenesis of IBD is only partially understood; complex interactions between the immune system, enteric commensal bacteria and host genotype are thought to underlie the development of IBD[1]. The current guidelines for conventional IBD therapy recommend the use of aminosalicylates [sulphasalazine, 5-aminosalicylic acid (5-ASA)], corticosteroids, or immunosuppressive drugs (azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine A) according to the extent and severity of disease, the response to current or prior treatment, and the presence of complications[2]. The use of these drugs is limited by side effects, unresponsiveness or high relapse rates[3-8]. The introduction of anti-TNF-α drugs has changed the mode of therapy in patients with Crohn's disease (CD). Anti-TNF-α drugs induce clinical remission and significant endoscopic improvement. However, both immune-mediated adverse reactions and loss of efficacy with time have limited their use[9,10].

Currently, mucosal healing, which is referred to as "endoscopic remission", has emerged as an increasingly important therapeutic goal. In CD, mucosal healing is associated with a decreased need for hospitalization and surgery, lower costs, decreased side effects, increased rates of clinical remission, and improved quality of life. However, there are no published data on the ability of 5-ASA to induce mucosal healing in CD[11]. Among the 92% of CD patients who achieved clinical remission, 29% achieved endoscopic remission, and 13% achieved complete mucosal healing with oral prednisolone[12,13]. In

several studies with immunomodulators (azathioprine and methotrexate), the mucosal healing rates were between 11% and 62.5%[14-20]. In contrast, mucosal healing is achieved in approximately 30% of CD patients who receive anti-TNF therapies[21-25]. Briefly, 20%-30% of patients do not respond to biological therapy, and up to 70% of CD patients will undergo intestinal resection during the course of their disease. Hence, the available medical treatment options are still far from resulting in complete long-term remission and mucosal healing[26,27]. Therefore, it is critically important to identify new medical treatment options for IBD.

The protein tyrosine kinases (TKs) constitute a large family of homologous proteins that have an important role in regulating intracellular signal transduction pathways and control a range of fundamental cellular process, including growth, metabolism, differentiation, adhesion and apoptosis[28]. Imatinib, the best-known member of this class of drugs, is specific for TK receptor sites and suppresses the Abelson proto-oncogene (ABL), c-kit proto-oncogene, platelet-derived growth factor receptor (PDGFR), macrophage colony- stimulating factor receptor (c-fms), TNF-α, and inducible nitric oxide synthase[29]. Nilotinib is a highly potent and selective inhibitor of the wild- type BCR-ABL1, and it was developed for imatinib-resistant mutants[30-32]. Nilotinib reduces the levels of interleukin (IL)-6, IL-1β, TNF-α, tumor growth factor (TGF) β1, and PDGFR β more significantly than imatinib, and it has a potent antifibrotic effect[33]. More recently, in our previous study, we demonstrated that nilotinib has a significant effect on weight loss and macroscopic and microscopic pathological scores and causes significant mucosal healing in a rat trinitrobenzene sulfonic acid (TNBS)-induced colitis model[34].

The present study was planned based on the success of nilotinib demonstrated in our previous study, and we aimed to investigate the effect of nilotinib in a chronic enterocolitis rat model that was induced with indomethacin. For this purpose, we evaluated the efficacy of nilotinib on weight, macroscopic and microscopic pathological scores, TNF-α levels, PDGFR levels, and apoptotic index in rats with indomethacin-induced enterocolitis.

MATERIALS AND METHODS

Animals

Approval was obtained from the animal ethics council of Dokuz Eylul University Faculty of Medicine (DEUTF). The DEUTF Hospital Experimental Research Laboratory provided 21 female Wistar albino rats, weighing 226-243 g (mean weight, 241.09 g), for use in this study. The rats were divided into three groups, each consisting of seven rats (Table 1).

The rats were maintained at room at a temperature of 23 ± 2 ℃ under a 12-h light/dark cycle at the DEUTF Experimental Animal Laboratory. Before and during the study, the animals were fed a standard diet, and their weights were monitored daily. The animals were also allowed water *ad libitum*.

Table 1 Study groups and experimental design

Group	*n*	1-3 d	4-13 d
1	7	Physiological serum	Physiological serum
2	7	Indomethacin	Physiological serum
3	7	Indomethacin	Nilotinib

Experimental design

After 24 h of fasting, 0.25 mL of physiological serum was administered to the control group of rats through an orogastric tube. To induce chronic enterocolitis, the rats in the other two groups received 7.5 mg/kg indomethacin dissolved in 5% sodium bicarbonate and administered in a 0.5-mL volume subcutaneously, two times a day, for three days[35,36]. Across both groups of rats treated with indomethacin, one of the rats treated with only indomethacin was found dead on the sixth day of the experiment. A necropsy was not performed on this rat.

The indomethacin and control groups received saline placebo for 13 d through an orogastric tube. Nilotinib, administered 20 mg/kg/d (Novartis Pharma AG, Basel, Switzerland) in two divided doses, was administered to the nilotinib group of rats (n = 7) for 13 d through an orogastric tube, beginning on the same day as indomethacin administration.

Blood and tissue samples for pathological examination were obtained from all rats under ether anesthesia at the end of the 13-d period. All animals were then sacrificed by decapitation. The abdominal cavity was opened by a midline incision, and the stomach, small intestine and colon were dissected. The intestinal lumen was washed with saline and then removed and opened along its length. The intestinal tissue was then fixed with buffered formalin.

Pathological examinations

A pathologist, who was blinded to the group identity of the intestinal samples, performed the pathological evaluation of all tissue samples twice. The pathologist first examined the tissues macroscopically, recording the number and size of the ulcers noted. In addition, each small and large intestinal column was longitudinally opened according to the method reported by Vilaseca *et al*[37], and macroscopic scoring was performed. Tissue sections of the gross ulcerative lesions and surrounding normal mucosa were then stained with hematoxylin-eosin (HE). The pathologist then performed microscopic scoring according to the method reported by Dieleman *et al*[38].

Apoptosis

The pathologist then stained all tissue samples using the TUNEL method. Mucosal crypts and apoptotic

cells were counted along the surface epithelium under a microscope (Olympus DX51 Tokyo, Japan) at a magnification of × 400. Using the TUNEL technique, all cut sections were preserved with lysine for three nights at 37 ℃ and then treated for one night at 60 ℃ in an incubator. Thereafter, deparaffinization was performed with three changes with xylene (20 min) (first xylene in the incubator, and the others at room temperature). The tissue sections were then rehydrated by flushing with a series of alcohol solutions of decreasing degree (absolute, 96%, 80%, and 70%); the samples were then stored in distilled water for 5 min. After absorbing water from the edge of each section, proteinase K (Invitrogen, United States) was applied for 10 min at room temperature. The sections were then washed twice with phosphate-buffered solution (PBS) for a period of 2 min each. After drying the cross sections, 3% H_2O_2 (Merck, Germany) was applied for 5 min to inhibit tissue endogenous peroxidase, and the sections were then washed twice with PBS for 5 min each. The cross-section slices were then dried, and an equilibration buffer (ApopTag Plus peroxidase kit, Millipore, United States) was applied for 10 min at room temperature. A total of 55 μL of the enzyme terminal deoxynucleotidyl transferase was then applied to each cross section. The cross sections were cover-slipped (ApopTag Plus peroxidase kit, Millipore, United States) and incubated for 1 h at 37 ℃. Stop/wash buffer (ApopTag Plus peroxidase kit, Millipore, United States) was then applied to the sections that were removed from the incubator for 10 min at room temperature. The sections were then washed three times (for 1 min each) with PBS at room temperature, dried, and incubated with anti-streptavidin-peroxidase (ApopTag Plus peroxidase kit, Millipore, United States) at room temperature for 30 min. The sections were then washed with PBS four times for 2 min each to determine the visibility of the TUNEL reaction, and the samples were then stained with diaminobenzidine (DAB) (DAB-PLUS kit, Invitrogen, United States). After washing with distilled water, ground staining was performed using methyl green. The sections were then washed with three changes of distilled water. After three changes of the searing process with xylene for 20 min, closure was performed with Entella.

Tissue homogenization and measurement of the tissue serum TNF-α

The tissue samples obtained from the terminal ileum were placed in 2-mL microcentrifuge tubes and stored at -80 ℃ until further use. These tissues were then removed on the day of the study and warmed to 4 ℃. Then, 60-80-mg pieces were obtained from these samples and placed into a tube containing 5-mm-diameter stainless steel beads and phosphate buffer with a 1:7 ratio (pH 7.2). Microcentrifuge tubes were introduced into a pre-chilled TissueLyser LT device and replaced in a TissueLyser (Qiagen-Germany) tissue homogenization device. The frequency and time were adjusted to 50 and 5 min, respectively. The resulting homogenate was centrifuged for 10 min at 4 ℃ and 5000 × *g*. Next, an enzyme-linked immunosorbent assay (ELISA) was performed on tissue supernatants, and serum was obtained from centrifugation for identifying TNF-α in accordance with the manufacturer's recommendations (Invitrogen, Rat TNF-α, United States). Finally, the ELISA plates were spectrophotometrically evaluated at 450 nm (Biotech Synergy HT, United States).

PDGFR α and β levels

The PDGFR α and β levels were assessed through staining scores and compared among the groups by immunohistochemistry. For immunohistochemical staining, 2-3-micron sections were stored overnight in the incubator at 40 ℃. The following day, the sections were washed with xylene, in descending alcohol series, and distilled water for 20 min. Afterwards, the samples were boiled for 20 min in an EDTA solution at pH 8.0. The samples were then stored in DakoFlex peroxidase solution for 5 min and washed again with Tris-buffered saline. A primary antibody was then applied: PDGFR α, in a 1:100 dilution (NOVUS Biologicals, NBP1-19 423, United States) and PDGFR β, in a 1:50 dilution (NOVUS Biologicals, NBP1-19 473, United States). The samples were incubated for 30 min, washed with Tris buffer, stored in DakoFlex HRP solution for 20 min, washed with Tris buffer again, and stored in DakoFlex DAB for 7 min. These samples were then washed with Tris-buffered saline, kept under tap water for 5 min, stained with Mayer's hematoxylin solution for 10 min, washed with tap water for 1 min, rinsed in an alcohol series, and cleaned with xylene for 5-10 min.

The PDGFR α and β positivity was determined according to a devised scoring system. According to this system, a score of +1 was assigned if PDGFR α and β positivity was confirmed in inflammatory cells as well as in cells of the lamina propria, stroma, and submucosal endothelium. A score of +2 was assigned if PDGFR α and β positivity was confirmed in the lamina propria and submucosa. A score of +3 was assigned if PDGFR α and β positivity was confirmed with widespread staining in the ulcerated areas or inflammatory cells, fibroblasts, endothelial cells, submucosa, and mucosa of the surrounding tissue.

Statistical analysis

All statistical procedures were performed using SPSS software (version 15.0). The Kruskal-Wallis test was used for multigroup comparisons, while the Mann-Whitney *U* test was used to compare the means of two groups. A *P* value less than 0.05 was considered significant.

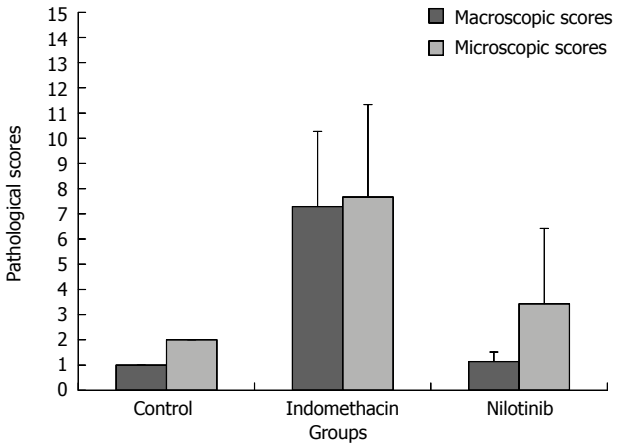

Figure 1 Small intestine macroscopic and microscopic scores.

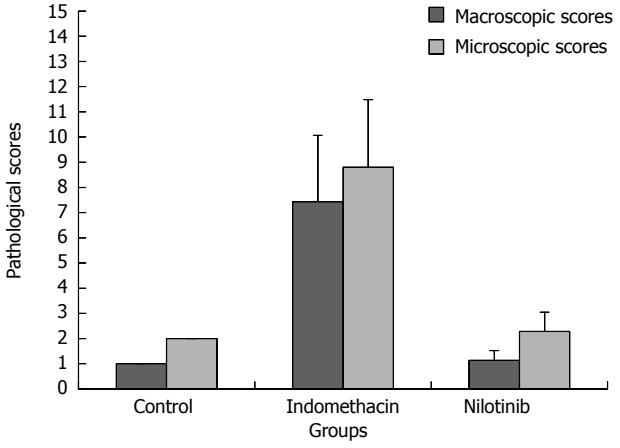

Figure 2 Colon macroscopic and microscopic scores.

RESULTS

On the first experimental day, the average rat weights were similar in all study groups. The average rat weights were determined daily. The average weight of the control group of rats increased to 14.14 g after 13 d. The indomethacin group of rats lost an average of 30 g throughout the study, and the nilotinib group of rats lost an average of 11 g. There was a significant difference among the groups with respect to average weight throughout the study (P = 0.002). The differences in the weight between the control and nilotinib groups and between the control and indomethacin groups were significant (+14.14 g and -11 g, respectively, P = 0.013 and +14.14 g and -30 g, respectively, P = 0.003). There was no significant difference in weight between the indomethacin and nilotinib groups (-30 g and -11 g, respectively; P = 0.085).

The mean macroscopic pathological score of the small intestine and colon of the control group was 1 ± 0. In the indomethacin group, the macroscopic pathological score of the small intestine was 7.29 ± 2.98 and that of the colon was 7.43 ± 2.64. On the other hand, in the nilotinib group, the mean macroscopic pathological scores of the small intestine and colon were the same, 1.14 ± 0.38 and 1.14 ± 0.38. Adhesion, strictures, broad-based ulcers and mesenteric cohesiveness were observed in the indomethacin group of rats. The control and nilotinib groups were similar in terms of the macroscopic scores of the small intestine and colon (P > 0.05). Macroscopic scores were significantly lower in the control and nilotinib groups than in the indomethacin group for the small intestine (1 and 7.29 ± 2.98, P = 0.003; 1.14 ± 0.38 and 7.29 ± 2.98, P = 0.005, respectively). The macroscopic scores were significantly lower in the control and nilotinib groups than in the indomethacin group for the colon (1 and 7.43 ± 2.64, P = 0.001; 1.14 ± 0.38 and 7.43 ± 2.64, P = 0.001, respectively; Figure 1 and 2; Figures S1 and S2).

The mean microscopic score of the small intestine and colon in the control group was 2 ± 0. The microscopic pathological scores of the small intestine and colon were 7.67 ± 3.67 and 8.80 ± 2.68, respectively, in the indomethacin group. In the nilotinib group, the mean microscopic pathological score of the small intestine was 3.43 ± 2.99, and that of the colon was 2.29 ± 0.76. The mean microscopic scores of the small intestine were significantly lower in the control and nilotinib groups than in the indomethacin group (2 ± 0 and 7.67 ± 3.67, P = 0.004; 3.43 ± 2.99 and 7.67 ± 3.67, P = 0.043, respectively). The control and nilotinib groups were similar in terms of the mean microscopic scores (P > 0.05). The mean microscopic scores of the colon were significantly lower in the control and nilotinib groups than in the indomethacin group (2 ± 0 and 8.80 ± 2.68, P = 0.001; 2.29 ± 0.76 and 8.80 ± 2.68, P = 0.003, respectively; Figures 1 and 2; Figures S1 and S2).

In the PDGFR α and β scoring system, the samples were classified as +1, +2, and +3, according to their staining properties. The PDGFR α scores of the small intestine and colon in the control group were 1 ± 0 and 1.14 ± 0.38, respectively. In the indomethacin group, the PDGFR α scores of the small intestine and colon were 2 ± 0.82 and 3 ± 0, respectively. The PDGFR α scores of the small intestine and colon in the nilotinib group were 1.43 ± 0.79 and 1.71 ± 0.49, respectively. There was a significant difference among the groups in the PDGFR α scores of the small intestine and colon (P = 0.026, P = 0, respectively). The PDGFR α scores of the colon were significantly lower in the control and nilotinib groups than in the indomethacin group (1.14 ± 0.39 and 3 ± 0, P = 0.001; 1.71 ± 0.49 and 3 ± 0, P = 0.001, respectively). The PDGFR α scores of the small intestine were significantly lower in the control group than in the indomethacin group (1 ± 0 and 2 ± 0.82, P = 0.009). The control and nilotinib groups as well as indomethacin and nilotinib groups were similar in terms of the PDGFR α scores of

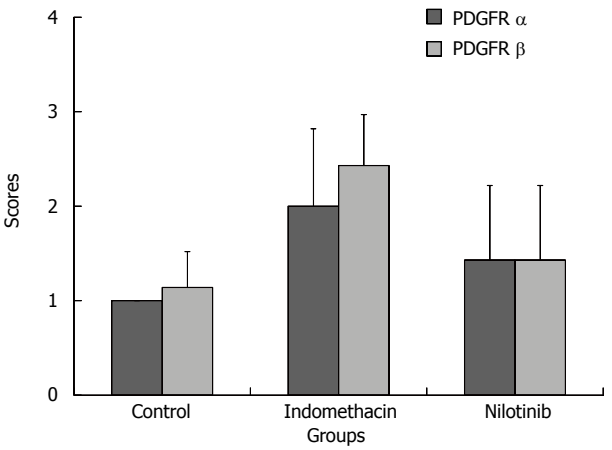

Figure 3 Small intestine platelet-derived growth factor receptor α and β scores. PDGFR: Platelet-derived growth factor receptor.

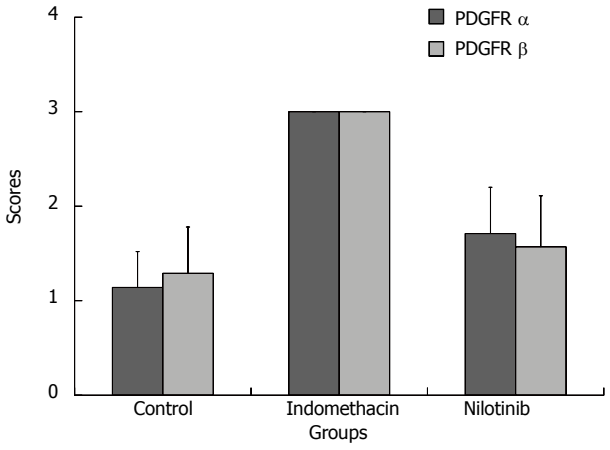

Figure 4 Colon platelet-derived growth factor receptor α and β scores. PDGFR: Platelet-derived growth factor receptor.

the small intestine ($P > 0.05$). The control and nilotinib groups were similar in terms of the PDGFR α scores of the colon ($P > 0.05$; Figures 3 and 4).

The mean PDGFR β scores of the small intestine and colon in the control, indomethacin, and nilotinib groups were 1.14 ± 0.38 and 1.29 ± 0.49; 2.43 ± 0.54 and 3 ± 0; and 1.43 ± 0.79 and 1.57 ± 0.54, respectively. There was a significant difference among all groups in terms of the mean PDGFR β scores of the small intestine and colon ($P = 0.004$, $P = 0.001$, respectively). The PDGFR β scores of the small intestine were significantly lower in the control and nilotinib groups than in the indomethacin group (1.14 ± 0.38 and 2.43 ± 0.54, $P = 0.002$; 1.43 ± 0.79 and 2.43 ± 0.54, $P = 0.021$, respectively). The PDGFR β scores of the colon were significantly lower in the control and nilotinib groups than in the indomethacin group (1.29 ± 0.49 and 3 ± 0, $P = 0.001$; 1.57 ± 0.54 and 3 ± 0, $P = 0.001$, respectively). The PDGFR β scores of the small intestine and colon in the control and nilotinib groups were similar ($P > 0.05$; Figures 3 and 4; Figure S3).

The mean serum TNF-α levels in the control, indomethacin, and nilotinib groups were 0.071 ± 0.003 pg/mL, 0.065 ± 0.005 pg/mL, and 0.083 ± 0.037 pg/mL, respectively. There was no significant difference observed among the groups in terms of the mean serum TNF-α levels ($P > 0.05$; Figure 5). The average tissue TNF-α levels in the control, indomethacin, and nilotinib groups were 0.145 ± 0.242 ng/mL, 0.268 ± 0.061 ng/mL, and 0.292 ± 0.086 ng/mL, respectively. There was a significant difference among all groups ($P = 0.002$). The tissue TNF-α levels were significantly lower in the control group than in the indomethacin and nilotinib groups (0.145 ± 0.242 ng/mL and 0.268 ± 0.061 ng/mL, $P = 0.004$; 0.145 ± 0.242 ng/mL and 0.292 ± 0.086 ng/mL, $P = 0.002$, respectively). However, there was no significant difference between the indomethacin and nilotinib groups in terms of the mean tissue TNF-α levels ($P > 0.05$; Figure 5).

The mean numbers of apoptotic cells in the small intestine and colon in the control, indomethacin, and nilotinib groups were 1.57 ± 0.79 and 1.57 ± 1.13; 2.50 ± 0.84 and 7.40 ± 2.88; 2.14 ± 1.46 and 4 ± 1.29, respectively. Although, a significant difference was observed among the groups in the colon ($P = 0.002$), there was no significant difference among the groups in the small intestine ($P > 0.05$). The numbers of apoptotic cells in the colon were significantly lower in the control group than in the indomethacin and nilotinib groups (1.57 ± 1.13 and 7.40 ± 2.88, $P = 0.004$; 1.57 ± 1.13 and 4 ± 1.29, $P = 0.007$, respectively). There was a significant difference between the indomethacin and nilotinib groups in terms of the mean number of apoptotic cells in the colon (7.40 ± 2.88 and 4 ± 1.29, $P = 0.038$; Figure 6).

DISCUSSION

Many management strategies that involve targeting particular pathways involved in the development of IBD have been developed[39,40]. However, unresponsiveness to medical treatment in IBD still poses a therapeutic challenge.

TK receptors play an important role in controlling most fundamental cellular processes, including cell cycle, migration, metabolism and survival, as well as cell proliferation and differentiation[41]. Nilotinib is a second-generation tyrosine kinase inhibitor that is 30-fold more potent than imatinib against BCL-ABL kinase. Nilotinib affects the cornerstone of the steps in the pathogenesis of IBD, including TNF-α, PDGFR and nitric oxide synthesis. In our previous study, we demonstrated that nilotinib has a significant effect on weight loss and macroscopic and microscopic pathological scores, and it also leads to significant mucosal healing in a TNBS-induced acute colitis rat model[34]. Until now, no reports in the literature

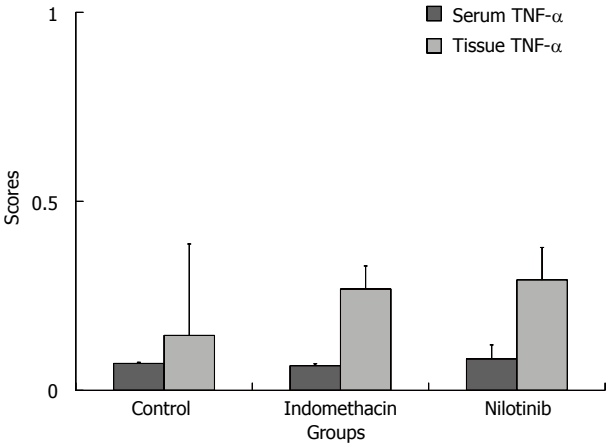

Figure 5 Serum and tissue tumor necrosis factor-α scores. TNF: Tumor necrosis factor.

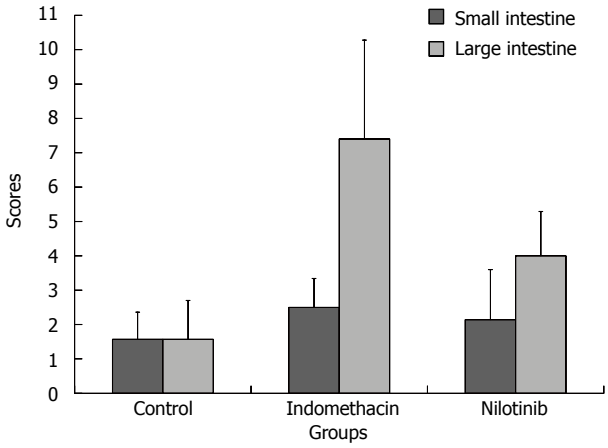

Figure 6 Apoptotic cell number.

had evaluated the efficacy of nilotinib in either an enterocolitis rat model or human enterocolitis. In the present study, we evaluated the efficacy of nilotinib in a chronic indomethacin-induced enterocolitis rat model.

In this study, the weights of the control and experimental rats were monitored daily. At the end of the study, the nilotinib group of rats lost less weight than the indomethacin group of rats, while the control rats gained weight. However, there was no significant difference in the weight changes between the indomethacin and nilotinib groups ($P > 0.05$). In our previous study, the nilotinib group rats lost significantly less weight than the TNBS group rats ($P = 0.047$)[34]. In another study by Cuzzocrea *et al*[42], weight loss was significantly reduced by seven days of treatment with a TK inhibitor, Tyrphostin AG 126, in a dinitrobenzene sulfonic acid-induced colitis animal model.

In recent years, mucosal healing, which is referred to as 'endoscopic remission,' has gained acceptance as a measure of disease activity and as an end point in clinical trials. In the present study, the macroscopic and microscopic pathological scores were significantly lower in the control and nilotinib groups than in the indomethacin group for the small intestine and colon ($P < 0.05$). The control and nilotinib groups were similar in terms of the macroscopic and microscopic scores of the small intestine and colon. These pathological scores were remarkable and indicate the significant mucosal healing effect of nilotinib on the small intestine and colon. These results parallel the results of previous studies. In our previous study, we demonstrated that nilotinib has a significant mucosal healing effect on a TNBS-induced rat model of colitis[34]. In another study conducted by Cuzzocrea *et al*[42], TK inhibitor treatment had a significant histological improvement compared with control rats. Although there are no human studies investigating the use of TK inhibitors in patients with IBD, the case report by Margo *et al*[43], in which there was long-standing remission of CD under imatinib therapy, supports this conclusion. Further experimental investigations can provide more definitive evidence for humans.

PDGF is related to the progression and repair of inflammation and is predictive of both oxidative stress and angiogenesis in the intestine. PDGF is released in response to inflammatory and thrombotic stimuli[44]. PDGF and its receptors are expressed in mononuclear inflammatory cells in areas of active inflammation in the ulcer base as well as in mucosal inflammation. It plays an important role in neovascularization in acute inflammation, the repair process and fibrogenesis in IBD[45]. Therefore, control of PDGFR β expression may be beneficial in chronic intestinal inflammation and prevent intestinal fibrosis. In our study, PDGFR β scores were significantly lower in the nilotinib group than in the indomethacin group in the small intestine and colon ($P < 0.05$), while the PDGFR β scores in the control and nilotinib groups were similar. These results parallel those of our previous study, which investigated the effects of nilotinib in a TNBS-induced model of rat colitis[34]. However, no previous studies have investigated the effect of TK inhibitors on PDGFR β in an enterocolitis animal model. The results of our study suggest that nilotinib demonstrates its effect on mucosal healing in enterocolitis by reducing PDGFR.

TNF-α is expressed on immune cells and plays a critical role in the immune response and pathogenesis of IBD. TNF-α affects the expression of adhesion molecules, fibroblast proliferation, procoagulant factors, initiation of cytotoxic, apoptotic and acute-phase responses[46]. The TNF-α level correlates with the clinical activity of IBD[47]. Therefore, the management of IBD has dramatically changed with the advent of biological therapies. In our study, the levels of serum and tissue TNF-α levels were similar in the nilotinib and indomethacin groups. Similarly, our previous study showed that nilotinib has no significant effect on the TNF-α levels in a TNBS-induced model of colitis in rats[34]. These results indicated that nilotinib might have no significant effect on the TNF-α levels

in intestinal tissue. However, previous studies have shown that TNF-α and IL-1β, both proinflammatory cytokines synthesized in the colon, are reduced with TK inhibitors[42,48]. TNF-α is an unstable molecule that is affected by environmental factors. In a previous study, serum and tissue TNF-α levels were only measured once, and the results may be more reliable if the samples are measured more often.

CD is the result of an imbalanced mucosal T cell response. The recent data have shown that the most powerful therapeutic approaches inhibit T cell survival by inducing apoptosis, and these approaches include effects on complex pathways. Several studies have shown that the absence of PDGFR β induces the intrinsic pathway of TNF-related apoptosis, resulting in ligand-induced apoptosis[49-51]. In the present study, no significant difference was observed among the groups in terms of the apoptotic index in the small intestine, while the apoptotic indexes in the nilotinib and indomethacin group of rats were higher than in the control group of rats. On the other hand, in the colon, the apoptotic indexes of the nilotinib and indomethacin groups were significantly higher than that of the control group. D'Argenio *et al*[52] reported on apoptotic cells and the expression levels of apoptotic proteins and in TNBS-induced colitis over a period of 4 wk. According to the study results, the apoptotic cell count was significantly decreased after the first week, according to the TUNEL method. The similar apoptotic scores that were detected in our study may be because the apoptotic cell peak could not be obtained after 14 d. Furthermore, similar results for the TNF-α levels and apoptosis scores in our study may also suggest that nilotinib has no significant effect on the TNF-α levels and apoptosis.

In conclusion, nilotinib has a significant healing effect on the macroscopic and microscopic pathologic scores and ensures considerable mucosal healing in the indomethacin-induced enterocolitis rat model. While nilotinib decreased the PDGFR α and β levels and apoptotic scores in the colon, it did not have a significant effect on the weight and TNF-α levels. Further experimental investigations could provide more definitive evidence for humans.

COMMENTS

Background

The pathogenesis of inflammatory bowel diseases (IBD) is only partially understood; complex interactions between the immune system, enteric commensal bacteria and the host genotype are thought to underlie IBD development. These particular pathways are targets for drugs used to treat IBD. Side effects, unresponsiveness, high relapse rates, immune-mediated adverse reactions and loss of efficacy with time have limited the use of these drugs. The protein tyrosine kinases (TKs) constitute a large family of homologous proteins that have an important role in regulating intracellular signal transduction pathways and control a range of fundamental cellular process, including growth, metabolism, differentiation, adhesion and apoptosis. To establish a new alternative treatment option, the authors selected a TK inhibitor drug, nilotinib, that affects TNF-α, platelet-derived growth factor receptor (PDGFR) and nitric oxide (NO) synthesis.

Research frontiers

Nilotinib is a TK inhibitor used as an anticancer drug that affects the cornerstone of the steps in the pathogenesis of IBD, including TNF-α, PDGFR and NO synthesis. The authors concluded that nilotinib has a significant healing effect on the macroscopic and microscopic pathologic scores and ensures considerable mucosal healing in the indomethacin-induced enterocolitis rat model. While nilotinib decreased the PDGFR α and β levels and apoptotic scores in the colon, it did not have a significant effect on weight or TNF-α levels.

Innovations and breakthroughs

Available medical treatment options are still far from resulting in complete long-term remission and mucosal healing in IBD. Therefore, it is critically important to identify new medical treatment options for IBD. Nilotinib is a strong TK inhibitor that affects the cornerstone of IBD pathogenesis. Previously, Ataca *et al* demonstrated that nilotinib prevents weight loss, decreases macroscopic and microscopic pathological scores, and improves mucosal healing in a trinitrobenzene sulfonic acid-induced colitis model in rats. In the present study, the authors evaluated the efficacy of nilotinib in a chronic indomethacin-induced enterocolitis rat model.

Applications

The results of this study suggest that nilotinib has a significant effect on the macroscopic and microscopic pathologic scores in the indomethacin-induced enterocolitis rat model. While nilotinib decreased the PDGFR α and β levels and apoptotic scores in the colon, it did not have a significant effect on weight or TNF-α levels. Nilotinib can demonstrate its effect on mucosal healing in enterocolitis by reducing PDGFR. These results suggest that nilotinib may be effective in patients with IBD. The findings of this study shed light on important considerations for future clinical practice. Therefore, further experimental investigations could provide more definitive evidence for humans.

Terminology

IBD are a group of idiopathic, chronic, and relapsing inflammatory conditions of the gastrointestinal tract. The protein TKs constitute a large family of homologous proteins that have an important role in regulating intracellular signal transduction pathways and control a range of fundamental cellular process, including growth, metabolism, differentiation, adhesion and apoptosis. TK inhibitors are drugs that block these pathways. Nilotinib is a highly potent TK inhibitor. Indomethacin-induced enterocolitis is well-established rat model of mucosal inflammation that has been used in the study of IBD pathogenesis.

Peer-review

This is a very well-designed study that focuses on an area of clinical need in the treatment of IBD. The authors investigated the ability of nilotinib to treat various clinical, laboratory and pathological parameters. The authors demonstrated that nilotinib decreased macroscopic and microscopic pathological scores and improved mucosal healing in an indomethacin-induced enterocolitis rat model. The authors showed that while nilotinib decreased PDGFR α and β levels and apoptotic scores in the colon, the treatment did not have a significant effect on weight or TNF-α levels.

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P- Reviewer: Benkoe T **S- Editor**: Yu J **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i44.12586

World J Gastroenterol 2015 November 28; 21(44): 12586-12592
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Basic Study

Hepatitis B virus infection and genotype in asymptomatic people from 10 ethnic groups in Yunnan, China

Yuan-Ying Shen, Wei Hou, Zhan-Qiu Yang, Wen Xiao

Yuan-Ying Shen, Wei Hou, Zhan-Qiu Yang, Institute of Virology, Medical School of Wuhan University, Wuhan 430071, Hubei Province, China

Yuan-Ying Shen, Dali University, Dali 671000, Yunnan Province, China

Wen Xiao, Institute of Eastern-Himalaya Biodiversity Research, Dali University, Dali 671000, Yunnan Province, China

Author contributions: Shen YY, Hou W and Yang ZQ designed the research; Shen YY and Hou W preformed the research; Shen YY and Yang ZQ contributed reagents/analytic tools; Shen YY and Xiao W analyzed the data-and wrote the paper; Hou W and Yang ZQ revised the paper.

Supported by National Nature Science Foundation of China, No. 30560136.

Institutional review board statement: The study was reviewed by Institutional Review Board of Dali University.

Institutional animal care and use committee statement: The manuscript did not relate to any animal use and care.

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript.

Data sharing statement: No additional data are available.

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Correspondence to: Zhan-Qiu Yang, Professor, Institute of Virology, Medical School of Wuhan University, No. 115 Dong Hu Road, Wuhan 430071, Hubei Province, China. yangzhanqiu@163.com
Telephone: +86-27-68759136
Fax: +86-27-68759136

Received: February 24, 2015
Peer-review started: February 26, 2015
First decision: March 26, 2015
Revised: April 12, 2015
Accepted: August 25, 2015
Article in press: August 25, 2015
Published online: November 28, 2015

Abstract

AIM: To evaluate the infection and genotype distribution of hepatitis B virus (HBV) in ethnic groups in Yunnan, China.

METHODS: Two thousand five hundred and eighty-four asymptomatic local people from 10 ethnic groups were investigated in Yunnan, China. Infection and genotype distribution were evaluated by serological and genetic methods. Genotyping was verified by sequencing. Ethnic genotype distribution was compared by proportion test.

RESULTS: Four types of infection model based on HBV serum markers were identified, and the average HBV infection rate was 5.7% in those asymptomatic local people. The genotype prevalence was 59.6% for B, 21.1% for C and 19.3% BC; subgenotypes Ba, Cs and Ce were identified in this study. Hepatitis B surface antigen-positive rate and the proportion of genotype B were significantly lower in ethnic groups with a northern origin compared to those with a southern origin (50% *vs* 73.9%, $P = 0.037$; 4.2% *vs* 10.5%, $P = 0.000$).

CONCLUSION: Genotype B is dominant and genotype BC has high occurrence in asymptomatic local ethnic groups in Yunnan. HBV infection status and genotype distribution may associate with ethnic origin.

Key words: Hepatitis B virus; Infection and genotype; Ethnic distribution; BC genotype; Yunnan; China

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Core tip: Hepatitis B virus (HBV) infection and genotype distribution were evaluated in asymptomatic local people from 10 ethnic groups in Yunnan, China. The genotype prevalence rate was 59.6% for B, 21.1% for C and 19.3% BC; hepatitis B surface antigen-positive rate and the proportion of genotype B were significantly lower in ethnic groups with a northern origin compared to those with a southern origin. Our results suggested that HBV infection status and genotype distribution may associate with ethnic origin. It may also give some hint on understanding virus evolution.

Shen YY, Hou W, Yang ZQ, Xiao W. Hepatitis B virus infection and genotype in asymptomatic people from 10 ethnic groups in Yunnan, China. *World J Gastroenterol* 2015; 21(44): 12586-12592 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12586.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12586

INTRODUCTION

Hepatitis B is a global public health threat. This chronic disease leads to the development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[1]. According to official World Health Organization website, one-third of the world's population, or approximately 2 billion people, have been infected with hepatitis B virus (HBV), and there are approximately 350 million people with chronic HBV worldwide. The situation is even worse in China: the prevalence of chronic HBV infection is up to 10%-20% in the population, which account for three-quarters of cases in the world[2], and half a million people are killed by HBV each year[3]. Although acute HBV infection has declined due to implementation of vaccination, HBV-related complications are still increasing[4].

Based on its genomic structure and sequence differences, HBV has been divided into nine genotypes, A-I, as well as corresponding subgenotypes[5-9]. The various HBV genotypes are associated with differences in pathogenicity[10], disease progression[11], and responses to antiviral drugs[12]. Studies have found that the distribution of HBV genotypes/subgenotypes has remarkable geographical characteristics[13], which may relate to the anthropologic history of the region[14].

Moreover, DNA recombination is a significant and relatively frequent event in the evolution of HBV[15]. There have been increasing reports regarding HBV hybrids or mixed genotypes[16], wherein part of the viral genome of a certain HBV genotype is replaced by the corresponding part of another HBV genotype. The existence of AD genotype is reported in Italy and South Africa, BC is identified in Southeast Asia, and CD is limited to China[16]. Reports in China show that mixed genotype occurs more in those regions with multi-ethnic society, suggesting that this genotype distribution may be related to both geography and ethnicity[17].

Yunnan is located in southwest China. It is characterized by the highest geographic diversity as well as the highest biodiversity and the most diverse mixture of ethnic groups in China[18]. The distinct geographical location and cultural background of Yunnan province result in the diverse epidemiological characteristics of blood-borne HBV infection in this region. However, ethnic distribution of infection and genotypes remains unclear[4].

In this study, we assessed the HBV genotype distribution and infection status in asymptomatic people from 10 ethnic groups in Yunnan, China; 6 of them (Bai, Lisu, Achang, Mosuo, Naxi and Dulong) were judged to originate from Shanxi, Qinghai and Gansu regions in northern China 4000-5000 years ago[18]. Because C dominates the HBV distribution in northern China while B is dominant in southern China[2], we hypothesized that genotype B dominates in Yunnan. Moreover, if historical origin impacted genotype distribution, those people with a northern ethnic background are likely to have a lower genotype B prevalence.

MATERIALS AND METHODS

Materials

Intravenous blood samples were collected from 2584 asymptomatic people (1153 males and 1431 females, villagers without any sign of HBV infection) from 10 ethnic groups in west Yunnan. Their ethnic background was identified with household registration. Only those with consistent ethnic records in 3 generations are included. Their sera were separated and stored at -20 ℃ for later analysis of the infection status and genotypes/subgenotypes.

We used the following toolkits for our analysis: ELISA kits (Shanghai Kehua Bio-engineering company); Taq DNA polymerase (Bio Bisic Inc); the restriction endonucleases *Stu* Ⅰ, *Hpa* Ⅰ, *Bst*E Ⅱ, and *Bcn* Ⅰ (MBI Fermentas); 10 × polymerase chain reaction (PCR) Buffer (Mg^{2+}) (Bio Bisic Inc.); $MgCl_2$ (Bio Bisic Inc.); medical virological Tris base (Bio Bisic Inc.); DNA Marker D (Bio Bisic Inc.); agarose gel (United States AMRESCO); boric acid, EDTA, dNTPs, and primers (Shanghai Sangon Inc.); PCR amplification instrumentation (Perkin Elmer 9600, United States);

Table 1 The primer sequences for identifying the hepatitis B virus genotypes/subgenotypes

Gene identification	PCR	Primer	Primer sequence	Site
Genotype (A-F)	1st PCR	P1 (s)	5′-TCA CCA TAT TCT TGG GAA CAA GA-3′	nt2823–2845
		S1-2 (as)	5′-CGA ACC ACT GAA CAA ATG GC -3′	nt685–704
	2nd PCR	B2 (s)	5′-GGC TCM AGT TCM GGA ACA GT-3′	nt67–86
	mixA	BA1R (as)	5′-CTC GCG GAG ATT GAC GAG ATG T-3′	nt113–134
		BB1R (as)	5′-CAG GTT GGT GAG TGA CTG GAG A-3′	nt324–345
		BC1R (as)	5′-GGT CCT AGG AAT CCT GAT GTT G-3′	nt 165–186
	mixB	BD1 (s)	5′-GCC AAC AAG GTA GGA GCT-3′	nt 2979–2996
		BE1 (s)	5′-CAC CAG AAA TCC AGA TTG GGA CCA-3′	nt 2955–2978
		BF1 (s)	5′-GYT ACG GTC CAG GGT TAC CA-3′	nt 3032–3051
		B2R (s)	5′-GGA GGC GGA TYT GCT GGC AA-3′	nt 3078–3097
Subgenotype B (B1 and B2)	1st PCR	PC1 (s)	5′-CAT GCA ACT TTT TCA CCT CTG CCT-3′	nt1813-1836
	2nd PCR	PC2 (s)	5′-ATT AGA CCT ATT GAT TGG AAA GT-3′	nt1861-1881
		COR-HBV (as)	5′-GAG TGC GAA TCC ACA CTC CA-3′	nt2285-2266
C (C1 and C2)	1st PCR	HBV964F (s)	5′-ATT AGA CCT ATT GAT TGG AAA GT-3′	nt964-986
	2nd PCR	HBV970F2 (s)	5′-CCT ATT GAT TGG AAA GTA TGT CA-3′	nt970-992
		HBV1272R (as)	5′-AGT ATG GAT CGG CAG AGG AG-3′	nt1272-1253

(s): Sense; (as): Anti-sense; M: A or C; Y: C or T; HBV: Hepatitis B virus; PCR: Polymerase chain reaction.

an electrophoresis groove (DYCR-31D, Beijing Liuyi Instrument Factory); and the Syngene Automatic Image Analysis System (GGMID2, Britain's Gene Inc).

Methods

Detection of serum markers for hepatitis B: Serum markers for hepatitis B were tested with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. The kit contained HBsAg, HBsAb, HBeAg, HBeAb and HBcAb.

HBV DNA extraction: HBV DNA was extracted using the methods described previously[19] and stored at -80 ℃.

Primer design and synthesis: Primer sequences were listed in Table 1, and primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai).

Identification of the HBV genotypes and subgenotypes: Genotypes were identified according to the methods described in an earlier study[20]. Specifically, the following procedure was followed with a 40 μL reaction system for the first round of PCR amplification. Cycle parameters were as follows: an initial 10 min denaturation at 94 ℃ followed by 40 cycles of amplification at 94 ℃ for 20 s, 55 ℃ for 20 s, and 72 ℃ for 60 s, and strand synthesis at 72 ℃ for 7 min. The second-round PCR was divided into two groups, A and B. Two milliliters of the first-round PCR products were used in the second round of PCR under the same reaction system but with the primers from group A (B2, BA1R, BB1R and BC1R2) and group B (B2R, BD1R, BE1R and BF1R). Both groups A and B were treated with the same cycle parameters, which were denaturation at 95 ℃ for 10 min, 20 cycles of amplification at 94 ℃ for 20 s, 58 ℃ for 20 s, and 72 ℃ for 30 s, followed by an additional 20 cycles of 94 ℃ for 20 s, 60 ℃ for 20 s, and 72 ℃ for 30 s, and an extension at 72 ℃ for 7 min. Finally, the PCR products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide and checked under UV light.

The HBV genotypes were determined based on the size of amplified fragments; products of 68 bp, 281 bp, 122 bp, 119 bp, 167 bp and 97 bp were considered as genotypes A, B, C, D, E and F, respectively. A mixed genotype was considered when its products consisted of any of the two aforementioned fragments.

Subgenotypes: The method for identifying subgenotypes was adapted from that described in the literature[20]. The first round of PCR amplification used a 20 μL reaction system containing 2.0 μL of 10 × buffer, 0.4 μL of 0.2 mmol/L dNTPs, 0.4 μL of each 0.2 μmol/L primer (genotype B: PC1 and COR and genotype C: HBV964F and HBV1272R), 2.0 μL of 0.5 U/μL Taq DNA polymerase, 3.0 μL of serum for template, and 11.8 μL ddH_2O. The following cycle parameters were used: denaturation at 94 ℃ for 1 min, 30 cycles of amplification at 94 ℃ for 40 s, 55 ℃ for 30 s, and 72 ℃ for 50 s, and a final extension at 72 ℃ for 7 min. The second-round PCR required 2 μL of the first-cycle PCR products as a template with the same reaction system and cycle parameters but different primers (genotype B: PC2 and COR and genotype C: HBV970F and HBV 1272R). The products (5 μL) of the second round were digested with the corresponding restriction endonucleases (Ba/Bj: *Stu* Ⅰ/*Hpa* Ⅰ; Cs/Ce: *Bst*E Ⅱ/*Bcn* Ⅰ), and the subgenotypes were identified according to the fragments of the digested products under a UV light following electrophoresis. A sequencing approach was adopted to determine the subgenotypes of the non-digested products.

Sequencing: To verify the accuracy of the genotype-specific PCR method, products from 5 samples of the first-round PCR, including 3 cases of genotype B and 2 cases of genotype C, were selected to determine

Table 2 Hepatitis B surface antigen-positive rates in the 10 ethnic groups in Yunnan Province

Ethnic group	Number	Age	Origin[1]	Gender		HBsAg	
				Male *n* (%)	Female *n* (%)	Positive	Rate (%)
Han	165	16.01 ± 14.47	-	87 (52.7)	78 (47.3)	7	4.2
Dai	459	38.41 ± 18.57	S	159 (34.6)	300 (65.4)	43	9.4[2]
Bulang	100	36.24 ± 18.11	S	54 (54.0)	46 (46.0)	7	7.0
Pumi	61	34.90 ± 18.44	S	33 (54.1)	28 (45.9)	15	24.6[2]
Achang	347	30.19 ± 18.46	N	154 (44.4)	193 (55.6)	16	4.6
Mosuo	234	31.61 ± 18.14	N	101 (43.0)	133 (57.0)	15	6.4[2]
Naxi	196	8.78 ± 18.32	N	100 (51.0)	96 (49.0)	2	1.0
Dulong	305	31.51 ± 18.66	N	143 (46.9)	162 (53.1)	10	3.3
Bai	401	32.78 ± 17.12	N	178 (44.4)	223 (55.6)	22	5.5
Lisu	316	18.24 ± 18.02	N	144 (45.6)	172 (54.4)	10	3.2
Total	2584	28.35 ± 18.66		1153 (44.6)	1431 (55.4)	147	5.7

[1]Historical ethnic origin; [2]Indicates those with higher than average values. N: From north China; S: From south China; -: Han has no specific origin; HBsAg: Hepatitis B surface antigen.

Table 3 The distribution of the genotypes and subgenotypes in different ethnic groups

Ethic group	Total	Source[1]	Genotype				Subgenotype		
			B	C	B + C	Sum	Ba	Cs	Ce
Han	165	-	1	0	1	2	1	0	0
Dai	459	S	13	4	1	18	13	3	0
Bulang	100	S	0	0	1	1	0	1	0
Pumi	61	S	4	0	0	4	3	0	
Achang	347	N	2	0	4	6	3	1	0
Mosuo	234	N	3	3	1	7	0	0	0
Naxi	196	N	1	0	0	1	1	0	0
Dulong	305	N	1	1	0	2	1	1	0
Bai	401	N	6	3	2	11	4	3	0
Lisu	316	N	3	1	1	5	0	1	1

[1]Historical ethnic origin. N: From north China; S: From south China; -: Han has no specific origin.

the sequence of the HBV S region. The PCR-amplified products were purified, cloned and directly sequenced by TAKARA Biotechnology (Dalian Co., Ltd). DNASTAR was used to analyze the homology between the sequencing genotype results and the standard GenBank strain sequences, and a phylogenetic tree was built.

Statistical analysis: χ^2 test and proportion test were performed with R Statistics (The statistical methods of this study were reviewed by Ren GP of Dali University).

Ethics statement

Written informed consent was obtained from all adult participants and from their parents or guardians for minors/children in the study [All procedures in this study were approved by the Medical Ethics Committee of Dali University (No. 2007005)].

RESULTS

Infection status

The overall positive rate of HBsAg in this study was 5.7% in average. Infection rates in Dai, Pumi, Bulang and Mosuo were higher than average, especially for Pumi (24.6%). Detailed infection status for the different ethnic groups is listed in Table 2.

The HBsAg positive rate was 10.5% in ethnic groups originating from south China and 4.2% for those with a northern origin, which had a significant difference (P = 0.000).

Genotype/subgenotype distribution

The genotype-specific PCR method was employed to detect the HBV genotypes. Fifty-seven samples were positive, including 34 cases of genotype B (59.6%), 12 cases of genotype C (21.1%), and 11 (19.3%) cases of BC. All ethnic groups had genotype B except Bulang. Achang had the highest BC occurrence. No C or BC was found in Pumi and Naxi. Detailed distribution of the genotypes and subgenotypes in different ethnic groups is listed in Table 3.

Genotype B accounted for 50% of those with a northern origin; C, 25%; and BC, 25%. For those ethnic groups with a southern origin, the genotype distribution was 73.9% for B, 17.4% for C, and 8.7% for BC. There was a statistically significance difference in the proportion of B genotype between groups of different origins (P = 0.037). No difference was noted in the genotype prevalence for genotypes C (P = 0.250) and BC (P = 0.061).

Regarding the subgenotypes, a total of 37 samples

Table 4 The distribution of the hepatitis B virus genotypes/subgenotypes in different serum marker models

Serum marker mode	Genotype				Subgenotype			
	B	C	B + C	Subtotal	Ba	Cs	Ce	Subtotal
HBsAg+HBeAg+HBcAb+	22	6	6	34	15	7	1	23
HBsAg+HBeAb+HBcAb+	7	4	2	13	6	3	0	9
HBsAg+HBcAb+	3	0	3	6	2	1	0	3
HBeAg+	2	2	0	4	1	1	0	2
Total	34	12	11	57	24	12	1	37

HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBcAb: Hepatitis B core antibody; HBeAb: Hepatitis B e antibody.

were genotyped; 24 of them were subgenotype Ba, while 12 were subgenotype Cs and 1 was subgenotype Ce.

Serum markers

There were 4 types of HBV serum infection marker model for all samples, namely, HBsAg+HBeAg+HBcAb+, HBsAg+HBeAb+HBcAb+, HBsAg+HBcAb+ and HBeAg+ (Table 4). Comparison of the genotypes and subgenotypes for the 4 models did not show statistically significant difference (P = 0.303; P = 1.000).

Genotype validation

The sequences of 2 standard strains of genotype C (EU306694 and EU439011) and 2 standard strains of genotype B (EU439019 and EU43902) from Yunnan province were downloaded from GenBank. Three cases of genotype B (No. KMS6330, KMS6337 and KMS6339) and 2 cases of genotype C (No. KMS6329 and KMS6333) in this study were compared with the standard strains. The homology of KMS6330, KMS6337 and KMS6339 with the standard B strain was 98%-99%, and their homology with the standard C strain was 87%-92%. The homology of KMS6329 and KMS6333 with the standard C strain was 98%-99% and 87%-94% with the standard B strain. Therefore, the genotype classification should be valid.

DISCUSSION

The overall positive rate of HBsAg in this study was 5.7% in average, which is lower than the average level in China (7.18%)[21]. However, it reached 24.6% in Pumi people. Moreover, all 4 sites with a positive rate higher than the average level were concentrated in a remote village of west Yunnan. This pattern is quite consistent with the distribution pattern of ethnic groups in Yunnan, which is characterized by a mixed distribution on a large scale and an isolated distribution on a small scale.

The HBV genotype distribution pattern in China is dominated by genotype C in the north and B in the south; main genotypes were C, B and BC, and their rates were 50.99%, 35.58%, and 6.07%, respectively[22]. The genotypes B and C distribution was consistent with this pattern, and genotype B was dominant with a prevalence of 59.6% in this study. However, Wang *et al*[4] and Kang *et al*[23] found that genotype C dominated in their samples. Wang *et al*[4] reported that genotype B (33.3%), genotype C (62.5%), genotype I (2.78%) and C/D (1.39%) in 80 samples of patients in middle and east Yunnan; Kang *et al*[23] reported 76.9% C, 15.4% B, 5.1% D and 2.5% I in 2216 samples from people who had a physical examination in Kunming city. Since Kunming is the capital of Yunnan province; thus, those studies focusing on patient instead asymptomatic people in big cities with large migration population may cause a biased conclusion. Genotype C proportion is much higher that reported by Zhu and Dong[23]. We think our study may represent the origin distribution of genotypes in Yunnan better, because all samples were collected from asymptomatic people in west Yunnan, where is underdeveloped than other part of Yunnan. Besides the overall distribution of genotype distribution in this study, comparison between ethnic groups with different origins also showed a pattern consistent with overall pattern in China. The prevalence of genotype B in ethnic groups with a northern origin was much lower than that with a southern origin (50% *vs* 73.9%). Thus, ethnic/genomic background may have driven the HBV genotype distribution of a certain human population. Although there was no difference in the distribution of genotype B, the BC distribution nearly showed a statistically significant difference (P = 0.061), which may be due to the small sample size and the fact that the study subjects were from an asymptomatic population.

A much higher prevalence of BC (19.3%) was found in this study, especially for those with a northern origin. This result is consistent with the 26.1% BC frequency reported in a previous study[17] in southwest China (Sichuan Province) that included minority groups (Tibetan people). Higher infection in ethnic groups with a southern background, and the lower infection rate in ethnic groups with a northern origin in a region (in which B dominates) may reflect the physical-anthropological factors contributing to the HBV infection distribution. This also suggested that recombination is an important way for HBV genotypes to adapt to people with different genomic backgrounds. The BC genotype may be a strategy that allows genotype C to survive; when the familiar hosts adapt to a different habitat, it can be a transition expression or another adaption type. One may expect

a higher prevalence of the mixed genotype in regions with ethnic group migration, which agrees well with the finding that the CD hybrid is the dominant genotype in a population with frequent migration[24]. A recent paper reviewed 16 studies on HBV genotype distribution in 20 minority groups in China, and it shows that recombination was found in 13/20 ethnic groups[25].

The HBV divergence in humans and apes was estimated to occur in the last 6000 to 7000 years[26]. However, most estimations of the time to the most recent ancestor of human HBV fell in the range of 2000-4000 years[15]. This is consistent with ethnic migration time estimation (4000-5000 years) based on a linguistics study in Yunnan[18]. Wang *et al*[4] suggested that distribution of HBV in Yunnan is also impacted by other provinces of China with an estimation around 1900s, when Yunnan started its development. Thus, HBV genotype distribution can be shaped by both historical genomic backgrounds of people and economic development.

Additionally, there were 37 cases of HBV sub-genotype positivity in the identified genotype samples, of which there were 24 cases of Ba subgenotype, 12 cases of Cs subgenotype and 1 case of Ce subgenotype. This is consistent with other studies in China[2]. However, we did not find the Bj subgenotype in this study, which is likely due to a small sample size.

In conclusion, our study not only found that the genotype distribution in asymptomatic people is related to their ethnic origin but also found different HBV infection rates in different ethnic groups. A recent study showed that the ethnogeographical project can link ethnic origins and HBV genotype distribution in patients[27]. Ethnic epidemiological studies with systematic spatial sampling that account for the migration history and socioeconomic factors (such as an ethnic group's travel), may improve our understanding of the evolution of HBV. Such studies will improve disease prevention and clinical treatment, because HBV transmission is affected by increasing interactions among different ethnic groups. More systematic sampled epidemiological research is needed in Yunnan, which is a hub between China and southeast Asia and a reserve representing historical issues.

COMMENTS

Background

Hepatitis B is a global public health threat. It is even worse in China. Hepatitis B virus (HBV) has been divided into nine genotypes, and various HBV genotypes are associated with differences in pathogenicity, disease progression, and responses to antiviral drugs. Studies have found that the distribution of HBV genotypes/subgenotypes has remarkable geographical characteristics, and it may relate to the anthropologic history of the region. Yunnan is located in southwest China. It is characterized by the highest geographic diversity as well as the highest biodiversity and the most diverse mixture of ethnic groups in China. However, ethnic distribution of infection and genotypes remains unclear.

Research frontiers

Ethnogeographical project can link ethnic origins and HBV genotype distribution in patients. Thus, ethnic epidemiological studies should be a hotspot to improve our understanding of the evolution of virus in the future.

Innovations and breakthroughs

Former studies focusing on patients in big cities with large migration population may cause a biased conclusion. This study focuses on asymptomatic people in an underdeveloped region, and included ethnic origin as a key factor to understand HBV genotype distribution. Results showed that HBV genotype distribution may relate to ethnic origin thousands years ago.

Applications

Results from this study may give some hint on evolution of HBV, and it also has potential contribution to the prevention and management of HBV infection.

Terminology

Hepatitis B is an infectious disease caused by the HBV which affects the liver. It can cause both acute and chronic infections.

Peer-review

This manuscript describes a prospective epidemiological study on the infection and genotype of hepatitis B virus in Yunnan, China. The authors have clearly outlined their hypothesis for the study. The study design and methods were described in a very detailed manner, especially regarding sample testing techniques.

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P- Reviewer: Celikbilek M, Koch TR, Mihaila RG, Xu R
S- Editor: Ji FF **L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12593

World J Gastroenterol 2015 November 28; 21(44): 12593-12604
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Basic Study

Potential effect of chronic *Helicobacter pylori* infection on glucose metabolism of Mongolian gerbils

Zhen Yang, Wei Li, Cong He, Chuan Xie, Yin Zhu, Nong-Hua Lu

Zhen Yang, Wei Li, Cong He, Chuan Xie, Yin Zhu, Nong-Hua Lu, Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

Author contributions: Yang Z, Li W and He C contributed equally to this work; Yang Z and Lu NH designed the research; Li W, Xie C and Zhu Y performed the research; He C analyzed the data and wrote the paper.

Supported by (in part) grants from the National Natural Science Foundation of China, No. 81060038, No. 81270479, and No. 81470832; the National Science and Technology Major Projects for "Major New Drugs Innovation and Development" of China, No. 2011ZX09302-007-03; the "Talent 555 Project" of Jiangxi Province, China; and the Graduate Innovation Fund of Jiangxi Province, No. YC2014-B021.

Institutional review board statement: The study was reviewed and approved by the First Affiliated Hospital of Nanchang University Institutional Review Board.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Nanchang University (2014-018).

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

Data sharing statement: No additional data are available.

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Correspondence to: Nong-Hua Lu, MD, Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Donghu District, Nanchang 330006, Jiangxi Province, China. lunonghua@ncu.edu.cn
Telephone: +86-791-88692705
Fax: +86-791-88623153

Received: April 16, 2015
Peer-review started: April 18, 2015
First decision: May 18, 2015
Revised: June 19, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: November 28, 2015

Abstract

AIM: To assess the effect of *Helicobacter pylori* (*H. pylori*) infection on metabolic parameters in Mongolian gerbils.

METHODS: A total of 40 male, 5- to 8-wk-old, specific-pathogen-free Mongolian gerbils (30-50 g) were randomly allocated into two groups: a control group (n = 20) and an *H. pylori* group (n = 20). After a two-week acclimation period, the control group was administered Brucella broth and the *H. pylori* group was challenged intra-gastrically five times every other day with approximately 10^9/CFU *H. pylori* ATCC43504 (CagA+, VacA+). Each group was then divided into two subgroups, which were sacrificed at either 6 or 12 mo. The control and *H. pylori* subgroups each contained 10 Mongolian gerbils. Body weight, abdominal circumference, and body length were measured, and body mass index (BMI) and Lee's index were calculated. Biochemical assays were used to detect serum indexes, including glucose, glycated hemoglobin (GHb), glycated hemoglobin A1c (HbA1c), triacylglycerol, and total cholesterol, using an automatic biochemistry analyzer. Inflammatory cytokines, including interleukin (IL)-1β, IL-2, IL-4,

IL-10, IL-12, tumor necrosis factor-α (TNF-α) and interferon (IFN)-γ, were assayed using ELISA. The expression of insulin and insulin-like growth factor 1 (IGF-1) was detected by immunohistochemistry, and islet apoptosis was measured using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

RESULTS: At each time point, body weight, abdominal circumference, BMI, and Lee's index were increased after *H. pylori* infection. However, these differences were not significant. *H. pylori* infection significantly increased the GHb (5.45 ± 0.53 *vs* 4.98 ± 0.22, $P < 0.05$) and HbA1c (4.91 ± 0.61 *vs* 4.61 ± 0.15, $P < 0.05$) levels at 12 mo. We observed no significant differences in serum biochemical indexes, including fasting blood glucose, triacylglycerol and total cholesterol, at 6 or 12 mo after infection. *H. pylori* infection significantly increased the expression of IGF-1 ($P < 0.05$). Insulin levels from the pancreas and the apoptotic rate of islet β-cells remained unchanged. Also, we observed no significant differences among cytokines levels, including IL-1β, IL-2, IL-4, IL-10, IL-12, TNF-α and IFN-γ. IL-4 was the only exception, which increased at 6 (44.36 ± 25.17 *vs* 17.38 ± 3.47, $P < 0.05$) and 12 mo (33.41 ± 10.00 *vs* 18.91 ± 5.31, $P < 0.05$) after *H. pylori* infection.

CONCLUSION: Long-term *H. pylori* infection is significantly associated with high levels of HbA1c in Mongolian gerbils, indicating a potential role of *H. pylori* infection in glucose dysregulation.

Key words: *Helicobacter pylori*; Glycated hemoglobin A1c; Glucose metabolism; Inflammatory cytokines

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Core tip: Accumulating evidence suggests a link between *Helicobacter pylori* (*H. pylori*) infection and type 2 diabetes although it is controversial. This study assessed the effect of chronic *H. pylori* infection on metabolic parameters in Mongolian gerbils. The results showed that the glycated hemoglobin and glycated hemoglobin A1c levels increased significantly after *H. pylori* infection while no obvious differences of other serum indexes including fast glucose, lipid and cytokines were observed. It is assumed that chronic *H. pylori* infection might affect glucose metabolism and the inflammatory cytokines does not appear to mediate the effect. Further studies are warranted to elucidate the underlying mechanisms.

Yang Z, Li W, He C, Xie C, Zhu Y, Lu NH. Potential effect of chronic *Helicobacter pylori* infection on glucose metabolism of Mongolian gerbils. *World J Gastroenterol* 2015; 21(44): 12593-12604 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12593.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12593

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a host-specific, bacterial pathogen that can establish a chronic infection within the human gastric mucosa. Chronic infection causes a variety of gastroduodenal diseases ranging from superficial gastritis and peptic ulcers to gastric cancer and mucosa-associated lymphoid tissue lymphoma[1,2]. Over the past several decades, a large number of epidemiological studies have revealed that the effects of *H. pylori* infections may not be confined to the digestive tract. These infections can be associated with extra-digestive pathologies, especially infections characterized by persistent, low-grade, systemic inflammation[3,4].

The incidence of type 2 diabetes mellitus (T2DM) is rising globally, and T2DM is responsible for an estimated 3.8 million adult deaths worldwide[5]. The pathogenesis of T2DM is complex, and the risk factors are associated with lifestyle (*e.g.*, diet, obesity, and physical activity), genetic background, and socioeconomic factors[6,7]. However, these factors provide only partial explanations, and recent evidence has indicated the pathological involvement of inflammation in T2DM. Thus, chronic infections may be another contributing factor[8]. Previous studies have observed a higher prevalence of *H. pylori* infection in diabetic subjects compared to non-diabetic subjects[9,10]. Other researchers, however, have proposed an insignificant or even opposite association between *H. pylori* infection and diabetes[11,12]. Therefore, the relationship between *H. pylori* infection and T2DM is unclear.

Mongolian gerbils have frequently been used to study the pathogenesis of *H. pylori* infection because these gerbils are susceptible to colonization and develop gastric diseases due to infection[13,14]. Furthermore, no studies have been performed regarding the link between *H. pylori* infection and diabetes in Mongolian gerbils. Thus, this study evaluated the effects of chronic *H. pylori* infection on glucose and lipid metabolism, serum levels of cytokines, insulin and insulin-like growth factor 1 (IGF-1) levels in the pancreas, and the apoptotic rate of islet β-cells *in vivo*.

MATERIALS AND METHODS

Animals and bacterial strains

A total of 40 male, 5- to 8-wk-old, specific-pathogen-free, Mongolian gerbils (30-50 g) were purchased from the Zhejiang Academy of Medical Sciences (Zhejiang, China). The animals were randomly allocated into two groups: a control group ($n = 20$) and an *H. pylori* group ($n = 20$). All animals were housed in air isolation cages (IVC-Ⅱ; Suzhou Fengshi Animal Equipment Co., Jiangsu, China) (12/12 h light/dark cycle; room temperature, 20-22 ℃; 55% relative humidity) with the same access to food and tap water. The control group was administered Brucella broth, and the *H. pylori* group was challenged intra-gastrically five times every other day with approximately 10^9/CFU *H. pylori*

ATCC43504 (CagA+, VacA+). Each group was then divided into two subgroups that were sacrificed at 6 or 12 mo. The control and *H. pylori* subgroups each contained 10 Mongolian gerbils. All protocols for animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. *H. pylori* infection was produced in mice in compliance with the institutional guidelines and regulations with an effort to minimize the number of animals used and their suffering.

Biochemical assays

At 6 and 12 mo, the animals were sacrificed after anaesthetization, and blood samples were obtained from the abdominal aorta for biochemical analysis. Body weight, abdominal circumference and body length were measured, and the body mass index (BMI) [body weight (g)/length^2 (cm^2)] and Lee's index [body weight (g) × 10^3/nose-to-anus length (cm)]$^{1/3}$ were calculated. Blood was placed in a centrifuge tube at room temperature and allowed to clot to obtain the serum. Serum was separated by centrifugation at 3000 rpm for 10 min. Serum glucose (Glu), glycated hemoglobin (GHb), glycated hemoglobin A1c (HbA1c), triacylglycerol (TG), and total cholesterol (TC) were assayed using an automatic biochemistry analyzer (Hitachi 7600, Japan). The serum levels of inflammatory cytokines, including interleukin (IL)-1β, IL-2, IL-4, IL-10, IL-12, tumor necrosis factor-α (TNF-α) and interferon (IFN)-γ, were assayed using ELISA kits (BD Bioscience, United States).

Histopathological examinations

To determine whether bacterial colonization had occurred in the stomach, whole stomachs were stored in 10% formaldehyde in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) overnight at 4 ℃ prior to paraffin embedding, and *H. pylori* infection was observed *via* Giemsa staining. The pancreases were stored in 10% formaldehyde for immunohistochemistry and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assays. Paraffin sections of 4 μm were cut with a microtome and stored at room temperature.

Immunohistochemistry assay

The primary antibodies used in this study were guinea pig polyclonal to insulin and rabbit polyclonal to IGF-1 (Abcam, United Kingdom). The anti-insulin antibody was diluted 1:100, and the anti-IGF-1 antibody was diluted 1:1000.

The paraffin sections were mounted on slides, dewaxed in xylene and sequentially dehydrated in 100%, 95% and 85% ethanol. The sections were stained following the PV-6000 Polymer Detection System (Zhongshan Goldenbridge, China) staining protocol. The sections were then washed in PBS, and endogenous peroxidase was blocked using 3% H_2O_2. After the specimens were incubated with the primary antibody overnight at 4 ℃, they were washed with PBS and incubated with polymer helper for 30 min and polyperoxidase-anti-mouse or rabbit IgG for 30 min. After the sections were washed with PBS, they were incubated with 3,3'-diaminobenzidin (DAB, Zhongshan Goldenbridge, China). The control sections were incubated with PBS instead of the primary antibodies (negative controls). The sections were counterstained with hematoxylin.

The stained sections were chosen, reviewed, and scored from five randomly selected high power fields (40 × objective lens) by two pathologists blinded to the histopathological data. Grading discrepancies were re-reviewed and discussed to obtain a final score. Epithelial cells with yellow or brown staining in the nucleus and/or cytoplasm were defined as positive for immunoreactivity. The percentages of immunoreactive cells from 100 cells in each field were averaged from five fields and scored as follows: 0 = 0%-5.0% immunoreactivity; 1 = 5.1%-25.0% immunoreactivity; 2 = 25.1%-50.0% immunoreactivity; 3 = 50.1%-75.0% immunoreactivity; and 4 = 75.1%-100% immunoreactivity. In addition, the immunostaining intensity was semi-quantitatively assessed (0 = negative, 1 = weak staining, 2 = moderate staining, and 3 = intense staining). The overall protein expression level was reported as a grade calculated from an integral score of "area × intensity" as follows: grade 1 = scores 0-2 (negative); grade 2 = scores 3-5 (weakly positive); grade 3 = scores 6-8 (moderately positive); and grade 4 = scores 9-12 (strongly positive)[15,16].

Apoptosis assay

Apoptosis was quantified using the TUNEL method with the DeadEnd™ colorimetric apoptosis detection system (Promega Corp., United States) according to the manufacturer's protocol. The number of TUNEL-positive cells was counted in 20 randomly selected fields per section under a microscope at 200-fold magnification. Apoptosis was calculated as the percentage of apoptotic nuclei (dark brown nuclei) versus the total nuclei of multinucleated TRAP-positive cells. A dark brown DAB signal indicated positive staining, and shades of blue-green to greenish-tan indicated a non-reactive cell[17,18].

Statistical analysis

The data are presented as mean ± SEM or percentages. Student's *t*-test (SPSS v.16.0 for Windows; SPSS, Inc., United States) and the chi-square test were used to detect statistical differences. $P < 0.05$ was considered significant.

RESULTS

Effects of H. pylori infection on body weight, body length, abdominal circumference, BMI and Lee's index in Mongolian gerbils

The Mongolian gerbils were successfully infected with *H. pylori* as confirmed by Giemsa staining. No animals

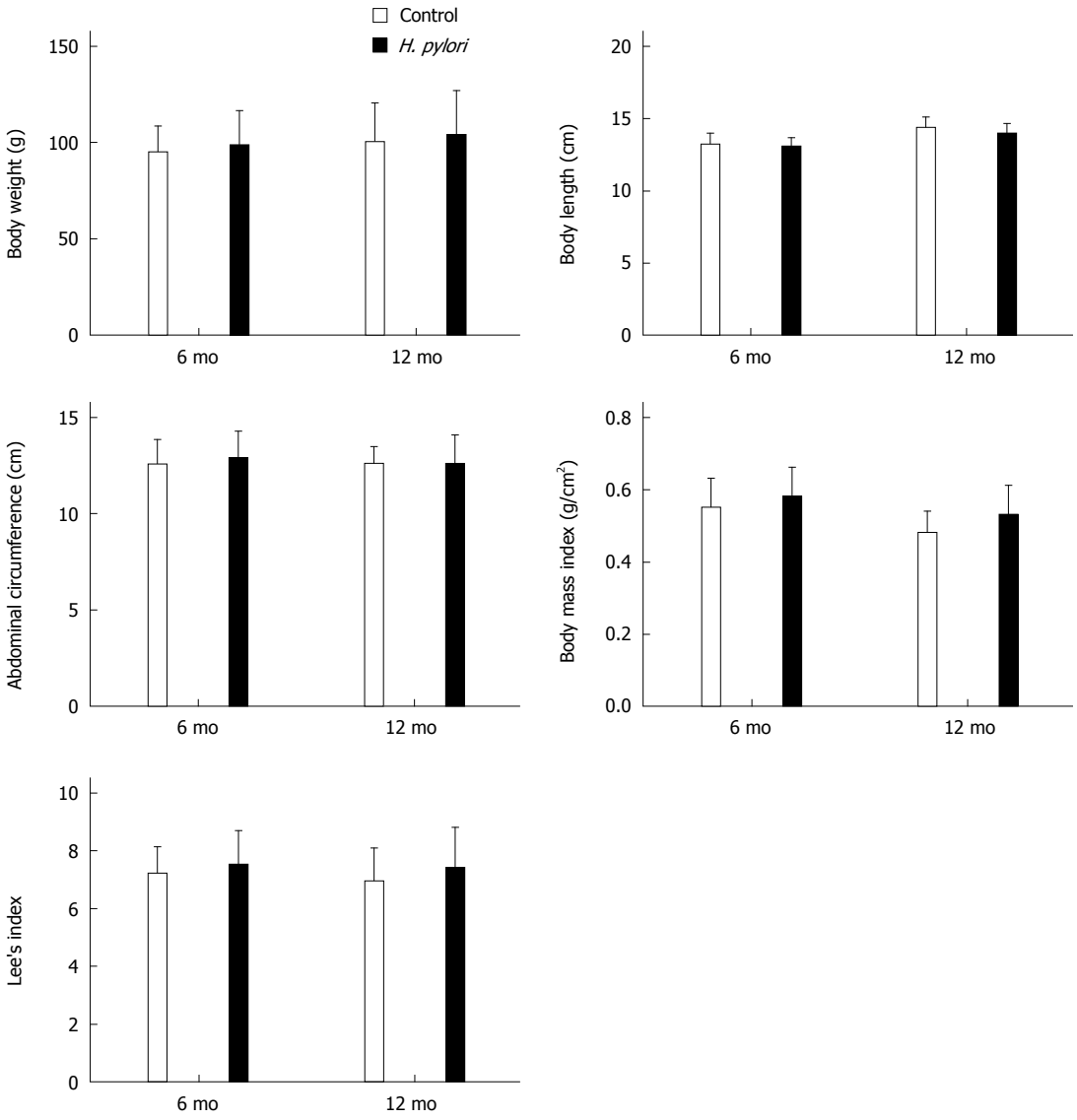

Figure 1 Effects of *Helicobacter pylori* infection on body indexes of Mongolian gerbils at different time points. The body weight, body length, and abdominal circumference were measured, and body mass index and Lee's index were calculated at 6 and 12 mo after *Helicobacter pylori* (*H. pylori*) infection. The data denote an upward trend of these parameters, although there were no significant differences ($P > 0.05$). Bars represent the mean ± SEM, $n = 8$-10 mice per group.

challenged with Brucella broth alone had detectable evidence of *H. pylori*. Body weight, body length, abdominal circumference, BMI and Lee's index were calculated at 6 and 12 mo after *H. pylori* infection in Mongolian gerbils (Figure 1). The body weight, abdominal circumference, BMI, and Lee's index increased after *H. pylori* infection at each time point. However, these differences were not significant ($P > 0.05$) (Figure 1).

H. pylori infection and serum levels of biochemical indexes in Mongolian gerbils

At 6 and 12 mo after *H. pylori* infection, the animals were sacrificed after anesthetization. The serum levels of biochemical indexes, including fasting Glu, TG, TC, GHb and HbA1c, were assayed using an automatic biochemistry analyzer. The serum Glu, TG, and TC levels were not significantly different between the control and *H. pylori* groups ($P > 0.05$), although there was a slight increase in TG and TC in the infected groups compared to the control groups (Figure 2). HbA1c, the major fraction of GHb, is an efficient glucose monitoring index for patients with diabetes. At 12 mo post-infection, the levels of both GHb (5.45 ± 0.53 *vs* 4.98 ± 0.22, $P < 0.05$) and HbA1c (4.91 ± 0.61 *vs* 4.61 ± 0.15, $P < 0.05$) were significantly increased in the infected group compared to the control group (Figure 2).

Effects of chronic H. pylori infection on the serum levels of inflammatory cytokines in Mongolian gerbils

It is commonly believed that the chronic inflammation induced by an *H. pylori* infection is a major link to T2DM. Thus, the serum levels of inflammatory cytokines, including IL-1β, IL-2, IL-4, IL-10, IL-12, TNF-α and IFN-γ, were assayed using ELISA kits. No significant

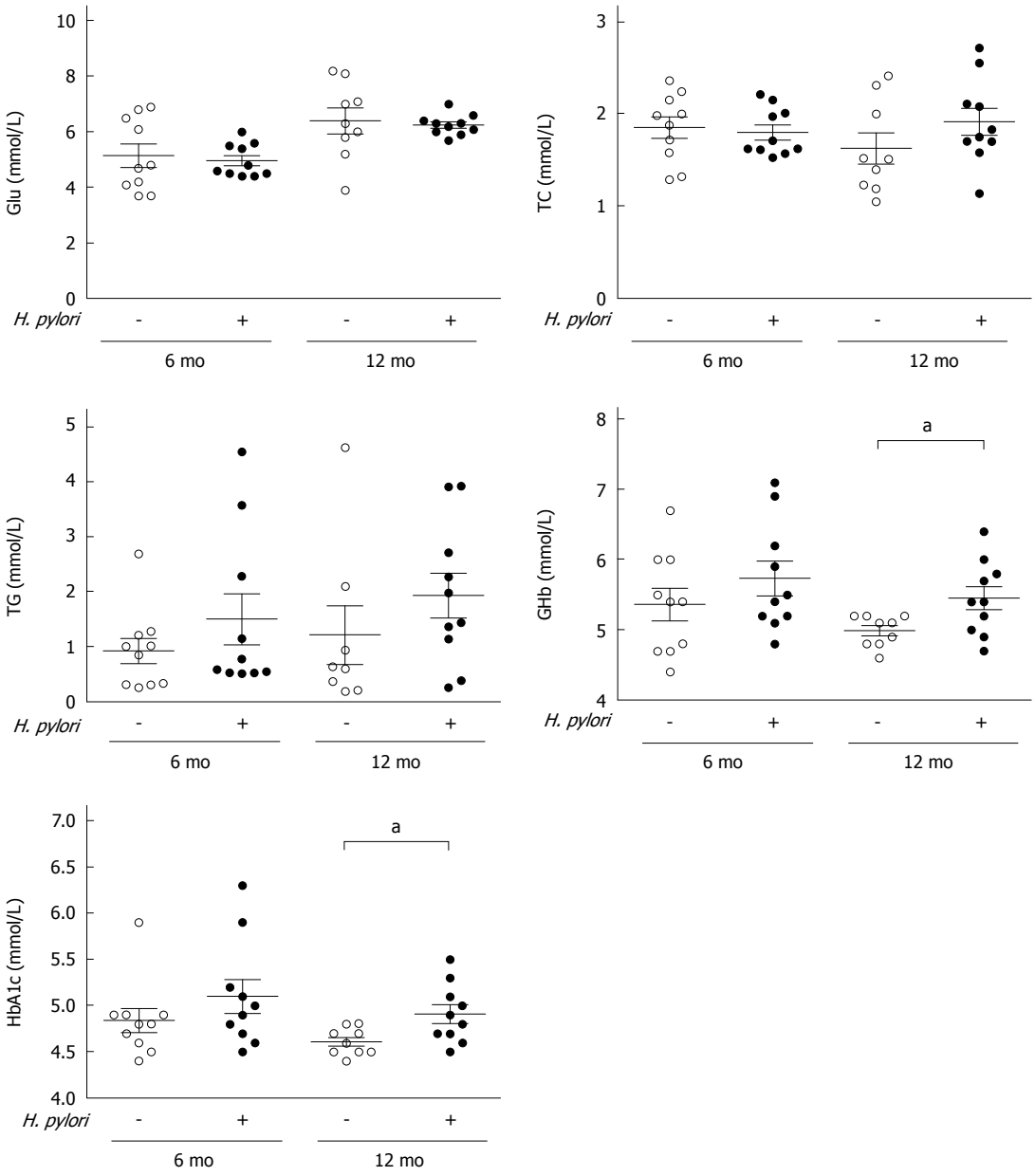

Figure 2 Measurements of serum biochemical parameters of Mongolian gerbils after *Helicobacter pylori* infection at different time points. Serum concentration of fasting glucose (Glu), triacylglycerol (TG), total cholesterol (TC), and glycated hemoglobin (GHb) and hemoglobin A1c (HbA1c) were measured at 6 and 12 mo after *Helicobacter pylori* (*H. pylori*) infection. Data are presented as mean ± SEM from eight to ten mice per group. $^{a}P < 0.05$ between *H. pylori* infected mice and their controls at 12 mo concerning GHb and HbA1c.

differences were observed ($P > 0.05$), except for the serum level of IL-4, which was significantly increased in the *H. pylori* group compared to the control group at both 6 (44.36 ± 25.17 *vs* 17.38 ± 3.47, $P < 0.05$) and 12 mo (33.41 ± 10.00 *vs* 18.91 ± 5.31, $P < 0.05$) (Figure 3).

H. pylori infection and pancreatic insulin and IGF-1 in Mongolian gerbils

Insulin is the key hormone for blood glucose regulation. Generally, normoglycemia is maintained by a balanced interplay between insulin action and secretion. IGF-1 is structurally similar to insulin and is also involved in the physiological regulation of nutrient intake, metabolism and tissue growth. Thus, IGF-1 contributes to the complex balance between anabolism and consumption. To investigate the effects of *H. pylori* on the expression of insulin and IGF-1 in gerbil pancreatic tissues, immunohistochemical staining was used. Insulin expression did not differ between the *H. pylori* group and the controls at 6 or 12 mo ($P > 0.05$) (Figure 4). At 6 mo, the expression of IGF-1 was significantly increased in the *H. pylori* group compared to the control groups ($P < 0.05$). No significant difference was observed at 12 mo ($P > 0.05$) (Figure 4).

Effects of chronic H. pylori infections on apoptosis of islet β-cells in Mongolian gerbils

Islet β-cell dysfunction is a critical factor during the pathogenesis of T2DM. β-cell mass may be decreased

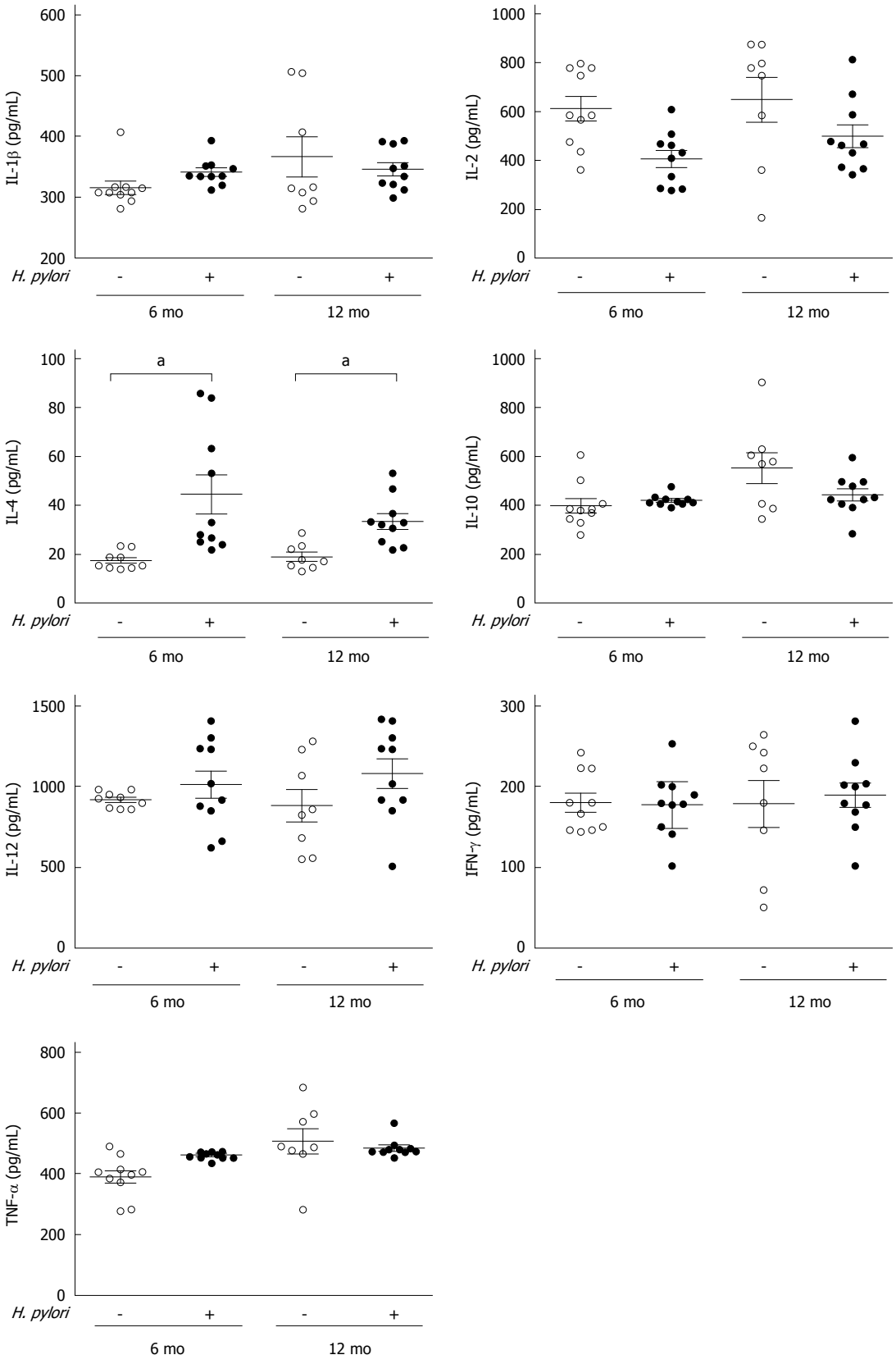

Figure 3 Effects of chronic *Helicobacter pylori* infection on the serum inflammatory cytokines in Mongolian gerbils at different time points. The serum levels of cytokines including IL-1β, IL-2, IL-12, IL-4, IL-10, IFN-γ, and TNF-α were measured by ELISA at 6 and 12 mo after *Helicobacter pylori* (*H. pylori*) infection. Data are presented as mean ± SEM from eight to ten mice per group. Cytokines were not significant different except IL-4 ($^aP < 0.05$ between *H. pylori* infected mice and their controls). IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon.

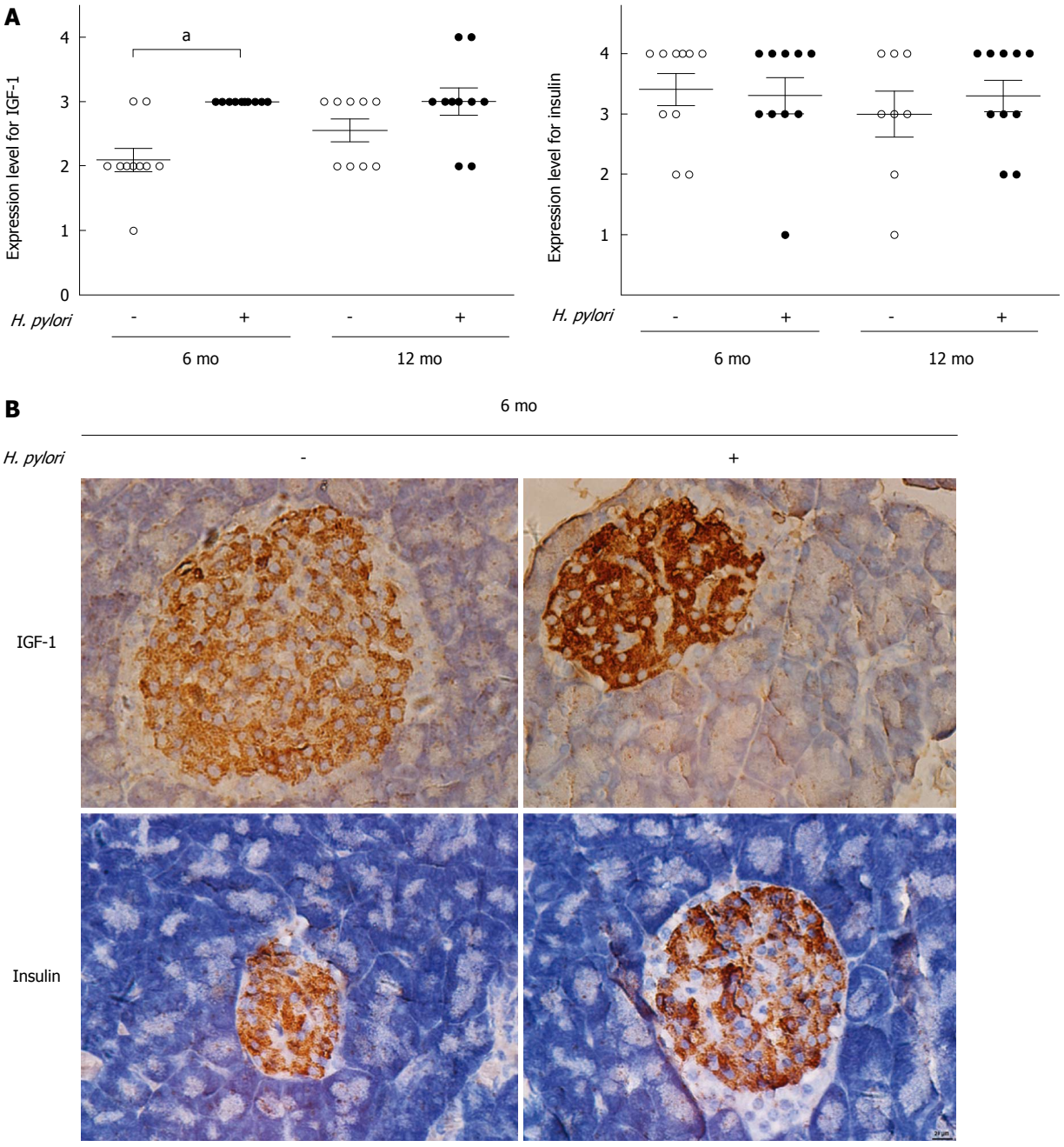

Figure 4 Effects of chronic *Helicobacter pylori* infection on the expression of insulin-like growth factor-1 and insulin in the pancreas of Mongolian gerbils. A: Immunohistochemistry scores of insulin-like growth factor-1 (IGF-1) and insulin were determined in the control and *Helicobacter pylori* (*H. pylori*) groups at 6 and 12 mo (n = 8-10). Data are expressed as the mean ± SEM. $^{a}P < 0.05$ between *H. pylori* infected mice at 6 mo and their controls concerning the expression of IGF-1; B: Representative immunohistochemical staining of IGF-1 and insulin expression in the pancreas of Mongolian gerbils at 6 mo after *H. pylori* infection (magnification × 400).

during T2DM, and the underlying cause may be increased β-cell apoptosis[19]. TUNEL assay was used to quantify the apoptotic rate of islet β cells at 6 and 12 mo after *H. pylori* infection in Mongolian gerbils. There was no significant difference in β-cell apoptosis between the *H. pylori* infected and control groups (P > 0.05) (Figure 5).

DISCUSSION

A growing body of epidemiological evidence supports a relationship between *H. pylori* infection and diabetes[9,10,20]. Simon *et al*[21] in 1989 first observed that the prevalence of *H. pylori* infection in patients with diabetes mellitus was significantly higher than that in asymptomatic controls. A recent meta-analysis also showed that the prevalence of *H. pylori* infection in patients with diabetes was higher than that of the control group[22]. Moreover, a recent prospective cohort study demonstrated that *H. pylori* infection leads to an increased rate of incident diabetes, which suggests a potential role for antibiotic and gastrointestinal

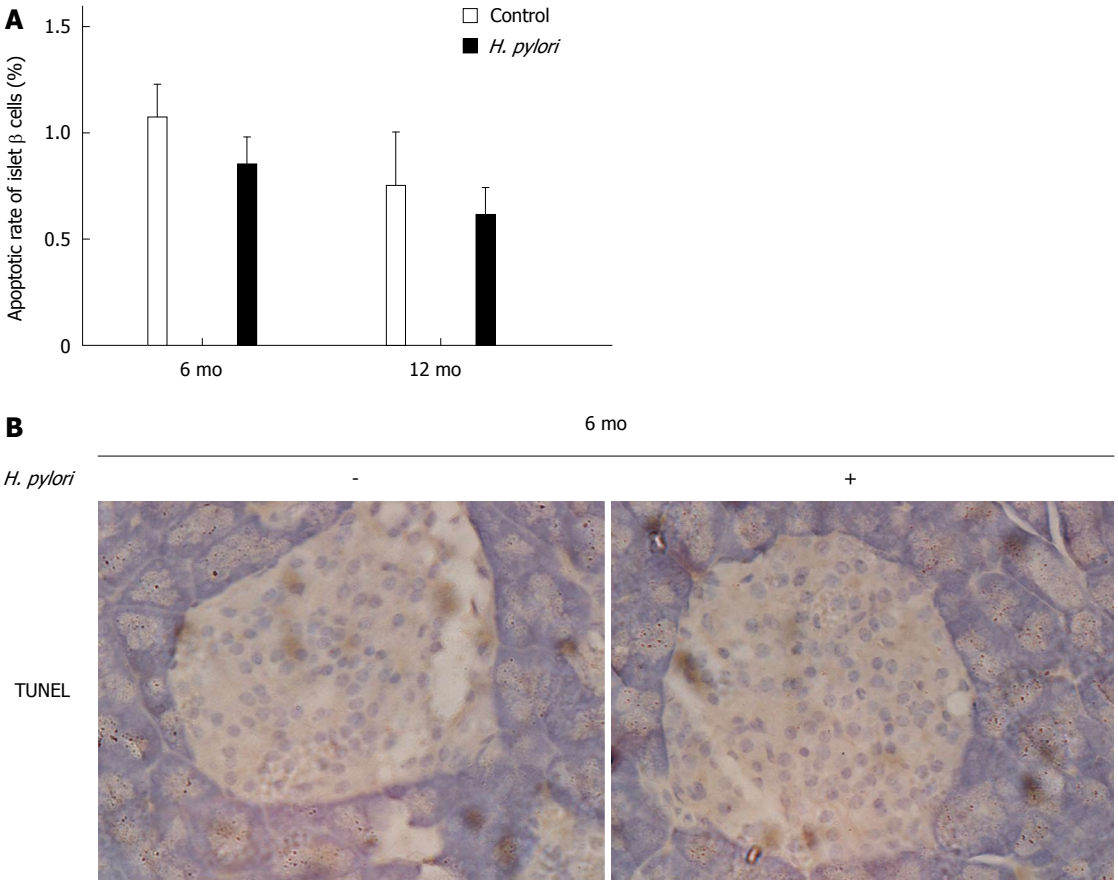

Figure 5 Effects of chronic *Helicobacter pylori* infection on the apoptosis of islet β-cells in Mongolian gerbils. A: Apoptotic rate of islet β-cells was detected by TUNEL assay in the control and *Helicobacter pylori* (*H. pylori*) groups at 6 and 12 mo. Data are expressed as the means ± SEM (n = 8-10 mice/group). $P > 0.05$ was observed between *H. pylori* infected mice and their controls; B: Representative images of islet β-cells in the pancreas of Mongolian gerbils at 6 mo after *H. pylori* infection by TUNEL staining (magnification × 400).

treatments to prevent diabetes[23]. These studies suggested that *H. pylori* infection might cause diabetes. Furthermore, patients with diabetes are more prone to *H. pylori* infections than non-diabetic individuals. There are several reasons for this phenomenon. First, the immune system of diabetic patients is compromised, leading to an increased susceptibility to *H. pylori* infection[24]. In addition, altered glucose metabolism may produce chemical changes in the gastric mucosa and reduce acid secretion and gastrointestinal mobility (thus promoting *H. pylori* colonization)[25]. However, other studies have denied this association[11,12]. To better understand whether *H. pylori* infection plays a role in diabetes etiology, research regarding diabetes biomarkers is needed.

HbA1c is the major fraction of GHb and results from non-enzymatic glycosylation. HbA1c reflects the integrated blood glucose levels during the preceding 3-4 mo. Thus, HbA1c levels are predictive of both the prevalence and incidence of diabetes and are a more stable measurement than fasting blood glucose for the diagnoses of pre-diabetes and diabetes[26-28]. Two large national surveys reported that *H. pylori* seropositivity, especially CagA+ strains, was associated with higher mean HbA1c levels and that there was a synergistic effect of *H. pylori* and BMI on increased HbA1c levels. These findings support a role for *H. pylori* in impaired glucose intolerance[29]. Similar results were observed by Hsieh *et al*[9]. The authors observed that long-term *H. pylori* infection was significantly associated with high HbA1c levels and a higher T2DM prevalence in Taiwanese patients.

In this study, we examined the levels of HbA1c in *H. pylori*-infected Mongolian gerbils and found a significant increase in HbA1c at 12 mo. This result indicates an association between *H. pylori* colonization and diabetes *in vivo*. However, fasting glucose was not significantly different between the *H. pylori* group and the control group (which is not contradictory to the significantly increased HbA1c levels). A previous study also reported a significant association between *H. pylori* infection and elevated HbA1c without changes in fasting glucose levels in humans[9]. Fasting glucose, which is a less stable measurement than HbA1c, is susceptible to changes in daily activities, such as diet content and exercise. These fluctuations may confound evaluations of the association between chronic *H. pylori* infection and glucose regulation. Moreover, fasting blood glucose levels markedly increase due to β-cell dysfunction and insulin secretion deficiency. Our study

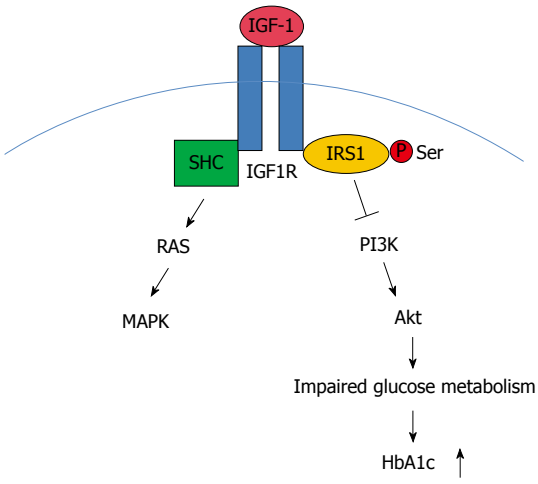

Figure 6 Insulin-like growth factor-1 signaling with regard to *Helicobacter pylori* associated glucose dysregulation. Insulin-like growth factor-1 (IGF-1), upon binding to its receptor IGF-1R, activates the intrinsic tyrosine kinase activity. IGF-1R then phosphorylates substrate proteins, including members of the IRS family such as IRS1 and Shc on selective tyrosine residues. Downstream to the receptors are two major pathways: the phosphatidylinositol-3 kinase (PI3K)/Akt pathway and MAPK pathway. IGF-1 acts *via* the MAPK pathway to mediate growth responses. The PI3K pathway is thought to have predominantly metabolic effects. We supposed that this pathway might play a part in *Helicobacter pylori* (*H. pylori*) associated abnormal glucose metabolism and the upregulation of HbA1c. SHC: Spontaneous human combustion.

showed that β-cell apoptosis and insulin expression in *H. pylori*-infected Mongolian gerbils were not different from those in the control group, which might contribute to the similarity in fasting glucose between the two groups. In contrast, another study demonstrated that gastric infection with some commensal strains of *H. pylori* ameliorates glucose homeostasis in mice and provides partial protection against some metabolic disorders[30]. The use of different bacterial and mice strains has produced inconsistent results, indicating that *H. pylori* may affect glucose metabolism in an individual/bacterial strain-dependent manner.

The mechanisms by which *H. pylori* infection increases HbA1c levels and potentiates the development of diabetes remain to be elucidated. Although insulin insensitivity is an early phenomenon, islet β-cell function declines gradually over time before the onset of clinical hyperglycemia and the occurrence of T2DM. IGF-1 is structurally similar to insulin and is also involved in the physiological regulation of nutrient intake, metabolism and tissue growth. Thus, IGF-1 contributes to the complex balance of anabolism and consumption. This balance can be disrupted during obesity and diabetes, and IGF-1 may be an important component[31]. It is commonly believed that pancreatic β-cell mass and function are crucial to whole body glucose regulation. Pancreatic islet cells produce IGF-1, which binds to IGF-1 receptors on β-cells[32]. IGF-1 was thought to be an important stimulus to pancreatic islet cell growth and to inhibit β-cell apoptosis[33,34]. The IGF-1 receptor and elements of the IGF-1 signal transduction pathway are expressed in pancreatic β-cells[35].

In our study, the expression of IGF-1 was significantly increased in *H. pylori*-infected Mongolian gerbils compared to the controls at 6 mo. No significant difference was observed at 12 mo. We assumed that there may be a time course accounting for the alteration in IGF-1 expression. At 6 mo, pancreatic IGF-1 probably increased to maintain normal HbA1c levels, and at 12 mo the HbA1c levels significantly increased due to the decompensation of pancreatic IGF-1. IGF-1, upon binding to its IGF-1 receptor, activates the intrinsic tyrosine kinase activity of the IGF-1 receptor[36]. The IGF-1 receptor then phosphorylates substrate proteins, including members of the IRS family, such as IRS1 and Shc, on selective tyrosine residues. Downstream of IGF-1 are two main pathways: phosphatidylinositol-3 kinase (PI3K)/Akt and MAPK[37]. IGF-1 acts *via* the MAPK pathway to mediate growth responses, and the PI3K pathway is thought to have predominantly metabolic effects, such as glucose uptake and Glut4 translocation. In addition to tyrosine phosphorylation, IRS proteins could undergo serine phosphorylation, which may attenuate signaling by decreasing normal tyrosine phosphorylation and promoting insulin resistance[38]. We propose that *H. pylori* infection-associated impaired glucose metabolism and HbA1c up-regulation may be mediated through the IGF-1 signaling pathway (Figure 6). However, insulin expression in the pancreas and the apoptotic rate of islet β-cells were not significantly different between the two groups, which suggests that the effects of *H. pylori* infection on glucose metabolism in Mongolian gerbils may not act through the injury of islet β-cells. It is well known that insulin resistance (IR) and abnormal insulin secretion are central to the development of T2DM, and IR is supposed to precede defects in insulin secretion[39]. A growing body of evidence has linked *H. pylori* infection to IR[40,41]. The limitation of our study is that we did not determine the insulin sensitivity of *H. pylori*-infected Mongolian gerbils at each time point. Therefore, it remains to be clarified whether *H. pylori* infection impairs glucose tolerance and subsequently breaks the balance of glucose metabolism.

Moreover, it is commonly believed that the chronic inflammation induced by *H. pylori* infection is the major link to T2DM. *H. pylori* infection, which leads to an increased production of lipopolysaccharides, may activate innate inflammatory processes[42]. Inflammatory processes correlate with elevated inflammatory cytokines, including C-reactive protein[43], IL-6 and TNF-α[44]. However, the inflammation hypothesis was not substantiated in our study. With the exception of IL-4, there were no significant differences in cytokines, including IL-1β, IL-2, IL-4, IL-10, IL-12, TNF-α and IFN-γ, between the *H. pylori* and the control groups. Interestingly, a similar phenomenon was reported by Jeon *et al*[23] who also found that serological evidence of *H. pylori* infection was associated with an increased

rate of incident diabetes in an elderly Latino cohort, and the inflammatory cytokines did not appear to mediate the effect. An alternative hypothesis is that *H. pylori*-induced gastritis can affect the secretion of "gastric" hormones, including leptin and ghrelin[45], which might predispose patients to diabetes.

There are conflicting data regarding the effect of *H. pylori* infection on serum lipids[46,47] and body indices, including body weight and BMI[48,49]. In the present study, TC and TG concentrations were not significantly different between Mongolian gerbils with *H. pylori* infection and the controls. The *H. pylori* group had an increased body weight, BMI and Lee' s index compared to the control group. However, these differences were not significant. A possible explanation for this observation may be in the putative manipulation of systemic ghrelin levels, which is the pivotal hormone regulating food intake and appetite. There are limited and conflicting data regarding the effects of *H. pylori* eradication on glucose metabolism and insulin sensitivity. Zojaji *et al*[50] showed that *H. pylori* treatment can improve the mean HbA1c and metabolic abnormalities in patients with T2DM. Thus, it may be beneficial for patients at risk of developing diabetes to be checked for *H. pylori* infections. Gen *et al*[51] also demonstrated that successful *H. pylori* eradication significantly decreased fasting insulin and HOMA-IR levels. To date, no study has investigated the effects of *H. pylori* eradication on glucose metabolism in Mongolian gerbils.

In conclusion, this study identified a significant association between chronic *H. pylori* infection and high HbA1c levels in Mongolian gerbils. Insulin expression in the pancreas and the apoptotic rate of islet β-cells were not significantly different after *H. pylori* infection. Inflammatory cytokines (IL-1β, TNF-α and IFN-γ) did not appear to mediate this effect. Further studies are warranted to explore the impact of *H. pylori* infections on insulin resistance and the corresponding mediating factors. The treatment of *H. pylori* infection is important for the prevention of gastric cancer. However, whether *H. pylori* infection is a risk factor for T2DM remains to be determined.

COMMENTS

Background

Epidemiological evidence has revealed that the outcome of *Helicobacter pylori (H. pylori)* infection may not be confined to the digestive tract, and that the infection can be associated with extra-digestive pathologies such as type 2 diabetes (T2DM). The studies regarding the relationship between *H. pylori* infection and T2DM are inconsistent and most in human. Thus, the authors assessed the effects of chronic *H. pylori* infection on metabolic parameters of Mongolian gerbils.

Research frontiers

Previous studies have found a higher prevalence of *H. pylori* infection in diabetic patients and recently a prospective cohort study further demonstrated that *H. pylori* infection leads to an increased rate of incident diabetes. Consequently, the current hotspot is whether *H. pylori* infection plays a role in the development of T2DM.

Innovations and breakthroughs

Epidemiological studies suggest that chronic *H. pylori* infection is associated with T2DM although it is still controversial. And there are few studies on the relationship between *H. pylori* infection and diabetes in animal models. This study found that chronic *H. pylori* infection significantly increased the glycated hemoglobin and glycated hemoglobin A1c levels in Mongolian gerbils. Besides, the expression of insulin growth factor-1 was significantly increased after *H. pylori* infection, with elevated serum level of IL-4.

Applications

These data provide evidence that long-term *H. pylori* infection is significantly associated with high levels of HbA1c in Mongolian gerbils, which is consistent with observations in human. Evidence supporting the role of *H. pylori* in the development of T2DM would provide *H. pylori* eradication as an easy and convenient preventive measurement for diabetes.

Terminology

H. pylori is a host-specific bacterial pathogen that establishes a chronic infection in the human gastric mucosa. T2DM is now becoming a global epidemic with risk factors associated with lifestyle, genetic background and socioeconomic factors.

Peer-review

This is an interesting study regarding the possible connection between *H. pylori* infection, glucose metabolism, HbA1c and diabetes. The methodology of the study is right, the results are interesting and the detailed discussion covers the topic and the research question.

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P- Reviewer: Diamantidis MD, Nakajima N
S- Editor: Yu J **L- Editor**: Wang TQ **E- Editor**: Liu XM

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12605

World J Gastroenterol 2015 November 28; 21(44): 12605-12611
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Case Control Study

Studied microRNA gene expression in human hepatocellular carcinoma by microRNA microarray techniques

Jian-Xiang Niu, Xing-Kai Meng, Jian-Jun Ren

Jian-Xiang Niu, Xing-Kai Meng, Jian-Jun Ren, Department of General Surgery of the Affiliated Hospital of Inner Mongolia Medical University, Huhehaote 010010, Inner Mongolia Autonomous Region, China

Author contributions: Niu JX and Ren JJ designed the research; Niu JX and Meng XK performed the research; Niu JX contributed new reagents; Meng XK contributed analytical tools; Niu JX and Ren JJ analyzed the data; Niu JX wrote the paper.

Supported by Inner Mongolia Medical College Millions of Science and Technology Project in 2011, No. NY2011bw004; the 2010 Inner Mongolia Health Bureau of Medical and Health Research Project, No. 2010038; Scientific Research Projects of the Inner Mongolia Autonomous Region High School in 2013, No. NJZY13416.

Institutional review board statement: This study was approved by the Medical Ethics Committee of Inner Mongolia Medical College.

Informed consent statement: All patients involved had previously provided their written, informed consent to have their clinical and pathogenic information used for research.

Conflict-of-interest statement: There was no conflict of interest for any of the authors.

Data sharing statement: There was no discussion of data sharing or its implications for the study participants.

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Correspondence to: Dr. Jian-Jun Ren, Department of General Surgery of the Affiliated Hospital of Inner Mongolia Medical University, No. 42 Zhaowuda Road, Saihan Region, Huhehaote 010010, Inner Mongolia Autonomous Region, China. renjianjunzml@163.com
Telephone: +86-471-3280800

Received: May 24, 2015
Peer-review started: May 25, 2015
First decision: June 25, 2015
Revised: July 26, 2015
Accepted: October 12, 2015
Article in press: October 13, 2015
Published online: November 28, 2015

Abstract

AIM: To achieve a better understanding of the molecular mechanisms of microRNA expression changes involved in hepatocellular carcinoma.

METHODS: In this research process, patients were not treated with antivirals, immunosuppressants or immunomodulators for at least 6 mo before collecting serum. The study population was composed of 35 outpatient hepatitis B virus (HBV) cases and 12 healthy control cases from the Affiliated Hospital of Inner Mongolia Medical University (Inner Mongolia, China) from July 2013 to April 2014. The 35 HBV cases were divided into two groups: a hepatocirrhosis group with 20 cases and a liver cancer group with 15 cases. All 35 cases carried HBsAg. The diagnostic criteria followed the European Association for the Study of the Liver 2012 (EASL2012) standards. MicroRNA (miRNA) was extracted from a control group of patients, a group with hepatocirrhosis and a group with liver cancer and its quality was analyzed using the human V2 microRNA expression beadchip. Cluster analysis and a radar chart were then applied to the miRNA changes.

RESULTS: The miRNA-qualified rate of human serum samples was 93%. The concentration of a single sample was > 200 ng/μL and the volume was > 5 μL.

All miRNA serum samples were uncontaminated by the genome. The Mann-Whitney test showed significant differences in miRNA between each group, with a detection *P*-value of < 0.05. Illumina software was set up with Diff Score set to ± 13, meaning that *P* = 0.001. There were significant changes in miRNA expression between the three groups. miRNA-183 was the most up-regulated, followed by miRNA-373. miRNA-129 and miRNA-188 were both strongly down-regulated and miRNA-378 was down-regulated a small amount. The liver cancer group had greater changes, which indicated that changes in miRNA expression levels were caused by hepatocirrhosis. The liver cancer disease course then further increased these changes. In the pentagon created by these five miRNAs, three groups showed significant deviation. The liver cancer group had a bigger deviation trend. The chart indicated that miRNA expression changes occurred in the hepatocirrhosis group, which increased in the liver cancer disease course and were irreversible.

CONCLUSION: There was a significant relationship between the irreversible up-regulation of miRNA-183/373 and down-regulation of miRNA-129/188/378 and incidences of hepatocirrhosis and liver cancer.

Key words: Hepatocellular carcinoma; MicroRNA; Expression; Microarray technologies; Radar chart

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Core tip: There was a better understanding of the molecular mechanisms of microRNA (miRNA) expression changes involved in hepatocellular carcinoma, associated patients with aggressive malignancy and poor prognosis. The 35 hepatitis B virus cases were divided into two groups, a hepatocirrhosis group with 20 cases and a liver cancer group with 15 cases. 12 healthy people were used as a control. There were significant changes in miRNA expression between the three groups. miRNA-183 was the most up-regulated, followed by miRNA-373. miRNA-129 and miRNA-188 were both strongly down-regulated and miRNA-378 was down-regulated a small amount. The liver cancer group had greater changes, which indicated that changes in miRNA expression levels were caused by hepatocirrhosis. The liver cancer disease course then further increased these changes.

Niu JX, Meng XK, Ren JJ. Studied microRNA gene expression in human hepatocellular carcinoma by microRNA microarray techniques. *World J Gastroenterol* 2015; 21(44): 12605-12611 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12605.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12605

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide, especially in East Asia and South Africa[1,2]. Due to its high malignant potential, HCC ranks as the third leading cause of cancer death in the world, resulting in almost 600000 deaths each year. Despite great improvements in treatment options, the long-term survival of patients with HCC remains unsatisfactory, with a 5-year survival rate of 20% to 30% reported in the literature. The treatment of advanced and metastatic HCC presents many challenging problems[3-5].

Carcinogenesis and the progression of HCC are multistage processes that involve many growth factors, oncogenes and tumor suppressor genes. Elucidating the molecular events underlying the tumorigenesis of HCC is important for its screening, prevention and treatment. In recent decades, many factors involved in the pathogenesis of HCC have been identified, including p53, Rb, PI3K, Akt, MAPK and many others[6-10]. However, the molecular mechanisms of HCC are still largely unknown.

Recent studies using DNA microarray techniques have identified unique gene expression profiles in hepatitis B and C virus-associated HCC[11,12]. Gene expression profiling also allows us to distinguish HCC from normal tissue or preneoplastic lesions and to evaluate metastatic or recurrent potential. These unique genes or gene products associated with malignant transformation and recurrent or metastatic potential may serve as molecular markers for early diagnosis and allow prediction of prognosis and responsiveness to therapy[13-15].

Although vaccination could reduce the incidence of hepatitis B, hepatocirrhosis and liver cancer remain major global health problems, the treatment of which poses many challenges. The World Health Organization reported 3.5 million new cases of chronic hepatitis in 2013 and 0.7 million deaths of chronic hepatitis patients from liver failure, cirrhosis and primary hepatocellular carcinoma[16-18]. The pathogenesis of HCC remains unclear. The inhibition microenvironment, comprising inhibitory receptors, inhibitory cells and immunosuppressant cytokines, can accelerate disease progression. In this study, the serum from hepatitis B virus (HBV) infected patients was used to analyze the different disease courses of HCC.

MicroRNAs (miRNAs) are short non-coding RNA molecules, similar to siRNAs. miRNAs consist of 20-25 nucleotides and their sequences are complementary to the 3′ untranslated regions of messenger RNAs. Binding results in inhibition of translation and gene silencing. Research has shown that thousands of human protein coding genes are regulated by miRNA[19-22].

It is suggested that miRNA plays an important role in the regulation processes of cell growth and development. miRNA is widely expressed in tissues and also in different types of cancer tissue. Research has shown that miRNAs can regulate the expression of target genes, thereby regulating liver cell proliferation and differentiation[20,21].

As such, miRNAs are significant in the ability of the liver to maintain normal physiological processes. To study the biological functions of miRNA, miRNA target genes must first be identified. Although miRNA has been studied intensely, the identification of miRNA target genes is still very challenging because each miRNA has hundreds of target genes and each miRNA can have a different regulatory process for each target gene. In recent years, studies have found that miRNA can bind to the 5′-UTR and the promoter region. This greatly increases the difficulty of studying miRNA but also provides ideas for understanding its complex biological functions[19-21].

The development of microarray technology, which allows us to undertake parallel analyses of many genes, has led to a new era in medical science. New genomic high-throughput technologies, such as RNA microarrays, considerably facilitate the molecular profiling of human tumors. Thousands of genes can now be analyzed in a simple hybridization microarray. The expression profile of a single tumor reflects the state of events of an individual malignancy at a certain time point. To generalize the findings and provide conclusive evidence for the involvement of a molecular alteration, it is often necessary to analyze several hundred tumors. Using traditional molecular pathology, such verification could take several months or even years to complete. To facilitate translational research in a large scale manner, technology was developed recently for making high density arrays of tumor tissue specimens. These arrays can be used for rapid miRNA evaluation of gene copy number and expression in thousands of tumors simultaneously. Current research is mostly concerned with the differentiation of one particular variable, such as HBV infection, environmental carcinogens, metastasis and recurrence, and sensitivity to chemo agents. It is rare for five kinds of gene expression profiling to be used. We have combined RNA microarray and TMA techniques to identify differentially expressed genes in the development and progression of human HCC[23-25]. In this study, we investigated the correlations between miRNA and clinical pathological characterization and our findings provide a comprehensive understanding of the molecular mechanisms of HCC and some new potential therapeutic targets.

MATERIALS AND METHODS

Survey and respondents

The study population was composed of 35 outpatient HBV cases and 12 healthy control cases from the Affiliated Hospital of Inner Mongolia Medical University (Inner Mongolia, China) from July 2013 to April 2014.

The 35 HBV cases were divided into two groups, a hepatocirrhosis group with 20 cases and a liver cancer group with 15 cases. All 35 cases carried HBsAg. The diagnostic criteria followed the European Association for the Study of the Liver 2012 (EASL2012) standards.

Patients with other hepatitis or HIV infections, other causes of liver damage, autoimmune disorders or neoplasm were excluded from the study, as well as pregnant or lactating women. In this research process, patients were not treated with antivirals, immunosuppressants or immunomodulators for at least 6 mo before collecting serum.

Medical Ethics Committee of Inner Mongolia Medical College approval was obtained and all patients involved had previously provided their written, informed consent to have their clinical and pathogenic information used for research.

Extracted miRNA

Blood samples were collected at the first hospital visit. Serum was separated and stored under -80 ℃. MiRNApure Mini Kit (Cat. No. CW0627) was purchased from CWBiotech Ltd. Nucleic acids and miRNA were extracted separately from the serum in accordance with the instructions.

Major equipment

Electrophoresis apparatus and slot were DYY-6B and CQU-200, the gel imaging instrument was Gel Doc 2000, and the spectrophotometer was NanoDrop 2000.

Statistical analysis

Logarithms were used to convert data with positive skew into a normal distribution. For homogenous data, analysis of variance, Student-Newman-Keuls and Pearson's correlation were used. For inhomogeneous data, Kruskal-Wallis, Games-Howell and Spearman's correlation analysis were used. All analyses were carried out using SPSS 17.0 software (SPSS Inc, Chicago, IL, United States). Values less than 0.05 were considered to be statistically significant.

RESULTS

Quality inspection of miRNA

The miRNA-qualified rate of human serum samples was 93%. Three serum samples did not meet the requirements. Optical densities at 260-280 nm were between 1.7 and 2.1. The concentration of a single sample was > 200 ng/μL and the volume was > 5 μL. All miRNA serum samples were uncontaminated by the genome. The results of electrophoresis of miRNA in a 1.2% agarose gel are shown in Figure 1, displaying the correct molecular weight and a high concentration of miRNA.

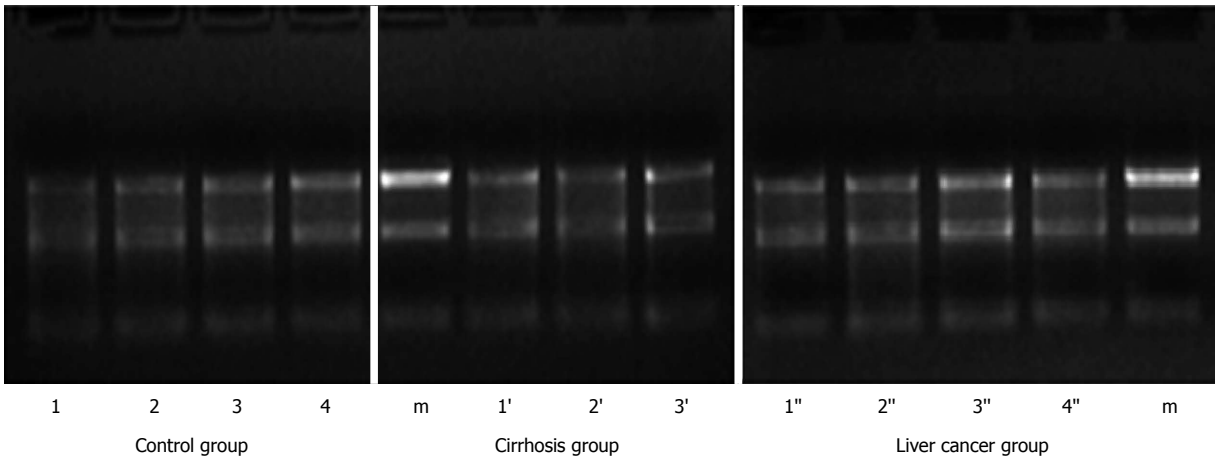

Figure 1 The result of electrophoresis of microRNA by 1.2% agarose gel. m: RNA marker; Arabic numbers: Representative of the specimen's number.

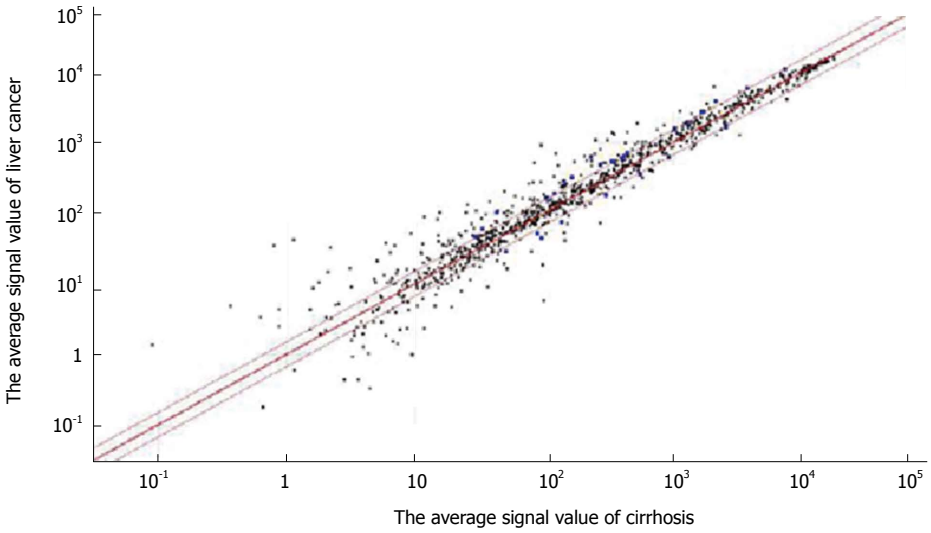

Figure 2 Scatter diagram of the calculation of microRNA expression by BeadArray reader software (Illumina).

miRNA gene expression profiling

Human miRNA from the Illumine Corporation were used as the gene chip, tested on the Illumine BeadArray. The human V2 miRNA expression beadchip was tested for quality control, including a negative control, a PAP control, query oligo annealing controls, mismatch controls, array hybridization controls and contamination controls.

Calculation of miRNA expression

GenomeStudio (Illumina) was used to calculate the signal value of each point on the gene chip after scanning by the BeadArray Reader software (Illumina). The Mann-Whitney test showed significant differences in miRNA between each group, with a detection p-value of < 0.05. Illumina software was set up with Diff Score set to ± 13, meaning that $P = 0.001$. The scatter diagram shown in Figure 2 was drawn by the software, without the negative logarithm loop. The red line is the cut-off line: the upper parts were > 1.5-fold and the lower parts were < 0.67-fold.

Cluster analysis

Cluster3.0 software was used to analyze the differences in miRNA between each group. MiRanda was used for miRNA target prediction (http://www.microrna.org) which is shown in Figure 3. Three groups showed significant changes in miRNA expression. The expression of five important miRNAs changed. miRNA-183 was the most up-regulated, followed by miRNA-373. miRNA-129 and miRNA-188 were greatly down-regulated and miRNA-378 was down-regulated a small amount.

The quantization map

The quantization map shows the expression difference in human serum between these five miRNAs. The radar chart in Figure 4 shows the changes in each miRNA in comparison to the baseline of the control group.

In the pentagon created by these five miRNAs, three groups showed significant deviation. The liver cancer group had a bigger deviation trend. The chart

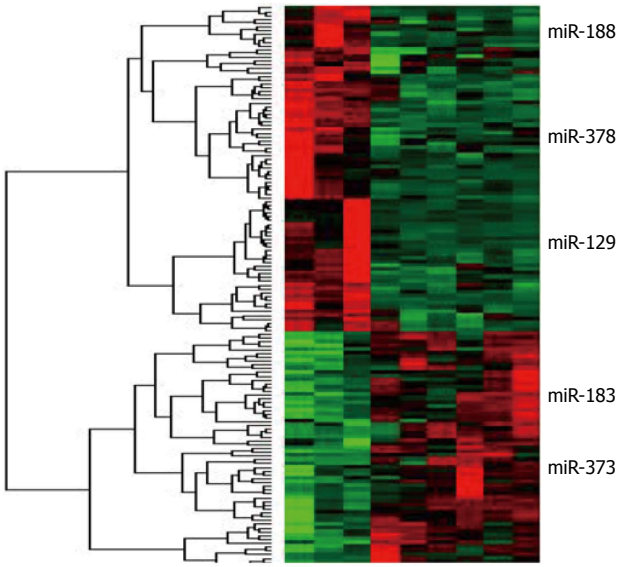

Figure 3 The results of the microRNA cluster analyzed.

indicated that miRNA expression changes occurred in the hepatocirrhosis group, which increased in the liver cancer disease course and were irreversible.

DISCUSSION

Hepatocellular carcinoma, an aggressive malignancy with poor prognosis and one of the most common tumors in humans, has become a leading cause of cancer-related death in adults from Asia and Africa. Despite advances in the diagnosis and treatment of HCC, the prognosis of patients with HCC remains dismal. The poor prognosis of HCC has been associated with recurrence and metastasis[17-24]. Therefore, a better understanding of the molecular mechanisms involved in HCC development and metastasis is needed.

Early diagnosis is essential for cancer prevention and control. Previously, we found that a specific course of liver cancer disease had a different protein expression and different miRNA regulation. Recurrent chromosomal aberrations are often observed in HCC but little is known about the role of functional non-coding sequences, particularly miRNA, at the chromosomal breakpoints[14-16].

miRNAs are small non-coding RNAs that function as key regulators of gene expression at the post-transcriptional level. They play important roles in cell proliferation, differentiation and apoptosis.

A study from Anhui Medical University investigated the functions of miRNA-183 in HCC and discussed the construction of an artificial miRNA cluster expression vector[26]. The miRNA-183 expression profile from HCC tumor tissues and adjacent normal liver tissues were compared using real-time PCR. The results showed that miRNA-183 was significantly up-regulated (2 to 300 fold) in 68% of tumors. The author suggested that

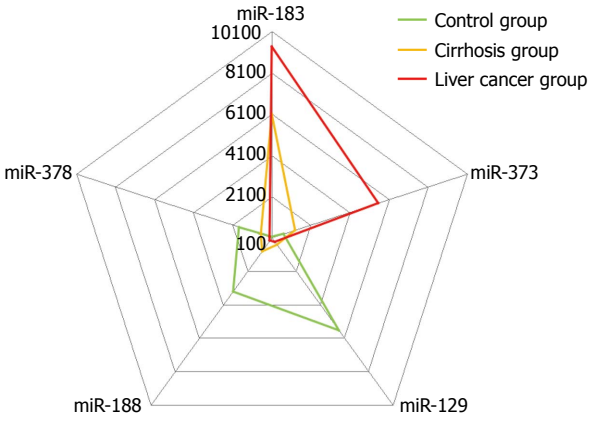

Figure 4 Radar chart showing the changes of each microRNA.

miRNA-183, which is up-regulated in HCC, repressed the expression of the tumor-suppressor PDCD4 post-transcriptionally and inhibited TGF-β1-induced apoptosis in human HCC cells. Therefore, miRNA-183 may play an important role in HCC development. Their artificial miRNA cluster efficiently expressed mature miRNA and miRNA procession suppressed the expression of the protein with miRNA host genes. Moreover, their artificial miRNA cluster on cell cycle arrest was more effective than single miRNAs.

Disturbance of miRNA expression and function results in tumors. Different miRNA have different roles as they regulate the expression of different target genes. miRNAs that originate from the same pre-miRNA transcript can have different functions, such as miRNA-371/miRNA-373. A study from Kunming University of Science and Technology showed that the molecular mechanisms of miRNA-373 related to the occurrence and development of tumors[27]. The real-time quantitative PCR results showed that there was a 6.684-fold decrease in miRNA-373 in BCSCs compared to MCF-7 cells and that EIF4A1 was a target gene of miRNA-373. However, our study found that more miRNA-373 was found in serum from patients with liver cancer and hepatocirrhosis, which may be due to differences in the cancer types.

The results from the bioinformatics prediction showed that miRNA-373 targeted genes involved in cell proliferation, apoptosis, cell cycle regulation, cell signal transduction, ontogenesis, tumor suppression and other closely related genes involved in tumor development and metastasis. These results indicate that some of the imbalance between the expression levels of key miRNAs might be important in breast and liver cancer occurrence.

A study from the National Engineering Center for Biochip in Shanghai investigated the diagnostic value of serum miRNA, including miRNA-129 in colorectal cancer (CRC)[28,29]. They identified 10 serum-specific miRNAs from patients with CRC. A set of serum-specific miRNAs, including miRNA-129, that are considered to be biomarkers for CRC detection were

validated by RFQ-PCR. The area under the receiver operating characteristic curve for this set of serum miRNAs reached a maximum value of 0.914 with a sensitivity of 77.78% and a specific sensitivity of 100% for CRC. The set of serum-specific miRNA-129s may become a group of feasible and effective indices in the screening and early diagnosis of CRC, which we also found in our study.

A study from Central South University found that miRNA-188 was frequently down-regulated in HCC[30]. miRNA-188 expression was correlated with clinicopathological characteristics and the prognosis of HCC. miRNA-188 inhibited HCC cell proliferation and metastasis *in vitro*. Its direct downstream target was AAC11.

A study from Soochow University found that miRNA-378 may suppress the growth characteristics of HBV-related HCC by directly targeting the IGF 1R 3′-UTR and inhibiting its expression[31].

Additionally, it was reported that 22 miRNAs were often amplified or deleted in HCC and that miRNA-151 was correlated with intrahepatic metastasis of HCC, which we did not find in our study[32]. miRNA-151 was often expressed together with its host gene FAK, focal adhesion kinase, which significantly increased HCC cell migration and invasion *in vitro* and *in vivo*, mainly through miRNA-151-5p, but not through miRNA-151-3p. As such, more research into miRNA, tumor invasion and metastasis of HCC is needed.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide, especially in East Asia and South Africa. MicroRNAs (miRNAs) are short non-coding RNA molecules, similar to siRNAs. Recent studies using DNA microarray techniques have identified unique gene expression profiles in hepatitis B and C virus-associated HCC. As such, a better understanding of the molecular mechanisms of miRNA expression changes involved in HCC is needed.

Research frontiers

A study from Anhui Medical University investigated the functions of miRNA-183 in HCC and discussed the construction of an artificial miRNA cluster expression vector. A study from Kunming University of Science and Technology showed that the molecular mechanisms of miRNA-373 related to the occurrence and development of tumors. A study from the National Engineering Center for Biochip in Shanghai investigated the diagnostic value of serum miRNA, including miRNA-129, in colorectal cancer. A study from Central South University found that miRNA-188 was frequently down-regulated in HCC.

Innovations and breakthroughs

A better understanding of the molecular mechanisms involved in HCC development and metastasis is needed. Early diagnosis is essential for cancer prevention and control. Previously, the authors found that a specific course of liver cancer disease had different protein expression and different miRNA regulation. Cluster analysis and a radar chart were applied to the miRNA changes. By using a gene chip, large-scale integration detection could be made at one time which was more meaningful for the same specimen.

Applications

Early diagnosis is essential for cancer prevention and control. The conclusion from the present study is helpful for early diagnosis and intervention. Drug development might be a process change of miRNA expression.

Peer-review

Overall, this study is well designed and the manuscript is well written.

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P- Reviewer: Maneesh D, Mulvihill SJ **S- Editor**: Yu J
L- Editor: Roemmele A **E- Editor**: Ma S

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12612

World J Gastroenterol 2015 November 28; 21(44): 12612-12619
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Retrospective Cohort Study

Laparoscopic gastric bypass *vs* sleeve gastrectomy in obese Korean patients

Ji Yeon Park, Yong Jin Kim

Ji Yeon Park, Yong Jin Kim, Department of Surgery, Soonchunhyang University Seoul Hospital, Seoul 140-743, South Korea

Ji Yeon Park, Department of Surgery, National Cancer Center, Goyang-si, Gyeonggi-do 410-769, South Korea

Author contributions: Kim YJ designed and performed the research; Park JY analyzed the data and wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Soonchunhyang University Seoul Hospital Institutional Review Board (SCHUH 2014-12-006).

Conflict-of-interest statement: Drs. Ji Yeon Park and Yong Jin Kim have no financial ties to disclose.

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Correspondence to: Yong Jin Kim, MD, PhD, Department of Surgery, Soonchunhyang University Seoul Hospital, 59, Daesagwan-ro, Yongsan-gu, Seoul 140-743, South Korea. yjgs1997@gmail.com
Telephone: +82-2-7099479
Fax: +82-2-7951687

Received: March 16, 2015
Peer-review started: March 17, 2015
First decision: May 18, 2015
Revised: May 26, 2015
Accepted: August 29, 2015
Article in press: August 31, 2015
Published online: November 28, 2015

Abstract

AIM: To compare the mid-term outcomes of laparoscopic sleeve gastrectomy (LSG) and laparoscopic Roux-en-Y gastric bypass (LRYGB) in obese Korean patients.

METHODS: All consecutive patients who underwent either LSG or LRYGB with primary to treat morbid obesity between January 2011 and December 2012 were retrospectively reviewed. Patients with a body mass index (BMI) $\geqslant$ 30 kg/m^2 with inadequately controlled obesity-related comorbidities (*e.g.*, diabetes, obstructive sleep apnea, hypertension, or obesity-related arthropathy) or BMI $\geqslant$ 35 kg/m^2 were considered for bariatric surgery according to the International Federation for the Surgery of Obesity-Asia Pacific Chapter Consensus statements in 2011. The decision regarding the procedure type was made on an individual basis following extensive discussion with the patient about the specific risks associated with each procedure. All operative procedures were performed laparoscopically by a single surgeon experienced in upper gastrointestinal surgeries. Baseline demographics, perioperative surgical outcomes, and postoperative anthropometric data from a prospectively established database were thoroughly reviewed and compared between the two surgical approaches.

RESULTS: One hundred four patients underwent LSG, and 236 underwent LRYGB. Preoperative BMI in the LSG group was significantly higher than that of the LRYGB group (38.6 kg/m^2 *vs* 37.2 kg/m^2, P = 0.024). Patients with diabetes were more prevalent in the LRYGB group (18.3% *vs* 35.6%, P = 0.001). Operating time and hospital stay were significantly shorter in the LSG group compared with the LRYGB group (100 min *vs* 130 min, P < 0.001; 1 d *vs* 2 d, P = 0.003), but the incidence of perioperative complications was similar between the groups (P = 0.351). The mean percentage of excess weight loss (%EWL) was 71.2% for LRYGB, while it was 63.5% for LSG, at mean follow-up periods of 18.0 and 21.0 mo, respectively (P = 0.073). The %EWL at 1, 3, 6, 12, 18, 24, and 36 mo was equivalent

between the groups. Four patients required surgical revision after LSG (4.8%), while revision was only required in one case following LRYGB (0.4%; P = 0.011).

CONCLUSION: Both LSG and LRYGB are effective procedures that induce comparable weight loss in the mid-term and similar surgical risks, except for the higher revision rate after LSG.

Key words: Morbid obesity; Bariatric surgery; Roux-en-Y gastric bypass; Sleeve gastrectomy; Weight loss

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Core tip: Both laparoscopic sleeve gastrectomy (LSG) and laparoscopic Roux-en-Y gastric bypass (LRYGB) are effective procedures that result in comparable weight loss in the mid-term with similar surgical risks in obese Korean patients. However, a larger number of patients required revisional surgery following LSG than LRYGB. The long-term complications encountered after each procedure differed significantly, and these complications were not negligible. Surgeons should provide a tailored surgical option for each patient that takes into consideration the possible risks, as the long-term complications may have a significant influence on the quality of life following the surgery.

Park JY, Kim YJ. Laparoscopic gastric bypass *vs* sleeve gastrectomy in obese Korean patients. *World J Gastroenterol* 2015; 21(44): 12612-12619 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12612.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12612

INTRODUCTION

Obesity is one of the most concerning health problems in the world today, imposing a considerable financial burden on society[1]. Consistent effort has been made to enable individuals to achieve weight loss and, concomitantly, to manage a variety of obesity-related comorbidities. However, none of the currently available conservative measures has succeeded in realizing these goals, and at the present time, bariatric surgery has proven to be the most effective method for achieving sustained weight loss[2].

Among the various available options for bariatric surgery, Roux-en-Y gastric bypass has been considered the gold standard for several decades. This procedure has a relatively long history compared to the other available procedures, qualified with sufficient data involving satisfactory long-term outcomes in terms of durable weight loss and resolution of comorbidities[3]. Recently, however, laparoscopic sleeve gastrectomy (LSG) has been rapidly gaining popularity as a stand-alone treatment for morbid obesity[4]. It is thought to be technically less demanding and to offer a potential benefit of reduced risk of long-term complications compared to laparoscopic Roux-en-Y gastric bypass (LRYGB). The trend of exponential increase in LSG is even noticeable in Asian countries where bariatric surgery has only recently been introduced[4], although results regarding the long-term efficacy of LSG are still lacking.

The present study aimed to evaluate the mid-term efficacy of LSG and LRYGB and to compare the results between the two procedures in obese Korean patients at a single center.

MATERIALS AND METHODS

All patients who were operated on at Soonchunhyang University Seoul Hospital, a tertiary referral medical center, between January 2011 and December 2012 were retrospectively reviewed. Of those, the patients who underwent either LRYGB or LSG with primary intent to treat morbid obesity were enrolled in the present study. Baseline, operative, and follow-up data from a prospectively established database were thoroughly reviewed and summarized. Approval for this review of hospital records was obtained from the Institutional Review Board (SCHUH 2014-12-006); the need for patient informed consent was waived.

Bariatric surgery candidates were selected according to the International Federation for the Surgery of Obesity-Asia Pacific Chapter Consensus statements in 2011[5]. As such, patients with a body mass index (BMI) $\geqslant$ 30 kg/m^2 with inadequately controlled obesity-related comorbidities (*e.g.*, diabetes, obstructive sleep apnea, hypertension, or obesity-related arthropathy) or with a BMI $\geqslant$ 35 kg/m^2 were considered for bariatric surgery. The decision regarding the procedure type was made on an individual basis following extensive discussion with the patient about the specific risks associated with each procedure. Patients received interdisciplinary education about potential surgical and nonsurgical options, possible outcomes, possible complications, and necessary postoperative lifestyle changes and nutritional supplementation.

Surgical procedures

All operative procedures were performed laparoscopically by a single surgeon experienced in upper gastrointestinal surgeries. Six trocars were used both in LSG and LRYGB; one 11-mm port for a scope at the umbilicus, two 12-mm ports, and three additional 5-mm ports. A 34 Fr bougie dilator was used for guidance during gastric resection in LSG. The lengths of the alimentary and biliopancreatic limbs were estimated at about 70-100 cm and 50 cm, respectively, and a 15-20 mm sized linear stapled gastrojejunostomy was established in LRYGB. Detailed surgical procedures were well described in our previously published study[6].

Table 1 Preoperative demographics and clinical characteristics of the enrolled patients

	LSG (n = 104)	LRYGB (n = 236)	P value[1]
Age (yr)	31 (25-38)	38 (29-46)	< 0.001
Sex			
Male	41 (39.4)	37 (15.7)	< 0.001
Female	63 (60.6)	199 (84.3)	
Body weight (kg)	107.0 (95.0-130.8)	100.0 (87.0-116.8)	< 0.001
BMI (kg/m^2)	38.6 (34.8-43.8)	37.2 (33.6-41.7)	0.024
Excess weight (kg)[2]	43.1 (33.5-62.6)	38.4 (28.1-52.6)	0.003
Comorbidities			
Diabetes	19 (18.3)	84 (35.6)	0.001
Hypertension	28 (26.9)	88 (37.3)	0.082
Dyslipidemia	64 (67.4)	116 (68.2)	0.892
OSA			
Confirmed	10 (10.5)	24 (14.1)	0.514
Suspicious	4 (4.2)	11 (6.5)	
Arthropathy	12 (12.6)	31 (18.2)	0.298
GERD	14 (13.5)	19 (8.1)	0.163
PCOS[3]	12 (19.0)	30 (15.1)	0.554
No. of comorbidities	1.5 (1-2)	2 (1-3)	0.031

Data are presented as n (%) or median (interquartile range). [1]Mann-Whitney U test for continuous variables and Pearson's χ^2 test or Fisher's exact test for categorical variables were applied; [2]The excess weight was calculated from the ideal weight using a BMI of 23 kg/m^2 as the upper limit of normal according to the World Health Organization recommended definition of obesity for Asians; [3]The incidence among female patients. LSG: Laparoscopic sleeve gastrectomy; LRYGB: Laparoscopic Roux-en-Y gastric bypass; BMI: Body mass index; OSA: Obstructive sleep apnea; GERD: Gastro-esophageal reflux disease; PCOS: Polycystic ovarian syndrome.

Postoperative data collection and follow-up data analysis

Patients returned to the outpatient clinic 2 wk after surgery and then every 3 mo for the first postoperative year to monitor weight loss, dysphagia or food intolerance, eating behavior, comorbidity status, and the presence of any complications. Follow-up frequency was then increased to every 12 mo after the first year. Telephone interviews were also used to monitor patients who were unable to visit the outpatient clinic.

The degree of weight loss was expressed as the percentage of total weight loss (%TWL) and the percentage of excess weight loss (%EWL), with the calculation of ideal body weight as that equivalent to a BMI of 23 kg/m^2 according to the World Health Organization (WHO)-recommended definition of obesity for Asians[7].

Statistical analysis

Statistical analysis was performed using SPSS version 18 for Windows (SPSS Inc., Chicago, IL, United States). Medians with interquartile ranges of the variables were calculated and compared between the two different procedures. The χ^2 test or Fisher's exact test was applied to analyze categorical variables, while Mann-Whitney U test was used for continuous variables. All tests were two-tailed and P values < 0.05 were considered significant.

Table 2 Surgical outcomes according to the surgical procedures

	LSG (n = 104)	LRYGB (n = 236)	P value[1]
Combined operation	5 (4.8)	19 (8.1)	0.282
Operating time (min)	100 (90-115)	130 (110-150)	< 0.001
Intraoperative blood loss	100 (50-150)	100 (50-200)	0.010
Length of hospital stay (d)	1 (1-2)	2 (1-2)	0.003
Intraoperative complication	1 (1.0)	4 (1.7)	> 0.999
Postoperative complication[2]			
No	95 (91.3)	202 (85.6)	0.351
Yes			
Mild	7 (6.7)	25 (10.6)	
Moderate	0 (0)	5 (2.1)	
Severe	2 (1.9)	4 (1.7)	
Re-admission	3 (2.9)	8 (3.4)	> 0.999

Data are presented as n (%) or median (interquartile range). [1]Mann-Whitney U test for continuous variables, and Pearson's χ^2 test or Fisher's exact test for categorical variables were applied; [2]The severity of postoperative complications were classified according to the Accordion Severity Grading System. LSG: Laparoscopic sleeve gastrectomy; LRYGB: Laparoscopic Roux-en-Y gastric bypass.

RESULTS

A total of 340 consecutive patients underwent either LSG or LRYGB for morbid obesity during the study period and were included in the study. One hundred four patients (30.6%) underwent LSG, while 236 patients (69.4%) underwent LRYGB. The demographic characteristics are shown in detail in Table 1. In the LSG group, the patients were younger, and the proportion of males was greater (P < 0.001 for both factors) than the LRYGB group. Preoperative BMI was 38.6 kg/m^2 [interquartile rage (IQR), 34.8-43.8] in the LSG group, which was significantly higher than the BMI of 37.2 kg/m^2 (IQR, 33.6-41.7) for the LRYGB group (P = 0.024). Patients with diabetes were more prevalent in the LRYGB group (35.6% *vs* 18.3%, P = 0.001), while the incidence of other obesity-related comorbidities was similar between the two groups.

The mean operating time and the length of hospital stay were significantly shorter in the LSG group than in the LRYGB group (100 min *vs* 130 min, P < 0.001; 1 d *vs* 2 d, P = 0.003; Table 2). There was one patient in whom the scheduled LRYGB was converted to LSG because of severe adhesions between small bowel loops associated with a previous history of panperitonitis. The left gastroepiploic vessels were injured during LSG in one patient, but there was no further evidence of ischemia. Technical failure of gastrojejunostomy reconstruction was encountered for four patients in the LRYGB group; successful laparoscopic revision was accomplished for all during the surgery. The incidence and severity of postoperative complications did not statistically differ between the groups (P = 0.351). Most complications in the LSG group were minor, involving operative wound or dietary problems; two severe complications were

Table 3 Anthropometric outcomes at last follow-up

	LSG (n = 104)	LRYGB (n = 236)	P value[1]
Mean follow-up period (mo)	21.0 (14.5-28.0)	18.0 (12.0-24.0)	0.012
At last follow up			
Body weight (kg)	81.5 (64.0-98.5)	72.0 (63.0-85.0)	< 0.001
BMI (kg/m^2)	28.5 (24.5-34.2)	27.3 (24.1-30.2)	0.014
%EWL (%)[2]	63.5 (44.8-88.0)	71.2 (53.7-91.1)	0.073
%TWL (%)	25.0 (19.4-32.9)	26.7 (20.0-32.4)	0.394
EWL < 50% at 1 year	18 (21.2)	23 (13.6)	0.148
Revision	5 (4.8)	1 (0.4)	0.011

Data are presented as n (%) or median (interquartile range). [1]Mann-Whitney U test for continuous variables and Pearson's χ^2 test or Fisher's exact test for categorical variables were applied; [2]A BMI of 23 kg/m^2 was adopted as the upper limit of normal to calculate %EWL according to the World Health Organization recommended definition of obesity for Asians. LSG: Laparoscopic sleeve gastrectomy; LRYGB: Laparoscopic Roux-en-Y gastric bypass; BMI: Body mass index; EWL: Excess weight loss; TWL: Total weight loss.

related to intra-abdominal bleeding in the immediate postoperative period that required reoperation to achieve hemostasis. Meanwhile, more than half of the complications (18/34, 52.9%) were associated with postoperative bleeding in the LRYGB group; 12 of these were mild, four were moderate, and two were severe complications. The overall incidence of postoperative bleeding was 7.6%, where two-thirds of the cases presented as luminal bleeding and one-third presented as intra-abdominal bleeding. Clinically significant hemorrhage requiring transfusion or invasive intervention occurred in 10 patients (4.2%) undergoing LRYGB. Other severe complications included one case of gastric pouch leakage and one intestinal obstruction; both of these required surgical intervention.

Patients were followed up for an average of approximately 21.0 mo and 18.0 mo in the LSG and LRYGB groups, respectively (Table 3). Although the postoperative BMI was significantly higher in the LSG group than in the LRYGB group (28.5 kg/m^2 *vs* 27.3 kg/m^2, P = 0.014) at the last follow-up, the %EWL and %TWL were similar between the groups (63.5% *vs* 71.2%, P = 0.073; 25.0% *vs* 26.7%, P = 0.394). The proportion of patients who had failed to achieve 50% of EWL 1 year postoperatively was larger in the LSG group (21.2%) than in the LRYGB group (13.6%), but the difference was not statistically significant (P = 0.148). Five patients in the LSG group (4.8%) required revisional surgery following the initial procedure because of intolerable *de novo* reflux disease (n = 2) and insufficient weight loss (n = 3). On the other hand, only one patient (0.4%) who had undergone LRYGB requested revision, RYGB reversal, due to malnutrition, and the rate of revision was significantly lower than in the LSG group (P = 0.011).

The chronological changes in anthropometric data during the follow-up period are shown in Figure 1. The body weight and BMI of the LSG group were generally

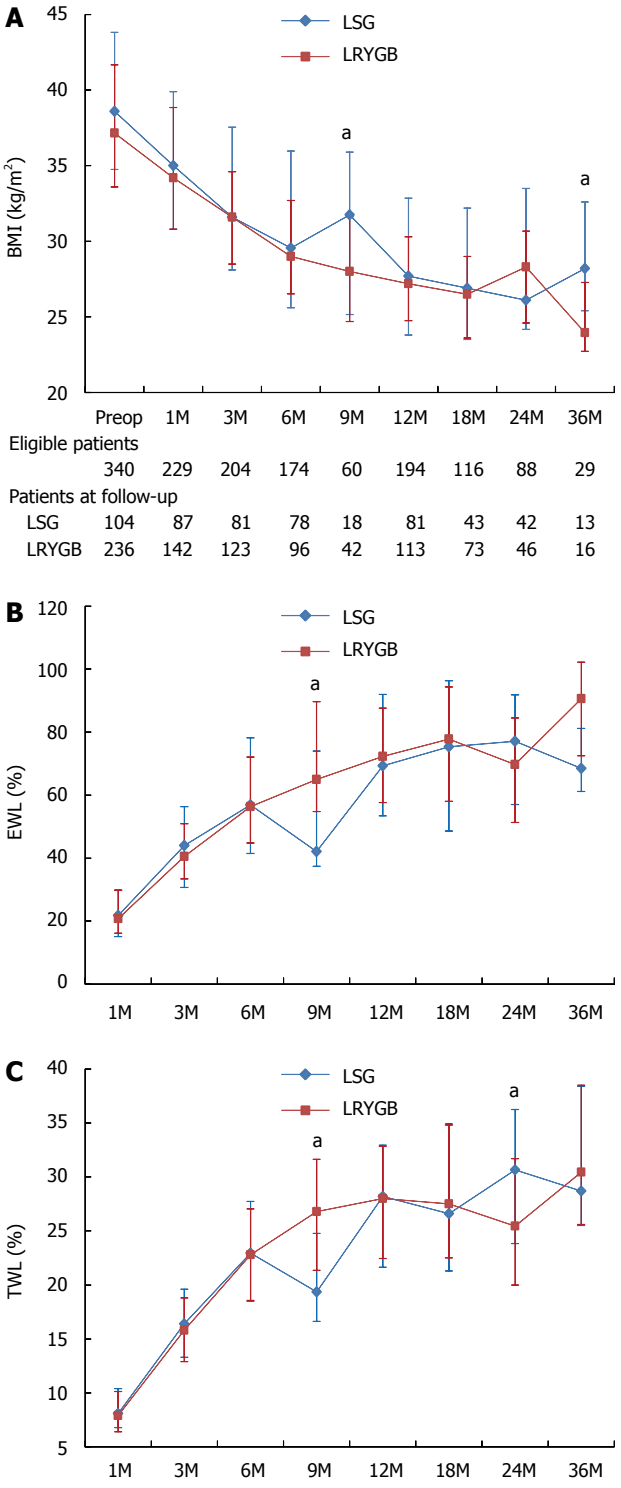

Figure 1 Chronological changes in anthropometric outcomes. A: Body mass index; B: Percentage of excess weight loss; C: Percentage of total weight loss. Medians are used to depict the values, and error bars indicate the interquartile range. [a]P < 0.05 between groups. LSG: Laparoscopic sleeve gastrectomy; LRYGB: Laparoscopic Roux-en-Y gastric bypass; BMI: Body mass index; EWL: Excess weight loss; TWL: Total weight loss.

higher than those of the LRYGB group throughout the study period. However, there were no significant differences between the LSG and LRYGB groups in %EWL and %TWL, which plateaued at around 80% and 30%, respectively, in both groups.

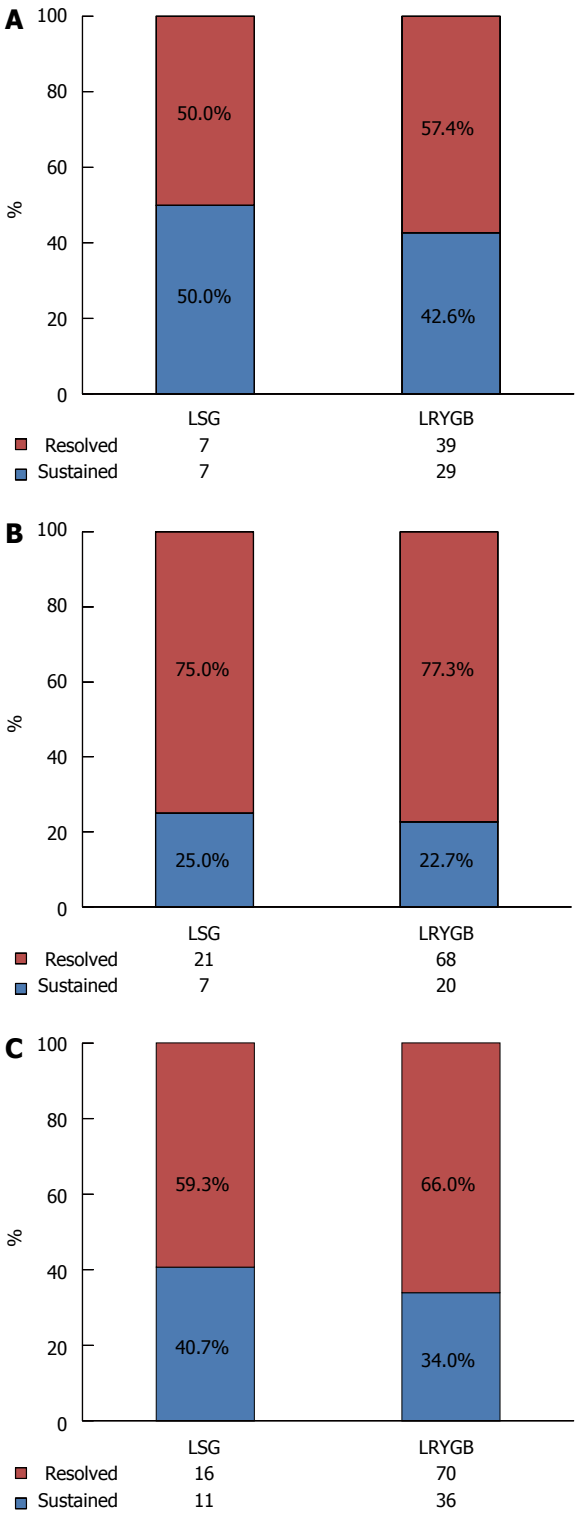

Figure 2 Resolution of comorbidities. A: Diabetes; B: Hypertension; C: Dyslipidemia. $P = 0.769$, 0.801, and 0.653, respectively.

The obesity-related comorbidities were resolved in a considerable number of patients in both groups. The overall resolution rates of the obesity-related comorbidities were 56.1% for type 2 diabetes, 76.7% for hypertension, and 64.7% for dyslipidemia in the entire study population. No difference was observed between the LSG and LRYGB groups regarding comorbidity resolution (Figure 2).

Table 4 Long-term complications (> 30 d) *n* (%)

LSG (n = 104)		LRYGB (n = 236)	
GERD	28 (26.9)	Marginal ulcer	64 (27.1)
Anemia	4 (3.8)	Confirmed by endoscopy	15 (6.4)
		Clinically suspicious	49 (20.8)
		Anemia	53 (22.5)
		GERD	11 (4.7)
		Peterson hernia	3 (1.3)
		Ventral hernia	3 (1.3)

LSG: Laparoscopic sleeve gastrectomy; LRYGB: Laparoscopic Roux-en-Y gastric bypass; GERD: Gastro-esophageal reflux disease.

Differences were observed between the groups in the types of long-term complications experienced (Table 4). Twenty-eight patients (26.9%) presented with gastroesophageal reflux symptoms following LSG; 24 of these suffered from *de novo* reflux symptoms after the surgery, and four showed aggravation of pre-existing gastroesophageal reflux disease (GERD). The most frequently encountered long-term complication following LRYGB was marginal ulcers. The clinical symptom-based incidence reached 27.1%, but only one-fourth of the cases were confirmed with endoscopic evaluation. Most of the symptoms associated with both reflux esophagitis and marginal ulcers were well managed with proton pump inhibitors (PPIs). However, two patients in the LSG group were converted to LRYGB due to intolerable reflux symptoms, and one patient in the LRYGB group developed panperitonitis owing to marginal ulcer perforation and required emergent laparotomic exploration to redo the gastrojejunostomy.

DISCUSSION

In the present study, both LSG and LRYGB were found to be effective bariatric procedures with similar surgical risks leading to equivalent weight loss outcomes and comorbidity resolution during the medium-term follow-up. To the best of our knowledge, this is the first report comparing LSG *vs* LRYGB in obese Korean patients, and we believe that this study will provide valuable information to better guide clinical decisions for individual obese patients in Korea.

Bariatric surgery is relatively new in East Asian countries, including Korea. There is a marked tendency in the region to prefer technically less demanding procedures, including laparoscopic adjustable gastric banding or LSG, over more complicated procedures such as LRYGB or biliopancreatic diversion[4]. This might be attributable to the surgeons' lack of experience as well as to the sufficient weight loss outcomes achieved by these relatively simple restrictive procedures. The surgeon in the current study first began to perform the technically less demanding LSG in 2008, and then, starting in 2011, gradually began to adopt the more

complicated LRYGB, after experience with 100 cases of LSG. In the present study, we enrolled patients who underwent surgery when the two surgical options were evenly offered to prospective candidates. The selection of the procedure type largely depended on the patient's decision after thorough discussion regarding the outcomes and potential risks of each procedure based on the historical data. However, LSG was prioritized in super obese patients with BMI over 50 kg/m^2 to reduce surgical risks with further staged operation in mind. This tendency has been reflected in the higher preoperative BMI of the LSG group compared to that of the LRYGB group.

LSG has been advocated for its technical simplicity and reduced surgical risks compared to LRYGB[8,9]. According to a recent meta-analysis by Zhang *et al*[10], LSG was shown to have statistically fewer major complications than LRYGB. The perioperative surgical outcomes in our series also suggested that LSG was technically less demanding than LRYGB, with a shorter operating time and hospital stay. Although the incidence of overall and severe complications did not statistically differ between LSG and LRYGB, the incidence of both did trend higher for LRYGB, and clinically significant bleeding requiring transfusion or reoperation developed more frequently following LRYGB. Given the disparity in surgical experience with LSG and LRYGB in our series, however, there is a chance that the complication rate of LRYGB can be further lowered with sufficient experience on the part of surgeons, and the trend toward higher complications may presumably recede.

In the present study, both procedures achieved a maximal %EWL of approximately 80% at between 12 and 18 mo postoperatively; this subsequently leveled off. These results are in line with the recently published literature reporting that the %EWL following LSG and LRGYB ranged from 60.0%-76.5% and 69.0%-76.6%, respectively, 1 year postoperatively, figures that were maintained as 60.0%-75.4% and 70.0%-73.0%, respectively, 2 years postoperatively[8,9,11,12]. The slightly higher %EWL in our study might be explained by the lower preoperative BMI of our study cohort, since %EWL is significantly influenced by initial BMI level[13]. The recent meta-analysis by Zhang *et al* found that the excess weight loss was similar between LSG and LRYGB in the early postoperative period, for up to 2 years[10]. The present study also revealed that LSG and LRYGB demonstrated almost equal efficacy in terms of %EWL during the study's 3 years of postoperative follow-up. Some studies with longer follow-up periods have suggested that weight regain is more prevalent in patients who have undergone LSG[11,14], but the number of patients who were followed up in the present study became too small after 2 years to allow a definite conclusion. Longer follow-up with a larger number of patients is necessary to determine whether the reduced weight would be maintained thereafter. Interestingly, the attrition rate was higher in the LRYGB group than in the LSG group throughout the follow-up period, with the exception of the third year. This finding might be attributable to the fact that the surgeon traced the patients undergoing LSG more rigorously in order to evaluate whether or not they required secondary operations.

A recent meta-analysis suggested that LSG and LRYGB showed equivalent efficacy in regard to resolution of most of the obesity-related comorbidities, except for diabetic control where LRYGB was superior to LSG[10]. The current study showed similar resolution rates of hypertension, dyslipidemia, and diabetes following both procedures. As shown by the preoperative clinical characteristics, the patients with diabetes in our study initially inclined toward LRYGB, expecting an additional metabolic effect from bypass. Therefore, there could be a selection bias from the beginning. In addition, the number of patients with diabetes in the LSG group was too small to allow a comprehensive comparison of the efficacy of the two procedures in diabetic control. Nonetheless, the resolution rate of diabetes in the current study was estimated to be less than 60% even following LRYGB, a figure which is much lower than the diabetes remission rate of 92%-95% reported in a recently published meta-analysis[15]. Ethnic differences in the characteristics of type 2 diabetes, such as early β-cell dysfunction, could be the reason for the decrease in effective diabetic control, despite equivalent %EWL, relative to the Western population-based studies[16].

The potential long-term complications can be an important issue when determining the type of surgical procedure for a given obese patient. The present study showed that patients undergoing LSG and LRGYB encountered different kinds of long-term complications following the surgery. LSG led to far less frequent nutritional problems, such as anemia, than LRYGB; but new onset GERD developed in about 23% of the patients. Although the majority of patients with pre-existing GERD (71.4% in the LSG group *vs* 94.7% in the LRYGB group) experienced symptom improvement along with weight loss following both procedures, the resolution rate was considerably lower in the LSG group, and some patients experienced endoscopically proven disease aggravation following LSG, similar to the results from a previous randomized trial[8]. On the other hand, marginal ulcer was one of the representative complications following LRYGB. The reported incidence varies significantly in the literature, ranging from 3.5% to 12.3%, depending on the definition and evaluation method[17-20]. The incidence of endoscopically confirmed marginal ulcers was 6.4% in the present study, which is consistent with previous reports. However, the actual incidence is expected to be higher, considering that only about half of the symptomatic patients were evaluated with endoscopy while the rest were managed with PPIs based on their symptoms. The incidence of marginal ulcer is reported to be as high as 27%-36% among

symptomatic patients[21,22]. Currently, plausible risk factors for marginal ulcer following LRGYB include technical factors, such as a long gastric pouch or non-absorbable suture materials, smoking, non-steroidal anti-inflammatory drugs, diabetes mellitus, and possibly *Helicobacter pylori* infection[19]. Although both post-LSG GERD and post-LRYGB marginal ulcers responded well to the PPI treatment, two LSG patients eventually required revisional surgery, and one LRYGB patient underwent emergent operation due to marginal ulcer perforation in our series. It is difficult to say which complications would be easier to manage. Nonetheless, surgeons should provide a tailored surgical option for each patient that takes into consideration the possible risks, as the long-term complications may have a significant influence on the quality of life following the surgery. We believe that LRYGB would be a better choice for the patients with symptomatic GERD preoperatively, while LSG would be recommended for those with poor compliance or for substance abusers, including heavy smokers.

There are several limitations to the present study. Above all, this study is a retrospective study based on prospectively collected data, and there could be a selection bias for each group, as shown in the preoperative demographics. Well-designed randomized trials are necessary to truly elucidate the differences between LSG and LRYGB. The attrition rate in our series was also quite high, a finding that seems to be a universal challenge among other institutions. Since bariatric surgery and its related examinations are not reimbursed at all in South Korea, the costs for the follow-up examinations must come directly from the patients. Patients are reluctant to cover all of the expenses for regular surveillance unless they feel that something is wrong, a situation which renders our follow-up data less reliable.

In conclusion, both LSG and LRYGB are effective procedures that yield comparable weight loss in the mid-term with similar surgical risks. However, a larger number of patients required revisional surgery following LSG. The long-term complications encountered after each procedure differ significantly, and these complications are not negligible. Longer follow-up periods are necessary to compare the long-term differences in weight loss and complications between LSG and LRYGB.

ACKNOWLEDGMENTS

Min Ju Soh cordially supported this study as a research coordinator.

COMMENTS

Background

Bariatric surgery is relatively new in East Asian countries, including South Korea. There is a marked tendency in the region to prefer technically less demanding and purely restrictive procedures, including laparoscopic sleeve gastrectomy (LSG), over more complicated procedures such as laparoscopic Roux-en-Y gastric bypass (LRYGB). Therefore, comparisons between LRYGB and LSG are still lacking from Asian countries to demonstrate of the efficacy of each procedure.

Research frontiers

The present study evaluated the mid-term efficacy of LSG and LRYGB and compared the results between the two procedures in obese Korean patients at a single center.

Innovations and breakthroughs

Both LSG and LRYGB were found to be effective bariatric procedures with similar surgical risks leading to equivalent weight loss outcomes and comorbidity resolution during the mid-term follow-up. To the best of our knowledge, this is the first report comparing LSG *vs* LRYGB in obese Korean patients.

Applications

This study will provide valuable information to guide clinical decisions for individual obese patients in Asian countries. LRYGB would be a better choice for the patients with symptomatic GERD preoperatively, while LSG would be recommended for those with poor compliance or for substance abusers, including heavy smokers.

Peer-review

The authors present a head-to-head comparison of laparoscopic sleeve gastrectomy and laparoscopic Roux-en-Y gastric bypass procedure as performed at a single Korean center. They performed a retrospective analysis of prospectively collected data. Overall the manuscript is well organized and very well written. The authors are to be commended for their work.

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P- Reviewer: Guidry CA, Pai SI **S- Editor**: Yu J
L- Editor: Filipodia **E- Editor**: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12620

World J Gastroenterol 2015 November 28; 21(44): 12620-12627
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Retrospective Cohort Study

Perihepatic nodes detected by point-of-care ultrasound in acute hepatitis and acute-on-chronic liver disease

I Che Feng, Szu Jen Wang, Ming Jen Sheu, Lok-Beng Koay, Ching Yih Lin, Chung Han Ho, Chi Shu Sun, Hsing Tao Kuo

I Che Feng, Ming Jen Sheu, Lok-Beng Koay, Ching Yih Lin, Chi Shu Sun, Hsing Tao Kuo, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chi Mei Medical Center, Yongkang District, Tainan 710, Taiwan

Szu Jen Wang, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chi Mei Medical Center, Liouying, Tainan 736, Taiwan

Chung Han Ho, Department of Medical Research, Chi Mei Medical Center, Yongkang District, Tainan 710, Taiwan

Hsing Tao Kuo, Department of Senior Citizen Service Management, Chia Nan University of Pharmacy and Science, Tainan 717, Taiwan

Author contributions: Feng IC and Wang SJ contributed equally for data collection, data analysis, and manuscript writing; Sheu MJ, Koay LB, Lin CY and Sun CS instructed the research; and Ho CH contributed to the data analysis; Kuo HT designed and performed the research.

Institutional review board statement: The study was approved by Institutional Review Board of the Chi Mei Medical Center.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at kuohsu2003@yahoo.com.tw.

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Correspondence to: Hsing-Tao Kuo, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chi Mei Medical Center, No. 901, Zhonghua Road, Yongkang District, Tainan 710, Taiwan. kuohsu2003@yahoo.com.tw
Telephone: +886-6-2812811-57111
Fax: +886-6-2750630

Received: April 5, 2015
Peer-review started: April 7, 2015
First decision: June 19, 2015
Revised: July 7, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: November 28, 2015

Abstract

AIM: To study the manifestations of perihepatic lymph nodes during the episode of acute hepatitis flare by point-of-care ultrasonography.

METHODS: One hundred and seventy-six patients with an episode of acute hepatitis flare (ALT value > 5 × upper normal limit) were enrolled retrospectively. Diagnosis of etiology of the acute hepatitis flare was based on chart records and serological and virological assays. The patients were categorized into two groups (viral origin and non-viral origin) and further defined into ten subgroups according to the etiologies. An ultrasonograpy was performed within 2 h to 72 h (median, 8 h). The maximum size of each noticeable lymph node was measured. Correlation between clinical parameters and nodal manifestations was analyzed

RESULTS: Enlarged lymph nodes (width ⩾ 5mm)

were noticeable in 110 (62.5%) patients, mostly in acute on chronic hepatitis B (54.5%). The viral group had a higher prevalence rate (89/110 = 80.9%) and larger nodal size (median, 7 mm) than those of the non-viral group (21/66 = 31.8%; median, 0 mm) (P < 0.001 for both). Meanwhile, there were significant differences in the nodal size between acute and chronic viral groups (P < 0.01), and between acute hepatitis A and non-hepatitis A viral groups (P < 0.001). In logistical regression analysis, the nodal width still showed strong significance in multivariate analysis (P < 0.0001) to stratify the two groups. The area under the curve of ROC was 0.805, with a sensitivity of 80.9%, a specificity of 68.2%, positive predictive value of 80.92%, negative predictive value of 68.18%, and an accuracy of 76.14%.

CONCLUSION: Point-of-care ultrasonography to detect perihepatic nodal change is valuable for clarifying the etiologies in an episode of acute hepatitis flare.

Key words: Point-of-care ultrasonography; Perihepatic lymph node; Acute on chronic hepatitis B; Acute hepatitis A; Acute hepatitis flare

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Core tip: The nodal manifestation in acute hepatitis flare has never been well studied especially in an endemic area of chronic hepatitis B. Our findings encouraged point-of-care ultrasonography to be performed as early as possible for clarifying the etiologies as well as early treatments. Those subjects with viral origin have a higher prevalence of nodal enlargement and larger nodal size as compared with those of non-viral origin. A 5 mm cut-off value for nodal shortest diameter (width) is convenient for defining nodal enlargement.

Feng IC, Wang SJ, Sheu MJ, Koay LB, Lin CY, Ho CH, Sun CS, Kuo HT. Perihepatic nodes detected by point-of-care ultrasound in acute hepatitis and acute-on-chronic liver disease. *World J Gastroenterol* 2015; 21(44): 12620-12627 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12620.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12620

INTRODUCTION

Acute hepatitis is often caused by one of several hepatotropic viruses, by systemic viral infections of the liver, or by drugs, toxic agents or hypoxia. Abruptly elevated alanine aminotransferase (ALT) level cannot be specific to any of the various causes. Hence, a definite diagnosis always depends on detailed history-taking, observations of clinical presentations and survey of serum markers, which is always time consuming and may delay targeting therapy toward the offending etiologies. Acute flare of chronic hepatitis has been observed in some viral hepatitis cases such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infections as well as autoimmune hepatitis and alcoholic hepatitis. The manifestations of acute flare in chronic hepatitis are usually similar to those of acute hepatitis, which makes a differentiation between these two scenarios sometimes difficult. Liver biopsy is therefore clinically important.

The enlarged lymph nodes around the hepatoduodenal ligament (perihepatic nodes) are prevalent in chronic infections with HBV or HCV. The size of the noticeable lymph nodes seemed to be histologically and serologically correlated with the severity of hepatic inflammation[1-4]. Our comprehensive studies demonstrated that the prevalence of nodal enlargements (nodal width $\geqslant$ 5 mm) in chronic HBV infection and chronic HCV infection groups was significantly increased (56.9% and 69.4%, respectively, P < 0.001 for both) when compared with that of non-viral type; take note that this difference was independent of serum aminotransferase levels. Meanwhile, the nodal width was the only significant parameter when viral and non-viral groups were compared (P = 0.031)[5]. During episodes of hepatitis flare, enlarged perihepatic lymph nodes are always depicted by ultrasonography, however, its clinical significance is not clear[6-9].

The growth of point-of-care ultrasonography has progressed since the 1990s, Point-of-care ultrasonography refers to ultrasonography performed and interpreted by the clinician at the bedside for a real-time patient analysis, allowing immediate notations that could be directly correlated with the patient's presenting signs and symptoms[10]. In this study, we tried to investigate whether the ultrasonographic assessment of perihepatic lymphadenopathy could facilitate the differential diagnosis in a cohort of patients with hepatitis flare of various etiologies.

MATERIALS AND METHODS

Subjects

From June 2003 to January 2013, patients with acute hepatitis flare presented to our institution were enrolled into this study. Inclusive criterion was abrupt elevation of alanine aminotransferase (ALT) > 250 U/L (> 5 × upper normal limit) with or without a known history of liver disease. All of the patients had undergone a pathophysical survey for the etiology, including routine abdominal sonographic examination and simultaneous measurement of the size of noticeable perihepatic lymph nodes. This study was designed as a retrospective study and the data were collected from chart review which was approved by the local ethics committee. The patients were categorized into two groups (viral origin and non-viral origin) and further defined into 10 subgroups according to the clinically diagnosed etiology. Diagnosis of acute hepatitis flare was based on chart records and serological and virological assays, which were

reviewed by two of the authors (Kuo and Wang). Acute hepatitis A (AHA) was diagnosed by the presence of anti-HAV IgM. Acute hepatitis B (AHB) was diagnosed by the presence of a high titer of anti-HBc IgM. Acute hepatitis C (AHC) was diagnosed by positive seroconversion of anti-HCV antibody. All diagnoses included recent possible infection bouts but no known history of liver disease. Acute flare in chronic hepatitis B (CHB) or C (CHC) was diagnosed by high HBV DNA or HCV RNA viral load with a known history of chronic HBV or HCV infection, and positive HBsAg or anti HCV for more than 6 mo, without superinfection with other virus. Acute flare in chronic autoimmune hepatitis (AIH) was diagnosed by positive antinuclear antibody (ANA) titer > 80 ×, and high serum IgG level (> 1.1 × upper normal limit) without a known history of viral infections. Alcoholic hepatitis (AH) was diagnosed by a known history of alcohol consumption, high serum gamma-glutamyl transpeptidase (rGT) level without biliary obstructive signs, and no known viral infections or other etiologies. Drug induced liver disease (DILI) was diagnosed by a known intake of hepatotoxic drug, without known viral infections or other etiologies, and complete recovery after cessation of the target drugs. Bacterial infective hepatitis was diagnosed by toxic infection with evidence of infective foci including acute biliary tract infection or sepsis. Ischemic hepatitis was diagnosed by an episode of hemodynamic collapse without known viral infections or other etiologies, and rapid recovery after restoration of hemodynamic status. Patients with superinfection on chronic viral hepatitis, acute non-viral flare in chronic viral hepatitis, known malignancy, poor visualization of sonography, a history of abdominal surgery, or acute liver failure with mortality were excluded.

Serologic and laboratory tests

White blood cell (WBC) count, platelet (PLT) count, ALT level, rGT level, total bilirubin level, alfa-fetoprotein (AFP) level and ANA titer were measured during acute flare stage using routine automated techniques at our clinical pathology laboratories. Serum hepatitis markers, including HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HAV IgM, anti-HBc IgM, anti-HDV, and anti-HCV, were tested using the EIA kit (Abbott Diagnostics, North Chicago, IL, United States). Serum HBV DNA was tested using Roche Cobas Amplicor HBV Monitor test (Roche COBAS TaqMan HBV Test, lower limit of detection: 69 or 1.84 log 10 copies/mL; 12 or 1.08 log 10 IU/mL, Roche Diagnostics, Pleasanton, CA, United States). Serum HCV-RNA levels were determined using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (Roche Diagnostics, Branchburg, NJ, United States), with a detection threshold between 600 and 850000 IU/mL.

Ultrasound detection of perihepatic lymph nodes

After diagnosis of acute hepatitis flare, an ultrasonograpy was performed within 2 h to 72 h (median, 8 h). Two ultrasound scanners (SSD-5000 Aloka, Tokyo, Japan and Xario SSA-660A, Toshiba Medical Systems, Tokyo, Japan) equipped with a 3.75-5 MHz convex probe and color Doppler imaging were used. After the completion of the standard scanning of the whole abdomen, any other solid structures beside the common hepatic artery and/or the main portal vein were specifically studied. One or more ovoid masses that were less echogenic than the liver parenchyma were detected as solid masses on the Doppler sonography; these masses were clearly separated from adjacent organs and vessels on transverse, sagittal, and oblique scans. The maximum size of each noticeable lymph node was measured on the longest axis (length) and the corresponding perpendicular short axis (width). In patients with multiple lymph nodes, the measured and calculated values were added for a total value, and the maximal values were used for analysis. An enlarged lymph node was defined as the nodal width $\geqslant$ 5 mm[5]. The concurrent liver parenchymal manifestation by sonography was recorded as early change (normal/fatty liver) or late change (chronic liver parenchymal disease/liver cirrhosis). The wall thickness of the gallbladder, which has been a sonographic marker of acute hepatitis, was also recorded as normal or thickened when the typical three-layer appearance was depicted. The prevalence and size of enlarged lymph nodes were compared between groups and subgroups. Correlation between clinical parameters and nodal manifestations was also evaluated for surveillance accuracy in this hepatitis flare cohort.

Statistical analysis

Clinical and biochemical characteristic patterns of patients are expressed as mean ± SD or median with interquartile range (IQR). Data between groups/subgroups were tested by *U* test (Mann-Whitney) and Wilcoxon test, and $P < 0.05$ was judged to be statistically significant. Sensitivities and specificities were given with 95% confidence intervals. Comparisons between groups/subgroups were made by Pearson's χ^2 test or Fisher's exact test for categorical variables. To find the independent factor, univariate logistic regression analysis was used to select possible variables as *P*-value < 0.1. Then, multiple logistic regression analysis was used to identify the independent factors. A *P*-value < 0.05 was considered significant in multiple logistic regression analysis. All analyses were performed using SPSS 20.0 statistical software.

RESULTS

There were a total of 203 patients who fulfilled the inclusive criteria and 27 patients were excluded due to variable reasons (9 for incomplete data, 8 for poor visualization of sonography, 4 for superinfection, 4 for unknown causes and 2 for mortality). One hundred and seventy-six patients were enrolled for this study,

Table 1 Nodal manifestations of variable etiologies in this study cohort, number of cases (*n*), gender distribution, nodal prevalence (WT ⩾ 5 mm) and nodal width (WT) values (medium and mean)

Diagnosis according to etiology	*n*	Gender (%) male/female	WT ⩾ 5 mm (Yes/No) (%)	WT, median (Q1, Q3)	WT, mean ± SD
Viral					
CHBwAE	77	64.9/35.1	77.9/22.1	6.5 (5, 8)	6.32 ± 3.245
CHCwAE	11	63.6/36.4	100/0	6.1 (5, 7)	6.79 ± 2.23
Acute A	5	60/40	100/0	13 (13, 14)	15.12 ± 7.76
Acute B	11	54.5/45.5	63.6/36.4	6.8 (4, 8.1)	6.35 ± 3.27
Acute C	6	50/50	100/0	8.15 (8, 9)	7.92 ± 1.41
Non-viral					
AIH	10	20/80	50/50	4.65 (0, 7)	3.83 ± 3.51
AH	9	88.9/11.1	44.4/55.6	3.3 (0, 7.6)	3.77 ± 3.84
DILI	24	37.5/62.5	29.2/70.8	0 (0, 5.1)	2.08 ± 2.90
Infective	13	30.8/69.2	30.8/69.2	0 (0, 5.2)	2.57 ± 3.16
Ischemic	10	70/30	10/90	0 (0, 5.2)	0.90 ± 1.91

CHBwAE: Chronic B hepatitis with acute flare; CHCwAE: Chronic C hepatitis with acute flare; Acute A: Acute hepatitis A; Acute B: Acute hepatitis B; Acute C: Acute hepatitis C; AIH: Autoimmune hepatitis; AH: Alcoholic hepatitis; DILI: Drug induced liver injury; Infective: Infective hepatitis; Ischemic: Ischemic hepatitis; WT: Width.

Table 2 Demographic differences between viral and non-viral groups

	Viral group (*n* = 110)	Non-viral group (*n* = 66)	*P* value
Gender (%) male/female	62.7/37.3	45.5/54.5	0.025
Age (years), mean ± SD	40 ± 14.63	52.47 ± 17.82	< 0.001
ALT (IU/L), median (Q1-Q3)	1055 (516.8-1869)	821 (316-1520)	0.022
Total bilirubin (mg/dL), median (Q1-Q3)	1.57 (0.72-5.15)	2.49 (0.98-5.92)	0.335
AFP (ng/mL), median (Q1-Q3)	8.22 (4.60-18.83)	4.7 (0-7.6)	< 0.001
WBC (/μL), median (Q1-Q3)	5900 (5000-7500)	7200 (5700-9500)	0.003
PLT (10^3/μL), median (Q1-Q3)	186 (151-251)	206.5 (142-263)	0.543
WT, median (Q1-Q3)	7 (5-8.9)	0 (0-5.4)	< 0.001
Gallbladder wall thickening (%) (Yes/No)	32.7/67.3	30.3/69.7	0.738
Echogenecity (%) (1/0)	30.6/69.4	20.3/79.7	0.142
LN5 (%) (Yes/No)	80.9/19.1	31.8/68.2	< 0.001

Clinical and biochemical characteristics of patients are expressed as median. Data were tested by *U* test (Mann-Whitney) and Wilcoxon test, and $P < 0.05$ was judged to be statistically significant. WT: Value of nodal width; Echogenecity: Chronic liver parenchymal change; 1: Positive, 0: Negative; LN5: Nodal width ⩾ 5 mm. ALT: Alanine aminotransferase; AFP: α-fetoprotein; PLT: Platelet; WBC: White blood cell.

including cases of CHB with acute exacerbation (*n* = 77), CHC with acute exacerbation (*n* = 11), AHA (*n* = 5), AHB (*n* = 11), AHC (*n* = 6), AIH (*n* = 10), AH (*n* = 9), DILI (*n* = 24), infective hepatitis (*n* = 13), and ischemic hepatitis (*n* = 10) (Table 1). They were further divided into a viral origin group (*n* = 110) and a non-viral origin group (*n* = 66) (Table 2).

Enlarged lymph nodes (width ⩾ 5 mm) were noticeable in 110 patients with a total prevalence rate of 62.5%, most of which were diagnosed in CHB patients with acute flare (66/110 = 54.5%). The prevalence rates in all subgroups were as follows: CHB with acute flare (60/77 = 77.9%), CHC with acute flare (11/11 = 100%), AHA (5/5 = 100%), AHB (7/11 = 63.6%), AHC (6/6 = 100%), AIH(5/10 = 50%), AH (4/9 = 44.4%), DILI (7/24 = 29.2%), infective hepatitis (5/13 = 30.8%), and ischemic hepatitis (1/10 = 10%) (Table 1). The viral origin group had a higher prevalence rate (89/110 = 80.9%) than the non-viral origin group (21/66 = 31.8%) (Table 2) ($P < 0.001$). The median nodal width was 7 mm in the viral origin group and 0 mm in the non-viral origin group. In subgroups, the median nodal width was as follows: CHB with acute flare, 6.5 mm; CHC with acute flare, 6.1 mm; AHA, 13 mm; AHB, 6.8 mm; AHC, 8.15 mm; AIH, 4.65 mm; AH, 3.3 mm; DILI, 0 mm; infective hepatitis, 0 mm; and ischemic hepatitis, 0 mm. There were significant differences in nodal size between the viral and non-viral groups ($P < 0.001$), between the acute and chronic viral groups ($P < 0.01$), and between the AHA and non-AHA viral groups ($P < 0.001$) (Figure 1).

In logistical regression analysis, gender, age, WBC level and nodal width differed significantly between the viral and non-viral groups in univariate analysis ($P = 0.026$, < 0.0001, 0.003 and < 0.0001, respectively). The nodal width still had strong significance in multivariate analysis ($P < 0.0001$) (Table 3).

When using the value of nodal width to differentiate between the viral and non-viral groups, the area under the curve of ROC was 0.805 (Figure 2). If the cut-off value was 5 mm, it showed a sensitivity of 80.9%, a specificity of 68.2%, positive predictive value of 80.92%, negative predictive value of 68.18%, and

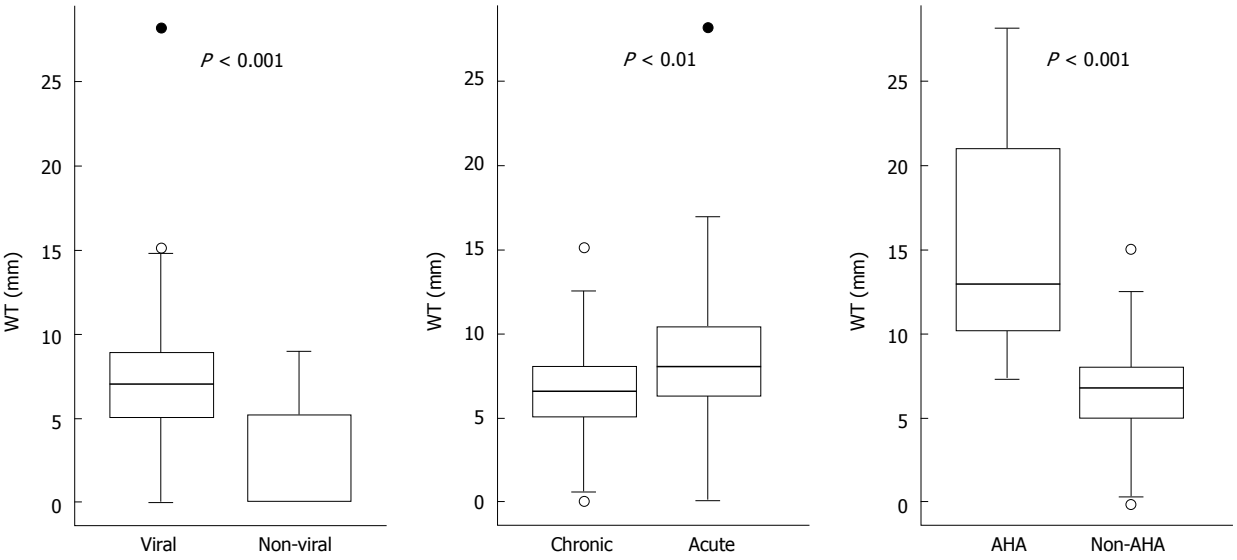

Figure 1 Nodal width differed significantly between viral and non-viral groups ($P < 0.001$), between acute and chronic viral groups ($P < 0.01$), and between acute hepatitis A and non-acute hepatitis A viral groups ($P < 0.001$). AHA: Acute hepatitis A; WT: The value of nodal width.

Table 3 Logistical regression analysis between viral and non-viral groups

	Univariate analysis			Multivariate analysis		
Variables	**OR**	**95%CI**	***P* value**	**OR**	**95%CI**	***P* value**
Gender (M/F)	2.020	1.087-3.753	0.026	0.280	0.108-0.725	0.009
Age (yr)	1.048	1.027-1.069	< 0.0001	1.033	1.001-1.065	0.04
ALT (IU/100 L)	0.982	0.956-1.008	0.982	0.973	0.929-1.019	0.24
Total bilirubin	1.011	0.961-1.064	0.665	1.239	1.096-1.401	0.001
AFP (ng/mL)	0.992	0.982-1.003	0.141	0.971	0.955-0.987	< 0.0001
WBC (/100 μL)	1.018	1.006-1.030	0.003	1.026	1.008-1.045	0.006
PLT (10^3/μL)	1.002	0.998-1.005	0.391	1.003	0.997-1.009	0.352
Gallbladder wall thickening	0.894	0.462-1.727	0.738	0.579	0.198-1.696	0.319
Echogenecity	0.579	0.278-1.207	0.579	0.803	0.272-2.370	0.691
WT	0.687	0.612-0.771	< 0.0001	0.627	0.530-0.741	< 0.0001

The gender, age, WBC level and the nodal width had significant differences in univariate analysis ($P = 0.026$, < 0.0001, 0.003 and < 0.0001, respectively). The nodal width remains strong significance in multivariate analysis ($P < 0.0001$). WT: Value of nodal width. ALT: Alanine aminotransferase; AFP: α-fetoprotein; PLT: Platelet; WBC: White blood cell.

an accuracy of 76.14%. A cut-off value of 3.7 mm resulted in a sensitivity of 89.1%, a specificity of 65.2% and the best accuracy rate of 80.14%.

DISCUSSION

In this study we found that the nodal enlargement was prevalence in acute hepatitis or acute flare on chronic liver disease especially in those caused by viral etiologies.

Among the various common etiologies, AHA manifested the most prominent nodal enlargement and ischemic hepatitis was of the least. The manifestations of perihepatic lymph nodes had been studied in liver disease of variable etiologies, mainly on chronic liver disease including AIH, primary biliary cirrhosis (PBC), CHB and CHC by ultrasound, CT and MRI and correlated to histopathology of the liver both by tissue biopsy and by graft from transplantation[2,3,11-19]. The accuracy of ultrasound detection of enlarged lymph nodes had been well validated by CT and MRI[19]. Correct sonographic detection of perihepatic lymph nodes has been confirmed by autopsy and verified by excision and histological examination during elective liver surgery with a high correlation coefficient ($r = 0.94$)[20].

However, there was less information about nodal change during acute hepatitis or acute flare on chronic liver disease. A small cohort study of acute hepatitis had been reported from Germany, where chronic viral hepatitis is not endemic[9]. Acute viral hepatitis had both a higher prevalence rate of noticeable nodes and larger nodal size than acute toxic liver disease.

Our study, within the endemic area of HBV and HCV infections, revealed that perihepatic lymphadenopathy (width ⩾ 5 mm) in patients with acute hepatitis flare may predict a viral etiology, mostly CHB with acute flare. This is eminent in cases when urgent antiviral

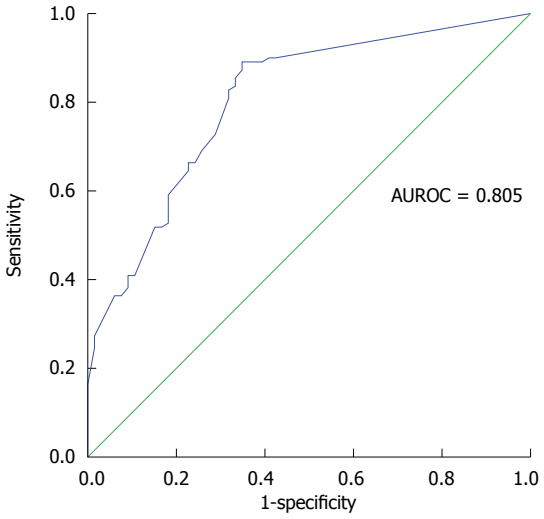

Figure 2 Area under the curve of ROC for the value of nodal width to discriminate between viral and non-viral groups was 0.805.

therapy is indicated, especially in those CHB patients with signs of hepatic decompensation. Ultrasound examination is naturally convenient, productive, affordable and safe. It can provide strong evidence before any results of laboratory examination are shown. Point-of-care ultrasonography should be widely promoted for its accurate depiction of the perihepatic nodal change during episode of acute hepatitis flare[10]. HBV and HCV are non-cytopathic viruses and thus immunologically mediated events are critical factors in the pathogenesis and outcome of these infections. The adaptive immune response mediates virtually all of the liver disease associated with viral hepatitis[21].

In this study, we found that AHA has more prominent nodal size than other acute viral hepatitis or chronic viral hepatitis with acute flare. AHA caused by primary infection with hepatitis A virus (HAV) is a common form of acute viral hepatitis. HAV is also a non-cytopathic virus, and hepatocyte injury is mediated by activated T cells in AHA. The frequency of circulating Tregs was reduced during AHA. A decrease in Treg numbers led to reduced suppressive activity of the Treg pool and consequently resulted in severe liver injury during AHA which was not observed in other acute liver diseases such as acute hepatitis B, acute hepatitis C and toxic/drug-induced hepatitis[22]. Thus, nodal reaction in acute hepatitis A could be a special sonographic detection for the diagnosis.

Immune mechanisms play a critical pathogenetic role in the majority of liver diseases, and are of crucial importance in particular infections and autoimmune disease affecting the liver[23]. Our study revealed variable prevalence and measurable size between variable non-viral etiologies of acute hepatitis flare, which may reflect the variable magnitudes of intrahepatic immune responses. Earlier studies of perihepatic lymph nodes revealed that autoimmune hepatobiliary diseases including PBC and AIH were associated with nodal enlargement. The nodal size and prevalence both correlated with the inflammatory severity[1,13]. We found that most of the non-viral hepatitis patients, however, presented with less prominent and less prevalent nodal enlargement than the viral group. Interestingly, four of nine alcoholic hepatitis patients also manifested with prominent nodal reaction, which, to our knowledge, has never been reported before. Immune response has been explored to be the mechanism of alcoholic liver disease. Immune responses were increased in the group of patients who are actively drinking or abstinent < 6 mo and although weaker, persisted in the abstinent patients[24]. The meaning of nodal enlargement in some alcoholic hepatitis cases requires further studies to evaluate its contribution to pathophysiology and management. The mechanism of acute ischemic hepatitis is more hemodynamic than immunologic and presents less nodal reaction (10% in the present study). In a patient with acute hepatitis flare with hemodynamic collapse, an enlarged perihepatic lymph node detected by ultrasound examination indicates etiologies other than ischemic hepatitis alone.

The mechanism of perihepatic lymph nodal reaction in liver disease, an important immune response, had not been well studied before. There was some evidence from previous studies of the celiac node, which is one of the main perihepatic lymph nodes. Matsuno *et al*[25] had identified the celiac node as a drainage pathway of the lymphatic macrophages from the hepatic sinusoids. In an animal study, orally administered antigen could induce antigen-specific regulatory T cells in the liver-draining celiac lymph node[26]. Recently, by using Evans Blue and purified dendritic cells, the celiac node was proved as lymphatic drainage of liver tissue in mice, which could reflect the intra-hepatic immune response appropriately[27]. Furthermore, a recent study about hepatitis B provided the evidence that the liver-draining lymph nodes induce an anti-HBV-specific immune response responsible for HBV clearance after hydrodynamic injection of HBV plasmid[28].

All of the above evidence suggested an immune responsive correlation between the perihepatic lymph nodes and the liver parenchyma. The nodal manifestations in variable liver diseases with different inflammatory stages merit further evaluations.

The issues for measurements of the nodal size and detectable number are critical when the magnitude of immune response is concerned. With inconsistent correlations, however, associations of the nodal size and number with serum parameters of cytolysis, severity of histologic damage, viremia, and a high CD8 lymphocyte level have been reported in many CHC studies[4,15,20]. Previously, the nodal size was measured by the nodal maximal diameter (long axis or short axis) or area (long axis multiplies short axis) with a maximal cut in transverse view or sagittal view[13,14]. A more convincing measurement was to calculate the nodal volume assumed with an ellipsoid shape[20]. However,

an enlarged node around the common hepatic artery does not necessary form as an ellipsoid shape[29,30]. The detectable number is also influenced by various factors including the operator's experience, definition of nodal enlargement and the resolution power of the machine. In our previous large series study, a nodal width (the short diameter) greater than 5 mm has the same predictable and more convenient value than the nodal volume. Recently, Shu *et al*[30] also noted that enlarged lymph nodes (more than 5 mm in the short diameter) could be found in about 90% of CHB patients by using MRI, which also proved that nodal width is related to the degree of liver inflammation (grade), when compared with liver biopsy pathology report. In this cohort of patients with acute hepatitis flare, nodal width also provided a better discrimination between viral and non-viral subgroups with the AUROC of 0.805. Actually, in this study cohort, a cut-point of 3.7 mm had a sensitivity of 89.1%, a specificity of 65.2% and a better accuracy rate of 80.14% than that of 5 mm. However, 5 mm may be clinically more practical for operation and no analytical difference was noted when either 5 mm or 3.7 mm was applied.

Limitation

It is difficult to make a correct differential diagnosis only by history-taking and laboratory data without pathology between AHB and CHB or between AHC and CHC. None of our patients has undergone a liver biopsy for diagnosis and some patients in the acute subgroup may belong to the chronic hepatitis with acute flare subgroup if a non-detectable chronic infection was present. However, the liver biopsy during acute flare hepatitis might be contraindicated due to impaired coagulative functions.

Based on our observations, point-of-care ultrasonography to detect perihepatic nodal change when performed in acute flare hepatitis is valuable to differentiate the etiologies of viral origins from non-viral origins. In an endemic area of HBV infection, CHB with acute flare presents the most prevalent nodal enlargement. In a hemodynamic critical patient with acute hepatic flare, an enlarged perihepatic lymph node detected by ultrasonography indicates etiologies other than ischemic hepatitis alone.

COMMENTS

Background

The enlarged lymph nodes around the hepatoduodenal ligament are prevalent in chronic liver diseases including chronic viral hepatitis and immune associated liver disease. The size of the noticeable lymph nodes seemed to be histologically and serologically correlated with the severity of hepatic inflammation, thus, they had been used to predict the effect of treatments and disease prognosis.

Research frontiers

Acute hepatitis flare is a life threatening disease and has variable etiologies that need different treatments. The perihepatic nodal appearances have not well been understood. Point-of-care ultrasonography to evaluate the perihepatic nodes may provide valuable evidence for episodes of acute hepatitis flare.

Innovations and breakthroughs

Enlarged lymph nodes were noticeable in 62.5% of acute hepatitis flare episodes and mostly acute on chronic hepatitis B (54.5%) in an endemic area of chronic hepatitis B. The viral group has a higher prevalence rate (80.9%) and larger nodal size (median width, 7 mm) than the non-viral group (31.8%; median width, 0 mm). Meanwhile, there were significant differences in the nodal size between acute and chronic viral groups ($P < 0.01$), and between acute hepatitis A and non-hepatitis A viral groups ($P < 0.001$). In logistical regression analysis, the nodal width still showed strong significance in multivariate analysis ($P < 0.0001$) to stratify the viral and non-viral groups. When a 5 mm cut-off value for nodal width was used, the diagnostic performance for differentiating the viral and non-viral groups was as follows: AUROC, 0.805; sensitivity, 80.9%; specificity, 68.2%; positive predictive value, 80.92%; negative predictive value, 68.18%; and accuracy, 76.14%.

Applications

In a victim of acute hepatitis flare, point-of-care ultrasonography to detect perihepatic nodes contributes an initial differential diagnosis of viral and non-viral origins. In an endemic area of chronic hepatitis B, an enlarged perihatic node usually directs acute-on-chronic hepatitis B. The appearance of multiple enlarged perihepatic nodes should lead one to diagnose acute hepatitis A.

Terminology

The perihepatic lymph nodes consist of hepatic portal lymph nodes, celiac lymph nodes, posterior mediastinal lymph nodes, supradiaphragmatic lymph nodes, and parasternal lymph nodes. Inflammatory process in the liver frequently leads to hyperplasia of regional lymph nodes. Point-of-care ultrasonography refers to ultrasonography performed and interpreted by the clinician at the bedside for a real-time patient analysis, allowing immediate notations that could be directly correlated with the patient's presenting signs and symptoms.

Peer-review

Overall this is a good paper.

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P- Reviewer: Hall TR **S- Editor**: Ma YJ **L- Editor**: Wang TQ
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DOI: 10.3748/wjg.v21.i44.12628

World J Gastroenterol 2015 November 28; 21(44): 12628-12634
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Retrospective Study

Peripheral portal vein-oriented non-dilated bile duct puncture for percutaneous transhepatic biliary drainage

Hiroaki Shimizu, Atsushi Kato, Tsukasa Takayashiki, Satoshi Kuboki, Masayuki Ohtsuka, Hideyuki Yoshitomi, Katsunori Furukawa, Masaru Miyazaki

Hiroaki Shimizu, Atsushi Kato, Tsukasa Takayashiki, Satoshi Kuboki, Masayuki Ohtsuka, Hideyuki Yoshitomi, Katsunori Furukawa, Masaru Miyazaki, Department of General Surgery, Chiba University Graduate School of Medicine, Chiba City, Chiba Prefecture 260-8670, Japan

Author contributions: Shimizu H designed and performed the research and wrote the paper; Kato A, Takayashiki T and Kuboki S performed the research and contributed to the analysis; Ohtsuka M, Yoshitomi H, Furukawa K and Miyazaki M provided clinical advice and supervised the report.

Institutional review board statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Chiba University Hospital. Because of the retrospective design, approval of the ethic commission was not always required.

Informed consent statement: Informed consent was obtained from all individual participants as to the percutaneous biliary drainage as part of the treatment for biliary disorders.

Conflict-of-interest statement: We have no potential conflicts of interest to declare.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at h-shimizu@faculty.chiba-u.jp. Consent was not obtained from the study participants because the present data are retrospective, de-identified, and anonymized; therefore, the risk of identification is low.

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Correspondence to: Hiroaki Shimizu, MD, Associate Professor, Department of General Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba Prefecture 260-8677, Japan. h-shimizu@faculty.chiba-u.jp
Telephone: +81-43-2227171
Fax: +81-43-2262552

Received: April 10, 2015
Peer-review started: April 11, 2015
First decision: June 19, 2015
Revised: July 31, 2015
Accepted: September 28, 2015
Article in press: September 30, 2015
Published online: November 28, 2015

Abstract

AIM: To evaluate the efficacy of peripheral portal vein (PV)-oriented non-dilated bile duct (BD) puncture for percutaneous transhepatic biliary drainage (PTBD).

METHODS: Thirty-five patients with non-dilated BDs underwent PTBD for the management of various biliary disorders, including benign bilioenteric anastomotic stricture (n = 24), BD stricture (n = 5) associated with iatrogenic BD injury, and postoperative biliary leakage (n = 6). Under ultrasonographic guidance, percutaneous transhepatic puncture using a 21-G needle was performed along the running course of the peripheral targeted non-dilated BD (preferably B6 for right-sided approach, and B3 for left-sided approach) or along the accompanying PV when the BD was not well visualized. This technique could provide an appropriate insertion angle of less than 30° between the puncture needle and BD running course. The puncture needle was then advanced slightly beyond the accompanying PV. The needle tip was moved slightly backward while injecting a small amount of contrast agent to obtain the BD image, followed by insertion of a 0.018-inch guide wire (GW). A drainage catheter was then placed using

a two-step GW method.

RESULTS: PTBD was successful in 33 (94.3%) of the 35 patients with non-dilated intrahepatic BDs. A right-sided approach was performed in 25 cases, while a left-sided approach was performed in 10 cases. In 31 patients, the first PTBD attempt proved successful. Four cases required a second attempt a few days later to place a drainage catheter. PTBD was successful in two cases, but the second attempt also failed in the other two cases, probably due to poor breath-holding ability. Although most patients (n = 26) had been experiencing cholangitis with fever (including septic condition in 8 cases) before PTBD, only 5 (14.3%) patients encountered PTBD procedure-related complications, such as transient hemobilia and cholangitis. No major complications such as bilioarterial fistula or portal thrombosis were observed. There was no mortality in our series.

CONCLUSION: Peripheral PV-oriented BD puncture for PTBD in patients with non-dilated BDs is a safe and effective procedure for BD stricture and postoperative bile leakage.

Key words: Percutaneous transhepatic biliary drainage; Cholangitis; Obstructive jaundice; Non-dilated bile duct

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Core tip: Percutaneous transhepatic biliary drainage (PTBD) offers a valuable alternative for access to the biliary system when endoscopic biliary drainage is impossible or infeasible. PTBD is generally performed in jaundiced patients with dilated bile ducts (BDs). However, some patients inevitably require PTBD even in the absence of dilated BD. Achieving needle access to the non-dilated BD is a challenging procedure. The present study reported on detailed technical aspects of peripheral portal vein-oriented BD puncture for PTBD in patients with non-dilated BDs, and also examined the safety and success rates of this procedure.

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INTRODUCTION

At present, endoscopic retrograde biliary drainage (ERBD) is the first-line treatment for patients with obstructive jaundice caused by benign[1,2] or malignant bile duct (BD) stricture[3-5]. However, the endoscopic transpapillary approach can often be difficult or even impossible in patients undergoing distal gastrectomy with Billroth Ⅱ reconstruction or bilioenteric Roux-en Y anastomosis after extrahepatic BD resection[6,7] in spite of recent improvement of balloon-enteroscope-assisted ERBD[8]. In such cases, percutaneous transhepatic biliary drainage (PTBD) offers a valuable alternative for access to the biliary system. PTBD still plays an important role[9,10], when an endoscopic approach is unsuccessful or infeasible[11,12].

PTBD is generally performed in jaundiced patients with dilated intrahepatic BD[13,14]. As a result, BD cannulation is easily achieved in most cases. However, PTBD in patients without intrahepatic BD dilatation is often very difficult[15-18] because the drainage catheter has to be inserted into the very small-caliber BD. A recent quality improvement guideline for PTBD reported that the threshold of success rate for non-dilated ducts was 65%[19]. However, some cases inevitably require PTBD even in the absence of a dilated intrahepatic BD, such as in cases of recurrent cholangitis resulting from BD stricture or bilioenteric anastomotic stricture, particularly when signs of a septic or pre-septic condition are present. Furthermore, when patients encounter biliary leakage from the resected surface of the liver after hepatectomy or biliary leakage associated with BD injury during laparoscopic cholecystectomy (Lap-C)[20,21], the intrahepatic BD remains non-dilated in most cases[22,23]. In such cases, PTBD can be technically demanding, but may offer the most simple and effective treatment for postoperative biliary leakage[24-26].

The present study reported on the detailed technical aspects of peripheral portal vein (PV)-oriented BD puncture for PTBD in patients with non-dilated intrahepatic BDs. We also evaluated the safety and success rates of our PTBD procedure in patients with non-dilated BDs.

MATERIALS AND METHODS

Between 2001 and 2014, PTBD procedures were performed for 405 patients at our institution, including 35 patients with non-dilated intrahepatic BDs (20 men and 15 women; mean age, 64.5 years; range, 38-75 years). Baseline characteristics of the patients are shown in Table 1. Non-dilated BDs were defined as peripheral BDs measuring < 2 mm in diameter or by visualization of peripheral BDs smaller than the adjacent PV[27], based on ultrasonography (US). Twenty-seven patients had previously undergone operations at other hospitals and were referred to our institution due to recurrent cholangitis or biliary leakage after surgery.

Underlying pathologies were as follows: 24 patients with benign bilioenteric anastomotic stricture after extrahepatic BD resection due to BD injuries during Lap-C (n = 14) or open cholecystectomy (n = 2), after pancreatoduodenectomy (n = 4) or after living

Table 1 Baseline characteristics of the patients (*n* = 35)

Characteristic	
Gender	
Male/female	20/15
Age (yr)	64.5 (38-75)[1]
T-BiL (mg/dL)	3.8 (1.4-8.2)
ALP (IU/L)	784 (236-2082)
Cholangitis	
Present/absent	9/26
Hepatolithiasis	
Present/absent	10/15
Indication for biliary drainage *n* (%)	
Bilioenteric anastomotic stricture	24 (68)
Bile duct stricture	5 (15)
Bile leak after initial surgery	6 (18)

[1]mean (range). T-BiL: Total bilirubin; ALP: Alkaline phosphatase.

donor liver transplantation (*n* = 4); 5 patients with benign BD stricture after BD primary repair due to BD injury during Lap-C (*n* = 3) or with primary sclerosing cholangitis (*n* = 2); and 6 patients with bile leakage after hepatectomy (*n* = 3), Lap-C (*n* = 2) or living donor liver transplantation (*n* = 1) (Table 2). US and multidetector-row computed tomography (MDCT) were performed for all patients before PTBD procedures. The position of the targeted peripheral bile duct in relation to the accompanying PV was evaluated by MDCT. The total of 35 cases included 7 cases in which endoscopic approaches had failed, including 4 cases with double-balloon enteroscopy due to Roux-en-Y reconstruction. In the remaining 28 cases, the endoscopic approach had not been attempted because of the postoperative reconstructed anatomy.

Peripheral PV-oriented BD puncture technique with two-step guide-wire method

PTBD procedures were performed under local anesthesia with mild sedation. The most appropriate BD for targeted puncture was the B6 peripheral branch for the right-sided approach (Figures 1 and 2) or B3 for the left-sided approach, because the distance between the skin and puncture site of the BD was short and running course of the target BD was mostly straight from the hepatic hilus to the peripheral puncture site.

Under ultrasonographic guidance, the non-dilated peripheral branch of B6 or B3 was punctured with a 21-G needle (PTCD Two Step Drainage Set; Cook, Tokyo, Japan) along the running course of the BD or accompanying PV in the case when the targeted BD was not well visualized (Figure 1A), and the puncture needle was advanced slightly beyond the accompanying PV. After removal of the inner stylet, the needle tip was moved slightly backward while injecting a very small amount of contrast agent (0.5-1.0 mL) to obtain a BD image (Figure 1B), because the back flow of bile could not be obtained in most cases with the non-dilated BD. Once a BD image was obtained, contrast agent was quickly injected to acquire a clear image of the hilar BD. A 0.018-inch guide-wire (GW) was then advanced carefully into the BD while controlling the needle tip (Figure 1C). During this process, the insertion angle between the puncture needle and running course of the BD (Figure 3) is very important. The angle should be less than 30°, otherwise, insertion of the assembly set catheter (PTCD Two Step Drainage Set; Cook) into the BD over the thin, 0.018-inch GW may become quite difficult.

Table 2 Outcomes of percutaneous transhepatic biliary drainage in patients with nondilated intrahepatic duct *n* (%)

Technical success	33 (94.3)
First PTBD	31
Second PTBD[1]	2
PTBD procedure-related complication	5 (14.3)
Transient hemobilia	1
Cholangitis	4

[1]Second PTBD attempt was performed a few days later in all cases. PTBD: Percutaneous transhepatic biliary drainage.

After the thin GW was inserted and locked at a secure position in the BD, the 21-G puncture needle was removed. The assembly set (PTCD Two Step Drainage Set; Cook), consisting of a metallic cannula and inner sheath and outer sheath catheters, was slowly inserted over the thin GW, and advanced into the targeted BD. The metallic cannula and inner sheath catheter were then removed, leaving the outer sheath catheter behind in the BD. Cholangiography was performed to confirm that this catheter had been correctly placed into the BD. A 0.035-inch hydrophilic GW (Radifocus, Terumo, Tokyo, Japan) was then inserted to a secure position in the biliary system. A 7-Fr catheter with a distal curve (Seeking catheter; Hanako Medical, Saitama, Japan) was then inserted along the GW (Figure 1D) and the BD stricture or anastomotic stricture was crossed using the GW. A final 8-Fr drainage tube (Straight; Hanako Medical) with side halls was advanced to the appropriate position for internal-external drainage (Figure 1D). After an interval of several days, dilation of the stricture site was performed with a balloon catheter and plastic dilator (Cook). The drainage catheter was exchanged in size up to 14-18 Fr, and kept for 3-6 mo after the first PTBD placement to avoid re-stricture. However, in this study, PTBD procedures were considered as successful when the PTBD catheter was successfully inserted into the BD.

RESULTS

PTBD was successful in 33 (94.3%) of the 35 patients with non-dilated intrahepatic BDs. In 31 patients, the first PTBD attempt proved successful. Four cases required a second attempt a few days later to place the drainage catheter. This second attempt was successful for two cases, but failed in the other two cases,

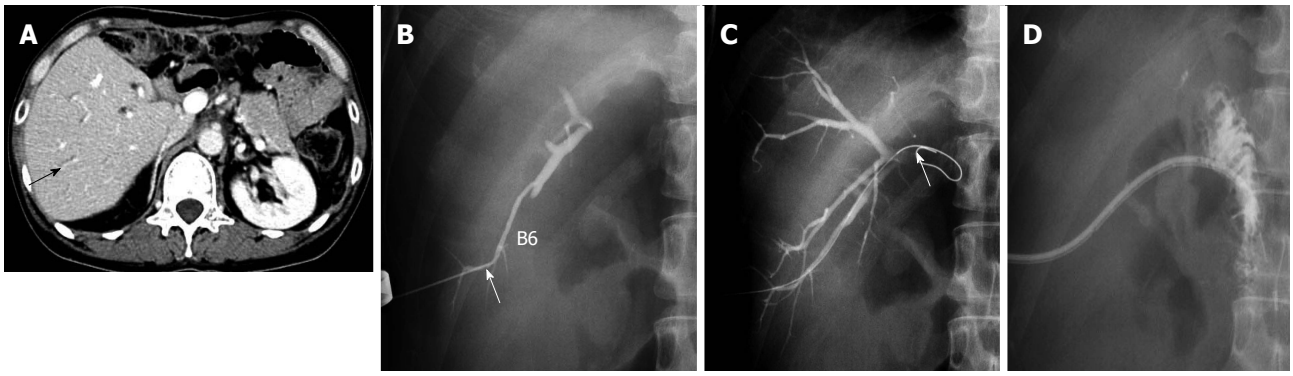

Figure 1 A 57-year-old man who had undergone living donor liver transplantation with right posterior sectional graft 8 mo earlier presented with recurrent cholangitis due to stricture of the bilioenteric anastomosis. A: Computed tomography shows that the intrahepatic bile duct (BD) is not dilated. The arrow indicates a peripheral branch of B6; B: Under ultrasonographic guidance, a non-dilated peripheral branch of B6 is punctured along its running course with a 21-G needle; C: While controlling the needle tip, a 0.018-inch guide-wire (GW) is inserted carefully into the BD; D: When the hydrophilic 0.035-inch GW crosses the anastomotic stricture, a 7-Fr catheter with distal curve crosses the stenotic bilioenteric anastomosis and advances into the jejunal loop.

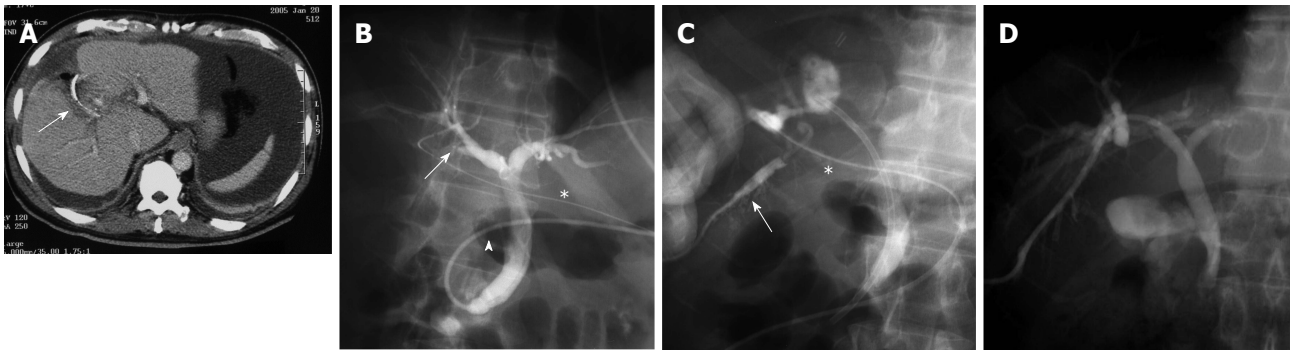

Figure 2 A 65-year-old man who had undergone segment 5 resection and radiofrequency ablation in segment 1 for hepatocellular carcinoma presented with bile leakage after surgery. A: Computed tomography shows non-dilated intrahepatic bile ducts and an intraperitoneal drainage tube (arrow) placed at the time of surgery; B: Cholangiogram via the endoscopic nasobiliary drainage tube (arrowhead) reveals stricture of the posterior sectional bile duct (arrow). Asterisk shows the intraperitoneal drain placed at the time of surgery; C: Non-dilated peripheral B6 (arrow) is punctured with a 21-G needle. Asterisk shows the intraperitoneal drain placed at the time of surgery; D: An 8-Fr biliary drainage tube is advanced through the strictured right posterior sectional bile duct and placed from B6 to the common bile duct.

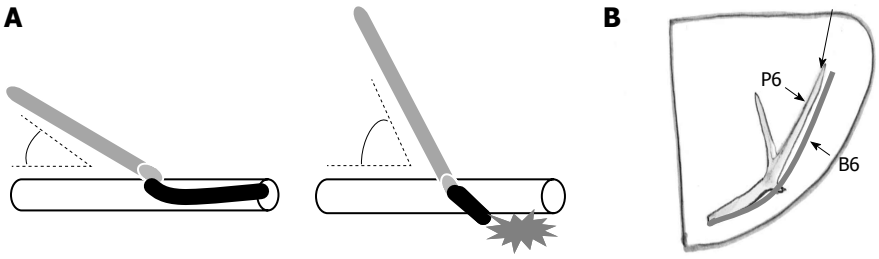

Figure 3 This technique increased the possibility of the puncture needle crossing the targeted non-dilated bile duct, and also provided an appropriate angle to insert the drainage catheter for the next step. A: The insertion angle between the puncture needle and running course of the bile duct (BD) should be less than 30°; B: Under ultrasonographic guidance, percutaneous transhepatic puncture is performed along the running course of the targeted peripheral non-dilated BD (B6 for right-sided approach) or along the accompanying portal vein (P6) when the BD is not well visualized. This technique can provide an appropriate insertion angle of less than 30° between the puncture needle and BD running course.

probably due to poor breath-holding ability (Table 2). A right-sided approach *via* B6 was performed in 23 cases, and *via* B5 in 2 cases, while a left-sided approach was performed through B3 in 9 cases, and B2 in 1 case.

Although most patients (n = 26) had been suffering from cholangitis with fever including septic condition in 8 cases before PTBD, only 5 (14.3%) of 35 patients encountered PTBD procedure-related complications, such as transient hemobilia and cholangitis (Table 2). No major complications such as bilioarterial fistula or portal thrombosis were observed. There was no

mortality in our series.

DISCUSSION

Successful biliary drainage is essential for the management of recurrent cholangitis due to BD stricture or bilioenteric anastomotic stricture[1,2], and also for effective treatment of postoperative biliary leakage[24-26]. Although ERBD is currently the first-line treatment, endoscopic transpapillary access to the biliary system can often prove difficult, because of postoperative anatomical conditions such as with Billroth Ⅱ reconstruction or bilioenteric Roux-en-Y anastomosis[6,7]. In such cases, PTBD offers a valuable alternative for accessing the biliary system[11,12]. Surgical management may also be one of the options for the treatment of BD or bilioenteric anastomotic stricture. However, surgical re-exploration and repair of the biliary system, such as BD reconstruction or bilioenteric re-anastomosis, can be extremely difficult and complex in patients with long-lasting cholangitis[28-30], particularly due to Bismuth type Ⅲ or Ⅳ[31] biliary stricture[32,33].

Non-dilated intrahepatic BDs have generally been defined as peripheral BDs measuring < 2 mm in diameter or by visualization of a BD smaller than the adjacent PV[27] based on US. According to previous reports, the success rate for PTBD in patients with non-dilated BDs has ranged from 75% to 90%[17,18,24-26], and complication rates were also higher as compared to patients with dilated BDs[16]. According to the recent quality improvement guideline for PTBD[19], the threshold of success rate for non-dilated ducts is 65%, and the rates of major complications, such as hemorrhage and sepsis, are 5%. In our series, PTBD was technically successful in 94.3% of patients without any major complications. In cases with non-dilated BDs, BD puncture is generally performed much closer to the hepatic hilum, because the target BD is simply larger. However, BD puncture close to the hepatic hilum may carry a high risk of vascular complications, including bilioarterial fistula and PV thrombosis. Peripheral BD puncture is therefore preferable to central BD puncture, but is technically demanding, resulting in lower success rates. Funaki *et al*[27] clearly reported that many BD punctures and passes were required for BD cannulation in patients with non-dilated BDs.

With recent improvements of the resolution of US, peripheral BD can be visualized in most cases. However, after a failure of the first BD puncture, the target non-dilated BD was not well visualized in most cases. In such situations, we punctured along the peripheral PV running parallel with a target BD using a 21-G needle. This technique increased the possibility of the puncture needle crossing the targeted non-dilated BD, and also provided an appropriate angle to insert the drainage catheter for the next step (Figure 3). A similar puncture technique was reported by Lee *et al*[15], who termed the procedure the "parallel technique". Our series successfully performed the PTBD procedure using a 21-G puncture needle with a two-step GW method exchanging from a 0.018-inch GW to a 0.035-inch GW. Furthermore, no major complications such as bilioarterial fistula or PV thrombosis after PTBD procedure were encountered in this series. Minor PTBD-related complications included transient hemobilia and cholangitis, with a morbidity rate of 14.2%. Accordingly, the most advantageous point of this technique is the safe procedure, as the puncture site is very peripheral and major complications such as portal thrombosis or bilioarterial fistula are unlikely to occur. On the other hand, this procedure may be technically demanding. Therefore, it is better for a biliary physician, surgeon or radiologist who is familiar with the biliary anatomy and has mastered ordinary PTBD techniques to perform this procedure.

In conclusion, peripheral PV-oriented BD puncture with two-step GW method for PTBD is safe and feasible, offering high success rates in patients with non-dilated intrahepatic BDs. This procedure is useful and effective for the management of BD or bilioenteric anastomosis strictures and postoperative biliary leakage.

COMMENTS

Background

At present, endoscopic retrograde biliary drainage is the first-line treatment for patients with obstructive jaundice caused by benign or malignant bile duct (BD) stricture. However, the endoscopic transpapillary approach can often prove difficult or even impossible in patients undergoing distal gastrectomy with Billroth Ⅱ reconstruction or bilioenteric Roux-en Y anastomosis after extrahepatic BD resection. Percutaneous transhepatic biliary drainage (PTBD) offers a valuable alternative for access to the biliary system when endoscopic biliary drainage is impossible or infeasible. PTBD is generally performed in jaundiced patients with dilated BDs. However, some cases inevitably require PTBD even in the absence of a dilated BD. Achieving needle access to the non-dilated BD is challenging. The present study reported on the detailed technical aspects of peripheral portal vein (PV)-oriented BD puncture for PTBD in patients with non-dilated BDs, and the safety and success rates of this procedure.

Research frontiers

According to previous reports, the success rate for PTBD in patients with non-dilated BDs has ranged from 75% to 90%, and complication rates were also higher as compared to patients with dilated BDs. According to the recent quality improvement guideline for PTBD, the threshold of success rate for non-dilated ducts is 65%, and the rates of major complications, such as hemorrhage and sepsis, are 5%.

Innovations and breakthroughs

In cases with non-dilated BDs, BD puncture is generally performed much closer to the hepatic hilum because the target BD is simply larger. However, BD puncture close to the hepatic hilum may carry a high risk of vascular complications. Peripheral BD puncture is therefore preferable to central BD puncture, but is technically demanding, resulting in lower success rates. Under ultrasonographic guidance, percutaneous transhepatic puncture using a 21-G needle was performed along the running course of the peripheral targeted non-dilated BD (preferably B6 for a right-sided approach, and B3 for a left-sided approach) or along the accompanying PV when the BD was not well visualized. This strategy increased the probability of non-dilated BD puncture without risk and could also provide an appropriate insertion angle of less than 30° between the puncture needle and BD running course. In these series, PTBD was technically successful in 94.3% of patients without any major complications.

Applications

Peripheral PV-oriented BD puncture with two-step guide wire method for PTBD is safe and feasible, offering high success rates in patients with non-dilated intrahepatic BD. This procedure is useful and effective for the management of BD or bilioenteric anastomosis strictures and postoperative biliary leakage.

Terminology

Non-dilated bile ducts (BDs): peripheral BDs measuring < 2 mm in diameter or by visualization of peripheral BDs smaller than the adjacent PV based on ultrasonography.

Peer-review

The authors of this paper evaluated the efficacy of peripheral PV-oriented non-dilated BD puncture for PTBD. In their series, PTBD was technically successful in 94.3% of patients without any major complications. Peripheral PV-oriented non-dilated BD puncture for PTBD is therefore a safe and effective procedure for BD stricture and postoperative bile leakage, with a high success rate.

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P- Reviewer: Choe BH, Mendez-Sanchez N, Shih SC
S- Editor: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12635

World J Gastroenterol 2015 November 28; 21(44): 12635-12643
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Retrospective Study

Management and associated factors of delayed perforation after gastric endoscopic submucosal dissection

Haruhisa Suzuki, Ichiro Oda, Masau Sekiguchi, Seiichiro Abe, Satoru Nonaka, Shigetaka Yoshinaga, Takeshi Nakajima, Yutaka Saito

Haruhisa Suzuki, Ichiro Oda, Masau Sekiguchi, Seiichiro Abe, Satoru Nonaka, Shigetaka Yoshinaga, Takeshi Nakajima, Yutaka Saito, Endoscopy Division, National Cancer Center Hospital, Tokyo 104-0045, Japan

Author contributions: Suzuki H and Oda I designed the study, analyzed and interpreted the data, and wrote the draft; Sekiguchi M, Abe S, Nonaka S, Yoshinaga S, Nakajima T, and Saito Y contributed to the critical revisions of the article for important intellectual content; all authors had final approval of this article.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: No conflict of interest was declared by the authors.

Data sharing statement: No additional data are available.

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Correspondence to: Haruhisa Suzuki, MD, Endoscopy Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. harusuzu@ncc.go.jp
Telephone: +81-3-35422511-7794
Fax: +81-3-35423815

Received: May 25, 2015
Peer-review started: May 27, 2015
First decision: June 19, 2015
Revised: July 15, 2015
Accepted: September 30, 2015
Article in press: September 30, 2015
Published online: November 28, 2015

Abstract

AIM: To identify the actual clinical management and associated factors of delayed perforation after gastric endoscopic submucosal dissection (ESD).

METHODS: A total of 4943 early gastric cancer (EGC) patients underwent ESD at our hospital between January 1999 and June 2012. We retrospectively assessed the actual management of delayed perforation. In addition, to determine the factors associated with delayed perforation, after excluding 123 EGC patients with perforations that occurred during the ESD procedure, we analyzed the following clinicopathological factors among the remaining 4820 EGC patients by comparing the ESD cases with delayed perforation and the ESD cases without perforation: age, sex, chronological periods, clinical indications for ESD, status of the stomach, location, gastric circumference, tumor size, invasion depth, presence/absence of ulceration, histological type, type of resection, and procedure time.

RESULTS: Delayed perforation occurred in 7 (0.1%) cases. The median time until the occurrence of delayed perforation was 11 h (range, 6-172 h). Three (43%) of the 7 cases required emergency surgery, while four were conservatively managed without surgical intervention. Among the 4 cases with conservative management, 2 were successfully managed endoscopically using the endoloop-endoclip technique. The median hospital stay was 18 d (range, 15-45 d). There were no delayed perforation-related deaths. Based on a multivariate analysis, gastric tube cases (OR = 11.0; 95%CI: 1.7-73.3; P = 0.013) were significantly associated with delayed perforation.

CONCLUSION: Endoscopists must be aware of not only the identified factors associated with delayed perforation, but also how to treat this complication

effectively and promptly.

Key words: Early gastric cancer; Endoscopic submucosal dissection; Delayed perforation; Emergency surgery; Conservative management

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Core tip: In this study, delayed perforation occurred in 0.1% (7 cases) of 4943 early gastric cancer patients undergoing endoscopic submucosal dissection (ESD); 43% (3 cases) of these cases required emergency surgery. This study also showed that the gastric tube was an independent risk factor associated with delayed perforation. This study is significant because it clarified both the clinical management and risk factors of delayed perforation based on data from a large series of consecutive patients undergoing ESD. Endoscopists must be aware of not only the identified factors associated with delayed perforation, but also how to treat this complication effectively and promptly.

Suzuki H, Oda I, Sekiguchi M, Abe S, Nonaka S, Yoshinaga S, Nakajima T, Saito Y. Management and associated factors of delayed perforation after gastric endoscopic submucosal dissection. *World J Gastroenterol* 2015; 21(44): 12635-12643 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12635.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12635

INTRODUCTION

Endoscopic submucosal dissection (ESD) is widely used in East Asia (*e.g.*, Japan and Korea) as an initial treatment for early gastric cancer (EGC) with a negligible risk of lymph node (LN) metastasis, even for cases that involve large and ulcerative lesions[1-4]. The therapeutic outcomes of gastric ESD are excellent; however, there are still cases of various complications, such as bleeding and perforation[5,6]. ESD procedure-related perforations can be subdivided into perforations that occur during gastric ESD and delayed perforations occurring after the completion of gastric ESD. Most perforations occur during gastric ESD, and the risk of perforation reportedly ranges from 1.2% to 9.6% for gastric ESD[5-12]. The majority of perforation cases can be treated conservatively using complete endoscopic closure with endoclips[5,6,13]. In contrast, delayed perforation is a rare (with an incidence of 0.43% to 0.45%) but serious complication that sometimes requires emergent surgery[6,14-20]. Under these circumstances, the actual clinical management and the associated factors of delayed perforation induced by gastric ESD should be clarified to minimize its incidence and to treat this complication effectively and promptly. Although several reports have described delayed perforation after gastric ESD, no published report has thoroughly evaluated the various factors associated with delayed perforation based on data from a large series of consecutive EGC patients undergoing ESD[14-20]. Therefore, we attempted to identify the actual clinical management and the associated factors of delayed perforation induced by ESD for EGC based on our extensive clinical experience.

MATERIALS AND METHODS

Patients

A total of 4943 EGC patients (male:female ratio, 3.9:1; median age, 69 years; range, 27-96 years) underwent ESD at our hospital between January 1999 and June 2012. The clinicopathological findings of these 4943 EGC patients are shown in Table 1. In our hospital, according to the Japanese gastric cancer treatment guideline, ESD is generally performed based on two independent sets of clinical indications: absolute indications for standard treatment, and expanded indications for investigational treatment[3]. Furthermore, ESD is also performed for a small number of patients with locally recurrent EGC or EGC lesions outside the clinical indications for ESD, particularly gastric tube cases, because the mortality rate for surgical resection is remarkably high[21-24]. The definitions for the characteristics of EGC lesions, such as status of the stomach (normal stomach/remnant stomach after a gastrectomy/gastric tube after an esophagectomy), lesion location (upper/middle/lower third of the stomach), gastric circumference (greater curvature/lesser curvature/anterior wall/posterior wall), tumor size, depth of invasion [mucosa (M)/submucosa (SM)], presence of ulcerations, and histological type (differentiated-type/undifferentiated-type), were based on the Japanese classification of gastric carcinoma and the Japanese gastric cancer treatment guidelines[3,25]. The term "gastric tube" refers to a stomach conduit that has been pulled up into the thorax for use as an esophageal substitute after an esophagectomy[23,24]. The histological type was defined according to the major histological features of the lesion. Differentiated-type adenocarcinoma included tubular adenocarcinoma and papillary adenocarcinoma, while undifferentiated-type adenocarcinoma included poorly differentiated adenocarcinoma, signet-ring cell adenocarcinoma and mucinous adenocarcinoma.

ESD procedure

The ESD procedure began with the identification of the lesion and the marking of dots at a distance of about 5 mm outside of the lesion. After submucosal injection using a saline solution or sodium hyaluronate (MucoUp; Johnson & Johnson Corp., Tokyo, Japan) with epinephrine, a 1- to 2-mm precut was made with an electrosurgical needle knife (KD-1L-1; Olympus Optical, Tokyo, Japan) or the Dual knife (KD-650Q; Olympus

Table 1 Clinicopathological findings of 4943 early gastric cancer patients undergoing endoscopic submucosal dissection

Clinicopathological finding	*n* (%)
Age (yr)	
median (range)	69 (27-96)
< 70	2608 (52.8)
⩾ 70	2335 (47.2)
Sex	
Male	3930 (80.0)
Female	1013 (20.0)
Chronological periods	
1st period: 1999-2005	2285 (46.2)
2nd period: 2006-2012	2658 (53.8)
Clinical indications	
Absolute indications	2884 (58.3)
Expanded indications	1737 (35.1)
Locally recurrent EGC	141 (2.9)
Outside indications	181 (3.7)
Stomach status	
Normal stomach	4704 (95.2)
Remnant stomach	152 (3.1)
Gastric tube	87 (1.7)
Location	
Upper	904 (18.3)
Middle	2100 (42.5)
Lower	1939 (39.2)
Circumference	
Greater curvature	807 (16.3)
Lesser curvature	2005 (40.6)
Anterior wall	963 (19.5)
Posterior wall	1168 (23.6)
Size (mm)	
median (range)	15 (0.4-120)
⩽ 20	3457 (69.9)
> 20	1486 (30.1)
Depth of invasion	
M	4075 (82.4)
SM	868 (17.6)
Ulceration	
Absent	4073 (82.4)
Present	870 (17.6)
Histological type	
Differentiated	4581 (92.7)
Undifferentiated	362 (7.3)
Type of resection	
En bloc resection	4859 (98.3)
Piecemeal resection	84 (1.7)
Procedure time (h)	
mean ± SD	1.4 ± 1.1
< 2	3811 (77.1)
⩾ 2	1132 (22.9)

EGC: Early gastric cancer; M: Mucosa; SM: Submucosa.

Optical, Tokyo, Japan), followed by a circumferential mucosal incision outside the marking dots with an insulation-tipped (IT) diathermy knife (KD-610L; Olympus Optical, Tokyo, Japan) or IT knife 2 (KD-611L; Olympus Optical, Tokyo, Japan). The submucosal layer was then dissected using an IT knife or an IT knife 2 after an additional submucosal injection. Cases with bleeding during or after the ESD procedure were controlled by coagulating the bleeding vessels with the IT knife itself and/or hemostatic forceps [Coagrasper (FD-410LR; Olympus Optical, Tokyo, Japan) and Radial Jaw hot biopsy forceps (Boston Scientific Japan, Tokyo, Japan)], or by grasping them with endoclips. The set-up for the high-frequency generators for ESD along with the IT knife for early gastric cancer (ICC200 Erbe Elektromedizin, Tübingen, Germany, ESG100 Olympus Medical and VIO300D Erbe Elektromedizin, Tübingen, Germany) is shown in Table 2. The risks and benefits of ESD were thoroughly explained to each patient, and written informed consent was obtained from them in accordance with our institutional protocols prior to treatment.

The procedure time was defined as the time from circumferential marking around the lesion to the completion of the ESD procedure. An *en bloc* resection was defined as a one-piece resection, and a piecemeal resection was defined as the removal of a lesion in more than one piece[3,25].

Assessments of actual clinical management and associated factors of delayed perforation

We retrospectively assessed the incidence of delayed perforation and the actual clinical management of this complication, including the need for emergency surgery, the methods of conservative management, and the median hospital stay. For the cases with delayed perforation requiring emergency surgery, the reason for the emergency surgery was also clarified. Finally, to determine the factors associated with delayed perforation induced by gastric ESD, after excluding 123 (2.5%) EGC patients with perforations that occurred during the ESD procedure, we retrospectively analyzed the following clinicopathological factors among the remaining 4820 EGC patients by comparing the ESD cases with delayed perforation with the ESD cases without perforation: age (< 70 years *vs* ⩾ 70 years), sex (male *vs* female), chronological periods (1st period: 1999-2005 *vs* 2nd period: 2006-2012), clinical indications for ESD (absolute indications *vs* expanded indications *vs* locally recurrent EGC *vs* outside indications), status of the stomach (normal stomach *vs* remnant stomach after gastrectomy *vs* gastric tube after esophagectomy), lesion location (upper/middle *vs* lower), gastric circumference (greater curvature *vs* lesser curvature *vs* anterior wall *vs* posterior wall), tumor size (⩽ 20 mm *vs* > 20 mm), depth of invasion (M *vs* SM), presence/absence of ulceration, histological type (differentiated-type *vs* undifferentiated-type), type of resection (*en bloc* resection *vs* piecemeal resection), and procedure time (< 2 h *vs* ⩾ 2 h).

Definition of delayed perforation induced by gastric ESD

Delayed perforation was identified by the sudden appearance of symptoms of peritoneal or mediastinal pleura irritation (gastric tube case) after the completion of gastric ESD, with free air visible on X-ray or computed tomography (CT) images and/or with a gross defect observed endoscopically, although endoscopically visible perforations did not occur

Table 2 Set-up for the high-frequency generators for endoscopic submucosal dissection along with the IT knife for early gastric cancer

Procedure	Device	Mode	Output
ICC200			
Marking	Needle knife	Forced coag	20W
Precutting	Needle knife	ENDO CUT	Effect 3, 80W
Mucosal incision	IT knife Needle knife	ENDO CUT	Effect 3, 80W
Submucosal dissection	IT knife	ENDO CUT Forced coag	Effect 3, 80W 50W
	Needle knife	ENDO CUT Forced coag	Effect 3, 80W 50W
Endoscopic hemostasis	IT knife Needle knife Hot biopsy Coagrasper	Forced coag Soft coag	50W 80W
ESG100			
Marking	Needle knife	Forced coag 1	20W
Precutting	Needle knife	Pulse cut slow	40W
Mucosal incision	IT knife Needle knife	Pulse cut slow	40W
Submucosal dissection	IT knife	Pulse cut slow Forced coag 2	40W 50W
	Needle knife	Pulse cut slow Forced coag 2	40W 50W
Endoscopic hemostasis	IT knife Needle knife Hot biopsy Coagrasper	Forced coag 2 Soft coag	50W 80W
VIO300D			
Marking	Needle knife	Swift coag	Effect 2, 50W
Precutting	Needle knife	ENDO CUT I	Effect 2, CUT duration 2, CUT interval 3
Mucosal incision	IT knife	ENDO CUT I or Q	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT	Effect 4, 50W
	Needle knife	ENDO CUT I	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT	Effect 4, 50W
Submucosal dissection	IT knife	ENDO CUT I or Q	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT Swift coag	Effect 4, 50W Effect 5, 50W
	Needle knife	ENDO CUT I	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT Swift coag	Effect 4, 50W Effect 5, 50W
Endoscopic hemostasis	IT knife Needle knife Hot biopsy Coagrasper	Swift coag Soft coag	Effect 5, 50W Effect 5, 80W

during the ESD procedure and no remarkable clinical symptoms were observed, suggesting perforation, just after the ESD procedures.

Statistical analysis

The Fisher exact test or the χ^2 test was used for the univariate analyses to assess the above-mentioned clinicopathological factors by comparing the ESD cases with delayed perforation with the ESD cases without perforation. We performed a multivariate analysis for clinicopathological factors that were significant in univariate analyses. A logistic regression analysis was used for the multivariate analysis. All the statistical analyses were performed using the statistical analysis software SPSS, version 20 (SPSS Japan Inc., Tokyo, Japan). A P-value < 0.05 was considered statistically significant.

RESULTS

Incidence and actual management of delayed perforation

Delayed perforation occurred in 7 (0.1%) ESD cases (Table 3). The median time until the occurrence of delayed perforation was 11 h (range, 6-172 h). As for the management of the delayed perforations, 3 (43%) of the 7 delayed perforation cases underwent emergency surgery, while 4 were conservatively managed with nasogastric tube placement, fasting, and the use of intravenous antibiotics and proton pump inhibitors. Two of the 3 patients who required emergency surgery received an omentoplasty or simple closure of the perforation hole; however, one patient underwent a distal gastrectomy because the ESD was evaluated as a non-curative resection. The reason for the emergency surgery in these three cases was panperitonitis with remarkable clinical symptoms, such as diffuse and severe tenderness and/or defense musculaire. Among the 4 cases treated with conservative management, 2 were successfully managed endoscopically using an endoloop-endoclip technique[23,26]. In this technique, the endoloop snare was anchored with some clips to the normal mucosa around the delayed perforation defect[26]. The endoloop snare was tightened slightly, approximating the borders of the defect. Finally, additional clips were placed to achieve complete closure. The median hospital stay in the delayed perforation cases was 18 d (range, 15-45 d). No delayed perforation-related deaths occurred in this series.

Factors associated with delayed perforation

Based on univariate analyses, outside clinical indications, gastric tube cases, location in the upper/middle third of the stomach, and procedure time ⩾ 2 h were significantly associated with a delayed perforation (Table 4). No significant difference between the rates of delayed perforation was observed when the absolute indications (0.1%) and expanded indications (0.1%) were applied. Using a multivariate analysis for these cases, gastric tube cases (OR = 11.0; 95%CI: 1.7-73.3; P = 0.013) were found to be significantly associated with delayed perforation (Table 4).

A representative case (Case 4 in Table 3) with delayed perforation is shown in Figures 1-5. A 64-year-old woman underwent surveillance endoscopy after an esophagectomy for esophageal cancer. The endoscopy showed a superficial depressed EGC lesion, 33 mm

Table 3 Clinical management of delayed perforation induced by gastric endoscopic submucosal dissection

Case	Age (yr)	Sex	Stomach status	Time until the occurrence of delayed perforation (h)	Panperitonitis or severe mediastinitis	Management of delayed perforation	Hospital stay (d)
1	68	Male	Gastric tube	11	Absent	Conservative management	45
2	75	Male	Normal stomach	35	Absent	Conservative management	18
3	80	Male	Normal stomach	6	Absent	Conservative management with endoloop-endoclip technique	18
4	64	Female	Gastric tube	7	Absent	Conservative management with endoloop-endoclip technique	25
5	73	Male	Normal stomach	9	Present (Panperitonitis)	Emergency surgery	15
6	62	Female	Normal stomach	27	Present (Panperitonitis)	Emergency surgery	18
7	56	Female	Normal stomach	172	Present (Panperitonitis)	Emergency surgery	15

Table 4 Factors associated with delayed perforation induced by gastric endoscopic submucosal dissection *n* (%)

Clinicopathological finding	Univariate analysis			Multivariate analysis, OR (95%CI), *P* value
	ESD cases without perforation (*n* = 4813)	ESD cases with delayed perforation (*n* = 7)	*P* value	
Age (yr)			1.00	-
< 70	2538 (99.8)	4 (0.2)		
⩾ 70	2275 (99.9)	3 (0.1)		
Sex			0.16	-
Male	3828 (99.9)	4 (0.1)		
Female	985 (99.7)	3 (0.3)		
Chronological periods			1.00	-
1st period: 1999-2005	2194 (99.9)	3 (0.1)		
2nd period: 2006-2012	2619 (99.8)	4 (0.2)		
Clinical indications			0.02	NS
Outside indications	169 (98.8)	2 (1.2)		
Other indications[1]	4644 (99.9)	5 (0.1)		
Stomach status			0.006	11.0 (1.7-73.3), 0.013
Normal stomach/Remnant stomach	4732 (99.9)	5 (0.1)		
Gastric tube	81 (97.6)	2 (2.4)		
Location			0.047	NS
Upper/Middle	2894 (99.8)	7 (0.2)		
Lower	1919 (100)	0 (0.0)		
Circumference			0.09	-
Greater curvature	774 (99.6)	3 (0.4)		
Others[2]	4039 (99.9)	4 (0.1)		
Size (mm)			0.43	-
⩽ 20	3395 (99.9)	4 (0.1)		
> 20	1418 (99.8)	3 (0.2)		
Depth of invasion			0.34	-
M	3988 (99.9)	5 (0.1)		
SM	825 (99.8)	2 (0.2)		
Ulceration			0.34	-
Absent	3982 (99.9)	5 (0.1)		
Present	831 (99.8)	2 (0.2)		
Histological type			0.09	-
Differentiated	4466 (99.9)	5 (0.1)		
Undifferentiated	347 (99.4)	2 (0.6)		
Type of resection			1.00	-
En bloc resection	4743 (99.9)	7 (0.1)		
Piecemeal resection	70 (100)	0 (0.0)		
Procedure time (h)			0.046	NS
< 2	3758 (99.9)	3 (0.1)		
⩾ 2	1055 (99.6)	4 (0.4)		

[1]Other indications, absolute indications, expanded indications and locally recurrent early gastric cancer; [2]Others, lesser curvature, anterior wall and posterior wall. ESD: Endoscopic submucosal dissection; M: Mucosa; SM: Submucosa; NS: Not significant.

in size, at the greater curvature of the upper gastric body of the gastric tube (Figure 1). The estimated tumor depth was the submucosa, and a biopsy revealed a poorly differentiated adenocarcinoma. ESD was performed for this lesion as a diagnostic procedure, and an *en bloc* resection with negative margins was achieved without any complications. As for the mucosal defect just after the completion

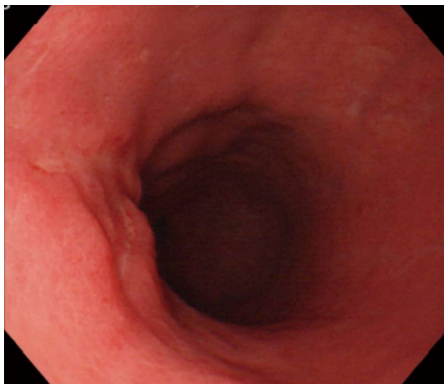

Figure 1 A superficial depressed early gastric cancer lesion located at the greater curvature of the upper gastric body of the gastric tube.

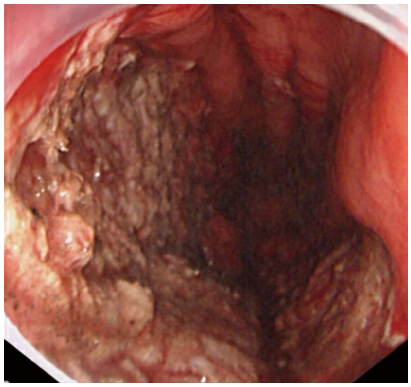

Figure 2 Mucosal defect just after the completion of endoscopic submucosal dissection (60 mm in size and half circumference).

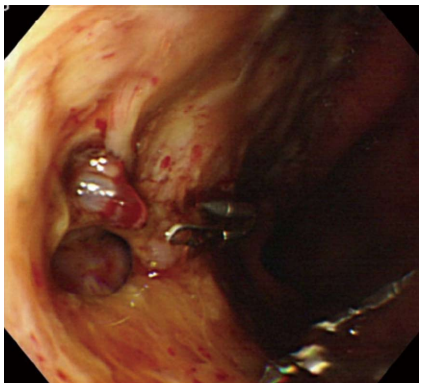

Figure 3 A delayed perforation occurred 7 h after endoscopic submucosal dissection.

of ESD, the size of the defect was 60 mm, and the circumferential extent of the defect was one half of the lumen of the gastric tube. At the proximal edge of the ulceration, severe damage to the surface of the muscularis propria as a result of electrical cautery was seen, but no remarkable clinical symptoms, suggesting perforation, were observed (Figure 2). Seven hours after the ESD, a delayed perforation occurred with chest pain (Figure 3). However, this patient did not develop severe mediastinitis, so endoscopic closure

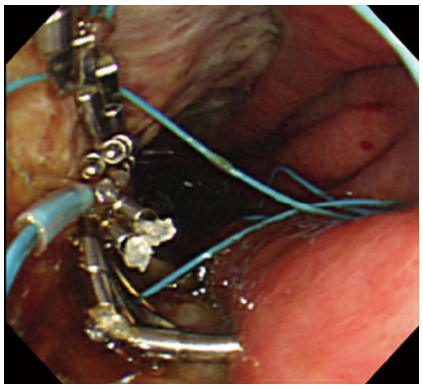

Figure 4 The delayed perforation was successfully closed using the endoloop-endoclip technique.

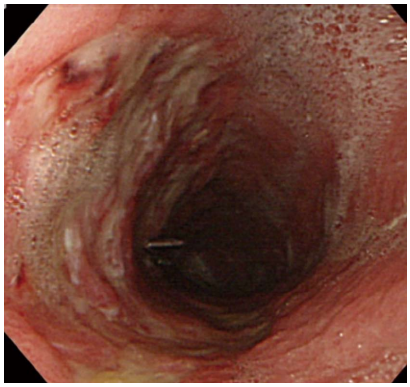

Figure 5 The delayed perforation had almost completely healed 15 d after endoscopic submucosal dissection.

using the endoloop-endoclip technique was attempted and successfully performed (Figure 4). In detail, the endoloop snare was anchored with some clips to the normal mucosa around the delayed perforation defect[23,26]. The endoloop snare was tightened slightly, which approximated the borders of the defect. To achieve complete closure, two endoloop snares with additional clips were needed. The delayed perforation had almost completely healed 15 d after ESD (Figure 5) and finally, the patient was conservatively managed and was discharged 25 d after ESD.

DISCUSSION

Delayed perforation is reported to be a rare (incidence of 0.43% to 0.45%) but serious complication induced by gastric ESD that can sometimes require emergent surgery[6,14-20]. Although several reports have described delayed perforation after gastric ESD, no published report has thoroughly evaluated the various factors that are associated with delayed perforation based on data from a large series of consecutive EGC patients undergoing ESD[6,14-20]. Therefore, the present study is significant because it clarified both the actual clinical management and the associated factors of delayed perforation induced by ESD for EGC based on data

from a large series of consecutive patients undergoing gastric ESD.

In the present study, delayed perforation after gastric ESD occurred in 7 (0.1%) ESD cases, and 3 (43%) of these 7 cases required emergency surgery. Another report from Hanaoka *et al*[14] described 6 (0.45%) cases with delayed perforation among 1329 EGC lesions, and 5 (83%) of these 6 cases underwent emergency surgery. In addition, Kato *et al*[16] reported 2 (0.43%) cases of delayed perforation occurring after the completion of ESD among 468 cases of gastric non-invasive neoplasia, both of which required emergency surgery. Several case reports of delayed perforation after gastric ESD that were successfully managed conservatively have also been reported[15,17,18,20]. Thus, although a small number of cases of delayed perforation might be successfully managed conservatively (9 among 21 delayed perforation patients, including 14 patients in previous reports[14-20] and our 7 patients, were successfully managed conservatively), we need to remember that in delayed perforation cases, emergency surgery may be required with a high probability and conservative management might not always be feasible. In the near future, the establishment of effective conservative treatments may reduce the rate of delayed perforation cases requiring emergency surgery[20]. The early recognition of the onset of delayed perforation after the sudden appearance of symptoms of peritoneal or mediastinal pleura irritation (gastric tube cases) within 24 h after gastric ESD followed by prompt conservative treatment may be useful for avoiding emergency surgery. In the case of delayed perforation without any findings of panperitonitis or severe mediastinitis (gastric tube cases), the endoloop-endoclip technique under CO_2 insufflation might make it possible to close defects of the gastric wall caused by delayed perforation in a conservative manner, as in our representative case[23,26,27]. CO_2 insufflation has increasingly been used instead of air insufflation to minimize pneumoperitoneum caused by perforation[27].

The results of the present study also showed that gastric tube cases were an independent risk factor associated with delayed perforation after ESD, based on a large consecutive series of EGC patients. Hanaoka *et al*[14] reported that 5 out of 6 delayed perforations occurred in the upper third of the stomach; however, this report represented a case series of delayed perforations without any assessment of the risk factors associated with delayed perforation by comparing the ESD cases with delayed perforation with ESD cases without perforation. The reason for the high frequency of delayed perforations in the gastric tube was uncertain, but reduced vascular circulation of the reconstructed gastric tube may have resulted in slower ESD ulcer healing[23]. In addition, Hanaoka *et al*[14] reported that the mechanism of delayed perforation was thought to be due to electrical cautery during the submucosal dissection or repeated coagulation causing ischemic changes to the gastric wall, resulting in necrosis. Furthermore, Onogi *et al*[28] reported the existence of a "transmural air leak" after gastric ESD, as detected by a CT examination. In the present study, we cannot rule out the possible existence of severe damage to the surface of the muscularis propria with a transmural air leak, since we did not perform a CT examination in most of the cases undergoing gastric ESD. Thus, there might be a possibility of developing delayed perforation from severe damage to the surface of the muscularis propria with transmural air leaks after the ESD procedure. More recently, the feasibility and effectiveness of ESD for gastric tube cancer after esophagectomy have been reported[23,24]. Thus, awareness of this finding is important before the widespread use of this treatment, and in cases of ESD for gastric tube cancer, it might be better to avoid excessive electrical cautery during submucosal dissection or repeated coagulation so as to prevent delayed perforation.

Our study had several limitations. First, the results of the present study were based on retrospective assessments of the medical records of patients with gastric ESD, although these data were based on a large consecutive series of gastric ESDs. Second, the present study was conducted at a single major referral cancer center in a large metropolitan area of Japan with many highly experienced endoscopists with specific expertise in ESD. Thus, a prospective multicenter study is required for a more precise evaluation of the actual clinical management and the associated factors of delayed perforation induced by gastric ESD. Several multicenter prospective cohort studies on gastric ESD are currently underway[29-31].

In conclusion, endoscopists must be aware of not only the identified factors associated with delayed perforation induced by gastric ESD, but also how to treat this complication effectively and promptly.

ACKNOWLEDGMENTS

We thank Dr. Hiroyuki Ono and Dr. Takuji Gotoda (our mentors of National Cancer Center Hospital in Tokyo) for their efforts in developing ESD.

COMMENTS

Background

Delayed perforation after gastric endoscopic submucosal dissection (ESD) is a rare but serious complication that sometimes requires emergent surgery. Therefore, the actual clinical management and the associated factors of delayed perforation after gastric ESD should be clarified to minimize its incidence and to treat this complication effectively and promptly.

Research frontiers

Although several reports have described delayed perforation after gastric ESD, no published report has thoroughly evaluated the various factors associated with delayed perforation in addition to the actual clinical management of this complication based on data from a large series of consecutive patients undergoing gastric ESD.

Innovations and breakthroughs

The early recognition of the onset of delayed perforation after the sudden appearance of symptoms of peritoneal or mediastinal pleura irritation (gastric tube cases) within 24 h after gastric ESD followed by prompt conservative treatment may be useful for avoiding emergency surgery. In addition, in cases of ESD for gastric tube cancer, it might be better to avoid excessive electrical cautery during submucosal dissection or repeated coagulation so as to prevent delayed perforation.

Applications

The results of the present study suggest that endoscopists should be aware of not only the identified factors associated with delayed perforation, but also how to treat this complication effectively and promptly.

Terminology

Bleeding and perforation are major complications of gastric ESD. ESD-related perforations can be subdivided into perforations that occur during gastric ESD and delayed perforations occurring after the completion of gastric ESD. Most perforations occur during gastric ESD, and the majority of perforation cases can be treated conservatively using complete endoscopic closure with endoclips. In contrast, delayed perforation is a rare but serious complication that sometimes requires emergent surgery.

Peer-review

This study is significant because it clarified both the actual clinical management and the associated factors of delayed perforation based on data from a large consecutive series of patients undergoing gastric ESD.

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P- Reviewer: Chung JW, Gonzalez N, Kim GH, Man-I M, Matsumoto
S- Editor: Yu J **L- Editor**: Wang TQ **E- Editor**: Ma S

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12644

World J Gastroenterol 2015 November 28; 21(44): 12644-12652
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Retrospective Study

Single center experience in selecting the laparoscopic Frey procedure for chronic pancreatitis

Chun-Lu Tan, Hao Zhang, Ke-Zhou Li

Chun-Lu Tan, Hao Zhang, Ke-Zhou Li, Department of Pancreatic Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Li KZ designed the research; Tan CL and Zhang H collected data and wrote the paper.

Conflict-of-interest statement: The authors have no conflicts of interest or financial ties to disclose.

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Correspondence to: Ke-Zhou Li, PhD, Department of Pancreatic Surgery, West China Hospital, Sichuan University, No. 37 Guo Xue Xiang, Chengdu 610041, Sichuan Province, China. huaxipancreas@163.com
Telephone: +86-28-85422474
Fax: +86-28-85422474

Received: February 2, 2015
Peer-review started: February 5, 2015
First decision: April 24, 2015
Revised: May 14, 2015
Accepted: August 28, 2015
Article in press: August 31, 2015
Published online: November 28, 2015

Abstract

AIM: To share our experience regarding the laparoscopic Frey procedure for chronic pancreatitis (CP) and patient selection.

METHODS: All consecutive patients undergoing duodenum-preserving pancreatic head resection from July 2013 to July 2014 were reviewed and those undergoing the Frey procedure for CP were included in this study. Data on age, gender, body mass index (BMI), American Society of Anesthesiologists score, imaging findings, inflammatory index (white blood cells, interleukin (IL)-6, and C-reaction protein), visual analogue score score during hospitalization and outpatient visit, history of CP, operative time, estimated blood loss, and postoperative data (postoperative mortality and morbidity, postoperative length of hospital stay) were obtained for patients undergoing laparoscopic surgery. The open surgery cases in this study were analyzed for risk factors related to extensive bleeding, which was the major reason for conversion during the laparoscopic procedure. Age, gender, etiology, imaging findings, amylase level, complications due to pancreatitis, functional insufficiency, and history of CP were assessed in these patients.

RESULTS: Nine laparoscopic and 37 open Frey procedures were analyzed. Of the 46 patients, 39 were male (85%) and seven were female (16%). The etiology of CP was alcohol in 32 patients (70%) and idiopathic in 14 patients (30%). Stones were found in 38 patients (83%). An inflammatory mass was found in five patients (11%). The time from diagnosis of CP to the Frey procedure was 39 ± 19 (9-85) mo. The BMI of patients in the laparoscopic group was 20.4 ± 1.7 (17.8-22.4) kg/m^2 and was 20.6 ± 2.9 (15.4-27.7) kg/m^2 in the open group. All patients required analgesic medication for abdominal pain. Frequent acute pancreatitis or severe abdominal pain due to acute exacerbation occurred in 20 patients (43%). Pre-operative complications due to pancreatitis were observed in 18 patients (39%). Pancreatic functional insufficiency was observed in 14 patients (30%). Two laparoscopic patients (2/9) were converted. In seven successful laparoscopic cases, the mean operative time was 323 ± 29 (290-370) min. Estimated intra-operative

blood loss was 57 ± 14 (40-80) mL. One patient had a postoperative complication, and no mortality was observed. Postoperative hospital stay was 7 ± 2 (5-11) d. Multiple linear regression analysis of 37 open Frey procedures showed that an inflammatory mass (P < 0.001) and acute exacerbation (P < 0.001) were risk factors for intra-operative blood loss.

CONCLUSION: The laparoscopic Frey procedure for CP is feasible but only suitable in carefully selected patients.

Key words: Chronic pancreatitis; Frey procedure; Laparoscopic surgery; Surgical outcome; Pain

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Core tip: The Frey procedure is an effective treatment for the relief of pain in chronic pancreatitis. Although the open Frey procedure is well described, the laparoscopic Frey procedure is rarely reported in the literature. Here, we share our experience with nine of these cases and discuss reasons for procedural failure. In addition, we describe the criteria for candidate selection and the results from a data review of open Frey cases.

Tan CL, Zhang H, Li KZ. Single center experience in selecting the laparoscopic Frey procedure for chronic pancreatitis. *World J Gastroenterol* 2015; 21(44): 12644-12652 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12644.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12644

INTRODUCTION

Chronic pancreatitis (CP) is a benign inflammatory disease characterized by the progressive conversion of pancreatic parenchyma to fibrous tissue. For the majority of CP patients, pain is the decisive symptom, and it causes much discomfort in their daily life. The management of CP is challenging, and most patients remain symptomatic despite medical therapy. When medical management fails, the next step is usually endoscopic interventions. Surgical intervention is the last resort when other treatments have failed, the severity of disease has progressed substantially, and pain is unmanageable.

Endoscopic therapy comprises pancreatic and biliary sphincterotomy, stricture dilation and stenting, stone extraction, and lithotripsy. However, not all patients are fit for endoscopic therapy. This approach requires careful patient selection and detailed evaluation of the pancreatic duct anatomy[1]. In fact, many patients choose endoscopic therapy, which is unsuitable, instead of surgical intervention, as it is minimally invasive. Surgical interventions seem to improve pain control[2,3], and it is necessary to develop a minimally invasive approach for surgical intervention in patients with CP.

In 2013, we performed the laparoscopic Frey procedure in selected patients with CP. In the beginning, we found it difficult due to the high rate of conversion because of extensive bleeding. Here, we summarize the outcome of nine laparoscopic cases and analyze the data for risk factors related to extensive bleeding in 37 CP consecutive patients.

MATERIALS AND METHODS

This is a retrospective study of the Frey procedure for CP. The outcome of laparoscopic cases were summarized, and open cases in this study were analyzed for risk factors related to extensive bleeding, which was the major reason for conversion during the laparoscopic procedure. After Institutional Review Board approval, a retrospective review of our database was performed for all consecutive patients undergoing duodenum-preserving pancreatic head resection (DPPHR) from July 2013 to July 2014, at West China Hospital, Sichuan, China. Patients who underwent the Frey procedure for CP were included in the present study. Indications for the Frey procedure included unmanageable pain after medical or endoscopic therapy, suspected malignant lesion, and stones in the distal pancreatic duct. Patients without malignant lesions were considered for the laparoscopic approach, and the Frey procedure was carried out by surgeons with substantial experience with total laparoscopic pancreaticoduodenectomy (PD) (> 20 cases) and significant experience in the open Frey procedure. Patients with malignant disease, which was proven during or after surgery, were excluded. Laparoscopic cases were described in this study and summarized for a single experience. Data on the laparoscopic cases, including age, gender, body mass index (BMI), American Society of Anesthesiologists score, imaging findings, inflammatory index [white blood cells, interleukin (IL)-6 and C-reaction protein (CRP)], visual analogue score (VAS) score during hospitalization and outpatient visit, history of CP, operative time, estimated blood loss, and postoperative data (postoperative mortality and morbidity, postoperative length of hospital stay) were obtained. The risk factors related to extensive intra-operative bleeding were analyzed in the open cases and included age, gender, etiology, imaging findings, level of amylase, complications due to pancreatitis, functional insufficiency, and history of CP.

Technique

The patients were placed in the modified lithotomy reverse Trendelenburg position with thighs parallel to the ground. The operating surgeon stood between the legs of the patient. The camera surgeon stood

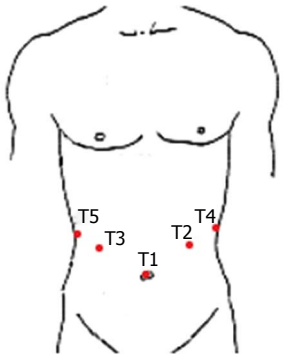

Figure 1 Placement of the 5 ports. T1: 12 mm telescope trocar in the navel; T2: 12 mm trocar along the left midclavear lines; T3: 12-mm trocar along the right midclavear lines; T4: 5 mm trocar along the left anterior axillary line; T5: 5 mm trocar along the right anterior axillary line.

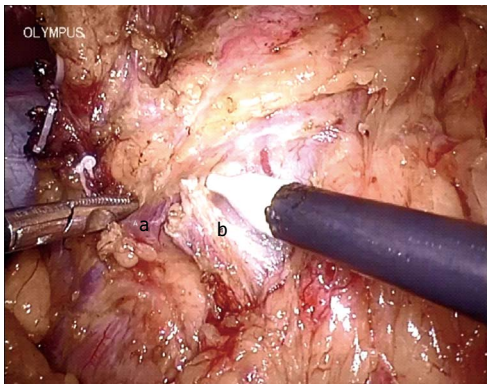

Figure 2 Skeletonized gastrocolic trunk and its branches. a: Anterior superior pancreaticoduodenal vein; b: Right colic vein.

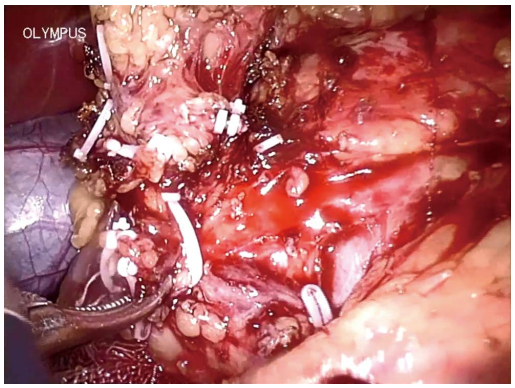

Figure 3 Resection of the branches of the gastrocolic trunk to expose the whole pancreatic head.

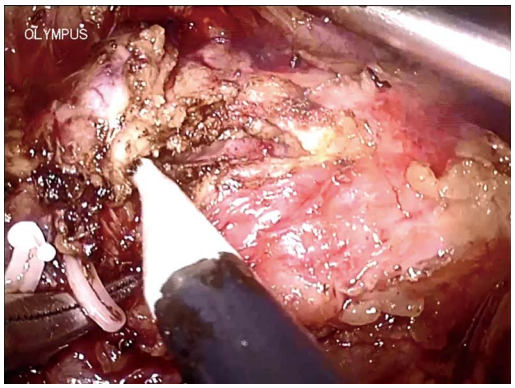

Figure 4 The pancreatic duct was opened longitudinally using the electrosurgical hook.

on the right side of the patient and two assistant surgeons were on the left and right side of the patient, respectively. The port positions are shown in Figure 1. Five trocars were used: a 12 mm telescope trocar in the navel (T1); two 12 mm trocars along the left (T2) and right (T3) midclavear lines, lateral to the rectus muscles, 2 cm above T1; two 5 mm trocars on the left and right side, along the left (T4) and right (T5) anterior axillary line.

The gastrocolic omentum was mobilized to gain entry to the lesser sac. The gastrohepatic omentum was opened to visualize the caudate lobe. The stomach was hung using a urinary catheter and fixed beneath the xiphoid process. The gastroduodenal artery was clipped with white locking plastic vascular clips and then resected. Conversion was considered if it was difficult to find the gastroduodenal artery due to severe inflammation around the pancreas. The gastrocolic trunk and its branches were skeletonized, and then the branches were resected to expose the whole pancreatic head (Figures 2 and 3). Intra-operative ultrasound was used to locate the main pancreatic duct and the extent of stones. The pancreatic duct was opened longitudinally using an electrosurgical hook (Figure 4). Large ductal stones were extracted while opening the duct distally and proximally (Figure 5). The parenchyma of the pancreatic head with a depth to the posterior wall of the Wirsung duct was excavated using a harmonic scalpel (Ethicon Endo-Surgery, Johnson & Johnson Company, New Brunswick, NJ, United States), preserving parenchyma 0.5 cm wide close to the duodenum to prevent damage to the biliary duct in pancreatic parenchyma (Figure 6). The most difficult procedure using the laparoscope was finding the posterior wall of the Wirsung duct. It is advised that the section of the pancreatic parenchyma near the duodenum should be identified, which looks like a dilated orifice. This is the landmark that can indicate that enough of the pancreatic head tissue has been excavated. Usually, this is very difficult to perform even in open surgery, especially in patients with an enlarged pancreatic head. If this is not found, the depth of excavation should exceed the Santorini duct. Stones in the uncinatus process should be excavated. The resected pancreatic tissue was then sent for frozen section examination. Intra-operative ultrasound was then used again to identify residual stones in small branch ducts. All stones visible to the naked eye were removed.

The transverse mesocolon was raised cephalad

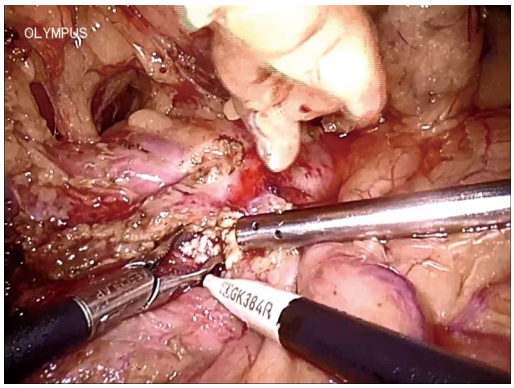

Figure 5 Large ductal stones are extracted while opening the duct distally and proximally.

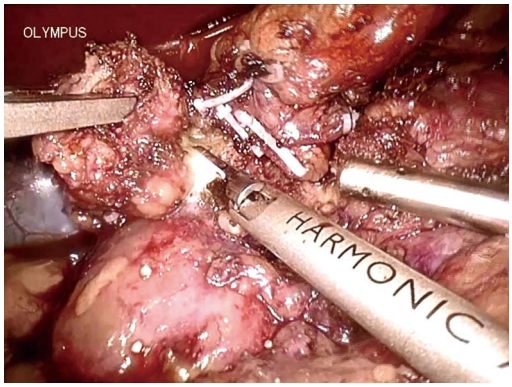

Figure 6 The parenchyma of the pancreatic head is excavated using a harmonic scalpel.

to identify the ligament of Treitz, and the pancreatic limb was measured to 40 cm. The small bowel was cut using an endolinear cutter (Ethicon Endo-Surgery, Johnson & Johnson Company). The Roux limb was marked and measured 40-60 cm according to the length of the opened pancreatic duct to allow tension-free retrocolic positioning to the lesser sac. A stapled side-to-side jejunojejunostomy was created using the endolinear cutter. The mesenteric defect was closed to prevent a potential internal hernia.

Careful staunching of bleeding was performed before pancreaticojejunostomy. The bowel was opened longitudinally and a one layer side-to-side pancreaticojejunostomy was completed with interrupted sutures (Figure 7). A drain was then placed beside the anastomosis.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Multiple linear regression analysis was used to discriminate risk factors related to operative blood loss. For all analyses, $P < 0.05$ was considered significant. All analyses were performed using SPSS version 18.0 software (SPSS Company, Chicago, IL, United States).

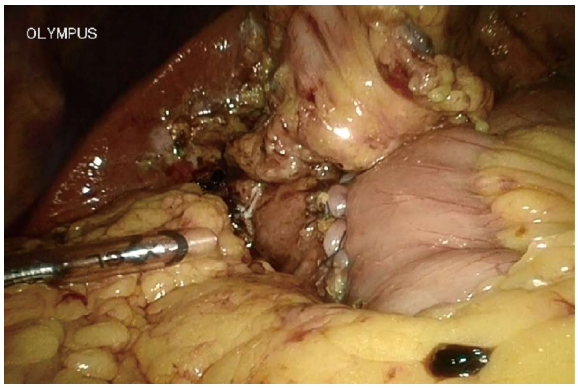

Figure 7 One layer side-to-side pancreaticojejunostomy completed with interrupted sutures.

RESULTS

Fifty-seven patients underwent DPPHR from July 2013 to July 2014. Nine patients underwent DPPHR for low malignant tumors and were excluded. Two patients were excluded due to malignant lesions confirmed after surgery. In total, 46 patients were included in this study. The laparoscopic Frey procedure was performed by one surgeon in nine patients with a mean age of 50 years. These nine patients were not suitable for endoscopic management during pre-surgery assessment, eight due to stones in the distal part of the pancreatic duct and one due to an inflammatory lesion in the pancreatic head. The open Frey procedure was performed by different surgeons in 37 patients with a mean age of 45 years.

Of these 46 patients, 39 were male (85%) and seven were female (16%). The etiology of CP was alcohol in 32 patients (70%) and idiopathic in 14 patients (30%). Stones were found in 38 patients (83%). An inflammatory mass was found in five patients (11%). The time from diagnosis of CP to the Frey procedure was 39 ± 19 (9-85) mo. BMI of patients in the laparoscopic group was 20.4 ± 1.7 (17.8-22.4) kg/m^2 and was 20.6 ± 2.9 (15.4-27.7) kg/m^2 in the open group. All patients required analgesic medication due to abdominal pain. Frequent acute pancreatitis or severe abdominal pain due to acute exacerbation occurred in 20 patients (43%). Pre-operative complications due to pancreatitis were observed in 18 patients (39%). Pancreatic functional insufficiency was observed in 14 patients (30%). The mean pre-operative VAS in the laparoscopic patients was 7.1 ± 0.8 (6-8) before surgery and was 1.1 ± 0.9 (0-2) at the 3 mo follow-up period. The demographics and peri-operative characteristics of the patients are listed in Tables 1 and 2.

Two patients were converted in the laparoscopic group. Seven patients were treated successfully with a mean operative time of 323 ± 29 (290-370) min. Estimated intra-operative blood loss (EBL) was

Table 1 Demographics and peri-operative characteristics of nine laparoscopic patients

Patient	Sex	Age (yr)	BMI (kg/m^2)	Etiology	ASA	VAS	Pancreatitis	M-ANNHEIM	M-ANNHEIM score
1	Male	54	17.8	Alcohol	Ⅱ	6	No	IIb	9
2	Male	44	20.4	Idiopathic	Ⅱ	7	No	Ib	7
3	Male	60	18.5	Alcohol	Ⅲ	7	No	IIb	14
4	Male	52	21.4	Alcohol	Ⅲ	8	Yes	Ib	10
5	Male	56	19.3	Alcohol	Ⅱ	7	No	IIb	14
6	Male	46	22.1	Alcohol	Ⅱ	6	No	Ib	9
7	Male	42	19.7	Alcohol	Ⅱ	8	No	Ib	9
8	Male	56	21.8	Idiopathic	Ⅲ	8	No	IIb	14
9	Male	36	22.4	Idiopathic	Ⅱ	7	No	Ib	9
	Amylase	WBC (× 10^9/L)	CRP	IL-6	Pancreatic duct (mm)	Stones	Inflammatory mass	History of CP (mo)	
1	68	5.1	No	No	8	Yes	No	62	
2	48	6.14	No	No	5	No	Yes	42	
3	47	4.11	No	No	10	Yes	No	85	
4	101	5.3	No	No	8	Yes	No	32	
5	78	6.84	No	No	9	Yes	No	76	
6	53	4.7	No	No	8	Yes	No	47	
7	55	5.1	No	No	9	Yes	No	56	
8	66	6.44	No	No	11	Yes	No	80	
9	44	5.3	No	No	9	Yes	No	32	

M-ANNHEIM: A classification system for chronic pancreatitis, Ib indicates recurrent or chronic abdominal pain without pancreatic insufficiency, IIb indicates isolated exocrine (or endocrine) pancreatic insufficiency with pain; ASA: American Society of Anesthesiologists; VAS: Visual analogue score; WBC: White blood cell; IL: Interleukin; CRP: C-reactive protein; CP: Chronic pancreatitis.

Table 2 Demographics and peri-operative characteristics of 37 open surgery patients

Male/Female	30/7
Age (yr)	45 ± 11 (27-70)
Body mass index	20.6 ± 2.9 (15.4-27.7)
Etiology	
Alcohol	26 (70)
Idiopathic	11 (30)
Pancreatic stone	30 (81)
Pancreatic mass	4 (11)
Acute exacerbation	19 (51)
History of diagnosis of CP (mo)	34 ± 17 (9-85)
Amylase elevated over 3 times	4 (11)
Complications due to pancreatitis	
Pancreatic pseudocyst	12 (32)
Bile duct stenosis	5 (14)
PPH	1 (3)
Functional insufficiency	
IGT/Diabetes mellitus	10 (27)
Malabsorption	1 (3)

CP: Chronic pancreatitis; PPH: Postpancreatectomy hemorrhage; IGT: Impaired glucose tolerance.

57 ± 14 (40-80) mL. Postoperative complications occurred in one patient who had postpancreatectomy hemorrhage (PPH) and recovered without treatment. No mortality was observed in the laparoscopic group. Postoperative hospital stay was 7 ± 2 (5-11) d (Table 3). Postoperative morbidity was 11% (4/37) in the open group, No mortality was observed. EBL was 293 ± 247 (70-1100) mL. Multiple linear regression analysis of the 37 open Frey procedures showed that an inflammatory mass ($P < 0.001$) and acute exacerbation ($P < 0.001$) were risk factors for intra-operative blood loss (Table 4) among the risk factors listed in Table 2.

DISCUSSION

The mechanism of pain in CP remains unclear and is debated[4,5]. Several hypotheses have been proposed, and it is thought that pain is probably the result of a combination of these concepts. They comprise intraductal and interstitial hypertension and neurogenic and central sensitization theories[6,7]. It has been shown than simple drainage procedures do not ensure sufficient pain relief in patients with enlargement of the pancreatic head[8,9]. Therefore, resection of the pancreatic head should be a central feature of any surgical procedure. The Frey procedure improves the overall pancreatic ductal drainage by decompressing both the main and small ducts in the pancreatic head. Moreover, most of the pancreatic head, which is thought to be the "pacemaker" of pain, is removed during this procedure[10]. Randomized controlled trials have demonstrated that PD is effective in controlling CP symptoms. However, the high morbidity and mortality associated with this procedure are considered preventable in this benign disease[11,12]. The 15 year long-term effectiveness of the Frey procedure was comparable to pylorus-preserving PD and resulted in permanent pain control. However, quality of life was better after the Frey procedure with regard to physical status[13]. When comparing the Beger procedure with the Frey procedure, the latter procedure was associated with fewer complications and equivalent pain relief, functional insufficiency, and quality of

Table 3 Postoperative characteristics of nine laparoscopic Frey procedures

Patient	Converted	Operative time (min)	EBL (mL)	Complications	Mortality	LOS	EBL (3-mo)
1	No	370	50	No	No	5	2
2	Yes	-	-	-	-	-	-
3	No	290	60	No	No	7	1
4	No	-	-	-	-	-	-
5	No	310	50	PPH (grade A)	No	11	0
6	No	330	70	No	No	6	2
7	No	350	50	No	No	7	2
8	No	300	40	No	No	5	0
9	No	310	80	No	No	6	1

PPH: Postpancreatectomy hemorrhage; EBL: Estimated intra-operative blood loss.

Table 4 Results of multiple linear regression analysis of 37 open Frey procedures

Risk factors	Standard regression coefficient	*P* value
Inflammatory mass	0.559	0.00
Acute exacerbation	0.508	0.00

life[10,14,15].

CP is a common inflammatory disease with the principal symptom of chronic pain, which can reduce quality of life and results in the inability to work. A 5-fold increase in mortality in CP patients was observed compared with a population without CP[16]. Despite improvements in conservative, interventional, and surgical procedures, the treatment of CP remains challenging[17]. Medical treatment is currently recommended first in order to avoid surgery. Surgical intervention is the last resort when other treatments have failed and the severity of the disease has increased substantially such that pain is unmanageable. At the end of the last century, several studies demonstrated that early surgical pancreatic drainage was beneficial for pain control and preservation of function[18-20]. A multicenter randomized controlled trial is now in progress by the Dutch Pancreatitis Study Group to evaluate the benefits, risks, and costs of early surgical intervention. Endoscopic treatment is less invasive than surgical intervention and is preferred by patients. Endoscopic therapy aims to remove obstructions (strictures or stones) in the main pancreatic duct. This approach requires careful patient selection and detailed evaluation of the pancreatic duct anatomy. In a retrospective analysis of 1000 patients treated with endoscopy, approximately 25% of patients underwent surgery for failure of pain relief after therapy[21]. Moreover, surgery has been shown to be superior to endoscopic treatment in two main randomized controlled trials[3,22]. However, some unsuitable patients choose endoscopic treatment as they are afraid of surgical intervention.

Laparoscopic surgery is now routinely performed for many pancreatic diseases, with the exception of CP[23-25]. We performed our first laparoscopic Frey procedure for CP in 2013. To date, we have carefully selected nine patients for the laparoscopic Frey procedure. Seven procedures were successful, while two procedures were converted to open surgery. Patient 2 was converted because we were unable to find the pancreatic duct, although intra-operative ultrasound identified segmental dilation of the main pancreatic duct. The pancreatic duct is the mark of depth for excavation during laparoscopy. Opening the pancreatic duct longitudinally is an important procedure for drainage during the Frey procedure. The pancreatic parenchyma is very thick, and the pancreatic duct is about 5 mm on pre-operative computed tomography (CT). Thus, a pancreatic duct width of over 8 mm was one of our criteria for laparoscopic candidates on the basis of our own experience. All patients met this criterion, and their pancreatic ducts were easily found during laparoscopy. Patient 4 was converted to the open procedure due to severe inflammatory pancreatic parenchyma. Blood oozed from the pancreas when the pancreatic head parenchyma was excavated. It was difficult to staunch the bleeding with electrocautery or suture during laparoscopy. Therefore, we immediately converted to the open procedure to avoid unmanageable bleeding during excavation of the pancreatic head.

The laparoscopic Frey procedure requires careful patient selection. The degree of pancreatic inflammation, which can lead to unmanageable bleeding, is the key factor in the success of this procedure. However, it is very difficult to judge how severe the inflammation is before surgery. We found that indicators of systemic inflammation, such as white blood cells, IL-6, and CRP in blood samples, were not sensitive for pancreatic inflammation. Patient 4 had a history of acute pancreatitis with two episodes per year in recent years. The last episode occurred 6 mo before surgery. We selected this patient for the laparoscopic Frey procedure because the level of amylase was normal and no sign of acute inflammation was seen on the CT image. Unfortunately, the patient was converted to the open procedure due to severe peri-pancreatic inflammation.

In order to identify risk factors for uncontrolled intra-operative bleeding, we obtained peri-operative data of 37 patients during the open Frey procedure.

Pre-operative factors, including age, BMI, gender, etiology, pancreatic stones, pancreatic mass, history of CP, amylase level, complications due to acute pancreatitis, and functional insufficiency, were evaluated in a multiple linear regression analysis. The results of multiple linear regression analysis showed that a pancreatic inflammatory mass and pre-operative acute exacerbation were related to the volume of intra-operative blood loss. These two factors usually indicate severe inflammation in the pancreatic head. These results were comparable to the findings during nine laparoscopic procedures. Although complications due to acute pancreatitis were not risk factors in our study, they were difficult to manage under laparoscopy. Thus, we chose the open procedure for patients with complications due to acute pancreatitis.

As laboratory examination can not indicate the degree of peri-pancreatic inflammation, CT imaging may be more suitable. In our successful cases, we found that fibrosis and calcification, which were easily observed on CT images, usually meant manageable bleeding during pancreatic excavation. This also indicated a low incidence of pancreatic fistula after surgery. According to a published report, the majority of pancreatic calcifications (56%) resulted in relapsing pain episodes[26].

The extent of excavation of pancreatic head parenchyma is questionable. The original Frey procedure involved coring out the anterior and posterior parenchyma of the main pancreatic duct in the pancreatic head and resecting as much of the parenchyma as possible, leaving only the posterior capsule of the head. We found this difficult to achieve during laparoscopy. The depth of parenchyma lacks an anatomic landmark. Injury to the common bile duct or portal vein frequently occurred during the original Frey procedure[27]. This is riskier during laparoscopy, as the main pancreatic duct is the only anatomic landmark during laparoscopy. Therefore, it is very important to open the main pancreatic duct during laparoscopy. In order to avoid injury to the common bile duct, we preserved parenchyma 0.5 cm wide close to the duodenum. We preserved parenchyma posterior to the main duct in the pancreatic head as much as possible to prevent injury to the superior mesenteric vein. The anatomic landmark for the depth of parenchyma excavated is the posterior wall of the Wirsung duct, similar to the modified Frey procedure[27]. It is difficult to find the posterior wall of the Wirsung duct during laparoscopy. It is advisable to find the section of the pancreatic parenchyma near the duodenum that looks like a dilated orifice. However, it is usually very difficult to do that even during open surgery, especially in patients with an enlarged pancreatic head. If it cannot be found, the depth for excavation should exceed the Santorini duct. Although our extent of resection is smaller than the ordinary Frey procedure, the VAS score decreased quickly. All seven patients showed significantly lower VAS score at 3 mo after surgery compared with before surgery. Sakata *et al*[28] advocated a "minimum Frey procedure" with limited resection of the anterior part of the pancreas head with a longitudinal pancreaticojejunostomy. These authors compared the effectiveness of the minimum Frey procedure with the original Frey procedure in terms of pain relief and preservation of endocrine and exocrine function. In the present study, the VAS score in laparoscopic patients decreased significantly at the 3 mo follow-up period. However, our reduced extent of excavation may not be suitable for candidates with an inflammatory mass in the pancreas head.

In the multiple linear regression analysis of 37 open Frey procedures, an inflammatory mass was a risk factor for intra-operative blood loss. The extent of excavation of the pancreatic head described above may not be enough for candidates with an inflammatory mass in the pancreatic head. Taking into consideration the two risk factors for massive bleeding and insufficient excavation, we think candidates with an inflammatory mass should undergo the open procedure.

Taking into account the outcomes of nine laparoscopic cases and the results of multiple linear regression analysis of 37 open cases, we suggest that the laparoscopic Frey procedure may only be suitable for patients with an obvious dilated pancreatic duct, an enlarged pancreatic head on CT, and an absence of an inflammatory mass, exacerbation, and complications due to pancreatitis.

In conclusion, the laparoscopic Frey procedure is feasible, but suitable only in carefully selected patients. It may only be suitable for patients with an obvious dilated pancreatic duct and enlarged pancreatic head on CT. If acute exacerbation, inflammatory mass, or complications due to pancreatitis exist before surgery, the laparoscopic procedure should not be considered.

COMMENTS

Background

Chronic pancreatitis (CP) is a benign inflammatory disease, characterized by the progressive conversion of pancreatic parenchyma to fibrous tissue. Patients with CP can have pain that reduces the quality of life. Surgical intervention is the last resort when other treatments have failed, the severity of disease has increased substantially, and pain is unmanageable. Here, the authors reported their experience regarding the laparoscopic Frey procedure for CP.

Research frontiers

Laparoscopic surgery is now routinely performed for many pancreatic diseases, with the exception of CP. There are few published studies on the use of this procedure for CP. Here, we summarize the outcome of nine cases using this procedure, discuss why this laparoscopic procedure is difficult in patients with CP, and how to select the right candidates.

Innovations and breakthroughs

The management of CP is challenging, and most patients remain symptomatic despite medical therapy. The endoscopic approach requires careful patient selection and detailed evaluation of the pancreatic duct anatomy. As one of the surgical therapies used for CP, the Frey procedure can provide permanent pain control. Laparoscopic surgery is now routinely performed for many pancreatic diseases, with the exception of CP. However, the laparoscopic

Frey procedure is rarely reported. The authors reported nine patients who underwent the laparoscopic Frey procedure, including seven successful cases, and highlighted the surgical process and the key points. According to their experience, conversion to the open procedure was usually due to severe peri-pancreatic inflammation, which could lead to uncontrolled bleeding during laparoscopy, or undetectable pancreatic duct. An inflammatory mass on CT and acute exacerbation were pre-operative risk factors for intra-operative blood loss. These findings were helpful in the pre-operative selection of candidates for laparoscopy.

Applications

This study shows that the laparoscopic Frey procedure is feasible, but only suitable for carefully selected patients. It may only be suitable for patients with an obvious dilated pancreatic duct and enlarged pancreatic head on CT. If acute exacerbation, inflammatory mass, or complications due to pancreatitis exist before surgery, the laparoscopic procedure should not be considered.

Terminology

The Frey procedure was first described in 1987 by Frey *et al* and combines partial resection of the head of the pancreas (resection) with lateral pancreatico-jejunostomy (drainage). The rationale for this hybrid procedure is that it improves the overall pancreatic ductal drainage by decompressing both the duct of Santorini and ducts in the uncinate process.

Peer-review

The true interest of this original paper is the scarce number of laparoscopic Frey procedures, with their difficulties and complications, reported in the literature. However, the short follow-up of the postsurgical evolution of patients makes it difficult to compare the evolution of the patient's pain after the laparoscopic approach compared with the standard Frey open procedure or with the results published in the literature.

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P- Reviewer: Olesen SS, Rabago L **S- Editor**: Yu J
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World J Gastroenterol 2015 November 28; 21(44): 12653-12659
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Retrospective Study

Xanthogranulomatous cholecystitis mimicking gallbladder carcinoma: An analysis of 42 cases

Yi-Lei Deng, Nan-Sheng Cheng, Shui-Jun Zhang, Wen-Jie Ma, Anuj Shrestha, Fu-Yu Li, Fei-Long Xu, Long-Shuan Zhao

Yi-Lei Deng, Shui-Jun Zhang, Fei-Long Xu, Long-Shuan Zhao, Department of Hepatopancreatobiliary Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, Henan Province, China

Nan-Sheng Cheng, Wen-Jie Ma, Anuj Shrestha, Fu-Yu Li, Department of Biliary Surgery, West China Hospital of Sichuan University, Chengdu 610000, Sichuan Province, China

Author contributions: Deng YL, Cheng NS and Zhao LS designed the research; Deng YL, Zhang SJ, Ma WJ and Zhao LS performed the research; Shrestha A, Li FY and Xu FL contributed new reagents or analytic tools; Deng YL, Zhang SJ, Ma WJ and Zhao LS analyzed the data; Deng YL, Cheng NS and Shrestha A wrote the paper.

Supported by The Science and Technology Support Project of Sichuan Province, No. 2014SZ0002-10.

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Correspondence to: Dr. Long-Shuan Zhao, Department of Hepatopancreatobiliary Surgery, The First Affiliated Hospital of Zhengzhou University, No. 1 Jian She Road, Zhengzhou 450000, Henan Province, China. dengyilei@163.com
Telephone: +86-371-66913114
Fax: +86-371-66916261

Received: January 2, 2015
Peer-review started: January 13, 2015
First decision: January 22, 2015
Revised: August 26, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: November 28, 2015

Abstract

AIM: To review and evaluate the diagnostic dilemma of xanthogranulomatous cholecystitis (XGC) clinically.

METHODS: From July 2008 to June 2014, a total of 142 cases of pathologically diagnosed XGC were reviewed at our hospital, among which 42 were misdiagnosed as gallbladder carcinoma (GBC) based on preoperative radiographs and/or intra-operative findings. The clinical characteristics, preoperative imaging, intra-operative findings, frozen section (FS) analysis and surgical procedure data of these patients were collected and analyzed.

RESULTS: The most common clinical syndrome in these 42 patients was chronic cholecystitis, followed by acute cholecystitis. Seven (17%) cases presented with mild jaundice without choledocholithiasis. Thirty-five (83%) cases presented with heterogeneous enhancement within thickened gallbladder walls on imaging, and 29 (69%) cases presented with abnormal enhancement in hepatic parenchyma neighboring the gallbladder, which indicated hepatic infiltration. Intra-operatively, adhesions to adjacent organs were observed in 40 (95.2%) cases, including the duodenum, colon and stomach. Thirty cases underwent FS analysis and the remainder did not. The accuracy rate of FS was 93%, and that of surgeon's macroscopic diagnosis was 50%. Six cases were misidentified as GBC by surgeon's macroscopic examination and underwent aggressive surgical treatment. No statistical difference was encountered in the incidence of postoperative complications between total cholecystectomy and subtotal cholecystectomy groups (21% *vs* 20%, $P > 0.05$).

CONCLUSION: Neither clinical manifestations and laboratory tests nor radiological methods provide a

practical and effective standard in the differential diagnosis between XGC and GBC.

Key words: Xanthogranulomatous cholecystitis; Gallbladder carcinoma; Frozen-section analysis; Cholecystectomy

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Core tip: Xanthogranulomatous cholecystitis (XGC) is a destructive inflammatory disease of the gallbladder that could mimic gallbladder carcinoma (GBC) in various ways. The aim of this study was to review and evaluate the diagnostic dilemma of XGC clinically. We concluded that neither clinical manifestations and laboratory tests nor radiological methods provide a practical and effective standard in the differential diagnosis between XGC and GBC. For XGC with suspected GBC, intra-operative frozen section analysis should be performed, and the benign diagnosis indicates that a simple cholecystectomy is appropriate. However, the final diagnosis still depends on the pathology.

Deng YL, Cheng NS, Zhang SJ, Ma WJ, Shrestha A, Li FY, Xu FL, Zhao LS. Xanthogranulomatous cholecystitis mimicking gallbladder carcinoma: An analysis of 42 cases. *World J Gastroenterol* 2015; 21(44): 12653-12659 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12653.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12653

INTRODUCTION

Xanthogranulomatous cholecystitis (XGC) is a rare variant of chronic cholecystitis characterized by severe proliferative fibrosis with infiltration of macrophages and foamy cells within the gallbladder wall. This condition is benign in nature but often shows a destructive inflammatory process[1,2]. The inflammatory infiltration and fibrosis cause the asymmetrical thickening of the gallbladder wall and the formation of multiple yellowish-brown nodules, which often extend into the neighboring organs, such as the liver, omentum and duodenum[1,2].

Due to the overlapping features between the two diseases, XGC is frequently misdiagnosed as gallbladder carcinoma (GBC) and usually undergoes an extended radical surgery[3,4]. Sometimes XGC might have a coexistent GBC[5]. Accurate diagnosis is important for the proper surgical management of these patients. To our knowledge, several non-invasive imaging and invasive techniques have been reported to differentiate XGC from GBC[6-10], but the dilemma still exists concerning the differential diagnosis in clinical practice, and the final diagnosis has to be dependent on the histological examination. In the present retrospective study, we analyzed the clinical, radiologic and surgical features of 42 cases of XGC misdiagnosed as GBC to review and evaluate the diagnostic dilemma of XGC clinically.

MATERIALS AND METHODS

From July 2008 to June 2014, a total of 142 cases with a pathological diagnosis of XGC were reviewed at our hospital, among which 42 (24 men and 18 women; male-to-female ratio, 4:3; mean age 59.9 years, age range 38-86 years) were misdiagnosed as GBC mainly by the presence of the focal or diffuse thickening of the gallbladder wall, and/or the mass protruding into the lumen based on preoperative radiographs, and/or intra-operative findings. Thirty-eight cases underwent contrast enhanced abdominal computed tomography (CT), and 4 cases underwent magnetic resonance imaging (MRI). The clinical characteristics, preoperative imaging, intra-operative findings, frozen section (FS) analysis, surgical procedures and histological features of these patients were collected and analyzed.

For each patient, intra-operative findings including inflammation of the gallbladder (atrophy, edema or gangrene), adhesions of the gallbladder to adjacent tissues, thickened gallbladder wall, gallstones, mass lesions, gallbladder internal fistula, enlarged regional lymph nodes and hepatic abscess were observed and recorded. And then, the excised gallbladder was opened along its longitudinal axis and the mucosa was examined macroscopically. If malignancy was suspected, suspicious areas would be labeled by the silk suture and sent for an FS analysis. Briefly, frozen sections of 6-μm thickness were cut using automated devices (Shandon Citadel 2000, Astmoor, United Kingdom). Subsequently, sections were stained with hematoxylin and eosin (HE), and the diagnosis was made by experienced pathologists. Simultaneously the direct contact between surgeons and pathologists was also established.

The gallbladder wall thickness of 1-2 mm was considered as normal, and a wall thickness of 3 mm or more on the imaging or intra-operative findings was considered thickened. GBCs were classified according to the TNM system as proposed by the American Joint Committee on Cancer. The study protocol was approved by the ethics committee review board of Sichuan University.

Statistical analysis

Data were analyzed using the SPSS v13.0 software package (SPSS, Chicago, IL, United States). Statistical comparisons between groups were performed using the Fisher's exact test. The least significant difference test was performed to compare two groups. A P-value < 0.05 indicated statistical significance.

RESULTS

The clinical presentations are summarized in Table

Table 1 The clinical presentations *n* (%)

Variable	No. of patients
Presenting signs and symptoms	
Pain	38 (90)
Chronic RUQ pain	26 (61)
Acute RUQ pain, Murphy's sign (+)	12 (28)
Abdominal distention	20 (48)
Anorexia	19 (45)
Jaundice	9 (21)
High grade fever (> 38 ℃)	6 (14)
Nausea and vomiting	5 (12)
RUQ mass	2 (5)
Laboratory findings	
Elevated WBC count	11 (26)
Elevated blood bilirubin level	9 (21)
Mild	9 (21)
Moderate	0
Severe	0
Increased CA199 (> 22 U/mL)	32 (76)
Increased CEA (> 4.0 ng/mL)	6 (14)

RUQ: Right upper quadrant; WBC: White blood cell; CEA: Carcinoembryonic antigen.

Table 2 Abdominal computed tomography or magnetic resonance imaging findings *n* (%)

Finding	No. of patients
Gallstones	22 (52)
Thickened gallbladder wall (> 3 mm)	42 (100)
Diffuse	27 (64)
Focal	15 (36)
Gallbladder distention with pericholecystic fluid	12 (29)
Heterogeneous enhancement of the gallbladder wall	35 (83)
Intramural hypo-attenuated nodules	11 (26)
Pericholecystic infiltration	
Blurred liver/gallbladder interface	37 (88)
Abnormal enhancement in hepatic parenchyma neighboring the gallbladder	29 (69)
Infiltration of other adjacent structures	16 (38)
Regional lymph node enlargement	11 (26)

1. The most common syndrome in these 42 patients was chronic cholecystitis, and patients with this clinical syndrome had chronic right upper quadrant (RUQ) pain (61%), abdominal distention (48%), and anorexia (45%). The second most common syndrome was acute cholecystitis, and these patients presented with acute RUQ pain (28%), high grade fever (14%), elevated WBC count (26%), nausea and vomiting (12%). Approximately 21% of the patients presented with mild jaundice, but only two patients were associated with choledocholithiasis. The RUQ mass was palpable in two (5%) patients. Increased CA199 level was observed in 32 (76%) patients and increased carcinoembryonic antigen (CEA) level was found only in 6 (14%) patients.

CT or MRI findings in 42 patients are summarized in Table 2. Only 4 (9%) of 42 patients underwent MRI, and studies suggest that the morphological appearance of the GBC in MRI was similar to that obtained by CT[11], so it seemed to be more appropriate to integrate the characteristic MRI findings with CT findings. The presence of a thickened gallbladder wall was noted in all 42 patients, with 64% of the patients having a diffuse thickening. It could also be seen that the majority (83%) of the thickened gallbladder walls showed a heterogeneous enhancement at the luminal surface. Furthermore, 26% of the markedly thickened gallbladder walls were associated with intramural hypo-attenuated nodules. Gallstones were detected in 22 (52%) patients, including two combined common bile duct (CBD) stones. Blurred liver/gallbladder interface was observed in 37 (88%) patients, 29 of whom presented with an abnormal enhancement in the hepatic parenchyma neighboring the gallbladder, which indicated hepatic infiltration. In addition, the infiltration of other adjacent structures could also be noted in 38% of the patients, including the omentum, colon, duodenum, and the antrum of the stomach. Enlarged lymph nodes were noted in 11 (26%) patients.

Open surgery was planned and performed in all 42 patients. Intra-operative findings confirmed the presence of a thickened gallbladder wall in all patients, and adhesions to the adjacent organs were seen in 40 (95.2%) patients (adhesions to the omentum in 32, the duodenum in 16, the colon in 12, and the stomach in 8). Eleven (26%) patients had the obscure Calot' s triangle anatomy. Twenty patients presented with acute cholecystitis including gallbladder wall edema (*n* = 15) and gangrenous cholecystitis (*n* = 5). Gallstones were observed in 23 (55%) patients, including two cases of combined CBD stones. Mass lesions were found in 12 (29%) patients. Other intra-operative findings are shown in Table 3.

The surgical procedures for the 42 XGC cases are shown in Figure 1. Intra-operative FS analysis was performed in 30 patients, revealing 28 benign and 2 malignant gallbladder lesions. Among 28 benign lesions, total cholecystectomy (TC) was performed on 14 patients and subtotal cholecystectomy (SC) on 10 patients. For two GBCs, only an external biliary drainage was carried out. Twelve cases of XGC did not undergo intra-operative FS analysis, whereas 6 "GBCs" were diagnosed by surgeon's macroscopic examination during surgery, and thereafter 4 patients received "radical resection of GBC", with 2 patients receiving an external biliary drainage. Another 6 patients were considered to be suffering from benign gallbladder disease by surgeon, including a combined CBD stone, hepatic abscess and Mirizzi's syndrome. Ultimately, specimens from all the 42 patients were examined pathologically after operation. Of the 14 patients who underwent TC, only 2 were definitively diagnosed with coexisting early GBC (T1a).

Of the 42 XGC cases reviewed, 12 patients underwent TC. SC was carried out only in 10 patients, leaving a part of the posterior wall adherent to the hepatic bed, or a part of the hartmann sac or the

Table 3 Intra-operative findings *n* (%)

Feature	No. of patients
Thickened gallbladder wall	42 (100)
Adhesions	40 (95)
Omentum	32 (76)
Duodena	16 (38)
Colon	12 (29)
Stomach	8 (19)
Obscure Calot's triangle anatomy	11 (26)
Gallbladder wall edema	15 (36)
Gangrenous cholecystitis	5 (12)
Gallstones	23 (55)
Combined CBD stones	2 (5)
Mass lesions	12 (29)
Enlarged regional lymph nodes	11 (26)
Gallbladder internal fistula	8 (19)
Mirizzi's syndrome	4 (10)
Cholecystoduodenal fistula	2 (5)
Gallbladder-transverse colon fistula	2 (5)
Hepatic abscess	3 (7)

CBD: Common bile duct.

gallbladder neck, due to obscure Calot's triangle anatomy and/or gangrenous cholecystitis with severe fibrotic adhesions and/or the high risk gallbladder bed in cirrhotic patients. Postoperative complications are summarized in Table 4. Comparison between the two groups showed no statistical difference in the incidence rates of complications (21% *vs* 20%, $P > 0.05$). However, there was one case of common bile duct injury which underwent a primary repair and "T-tube" drainage, and one case of duodenal injury repaired by omental patch and suture repair for the TC group, while there was no serious complication observed in the SC group. No deaths occurred in either group.

DISCUSSION

The pathogenesis of XGC is not fully understood till now. The presence of gallstones and biliary obstruction might play an important role, which causes the extravasation of the bile into the gallbladder wall *via* ruptured Rokitansky-Aschoff sinuses and/or ulcers of the surface mucosa[1,2]. Clinically, XGC not only often imitates GBC in various ways leading to the misdiagnosis of GBC, but it could also coexist with GBC[3-10]. In our study, 42 (29.6%) out of 142 cases of XGC were misdiagnosed as GBC either preoperatively or intra-operatively, but only 4 (9.5%) cases were corroborated by pathology, indicating a high rate of misdiagnosis. Only 4 (2.8%) of 142 XGC cases had the coexisting GBC.

Of these 42 XGC cases misdiagnosed as GBC, no specific symptoms were observed. These symptoms could be grouped roughly into three clinical syndromes. The first and most common syndrome was chronic cholecystitis, followed by acute cholecystitis. The third syndrome was a biliary-tract disease which mainly included jaundice, RUQ pain, and high grade fever. These vague and nonspecific symptoms are just similar to those of GBC[11], and usually not helpful in the differentiation of these two conditions. Of all the cases of XGC, 9 presented with mild jaundice in our study, which indicated that mild jaundice may be of important significance in differentiating XGC from GBC with moderate or severe jaundice. Increased presence of tumor markers such as CEA and CA 199 should raise suspicions of GBC. Some studies showed that the increased CA199 level (> 20 U/mL) had a 79.4% sensitivity and a 79.2% specificity, and the increased CEA level (> 4.0 ng/mL) had a 93% specificity, but only a 50% sensitivity[11,12]. However, the increased CA199 and CEA levels (76% and 14%, respectively) were also present in 42 XGC cases, which proved to be futile and of no clinical significance in the differential diagnosis of XGC from GBC.

Radiology was the only helpful modality to make a differential diagnosis between XGC and GBC. Several imaging studies have shown relative specificities in some CT or MRI features for the diagnosis of XGC, mainly including intramural hypo-attenuated nodules within the thickened walls, homogeneous enhancement of the mucosa, and absence of macroscopic hepatic invasion[6-10]. Combination of certain imaging findings could even provide excellent accuracy for the differentiation of both the conditions[7,8]. Despite the radiographic progressions in the XGC diagnosis, 29.6% of XGC cases were misdiagnosed as GBC by radiologists in our study. By analyzing imaging features of these 42 cases, the intramural hypo-attenuated nodule within the thickened wall was observed only in 26% of the cases, but abnormal enhancement in hepatic parenchyma neighboring the gallbladder was observed in 69% of cases, which indicated hepatic invasion. The infiltration of other adjacent structures and enlarged lymph nodes were also noted in several cases. These above imaging features were highly suggestive of GBC rather than XGC. Except for the concomitant liver metastases, there was too much overlap of the imaging findings between XGC and GBC to reliably differentiate between two entities. Furthermore, the rarity of XGC and the possibility of the coexisting GBC also contributed to the difficulty of the differential diagnosis. Therefore, pathological examination was necessary for the definitive diagnosis especially to rule out GBC.

Previously, needle biopsy guided by US or CT has been reported to be performed safely and accurately in differentiating XGC from GBC[13]. But this practice was solely dependent on both the operator's experience in obtaining the samples and a high quality pathology service. During surgery, if GBC could not be excluded, intra-operative FS analysis is worth recommending regardless of the possibilities of false-negative results. In our study, 30 gallbladder specimens were sent for an intra-operative FS analysis, showing GBC in 2 patients (the diagnosis was confirmed by pathology),

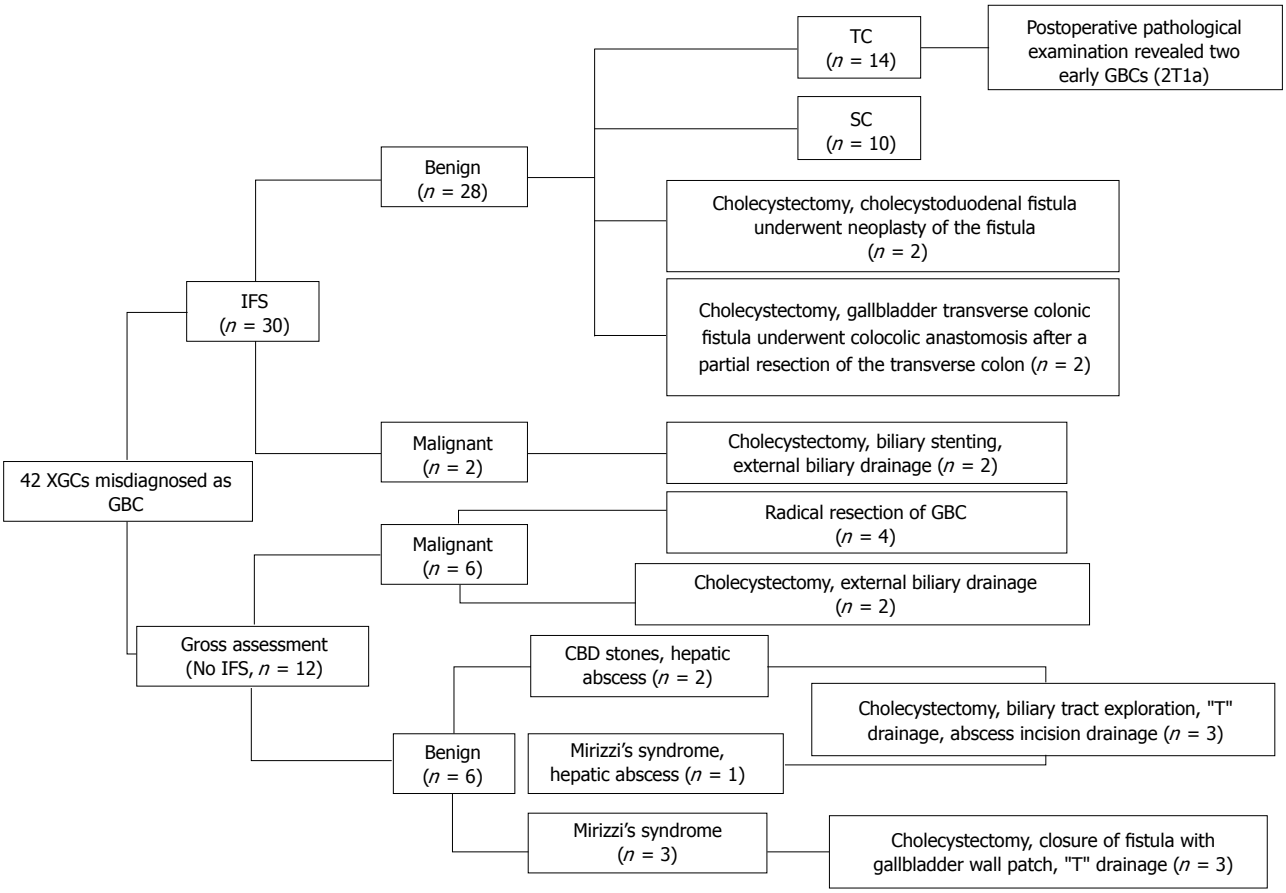

Figure 1 The surgical procedures for the 42 cases of xanthogranulomatous cholecystitis. XGC: Xanthogranulomatous cholecystitis; GBC: Gallbladder carcinoma; IFS: Intra-operative frozen section analysis; TC: Total cholecystectomy; SC: Subtotal cholecystectomy; CBD: Common bile duct.

Table 4 Comparison of complications for total and subtotal cholecystectomy *n* (%)

Complication	TC (*n* = 14)	SC (*n* = 10)	*P* value	Significance
Surgical site infection	1 (7)	1 (10)	1.000	NS
Retained stone	0	1 (10)	0.417	NS
Bile duct injury	1 (7)	0	1.000	NS
Duodenal injury	1 (7)	0	1.000	NS
Controlled bile leakage	0	0	-	NS
Biliperitoneum	0	0	-	NS
Hemoperitoneum	0	0	-	NS
Mortality	0	0	-	NS
Morbidity	3 (21)	2 (20)	1.000	NS

TC: Total cholecystectomy; SC: Subtotal cholecystectomy; NS: No significance.

and inflammatory lesions in 28 (but 2 of 28 patients had pathologically confirmed T1a GBC). The diagnostic accuracy of FS analysis was calculated to be around 93% (Table 5). The remaining 12 XGC cases did not undergo intra-operative FS analysis, and 6 of them were identified as GBC and the other 6 cases as benign lesions only by surgeon's macroscopic examination. However, 6 "GBCs" were not further confirmed by the final pathologic examination, showing that the accuracy of surgeon's macroscopic diagnosis of GBC in XGC was only 50% (Table 6). Although 2 patients had early GBCs (T1a) missed on FS analysis (underwent cholecystectomy alone), all 30 patients still received the appropriate surgical treatment on the basis of FS analysis results rather than pathologic examination[11,14]. However, 6 "GBCs" misdiagnosed by surgeons underwent aggressive surgical treatment, which may have been avoided if intra-operative FS analysis was also conducted.

Generally, a simple cholecystectomy is enough for XGC[1,2,15]. Sometimes, a complete resection of the gallbladder was not always judicious especially due to the obscure Calot's triangle anatomy and/or high risk gallbladder bed in cirrhotic patients. SC was a practical

Table 5 Diagnostic accuracy of frozen section analysis in 42 cases of xanthogranulomatous cholecystitis misdiagnosed as gallbladder carcinoma

Frozen section diagnosis	Definitive pathological diagnosis		
	Cancer	Benign	Total
Cancer	2	0	2
Benign	2	26	28
Total	4	26	30

Sensitivity: 50%; Specificity: 100%; Accuracy: 93%.

Table 6 Accuracy of surgeon's macroscopic diagnosis of gallbladder carcinoma in 42 cases of xanthogranulomatous cholecystitis

Surgeon's macroscopic diagnosis	Definitive pathological diagnosis		
	Cancer	Benign	Total
Cancer	0	6	6
Benign	0	6	6
Total	0	12	12

Sensitivity: 0; Specificity: 50%; Accuracy: 50%.

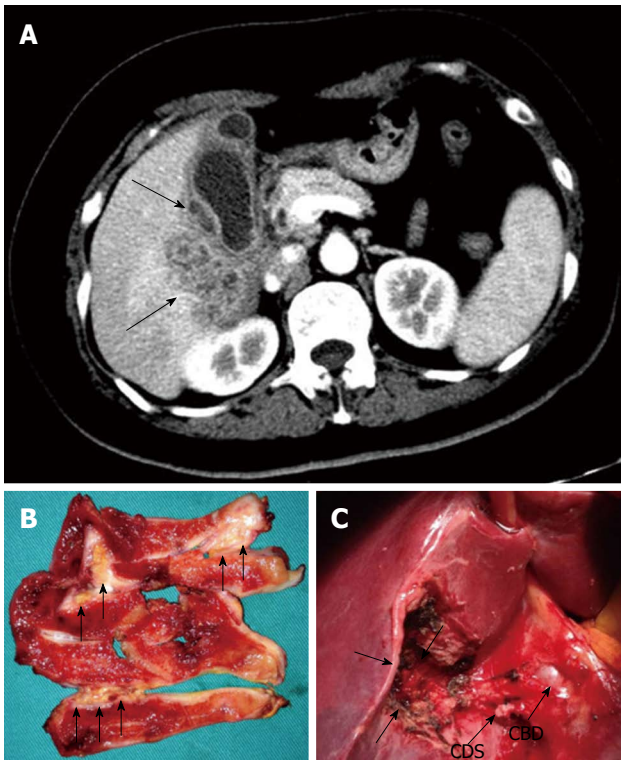

Figure 2 A 50-year-old woman with xanthogranulomatous cholecystitis misdiagnosed as gallbladder carcinoma based on computed tomography findings. A: Contrast-enhanced computed tomography shows the diffuse gallbladder wall thickening with intramural hypo-attenuated nodules (arrows) and heterogeneous enhancement in hepatic parenchyma neighboring gallbladder, indicating gallbladder carcinoma with direct liver invasion; B: Macroscopic examination of gallbladder specimen revealed multiple yellow nodules and bands in the wall (arrows). The gallbladder was sent for intra-operative frozen section (FS) analysis and demonstrated inflammatory lesions; C: Based on the FS diagnosis, a simple cholecystectomy was performed. Note that no macroscopic hepatic invasion was found (arrows). Ultimately, xanthogranulomatous cholecystitis was diagnosed by the pathological examination. CDS: Cystic duct stump; CBD: Common bile duct.

alternative to TC, leaving part of the hartmann sac, gallbladder neck, and part of the posterior wall adherent to the hepatic bed[16]. Table 4 shows that no statistical difference was encountered in the incidence of postoperative complications between the TC and SC groups, although two serious complications (common bile duct injury and duodenal injury) occurred in the TC group. SC should be a part of a surgeon's armamentarium in complicated XGC. However, coexisting GBC should be ruled out before SC was performed in XGC. No association with the GBC could be established in our 42 XGC cases. Therefore, an intra-operative FS analysis should be obtained in patients with suspected GBC, and the benign diagnosis indicates a more conservative surgical approach (Figure 2). However, we should always remember that a simple cholecystectomy, either TC or SC, could result in denying a patient the optimal surgical approach due to the existence of a false-negative result in FS analysis. If FS diagnosis was not confirmed by the final pathological examination, re-operation may be inevitable according to the oncologic point of view.

In conclusion, there was a significant overlap in various ways in clinical practice between XGC and GBC. Neither clinical manifestations, nor laboratory tests or radiological methods could provide an effective and practical standard in characterizing the differential diagnosis, and the occasional coexistence of two entities further resulted in a diagnostic confusion. The definitive diagnosis by all standards will still be dependent on the pathology. For XGC with suspected GBC, intra-operative FS analysis had a diagnostic accuracy of 93%, and the benign diagnosis indicated that a simple cholecystectomy was enough, *i.e.*, either TC or SC. Nevertheless, the benign diagnosis must be confirmed by a pathological examination, or else re-operation may be inevitable according to the oncologic point of view.

COMMENTS

Background

Xanthogranulomatous cholecystitis (XGC) was a destructive inflammatory disease of the gallbladder that could mimic gallbladder carcinoma (GBC) in various ways including clinical manifestations, imaging and intra-operative findings, and even coexisted with GBC, leading to a diagnostic dilemma.

Research frontiers

Due to the overlapping features between the two diseases, XGC is frequently misdiagnosed as GBC and usually undergoes an extended radical surgery. Sometimes XGC might have a coexistent GBC. Accurate diagnosis is important for the proper surgical management of these patients. Several non-invasive imaging and invasive techniques have been reported to differentiate XGC from GBC, but the dilemma still exists concerning the differential diagnosis in clinical practice, and the final diagnosis has to be dependent on the histological examination.

Innovations and breakthroughs

There is a significant overlap in various ways in clinical practice between XGC

and GBC. Neither clinical manifestations and laboratory tests nor radiological methods provide a practical and effective standard in the differential diagnosis between XGC and GBC. Moreover, the occasional coexistence of two entities further results in diagnostic confusion.

Applications

For XGC with suspected GBC, intra-operative frozen section analysis had a diagnostic accuracy of 93%, and the benign diagnosis indicates that a simple cholecystectomy is enough, *i.e.,* either total cholecystectomy or subtotal cholecystectomy. Nevertheless, the benign diagnosis must be confirmed by a pathological examination, or else re-operation may be inevitable according to the oncologic point of view.

Terminology

XGC is a rare variant of chronic cholecystitis characterized by severe proliferative fibrosis with infiltration of macrophages and foamy cells within the gallbladder wall. This condition is benign in nature but often shows a destructive inflammatory process. The inflammatory infiltration and fibrosis cause the asymmetrical thickening of the gallbladder wall and the formation of multiple yellowish-brown nodules, which often extend into the neighboring organs, such as the liver, omentum and duodenum.

Peer-review

This study is an interesting analysis for a rare inflammatory process mimicking gallbladder carcinoma.

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P- Reviewer: Mastoraki A **S- Editor**: Yu J **L- Editor**: Wang TQ **E- Editor**: Zhang DN

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World J Gastroenterol 2015 November 28; 21(44): 12660-12666
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Observational Study

Capsule enteroscopy is useful for the therapeutic management of Crohn's disease

João Santos-Antunes, Hélder Cardoso, Susana Lopes, Margarida Marques, Amadeu CR Nunes, Guilherme Macedo

João Santos-Antunes, Hélder Cardoso, Susana Lopes, Margarida Marques, Amadeu CR Nunes, Guilherme Macedo, Gastroenterology Department, Faculty of Medicine, Hospital de São João, 4200-319 Porto, Portugal

João Santos-Antunes, Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal

Author contributions: Santos-Antunes J wrote the paper, collected the data, elaborated the database, analyzed the statistics, and contributed to the interpretation and literature review; Cardoso H contributed to capsule reading, data collection, and study design; Lopes S contributed to patient follow-up and scientific collaboration; Marques M contributed to capsule reading and scientific collaboration; Nunes ACR contributed to patient follow-up and scientific collaboration; Macedo G contributed to study design, paper writing, and scientific revision of the manuscript.

Institutional review board statement: Due to its retrospective nature, no ethical concerns were raised for the writing of this manuscript.

Informed consent statement: Informed consent for CE and Patency capsule procedures was obtained for every patient. Due to its retrospective nature, no informed consent for the writing of this manuscript was applicable.

Conflict-of-interest statement: The authors state that they have no conflicts of interest to declare.

Data sharing statement: No additional data available.

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Correspondence to: João Santos-Antunes, MD, Department of Gastroenterology, Faculty of Medicine, Centro Hospitalar S. João. Alameda Prof. Hernani Monteiro, 4200-319 Porto, Portugal. joao.claudio.antunes@gmail.com
Telephone: +351-22-5513600
Fax: +351-22-5513601

Received: April 24, 2015
Peer-review started: April 26, 2015
First decision: June 2, 2015
Revised: June 30, 2015
Accepted: August 30, 2015
Article in press: August 31, 2015
Published online: November 28, 2015

Abstract

AIM: To analyze therapeutic changes in Crohn's disease (CD) patients following video capsule endoscopy (VCE) and to assess the usefulness of Lewis score and the Patency Capsule.

METHODS: Patency Capsule was performed in every patient that had indication for VCE, and those with negative patency did not undergo VCE. Patients with established CD that underwent VCE between January 2011 and February 2014 were selected for this study; those with suspected CD were excluded, independent of VCE results, since our purpose was to address differences in therapeutic regimen in CD patients before and after VCE. Patients with inconclusive VCE were also excluded. Patients had to be free of non-steroidal anti-inflammatories for at least 1 mo. Those patients who met these criteria were allocated into one of three groups: Staging group (asymptomatic CD patients that underwent VCE for staging of CD), Flare group (patients with active CD), or Post-op group (CD patients evaluated for post-operative recurrence). Lewis score was calculated for every VCE procedure. Statistical

analysis was performed to address the impact of VCE findings on the therapeutic management of CD patients and to evaluate the utility of the Lewis score.

RESULTS: From a total of 542 VCEs, 135 were performed in patients with CD. Patency capsule excluded nearly 25% of the patients who were supposed to undergo VCE. No videocapsule retention during VCE was reported. From these 135 patients, 29 were excluded because CD diagnosis was not established at the time of VCE. Therefore, a total of 106 patients were included in the final analysis. From these, the majority were in the Staging group (n = 73, 69%), and the remaining were in the Flare (n = 23, 22%) or Post-op (n = 10, 9%) group. Median time between diagnosis and VCE was 5.5 years. Overall, VCE determined changes in the treatment of 40% of patients: only 21% remained free of immunosuppressors after VCE compared to 44% before VCE (P < 0.001). The differences in therapy before and after VCE achieved statistical significance in the Staging and Flare groups. In addition, patients were significantly different when stratified regarding time since diagnosis to the date of VCE. A higher Lewis score was associated with therapeutic modifications (P < 0.0001); where a score higher than 1354 was related to 90% probability of changing therapy [area under the receiver operative characteristic (AUROC) 0.80 (95%CI: 0.69-0.88)].

CONCLUSION: VCE significantly changed the therapeutic management of CD patients, even in those with long-term disease. Systematic use of Patency capsule allowed for no videocapsule retention.

Key words: Capsule enteroscopy; Crohn's disease; Treatment modification; Patency capsule; Lewis score

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Core tip: Our work analyzed the therapeutic management of patients with Crohn's disease (CD) and concluded that a very significant proportion of patients modify their therapeutic regimens after performing video capsule endoscopy (VCE), even in those with long-term disease or those without symptoms. This finding highlights the importance of this procedure in the management of CD. The systematic use of Patency capsule is controversial; however, we showed in our study that after excluding patients with negative patency, who did not undergo VCE, none of the patients had video capsule retention during VCE, highlighting the importance of Patency capsule in this setting.

Santos-Antunes J, Cardoso H, Lopes S, Marques M, Nunes ACR, Macedo G. Capsule enteroscopy is useful for the therapeutic management of Crohn's disease. *World J Gastroenterol* 2015; 21(44): 12660-12666 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12660.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12660

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disease associated with mucosal and transmural inflammation of the bowel wall, and its diagnosis relies on the combination of clinical, endoscopic, radiologic and histopathological features.

Regarding endoscopic assessment, ileocolonoscopy is the first procedure for the establishment of the diagnosis. However, evaluation of the entire small bowel is mandatory, since it can change the therapeutic approach used and overall prognosis[1]. In this setting, international guidelines[1] regard cross-sectional studies, such as entero-computed tomography (CT) scan or magnetic resonance imaging (MRI), as first line tools, since they can evaluate extra-luminal features and characterize intra-abdominal adverse events related to CD, such as abscesses or fistulas. Video capsule endoscopy (VCE) is considered a second-line tool in those CD patients with atypical symptoms, in which imaging was negative[1].

Nevertheless, VCE is often used as a first-line study in the suspicion of CD, after ileocolonoscopy[2,3]. Its efficacy in detecting lesions in the upper small bowel seems higher than entero-CT or MRI, with similar accuracy for distal lesions[4-6].

Since the role of VCE in established CD is not completely defined, namely whether VCE is useful for treatment guidance, a few studies tried to evaluate its impact on determining treatment guidance and analyzing the therapeutic changes attributed to VCE[7-11]. However, some of these previous studies included a very low number of patients or had very short disease duration at the time of VCE, thereby compromising the interpretation of the results. Evaluation of possible changes in management includes searching for changes in inflammatory bowel disease (IBD) specific modification strategies, further radiologic or endoscopic studies, or even surgical interventions.

In this study, our main goal was to analyze the changes in the therapeutic regimen of patients with long-term CD after undergoing VCE. Additionally, we studied the impact of Lewis score in this setting and the number of videocapsule retentions with the systematic use of Patency capsule.

MATERIALS AND METHODS

All patients with established CD that underwent VCE since January 2011 to February 2014 were included in the study. Patients were assigned to one of three groups. The first group (Staging group) included patients with clinical remission who underwent VCE to assess disease extent or small bowel re-evaluation. The second group (Flare group) included patients who were undergoing re-evaluation because of a flare and had clinical deterioration or raised inflammatory markers. The third group (Post-op group) included

Table 1 Baseline characteristics of the patients included in the study *n* (%)

Population characteristics (*n* = 106)	Value
Male gender	47 (44)
Age - mean	40 ± 13 yr
Median time between diagnosis and VCE	5.5 (IQR 2-10) yr
Montreal classification	
Age at diagnosis	
A1: Below 17	7 (7)
A2: 17-40	84 (79)
A3: Above 40	15 (14)
Behavior	
B1: Non-stenosing/non-penetrating	86 (81)
B2: Stenosing	12 (11)
B3: Penetrating	8 (8)
Location	
L1: Terminal ileum	74 (70)
L2: Colonic	10 (9)
L3: Ileocolonic	22 (21)
+ L4: Upper disease	38 (36)
Treatment before VCE	
Anti-TNF	21 (20)
Immunosuppressors	38 (36)
Aminosalicylates only	40 (38)
No treatment	7 (6)

VCE: Video capsule endoscopy; TNF-α: Tumor necrosis factor α.

patients who were being evaluated for post-operative recurrence.

Patients with suspected CD in which VCE did not confirm the diagnosis or those with VCE considered inconclusive were excluded from the study. Also, patients with suspected CD in which VCE confirmed the diagnosis were also excluded, since our main goal was to evaluate changes in therapeutic regimens for CD before and after VCE. Patients had to be free of non-steroidal anti-inflammatories for at least 1 mo.

All the VCEs were performed after confirming small bowel patency using Agile Patency capsules (Given®, Imaging Ltd. Yoqneam, Israel), which were read 30 h after ingestion. VCEs were performed using PillCam® SB2 or SB3 capsules (Given®, Imaging Ltd.). On the previous day, patients were asked to follow a liquid diet and to perform a bowel preparation. On the day of the procedure, patients were on a clear-liquid diet for 6 h after swallowing the capsule. RAPID® Real-Time Viewer was performed in all patients after 2 h of ingestion, and domperidone 10 mg was prescribed if the capsule remained in the stomach. A new evaluation was performed 1 h later, and if the capsule was still retained in the stomach, a new dose of domperidone 10 mg was administered. If the medication failed, an upper endoscopy was performed to place the device in the duodenum.

All the exams were read by two experienced gastroenterologists using RAPID Reader®. Lewis score was calculated in order to assess the severity of the disease in all procedures, being classified as normal or clinical insignificant if lower than 135 points, mild disease between 135 and 790, and moderate/severe disease above 790, as described elsewhere[12].

In addition to demographic, clinical, and analytical data, medical therapy at the time of VCE and therapy modifications due to VCE were recorded. In order to simplify the results, the "Anti-tumor necrosis factor (TNF) group" included patients taking anti-TNF (infliximab or adalimumab) in monotherapy or combination therapy, and the "Immunosuppression group" included those under azathioprine (AZA) either in monotherapy or combination with 5-amynosalicilates (5-ASA); the remaining patients were under monotherapy with 5-ASA or had no therapy.

Changes in CD treatment in the Staging group and Post-op group were only attributable to VCE findings, since these patients were in clinical and analytical remission. Patients in the Flare group had clinical or analytical active disease, but statistical analysis was conducted to conclude if VCE findings were associated with changes in therapeutic regimen, independent of the flare itself.

Statistical analysis was performed using SPSS software version 22 (SPSS Inc., Chicago, IL, United States). Continuous variables were analyzed using T-student tests or Mann-Whitney test when normal distribution was not verified. Categorical variables were analyzed using Pearson's Chi-square, Fisher's exact tests or McNemar test as appropriate. Logistic regression was performed in order to assess variables independently associated with changes in therapeutic regimen. A *P* value below 0.05 was considered statistically significant.

RESULTS

Among the 542 VCEs performed during the analyzed period, 135 were performed in patients with CD, after positive patency was confirmed by Patency capsule (Patency capsule excluded nearly 25% of the patients who were supposed to perform VCE). From these 135 patients, 29 were excluded because they did not have established diagnosis of CD at the time of VCE. In total, 106 patients were included for the final analysis.

Most of the procedures were performed in patients within the Staging group (*n* = 73, 69%), with the remaining patients in the Flare (*n* = 23, 22%) and Post-op (*n* = 10, 9%) groups. Baseline characteristics are shown in Tables 1 and 2. Fifty-six percent were female, with mean age of 40 ± 13 years. Most patients (81%) had an inflammatory phenotype; 70% had isolated ileal disease. After VCE analysis, upper tract involvement was identified in 49 (46%) patients.

The median time between the diagnosis of CD and VCE was 5.5 [interquartil range (IQR) 2-10] years. Regarding disease activity (Lewis score), 51 (48%) had normal or clinical insignificant lesions (25% of the total procedures were normal), 14 (13%) had mild disease, and 41 (39%) had moderate to severe disease.

Table 2 Patient characteristics at baseline that could influence therapeutic changes after video capsule endoscopy

Variable	Univariate analysis	Multivariate analysis
Age	$P = 0.477$	-
Male gender	$P = 0.517$	-
Smoking	$P = 0.771$	-
C-reactive protein	$P = 0.188$	-
Disease time duration	$P = 0.073$	-
Age at diagnosis	$P = 0.097$	-
Ileal *vs* colonic *vs* ileocolonic disease	$P = 0.009$	$P = 0.367$
Disease behaviour	$P = 0.564$	-

Overall, VCE results guided changes in the treatment of 40% of the patients. At the time of VCE, 38% were under 5-ASA, 36% were under immunosuppressors, 20% were under Anti-TNF, and 6% had no treatment. After VCE, no patient remained without therapy; and the percentage of patients under 5-ASA decreased to almost half and those under AZA and anti-TNF rose significantly ($P < 0.0001$, Figure 1). Similarly, these results were significant when stratifying patients based on time between diagnosis and VCE (less than 1 year and more than 1, 5, and 10 years of the disease) (data not shown). Overall, only 21% of the patients remained free of immunosuppressors after VCE compared to 44% before VCE ($P < 0.001$).

When analyzing Lewis score, only 7% with normal or almost normal VCE changed therapy, whereas 67% changed therapy when VCE demonstrated moderate to severe disease ($P < 0.0001$, Figure 2). Those patients who changed therapy clearly had higher median Lewis score values (1446 *vs* 552, $P = 0.006$). Patients with a Lewis score higher than 1354 had a 90% probability of changing their medication [AUROC 0.80 (95%CI: 0.69-0.88)].

We found differences in the median Lewis score among the different groups. Patients in the Flare group had higher Lewis score values than the Staging (1648 *vs* 816, $P = 0.040$) and Post-Op (1648 *vs* 327, $P = 0.035$) groups. No significant differences were found between Staging and Post-op groups.

Regarding the indication for VCE, the percentage of patients under AZA was duplicated in the Staging group ($P < 0.0001$) after VCE, while in the Flare group, the number of patients doubled under anti-TNF ($P = 0.032$). In the Post-op group, there was an increase in the number of those taking AZA or anti-TNF, but it was not statistically different ($P = 0.133$, Figure 3).

When performing multivariate analysis, we found that the factors age, C-Reactive protein levels, smoking habits, or duration of the disease were not linked with changes in therapeutic regimen after VCE.

VCE was not retained in any of the patients. Furthermore, no clinical symptoms or other adverse events were reported in the patients where Patency capsule demonstrated negative patency.

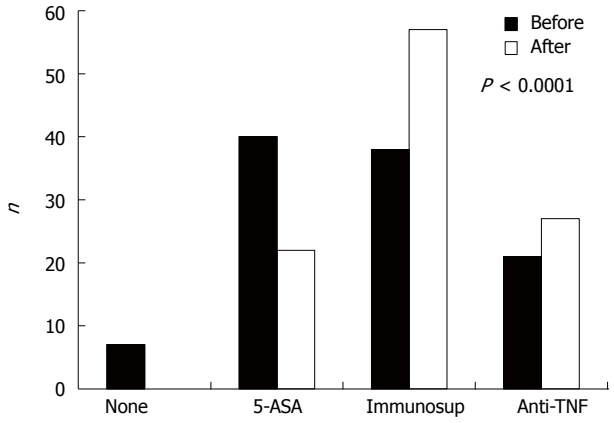

Figure 1 Therapeutic regimens for Crohn's disease before and after capsule enteroscopy. TNF: Tumor necrosis factor; 5-ASA: 5-aminosalicylic acid.

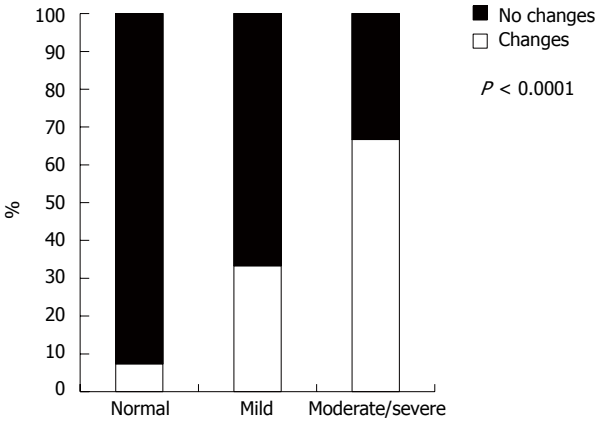

Figure 2 Changes in therapeutic regimen regarding the Lewis score calculated with video capsule endoscopy.

DISCUSSION

VCE is a valuable tool in the assessment of the small bowel[13-19], but its importance in the evaluation and follow-up of CD patients is not well established[20,21]. An indicator to determine the impact of this method on CD is modification of the therapeutic approach after VCE. Some studies address this issue, but there were some limitations.

A recent study[8] found that the number of patients with CD under anti-TNF or immunosuppressants rose after VCE. However, most of the patients underwent VCE in the first year of the disease; and, consequently, 48 of 50 patients were only on 5-ASA or steroids before VCE (only one was under AZA and one under anti-TNF), which makes this difference expectable. An advantage of our study is the inclusion of patients with long-term disease (median time 5.5 years) and patients whose CD is adequately managed, namely anti-TNF and immunosuppressants. Our results showed that VCE was decisive for therapeutic changes even in patients with more than 10 years of CD evolution,

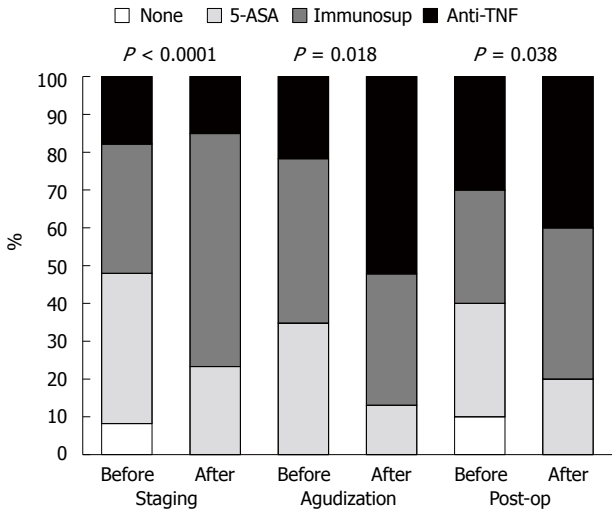

Figure 3 Therapeutic regimens before and after capsule enterosopy within the three analyzed groups. TNF: Tumor necrosis factor; 5-ASA: 5-aminosalicylic acid.

highlighting the importance of VCE in this pathology. Similar results were found in a previous study of 71 patients with CD that included subjects with long-term disease[10]. The treatment modification rate was even higher in a study performed in a pediatric population with CD[22].

VCE promoted changes in therapy in every group, although in the Post-op group a statistically significant difference was not achieved, probably due to the small amount of patients (n = 10). In fact, post-op evaluation is emerging as a potential indicator for VCE[23,24]. It is considered to have the same sensibility, specificity, and negative and positive predictive values[2] as colonoscopy. In addition to the latter being a more accessible procedure, VCE was previously shown to find more endoscopic recurrences than colonoscopy[25], with the advantage of allowing for proximal small bowel evaluation[26].

In the Staging and Flare groups, there was a clear and significant modification in therapeutic regimens after VCE, with a decrease in the number of patients under 5-ASA and an increase in patients under AZA (Staging group) and anti-TNF (Flare group). Downgrading of therapy was observed in three patients (infliximab to AZA), all in the Staging group. Those were patients with long-term remission who were under combination therapy and, after performing a VCE for address the possibility of anti-TNF withdrawal, had a normal exam. Previous studies have determined the utility of VCE for the assessment of small bowel mucosal healing after immunomodulator or biologic therapy[27,28], eventually contributing to a downgrade in CD therapy.

It should be noted that none of our patients were being treated with steroids. This was due to the duration of time between the flare and the realization of VCE. Patients in the Flare group started steroids as indicated, but when they came to undergo VCE, the steroid cycle was already completed. This delay in VCE could have been a problem in our analysis since some patients may escalate therapy upon flare before VCE, which could contribute to some attenuation in the differences between therapy before or after the VCE. Statistical differences were still found despite this time lag, making changes in therapy regimens more attributable to VCE findings than to clinical or analytical flare.

The importance of the VCE *per se* in the changes in therapeutic regimens was highlighted by the regression analysis. As observed, no other factor presented at baseline was independently related to therapeutic modifications. Age, gender, smoking habits, duration of the disease, and inflammatory markers at the time of the VCE were not determinant for therapeutic decisions, making therapeutic changes attributable to the results of VCE. Previous studies had already shown a weak correlation between VCE results and inflammatory biomarkers, making VCE very useful even in the absence of raised C-reactive protein or fecal calprotectin[11].

Lewis score can be a valuable tool for therapeutic management[29]; as expected, higher scores were related with more frequent changes in medical therapy, since they represent active disease requiring a more aggressive treatment.

All the VCEs were performed after confirming small bowel patency using Agile Patency capsules. This device proved to be a very useful tool for patients with known stenosis[30], but its systematic use, as we perform in our institution, is not consensual. International guidelines[2] state that the risk of capsule retention is high in patients with known CD, and, therefore, patency capsule or cross-sectional studies must be performed before VCE to exclude significant stenosis. In patients with suspected CD, the risk appears to be much less significant, and its use is controversial. Since patients with CD can have inflammatory changes in small bowel mucosa, raising the risk of capsule retention, we performed Patency capsule in every patient in this setting. In our Department, nearly 25% of the patients with CD do not perform videocapsule due to negative patency as assessed by Patency capsules. Consequently, we did not experience any videocapsule retention during VCE.

The main limitations of our study were the small number of patients in the Post-Group, which precluded significant results (although there was a clear trend towards therapeutic modifications after VCE), and its retrospective nature. Since this work was not designed to compare patients with and without VCE, we did not assess the differences in the follow-up between them. However, it is well known in the literature that a suboptimal treatment of CD could predispose to a worse outcome. Therefore, we strongly believe that therapy escalation, even in patients with clinical remission but with small bowel lesions detected by VCE, is of paramount importance for a better long-

term outcome of CD.

Overall, we concluded that VCE is a very powerful tool for evaluating CD in all groups of patients, including those with long-term disease under immunosuppressors and anti-TNF. It was decisive for treatment guidance, which ultimately can lead to an earlier introduction of immunosuppressors and anti-TNF therapy, consequently improving overall prognosis.

COMMENTS

Background

The role of video capsule endoscopy (VCE) in treatment guidance is not well established for Crohn's disease (CD). Previous studies have attempted to address this subject, but the data are still scarce, especially in patients with long-term disease.

Research frontiers

The authors' results demonstrated the utility of VCE and Patency capsule for the management of CD, namely for the guidance of medical therapy.

Innovations and breakthroughs

This study included patients with long-term CD and show how VCE can affect medical therapy. In addition, the clinical utility of patency capsule is well documented.

Applications

Patency capsule allowed for no VCE retention. A large proportion of patients with CD changed therapeutic regimen after VCE.

Terminology

Lewis score classification according to VCE findings: normal or clinical insignificant disease if lower than 135 points, mild disease between 135-790, and moderate/severe disease above 790 points.

Peer-review

The authors have reported the usefulness of VCE for the management of the therapeutic regimen in patients with CD. This manuscript is well-written.

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P- Reviewer: Oka S **S- Editor**: Ma YJ **L- Editor**: Filipodia
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DOI: 10.3748/wjg.v21.i44.12667

World J Gastroenterol 2015 November 28; 21(44): 12667-12675
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Prospective Study

Evaluation of a multiplex PCR assay for detection of cytomegalovirus in stool samples from patients with ulcerative colitis

Saifun Nahar, Atsushi Iraha, Akira Hokama, Ayako Uehara, Gretchen Parrott, Tetsuya Ohira, Masatoshi Kaida, Tetsu Kinjo, Takeshi Kinjo, Tetsuo Hirata, Nagisa Kinjo, Jiro Fujita

Saifun Nahar, Ayako Uehara, Gretchen Parrott, Tetsu Kinjo, Takeshi Kinjo, Tetsuo Hirata, Jiro Fujita, Department of Infectious, Respiratory, and Digestive Medicine, University of the Ryukyus, Okinawa 903-0215, Japan

Atsushi Iraha, Akira Hokama, Tetsuya Ohira, Masatoshi Kaida, Nagisa Kinjo, Department of Endoscopy, University of the Ryukyus, Okinawa 903-0215, Japan

Author contributions: Nahar S and Iraha A designed and performed the research; Nahar S drafted the manuscript; Hokama A and Parrott G reviewed the manuscript; Uehara A, Parrot G, Ohira T, Kaida M, Kinjo T, Kinjo T, Hirata T and Kinjo N provided technical support; and Hokama A and Fujita J supervised the project.

Institutional review board statement: All stool samples were taken after informed consent and ethical permission was obtained from patients participating in the study.

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

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Correspondence to: Akira Hokama, MD, PhD, Clinical Professor, Department of Endoscopy, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan. hokama-a@med.u-ryukyu.ac.jp
Telephone: +81-98-8951144
Fax: +81-98-8951414

Received: June 1, 2015
Peer-review started: June 3, 2015
First decision: July 10, 2015
Revised: August 3, 2015
Accepted: September 28, 2015
Article in press: September 30, 2015
Published online: November 28, 2015

Abstract

AIM: To evaluate a multiplex PCR assay for the detection of bacterial and viral enteropathogens in stool samples from patients with ulcerative colitis (UC).

METHODS: We prospectively analyzed 300 individuals, including immunocompetent patients, immunocompromised patients, and patients with UC. Stool samples were collected from the recto-sigmoid region of the colon by endoscopy. The samples were qualitatively analyzed for bacterial and viral enteropathogens with a multiplex PCR assay using a Seeplex® Kit. Additional clinical and laboratory data were collected from the medical records.

RESULTS: A multiplex PCR assay detected 397 pathogens (191 bacteria and 206 viruses) in 215 samples (71.7%). The most frequently detected bacteria were *Escherichia coli* H7, 85 (28.3%); followed by *Aeromonas* spp., 43 (14.3%); and *Clostridium perfringens*, 36 (12.0%) samples. The most prevalent viruses were Epstein-Barr virus (EBV), 90 (30.0%); followed by human herpes virus-6 (HHV-6), 53 (17.7%); and cytomegalovirus (CMV), 37 (12.3%) samples. The prevalence rate of CMV infection was significantly higher in the immunocompromised group than in the immunocompetent group ($P < 0.01$). CMV infection was more common in patients with UC (26/71; 36.6%)

than in the immunocompetent patients excluding UC (6/188; 3.2%) ($P < 0.01$). CMV infection was more prevalent in UC active patients (25/58; 43.1%) than in UC inactive patients (1/13; 7.7%) ($P < 0.05$). Among 4 groups which defined by the UC activity and immunosuppressive drugs, the prevalence rate of CMV infection was highest in the UC active patients with immunosuppressive drugs (19/34; 55.8%). Epstein-Barr virus (EBV) infection was more common in the immunocompromised patients excluding UC (18/41; 43.9%) than in the immunocompetent patients excluding UC (47/188; 25.0%) ($P < 0.05$). The simultaneous presence of CMV and EBV and/or HHV6 in UC active patients (14/58; 24.1%) was greater than in immunocompromised patients excluding UC (5/41; 12.2%) ($P < 0.05$).

CONCLUSION: The multiplex PCR assay that was used to analyze the stool samples in this study may serve as a non-invasive approach that can be used to exclude the possibility of CMV infection in patients with active UC who are treated with immunosuppressive therapy.

Key words: Polymerase chain reaction; Ulcerative colitis; Cytomegalovirus; immunosuppressive drugs; Epstein-Barr virus

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Core tip: Infection with cytomegalovirus (CMV) can cause exacerbation of ulcerative colitis (UC). Thus, early diagnosis of CMV infection is important. Although endoscopic biopsy is the best approach for the diagnosis of CMV infection, this procedure may be invasive for patients and damaging to the inflamed intestine. Our prospective study on the use of the qualitative multiplex PCR assay in stool samples revealed that CMV infection is significantly more prevalent in UC active patients with immunosuppressive drugs. The multiplex PCR assay for stool samples may prove useful, non-invasive method to exclude the presence of CMV infection in patients with active UC who are treated with immunosuppressive drugs.

Nahar S, Iraha A, Hokama A, Uehara A, Parrott G, Ohira T, Kaida M, Kinjo T, Kinjo T, Hirata T, Kinjo N, Fujita J. Evaluation of a multiplex PCR assay for detection of cytomegalovirus in stool samples from patients with ulcerative colitis. *World J Gastroenterol* 2015; 21(44): 12667-12675 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12667.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12667

INTRODUCTION

Cytomegalovirus (CMV), a double-stranded enveloped DNA virus and a member of β-*herpesviridae* family, commonly infects 40%-100% of adult populations[1] and 15.8%-34% of patients with inflammatory bowel disease (IBD) who are treated with steroids and/or other immunosuppressive drugs[2]. The eyes, lungs, central nervous system, liver and intestine are the primary target organs for CMV infection. Typically, individuals who are infected with CMV remain asymptomatic, but the infection may manifest with mild mononucleosis-like symptoms. CMV, like other herpes viruses, persists in a life-long latency coupled with a risk of intermittent reactivation in some situations, such as the following: recipients of solid organ transplants, patients undergoing hemodialysis, patients with HIV, and patients who are treated with steroids and other immunosuppressive drugs.

Typically, enteric infections are self-limiting and acute, but serious illness can occur in immunocompromised patients. The number of immunocompromised patients has been increasing dramatically in recent years due to the increased number of organ transplants, increased numbers of patients on hemodialysis, or infected with HIV, and widespread use of immunosuppressive drugs and steroids. Due to defective or altered cellular and humoral immunity, immunocompromised patients are more susceptible to infections. Any infection has the possibility to cause an overwhelming disease in these populations[3,4].

Patients with IBD such as ulcerative colitis (UC) and Crohn's disease (CD) are often immunosuppressed. Patients with severe, steroid-refractory or steroid-dependent states, as well as those treated with other biologic therapies undergo even more intensive immunosuppression. Together with the disease activity, these factors may contribute to the increased risk of colonic reactivation of latent CMV or CMV reinfection in patients with UC[5]. CMV can cause the exacerbation of UC, particularly in those with steroid-dependent/steroid- refractory diseases[2,6-8]. Histopathology and the identification of CMV DNA in colonic tissue by PCR or immunohistochemistry were recommended as the gold standard diagnostic tool for the diagnosis of CMV infection in immunosuppressed groups by the European Crohn's and Colitis Organization in 2009[9]. Although histopathology may be the most specific diagnostic approach, a biopsy is an invasive procedure that requires an endoscopic examination. In patients with UC, the inflamed colonic tissue is friable, which leads to an increased risk of hemorrhage or perforation during invasive procedures[10]. In addition, an extended length of time is required to obtain results of a histopathological analysis, and during this time, a given patient's condition may deteriorate clinically[11].

Molecular diagnostic tools based on the PCR method, have been developed to improve the detection of enteropathogens[12-14]. Of late, additional advances have been made to simultaneously identify enteropathogens using multiplex real-time PCR[15-18], but these methods can be low-throughput and expensive. Therefore, the implementation of a rapid, sensitive, powerful, and non-invasive molecular tool is necessary to determine

the etiology of diarrhea in UC patients. Only then will it be possible to provide early and specific interventions for the prevention and control of infections in both individuals and the community.

The aim of this prospective study was to evaluate the feasibility of a qualitative multiplex PCR assay of stool for the simultaneous detection of bacterial and viral enteropathogens, focusing on CMV infection for adult patients, including patients with UC, immunocompetent patients, and immunocompromised patients.

MATERIALS AND METHODS

Study population and specimens

We prospectively analyzed 300 patients who underwent colonoscopy at the Department of Endoscopy at the University of the Ryukyus Hospital from August 2014 to January 2015. Stool samples were collected endoscopically from the recto-sigmoid region of the colon and were transported to the laboratory. Stool samples were immediately processed for the multiplex PCR. Additional clinical and laboratory data were collected from medical records. UC activity was assessed using the Mayo scoring system[19].

DNA extraction

DNA was extracted using Ribospin™ vRD kit (GeneAll Biotechnology, Seoul, South Korea) according to manufacturer's instructions. Briefly, 300 μL of endoscopically collected stool was transferred into a 1.5-mL microcentrifuge tube followed by the addition of 500 μL of buffer to lyse the fecal matter. After an incubation of 10-15 min at room temperature, 700 μL of nucleic binding buffer was added; the solution was then vortexed and mixed. The resultant solution was transferred to a mini spin column and was centrifuged at 10000 *g* for 30-60 s. Total DNA was bound to the glass fiber membrane while the remaining impurities on the membrane were removed by two successive wash buffers. Pure DNA was eluted to a final volume of 50 μL of nuclease-free water. All procedures were performed at room temperature. The extracted DNA was stored at 4 ℃ for immediate use or at -20 ℃ for long-term use.

PCR amplification

The multiplex PCR was performed according to the manufacturer's instructions using a Seeplex® Meningitis V1, Diarrhea B1 and Diarrhea B2 ACE detection kits (Seegene, Seoul, South Korea). In regards to the Meningitis V1 ACE Detection assay, multiplex PCR was performed in a 20-μL total volume, which included 2 μL of each 10 × MV1 PM and 8-MOP solution, 1 μL of the Meningitis ACE internal control, 10 μL of the 2 × multiplex master mix and 5 μL of nucleic acid template. Negative and positive control samples were included in every PCR procedure and contained 5 μL of the Meningitis ACE NC and PC, respectively. DNA amplification was performed in an Eppendorf Mastercycler under the following conditions: an initial denaturation at 94 ℃ for 15 min, 94 ℃ for 30 s followed by 40 cycles at 63 ℃ for 1.5 min, 72 ℃ for 1.5 min with a final cycle at 72 ℃ for 10 min and then a hold at 4 ℃.

In regards to the Diarrhea B1/B2 ACE Detection assay, the multiplex PCR was performed in a 20-μL volume that contained 4 μL of 5 × DB1 PM, 3 μL of 8-MOP solution, 10 μL of 2 × master mix and 3 μL of nucleic acid template. Negative and positive controls samples were included in each PCR reaction and contained 3 μL of DB1 NC and DB1 PC, respectively, instead of nucleic acid.

All multiplex PCR mixtures underwent the same amplification conditions shown above. The Seeplex® Meningitis V1 kit is able to detect five pathogens in a single reaction tube including CMV, human herpes virus-6 (HHV-6), Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV-1), HSV-2, and varicella zoster virus (VZV). The Seeplex® Diarrhea B1 & B2 ACE detection assay permits the simultaneous amplification of the target DNA of the following: *Salmonella* spp. (*S. bongori* and *S. enterica*), Shigella spp. (*S. flexneri, S boydii, S. sonnei,* and *S. dysenteriae*), *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*), *Clostridium difficile toxin B, Campylobacter* spp. (*C. jejuni* and *C. coli*), *Clostridium perfringens* toxin, *Yersinia enterocolitica, Aeromonas* spp. (*A. salmonicida, A. sobria, A. bivalvium,* and *A. hydrophila*), *Escherichia coli* O157: H7, and Verotoxin-producing *E. coli* (VTEC). In total, we were able to test for 25 pathogens simultaneously.

Analysis of PCR product

PCR products were analyzed by microchip electrophoresis system using the DNA-1000 Reagent kit in the MCE-202 MultiNA machine (Shimadzu, Japan). The data was analyzed using MultiNA viewer software (Shimadzu, Japan).

Ethical considerations

This prospective study was approved by the institutional review board of the University of the Ryukyus, and written informed consent was obtained from all patients prior to their inclusion in the study.

Statistical analysis

Statistical comparisons were conducted by two-tailed χ^2 test with Yates' correction using SPSS (version 21.0, SPSS Inc., Chicago, IL, United States). A P value < 0.05 was considered statistically significant.

RESULTS

Study population

The clinical characteristics of the included patients

Table 1 Demographic and clinical characteristics of the patients *n* (%)

Characteristics	Number of patients 300 (100)
Gender	
Male	177 (59)
Female	123 (41)
Age in years	59.04 (16-86)
Underlying disease	
Immunocompromised	41 (13.7)
Ulcerative colitis	71 (23.7)
One or more bacteria/viruses detected	215 (71.7)
One or more bacteria detected	146 (48.7)
Number of bacteria detected	
1	108 (36.0)
2	33 (11.0)
3	5 (1.67)
One or more viruses detected	148 (49.3)
Number of viruses detected	
1	96 (32.0)
2	45 (15.0)
3	7 (2.3)
Bacteria detected	
Clostridium difficile toxin B	15 (5.0)
Salmonella spp.	4 (1.3)
Campylobacter spp.	3 (1.0)
Vibrio spp.	1 (0.3)
Clostridium perfringens	36 (12.0)
E. coli H7	85 (28.3)
E. coli O157	1 (0.3)
VTEC	1 (0.3)
Aeromonas spp.	43 (14.3)
Virus detected	
CMV	37 (12.3)
HHV6	53 (17.7)
EBV	91 (30.3)
HSV1	14 (4.7)
HSV2	6 (2.0)
VZV	6 (2.0)

E. coli: *Escherichia coli*; VTEC: Verotoxin-producing *E. coli*; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV6: Human herpes virus-6; VZV: Varicella zoster virus.

are summarized in Table 1. Three hundred patients were enrolled, including 41 immunocompromised patients and 71 patients with UC. The average age of the patients was 59.04 years (range 16-86 years), and there were more males (59%) than females. All of the UC active patients had diarrhea. A total of 300 samples collected by colonoscopy were examined. Among the 300 samples, multiplex PCR showed a positive reaction in 215 samples (71.7%) (Table 1). Among the 300 samples, one or more bacteria and viruses were identified in 146 (48.7%) and 148 (49.3%) samples, respectively. One bacterium was detected in 108 (36.0%) samples. Two and three other types of bacteria were detected in 33 (11.0%) and 5 (1.67%) samples, respectively. Ninety six (32.0%) had a single viral infection while 45 (15.0%) and 7 (2.3%) had 2 and 3 viruses, respectively. The most frequently detected bacteria were *E. coli* H7, 85 (28.3%); followed by *Aeromonas* spp., 43 (14.3%); *C. perfringens*, 36 (12.0%); and *C. difficile* Toxin B, 15 (5.0%). *Shigella* spp. and *Y. enterocolitica* were not

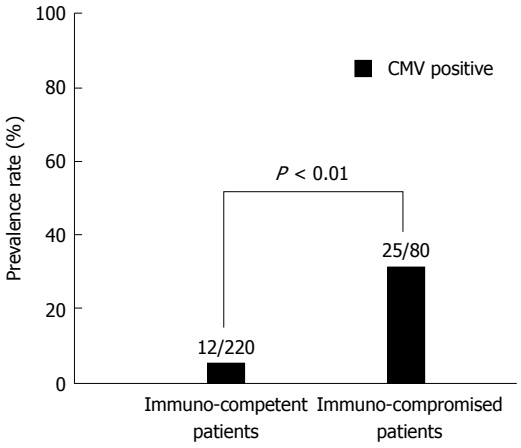

Figure 1 Comparison of prevalence rate of cytomegalovirus infection. The prevalence rate was significantly higher in the immunocompromised patients than in the immunocompetent patients. CMV: Cytomegalovirus.

detected. The most prevalent viruses were EBV, 91 (30.3%); followed by HHV-6, 53 (17.7%); and CMV, 37 (12.3%). The least prevalent viruses were HSV2, 6 (2.0%) and VZV, 6 (2.0%). Internal control was positive for all samples.

Patients who were treated with immunosuppressive drugs (*e.g.*, anticancer drugs, T-cell inhibitors, steroids), HIV patients, and patients on hemodialysis for chronic kidney disease were included in the immunocompromised group (80; 26.7%). Patients who were not treated with immunosuppressive drugs were incorporated into the immunocompetent group (220; 73.3%). The prevalence rate of CMV infection was significantly higher in the immunocompromised group than in the immunocompetent group ($P < 0.01$) (Figure 1). CMV infection was more common in patients with UC (26/71; 36.6%) than in the immunocompetent patients excluding UC (6/188; 3.2%) ($P < 0.01$) (Figure 2).

In UC patients, CMV infection was more prevalent in UC active patients (25/58; 43.1%) than in UC inactive patients (1/13; 7.7%) ($P < 0.05$) (Figure 3). UC patients were further categorized into 4 groups based on the UC activity and the administration of immunosuppressive drugs. The prevalence rate of CMV infection was highest among individuals in the active UC group who were treated with immunosuppressive drugs compared with individuals in the other 3 groups (Figure 4). Among the 58 patients with active UC, immunosuppressive drugs were prescribed for 34 patients. The CMV infection rate was significantly higher in those who were treated with immunosuppressive drugs (19/34; 55.8%) compared with those who were not treated with immunosuppressive drugs (6/24; 25.0%) ($P < 0.05$).

As for EBV infection, EBV infection was more common in the immunocompromised patients excluding UC (18/41; 43.9%) than in the immunocompetent patients excluding UC (47/188; 25.0%) ($P < 0.05$) (Figure 5). Figure 6 shows no significant differences

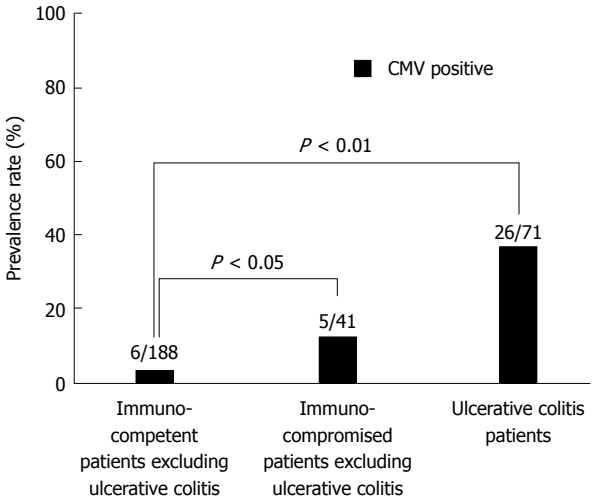

Figure 2 Comparison of prevalence rate of cytomegalovirus infection in 3 groups, including the immunocompetent patients excluding ulcerative colitis, the immunocompromised patients excluding ulcerative colitis, and ulcerative colitis patients. CMV: Cytomegalovirus.

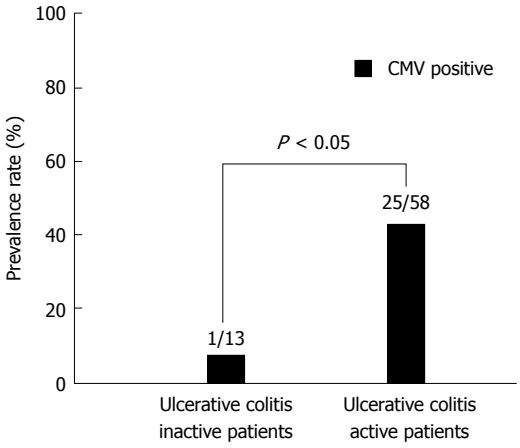

Figure 3 Comparison of prevalence rate of cytomegalovirus infection. The prevalence rate was significantly higher in ulcerative colitis active patients than in ulcerative colitis inactive patients. CMV: Cytomegalovirus.

of the prevalence rate of HHV6 infection among the groups. The overlapping prevalence patterns for CMV, EBV, and HHV6 are shown for UC active patients (Figure 7) and the immunocompromised patients excluding UC (Figure 8). The simultaneous presence of CMV and EBV and/or HHV6 in UC active patients (14/58; 24.1%) was greater than in immunocompromised patients excluding UC (5/41; 12.2%) ($P < 0.05$). No patients in our cohort were simultaneously infected with all three viruses.

DISCUSSION

Multiplex PCR has been widely applied to the diagnosis of gastrointestinal infectious diseases[18]. Notably, the Seeplex® Diarrhea ACE Detection assay has shown itself to be a rapid, sensitive, specific, and reliable diagnostic tool for the direct detection of the most common enteropathogens in stool samples[20]. Other multiplex PCR assays (*e.g.*, the FilmArray gastrointestinal panel and Luminex xTag gastrointestinal pathogen panel) have also yielded an increased percent positive rate compared with routine testing[21]. Based on these recent developments, we have conducted this prospective assay with the Seeplex® Diarrhea and Meningitis Detection method. We have analyzed (1) the results of a multiplex PCR assay as a rapid and non-invasive molecular diagnostic tool for the early diagnosis of CMV infection in patients with UC; (2) the prevalence rate of CMV infection in UC in comparison with control populations; and (3) the relationship of CMV infection with immunosuppressive drugs and disease activity. We have found that CMV infection is significantly more prevalent in the active UC group compared with the immunocompetent group and the immunocompromised group ($P < 0.05$), which further indicates the strong association of CMV infection in patients with active UC.

In addition, CMV infection is significantly correlated with the administration of immunosuppressive drugs among the patients with active UC ($P < 0.05$). UC patients who were treated with immunosuppressive drugs such as corticosteroids, tacrolimus, azathioprine, 6-mercaptopurine, or cyclosporine A, and those with existing inflammation are considered to be at an increased risk for the development of CMV infection[22-24]. Although some studies have suggested that CMV infection may be an occasional finding or that CMV may be an inactive participant in UC[22,25,26], CMV itself can be a major cause of exacerbation of UC. As a result, CMV subsequently leads to the worst clinical conditions seen among patients with UC[27]. Therefore, an early diagnosis of CMV infection in UC is crucial, and several modalities have been developed for the diagnosis of CMV infection.

Currently, the guidelines of the European Colitis and Crohn's Organization recommend the combined use of hematoxylin-eosin (HE) staining, immunohistochemical (IHC) staining with a CMV-specific monoclonal antibody and PCR for CMV in colonic tissue for the detection of colonic CMV infection in patients with UC[9]. IHC staining has a higher sensitivity than conventional histology (78%-93%) for the detection of CMV in colonic tissue[28].

Several studies have reported that the quantitative real-time PCR technique is a highly sensitive method for the diagnosis of CMV infection in inflamed colonic tissue compared with non-inflamed colonic tissue in the setting of UC[29]. The detection of CMV DNA in mucosal biopsies by PCR analysis has been regarded as the most sensitive assay for the diagnosis of CMV infection of the intestinal tract[30]. However, this technique requires invasive procedures such as sigmoidoscopy or colonoscopy to collect biopsy material and in some instances a physician may not be able to perform a colonoscopy. Furthermore, inflamed colonic tissue in cases of UC is mostly friable, edematous, and eroded

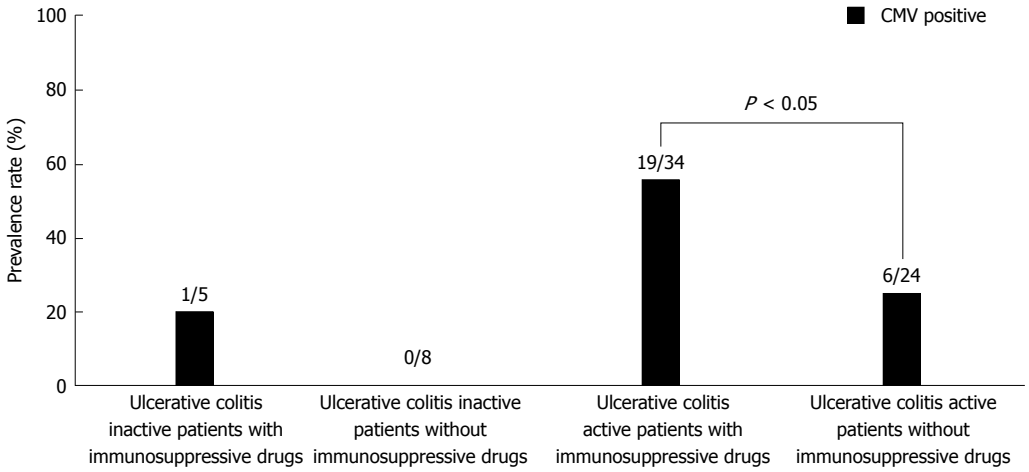

Figure 4 Comparison of prevalence rate of cytomegalovirus infection in 4 groups, including, ulcerative colitis inactive patients with immunosuppressive drugs, ulcerative colitis inactive patients without immunosuppressive drugs, ulcerative colitis active patients with immunosuppressive drugs, and ulcerative colitis active patients without immunosuppressive drugs. CMV: Cytomegalovirus.

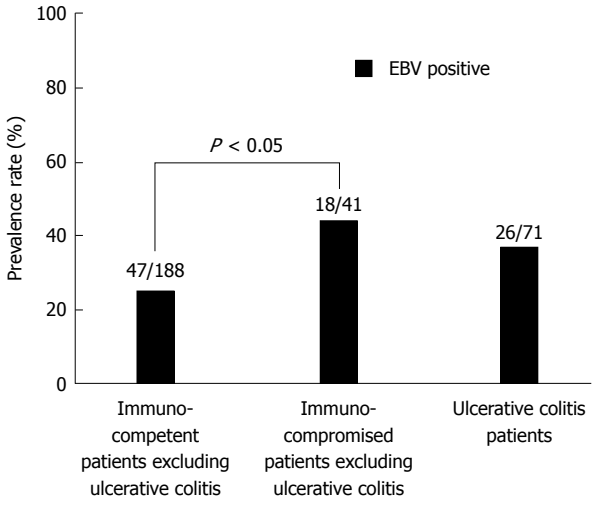

Figure 5 Comparison of prevalence rate of Epstein-Barr virus infection in 3 groups, including the immunocompetent patients excluding ulcerative colitis, the immunocompromised patients excluding ulcerative colitis, and ulcerative colitis patients. EBV: Epstein-Barr virus.

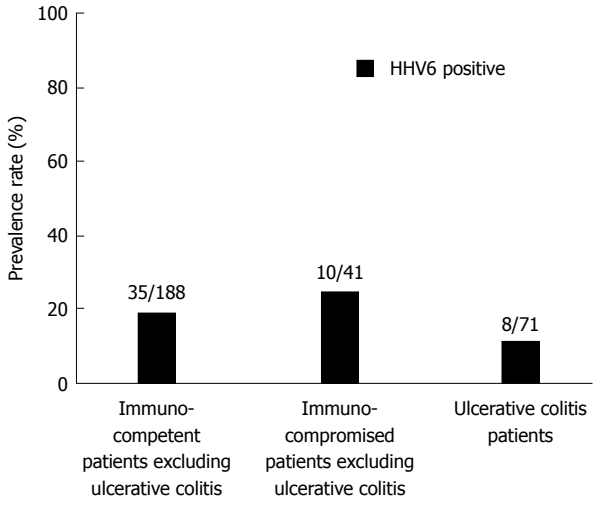

Figure 6 Comparison of prevalence rate of human herpes virus-6 infection in 3 groups, including the immunocompetent patients excluding ulcerative colitis, the immunocompromised patients excluding ulcerative colitis, and ulcerative colitis patients. HHV6: Human herpes virus-6.

and bleeds when touched. As a result, there is a high risk of bleeding during the collection of tissues. Additionally, endoscopic biopsy may lead to false-negative results due to sampling bias. To reduce sampling error, tissues in different locations along the colon are needed. Therefore, serology, a CMV antigenemia assay and PCR of blood samples for CMV are more convenient than invasive procedures[11,31]. Serological tests, such as the detection of CMV-specific IgM antibodies, have been shown to have a 100% sensitivity and a 99% specificity[32]. However, the presence of IgM antibodies can only reveal acute infection, and as such, cases of reactivation are often undiagnosed. Therefore, serology also has limited value in the diagnosis of CMV infection in patients with UC.

A recent study[11] evaluated the diagnostic performance of a CMV antigenemia assay and PCR of blood for the detection of CMV in patients with UC and showed lower sensitivities for the diagnosis of CMV infection compared with a previous study[10]. The presence of a CMV infection in the intestine cannot be ruled out in the case of a negative CMV antigenemia assay. In many cases, additional testing will be required to reach the diagnosis of CMV infection. The CMV antigenemia assay also has limited clinical value in the prediction of the reactivation of CMV infection in the gastrointestinal tract[33] and is therefore ineffective at preventing the development of CMV colitis. The CMV antigenemia assay is also relatively time consuming and demands expert pathologists to reduce subjective bias during interpretation of the slides. As the pp^{65} antigen for the CMV antigenemia assay is examined in blood leukocytes, it may also reveal false-negative results in leucopenic patients[27]. The diagnostic accuracy of CMV antigenemia may depend

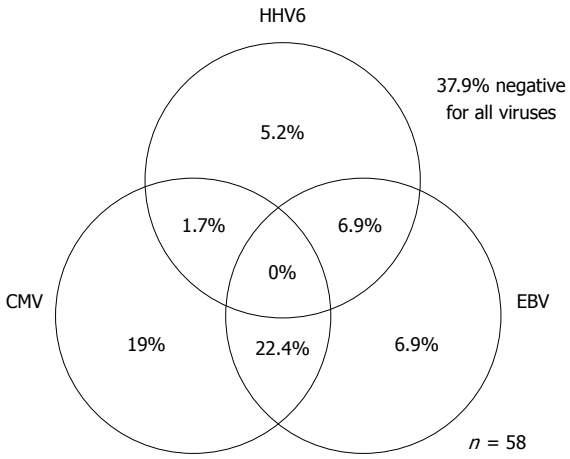

Figure 7 Overlapping prevalence patterns for cytomegalovirus, Epstein-Barr virus, and human herpes virus-6 infection in active ulcerative colitis patients. CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV6: Human herpes virus-6.

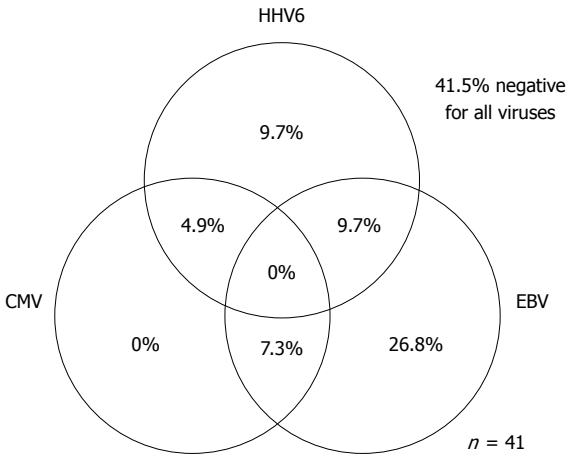

Figure 8 Overlapping prevalence patterns for cytomegalovirus, Epstein-Barr virus, and human herpes virus-6 infection in the immunocompromised patients excluding ulcerative colitis. CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV6: Human herpes virus-6.

on site of organ/tissue involvement[34]. Under these circumstances, the testing of stool samples by PCR was chosen as an alternative to these techniques.

We have found advantages in the detection of CMV DNA in stool samples by the multiplex PCR technique. Although this prospective study was designed so that fresh samples would be prepared from stool samples obtained by endoscopy, stool analysis is traditionally a non-invasive procedure. The procurement of tissues by biopsy may have potential risks when the intestine is inflamed. In addition, the stool may better reflect the CMV enterocolitis that is located in the proximal colon and small intestine. We are planning to analyse stool samples which are collected by the patients at home.

Several studies have evaluated CMV DNA in stool samples[26,35,36]. Ganzenmueller *et al*[37] have retrospectively evaluated quantitative real-time PCR in 66 fecal samples and intestinal biopsies for the diagnosis of CMV intestinal disease. Their study also compared CMV DNA in stool and colonic biopsies with the results of histopathology and the CMV antigenemia assay. In their study, the sensitivity and specificity of stool CMV DNA for the diagnosis of CMV intestinal disease are 67% and 96%, respectively. Therefore, CMV DNA in the stool is more sensitive and specific than the gold standard method (histopathology and IHC) for the diagnosis of CMV in inflamed colonic tissue.

Additional studies have evaluated the sensitivity, specificity and reliability of the multiplex PCR assay as a rapid molecular diagnostic tool for the investigation of the etiology of enteric infections in pediatric patients[20]. However, the diagnostic significance of the multiplex PCR assay in stool samples for the diagnosis of CMV infection in patients with UC has not yet been evaluated. In our prospective study, we evaluated the ability of a commercially available multiplex PCR assay to diagnose CMV infection in UC using stool samples. To our knowledge, this is the first large-scale prospective study that assesses the multiplex PCR assay as a diagnostic tool for the diagnosis of CMV in patients with active UC, and in both immunocompetent and immunocompromised patients. As for EBV and HHV6, this study has showed a possible synergistic role for these viruses in the pathogenesis in active UC activation, as suggested by the prior reports[38,39].

Our study has some limitations. First, we did not quantify the CMV DNA. However, in patients with UC, any positive result with respect to CMV is clinically significant without quantification of the viral load. Quantification of CMV in stool specimens is not feasible during specimen processing (*e.g.*, diluted versus non-diluted)[40], and positive results should be carefully considered, especially in patients with UC who are treated with immunosuppressive drugs[37]. Furthermore, this prospective study was not designed to compare the different modalities for the detection of CMV, including histopathology, CMV antigenemia assay, serology, and tissue CMV PCR. Herfarth *et al*[36] have shown that the sensitivity and specificity of the stool PCR analysis compared with PCR in mucosal biopsies were 83% and 93%, respectively. They have noted that it is not clear whether CMV DNA detected in the stool samples was due to leakage from the blood compartment into the intestinal tract, or if it was derived from intestinal CMV infection.

In conclusion, this prospective study proposes multiplex PCR as a successful, non-invasive diagnostic technique for rapid detection of CMV infection among UC patients in clinical in-patient and out-patient settings. Additionally, we present a new protocol for the broad analysis of the enteropathogens in stool samples in adult populations. This method will also help predict CMV infection prior to the development of intestinal symptoms, which is important for the prevention of exacerbation of UC by CMV reactivation. Positive PCR results may help to rapidly diagnose patients at a high risk for CMV infection. Further

studies are needed to confirm these results. Future studies that involve colonic biopsies will be needed to confirm the reliability of the multiplex PCR test results.

ACKNOWLEDGMENTS

The authors are grateful to clinical staff in the Department of Infectious, Respiratory, and Digestive Medicine and the Department of Endoscopy at the University of the Ryukyus.

COMMENTS

Background

Infection with cytomegalovirus (CMV) can cause exacerbation of ulcerative colitis (UC). Thus, early diagnosis of CMV infection is important. Although endoscopic biopsy is the best approach for the diagnosis of CMV infection, this procedure may be invasive for patients and damaging to the inflamed intestine.

Research frontiers

Molecular diagnostic advances have been made to simultaneously identify enteropathogens using multiplex polymerase chain reaction (PCR). The aim of this prospective study was to evaluate the feasibility of a qualitative multiplex PCR assay of stool for the simultaneous detection of bacterial and viral enteropathogens, focusing on CMV infection for adult patients, including patients with UC, immunocompetent patients, and immunocompromised patients.

Innovations and breakthroughs

This is the first large-scale prospective study that assesses the multiplex PCR assay in stool samples as a diagnostic tool for the diagnosis of CMV in patients with UC. This assay may prove useful, non-invasive method to exclude the presence of CMV infection in patients with active UC who are treated with immunosuppressive drugs.

Applications

This multiplex PCR as a successful, non-invasive diagnostic technique will help predict CMV infection prior to the development of intestinal symptoms, which is important for the prevention of exacerbation of UC by CMV reactivation.

Terminology

Multiplex PCR assay: A rapid, sensitive, specific, and reliable diagnostic tool for the simultaneous detection of bacterial and viral enteropathogens by PCR.

Peer-review

In this study, the authors detected CMV and other enteric pathogens using multiplex PCR in stool samples collected from 300 patients who underwent colonoscopy including patients with UC.

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P- Reviewer: Annese V, Zhang L **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Ma S

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12676

World J Gastroenterol 2015 November 28; 21(44): 12676-12685
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Prospective Study

Gastric cancer risk in relation to tobacco use and alcohol drinking in Kerala, India - Karunagappally cohort study

Padmavathy Amma Jayalekshmi, Soroush Hassani, Athira Nandakumar, Chihaya Koriyama, Paul Sebastian, Suminori Akiba

Padmavathy Amma Jayalekshmi, Natural Background Radiation Cancer Registry, Karunagappally, Kerala 690536, India

Padmavathy Amma Jayalekshmi, Paul Sebastian, Regional Cancer Center, Trivandrum, Kerala 695011, India

Soroush Hassani, Athira Nandakumar, Chihaya Koriyama, Suminori Akiba, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan

Author contributions: Jayalekshmi PA and Sebastian P conceived and designed the study; Hassani S, Nandakumar A, Akiba S and Jayalekshmi PA conducted the statistical analysis; Jayalekshmi PA, Hassani S, Nandakumar A, Koriyama C and Akiba S prepared the manuscript.

Supported by Department of Atomic Energy, Government of India; the Health Research Foundation, Japan; and Grants-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science and Technology of Japan, No. 12218231 and No. 1701503.

Institutional review board statement: The Regional Cancer Center, Thiruvananthapuram, India, initiated the cohort study in 1990 with the approval of ICMR, Government of India. The ethics committee of Kagoshima University Graduate School of Medical and Dental Sciences, Japan, also approved the present study.

Informed consent statement: All cohort members, or their legal guardian, provided verbal informed consent before study enrollment. Written informed consent was provided for cancer registry enrollment.

Conflict-of-interest statement: The authors confirm that this article content has no conflicts of interest.

Data sharing statement: Output files of the statistical analysis are available from the corresponding author at jayalekshminbrr@gmail.com.

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Correspondence to: Dr. Padmavathy Amma Jayalekshmi, Natural Background Radiation Cancer Registry, Karunagappally, Kerala 690536, India. jayalekshminbrr@gmail.com
Telephone: +91-476-2671833

Received: April 20, 2015
Peer-review started: April 21, 2015
First decision: June 19, 2015
Revised: July 13, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: November 28, 2015

Abstract

AIM: To assess the risk of gastric cancer (GC) in relation to tobacco use and alcohol drinking in the Karunagappally cohort in Kerala, South India.

METHODS: This study examined the association of tobacco use and alcohol drinking with GC incidence among 65553 men aged 30-84 in the Karunagappally cohort. During the period from 1990-2009, 116 GC cases in the cohort were identified as incident cancers. These cases were identified from the population-based cancer registry. Information regarding risk factors such as socioeconomic factors and tobacco and alcohol habits of cohort members were collected from the database of the baseline survey conducted during 1990-1997. The relative risks (RRs) and the corresponding 95% confidence intervals (95%CIs) for

tobacco use were obtained from Poisson regression analysis of grouped survival data, considering age, follow-up period, occupation and education.

RESULTS: Bidi smoking was associated with GC risk (P = 0.042). The RR comparing current versus never smokers was 1.6 (95%CI: 1.0-2.5). GC risk was associated with the number of bidis smoked daily (P = 0.012) and with the duration of bidi smoking (P = 0.036). Those who started bidi smoking at younger ages were at an elevated GC risk; the RRs for those starting bidi smoking under the age of 18 and ages 18-22 were 2.0 (95%CI: 1.0-3.9) and 1.8 (95%CI: 1.1-2.9), respectively, when their risks were compared with lifetime non-smokers of bidis. Bidi smoking increased the risk of GC among never cigarette smokers more evidently (RR = 2.2; 95%CI: 1.3-4.0). GC risk increased with the cumulative amount of bidi smoking, which was calculated as the number of bidis smoked per day x years of smoking (bidi-year; P = 0.017). Cigarette smoking, tobacco chewing or alcohol drinking was not significantly associated with GC risk.

CONCLUSION: Among a male cohort in South India, gastric cancer risk increased with the number and duration of bidi smoking.

Key words: Bidi smoking; Alcohol drinking; Gastric cancer; The Karunagappally cohort; Kerala; India

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Core tip: In South Asia, bidi smoking is a popular form of tobacco smoking. A bidi is 0.15-0.25 g of sun-dried tobacco flakes hand-wrapped in a temburni leaf. Bidi smoking has been shown to cause various cancers, such as cancers of the lung and oral cavity, by several epidemiological studies including the Karunagappally cohort study, one of the most important cohort studies in South Asia. However, only a few studies have examined the relation between bidi smoking and gastric cancer (GC) risk. Our results indicated that GC risk increased with the number and duration of bidi smoking. To our knowledge, the present study is the first cohort study to show an association between bidi smoking and GC risk.

Jayalekshmi PA, Hassani S, Nandakumar A, Koriyama C, Sebastian P, Akiba S. Gastric cancer risk in relation to tobacco use and alcohol drinking in Kerala, India - Karunagappally cohort study. *World J Gastroenterol* 2015; 21(44): 12676-12685 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12676.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12676

INTRODUCTION

Tobacco is used in different forms all around the world. The most common form of tobacco use worldwide is cigarette smoking, which is known to cause various cancers including gastric cancer (GC)[1]. In southern India, the most popularly smoked tobacco is bidi, which is made of sun dried flaked tobacco rolled into a conical shape in a dried rectangular piece of temburni leaf (*Diospyros melanoxylon*) with a thread securing the roll. As bidis are hand-rolled, the length and amount can vary; however, the difference is not large, and on average, a bidi in Karunagappally taluk, our study area, contains 0.15-0.25 g of tobacco leaves[1,2]. Tobacco-specific nitrosamine levels in the main stream smoke of bidis were reported to be as high as were those in cigarettes[3]. Bidi smoking has been found to be related to cancers of the oral cavity, lung, head and neck[4-10]. However, only a few studies have examined the relation between GC risk and bidi smoking, and their results are inconsistent[11-13].

Tobacco chewing is another common tobacco-related habit in many Asian countries, particularly in India. The chewing practices vary in different regions, and the most common combination used for chewing is areca nut, betel leaf, slaked lime and tobacco[14]. In the study area, tobacco chewing is always betel quid chewing. The International Agency for Research on Cancer (IARC) classified areca nut as a human carcinogen (Group 1) in 2004[14]. GC has been associated with the habit of chewing; an increase in GC was noted in the case of chewers with a habit of chewing tobacco alone[15]. However, the association that was noted in studies was inconsistent, warranting more in-depth studies[11,12,15,16].

Alcohol consumption is also known to cause various cancers, including cancers of the upper digestive tract and the liver[17]. Regarding the relation between alcohol drinking and GC risk, the IARC concluded in 2007 that the evidence is inconsistent[18]. A recent meta-analysis reported an increase in GC risk in alcohol drinkers, with a higher risk for heavy alcohol drinkers ($\geqslant$ 4 drinks per day) compared to non-drinkers[19]. In India, only a few studies have investigated the relation between alcohol drinking and GC risk[11,12,16].

Alcohol consumption is a common habit all over the world; however, commonly consumed beverages vary from country to country. In South India, the most popular alcoholic beverages are toddy and arrack. Toddy is prepared locally from the sap of coconut flowers or palm trees. This liquid is collected and allowed to ferment. This beverage has an alcohol content of approximately 5%-10%[20]. This preparation method is almost identical everywhere, including in Karunagappally. Arrack is a distilled beverage made from paddy, wheat, or palm sap, depending on the local availability. Jaggery, sugar or sugarcane is added to either one or a combination of these and boiled with water. This mixture is allowed to ferment, after which it is distilled. In Karunagappally taluk, arrack is made primarily from palm sap. This beverage contains approximately 25%-45% alcohol. With no legal control, some of the beverages were found

to have alcohol concentrations as high as 56%. In a study specifying the type of alcohol consumed, arrack drinkers and foreign liquor drinkers showed a significant association with an increased GC risk[11]. In that study, foreign liquors normally had 40% alcohol content.

The present cohort study investigated the association of tobacco use, alcohol drinking and socioeconomic status with GC in Karunagappally, Kerala, India.

MATERIALS AND METHODS

Base line survey

The area of the present study is Karunagappally taluk, which is an administrative unit consisting of 12 panchayats, in the coastal area of Kollam district in Kerala, South India, as described in our previous studies[8-10,21]. Based on the 1991 census, Karunagappally taluk, with an area of 192 km^2, had a total population of 385103 (193954 females and 191149 males). In the late 1980s, a cohort of all residents of Karunagappally was established with the objective of examining the risk of different cancer in association with natural radiation exposure, lifestyle and many other factors[8-10,21]. During the period from January 1st, 1990 to December 31st, 1997, every resident of Karunagappally taluk was surveyed for sociodemographic and other lifestyle-related factors as a part of the investigation. The face-to-face interview survey was conducted with the help of trained field investigators for each resident using a 6-page, standardized questionnaire. The questionnaire was constructed with specific questions to elicit factors such as household socioeconomic status, religion, education, income, and occupation along with lifestyle factors such as smoking and drinking habits, and dietary practices.

The questions pertaining to tobacco chewing, smoking and alcohol drinking included habits practices, and whether the person had a history of the habits in the past or was currently habituated. For the subjects with those habits, a detailed enquiry was made regarding the types of materials consumed, such as (1) pan, pan + tobacco, or tobacco alone in the case of tobacco chewing; (2) bidis or cigarettes in the case of smoking; and (3) and toddy, arrack or foreign liquors in the case of drinking. In addition, information was collected regarding the frequencies of smoking and tobacco chewing per day, the amount alcoholic beverages consumed in milliliters or liters per day, and the ages at starting and stopping those habits.

Study population

During the household survey, personal information on 359614 subjects from 71674 households (corresponding to 93% of the population and 94% of households in Karunagappally according to the 1991 census) were collected. There were 69943 men between the ages of 30-84 at the time of the interview. We excluded subjects younger than 30 because cancer incidence is low in this age group and the smoking effects will be apparent only decades after starting smoking. Subjects older than 84 were also excluded as the elderly tend not to seek medical attention for cancer, which can lower the completeness of case ascertainment and the diagnosis accuracy. The local Rare Earth factory workers were also excluded because of the possible occupational exposures (n = 1428). Additionally, subjects who were deceased or diagnosed with cancer before the base-line survey were excluded from the analysis (n = 136). Furthermore, we excluded subjects who died within 3 years after the interview because their health status might have affected their lifestyle. As a result, the statistical analysis was conducted on the remaining 65553 subjects[10].

Cancer case ascertainment

The present study analyzed cancer incidence among the Karunagappally cohort, during the 1990-2009 period. The regional cancer registry in Karunagappally taluk, which was initiated January 1st, 1990, registered the cancer cases[10,22]. Because this rural area does not have any center dedicated to cancer diagnosis or treatment, it was necessary to use an active registration method. We visited all health and medical facilities, in or outside the taluk, where cancer patients are attended to[23-26]. The registry reports are included in the IARC Cancer Incidence in Five Continents vol. Ⅶ-Ⅹ[23-26].

We obtained the death reports from the death registers of the Vital Statistics Division of each panchayat. To obtain supplemental information for determining the underlying cause of death, the cancer registry office started house visits of the deceased in 1997. The Death Certificate Only proportion was 13% during the 1991–1992 period[23], 10% and 11% for men and women, respectively, during the 1993–1997 period[24] and reduced to 4% and 5% for men and women, respectively, during the 1998–2002 period[25]. The mortality to incidence ratio (M:I %) for all cancers among men was 56% during the 2002-2003 period[25] and 53.8% during the 2006-2010 period[23] and was similar to those in other major cancer registries in this country[27].

To assess the extent of migration among cohort members, periodical door-to-door surveys of all the households in the 12 panchayats were conducted during the years 2001-2003 and 2008. The findings of those surveys were linked to incident cases through name, age, address, house number and so forth. These surveys showed that migration was negligible.

Statistical analysis

Statistical analysis was performed using the EPICURE program (DATAB; AMFIT)[28]. Poisson regression

Table 1 Sociodemographic features of study subjects (men) and their associations with gastric cancer

	n	Person-years	Gastric cancer cases	RR	95%CI	*P* value
Total	65553	900721				
Religion						> 0.5[2]
Hindu	47689	658303	88	1.0	Reference	
Muslim	11841	160475	19	0.9	0.5-1.5	
Christian	6023	81942	9	0.8	0.4-1.6	
Family income[3]						0.191[1]
< 500	4367	66066	10	1.1	0.6-2.2	
501-1200	19460	281461	41	1.0		
1201-2500	24794	332258	37	0.7	0.5-1.1	
2501-3500	10839	141566	14	0.6	0.3-1.0	
3500+	6093	79370	14	1.0	0.5-1.8	
Education						0.164[1]
Illiterate	4143	52337	7	0.5	0.2-1.1	
Primary school	16917	221434	49	1.0	Reference	
Middle school	17310	238947	30	0.7	0.5-1.2	
High school	20775	298699	25	0.7	0.4-1.1	
College	5703	79877	4	0.4	0.1-1.1	
Unknown	705	9427	1	0.5	0.1-3.9	
Occupation						0.008[2]
Farmers and fishermen	21683	302651	51	1.0	Reference	
Skilled workers	12445	169094	21	0.8	0.5-1.3	
White collars	13672	183094	28	0.8	0.5-1.2	
Others	17753	245882	16	0.4	0.2-0.7	

[1]The *P* for trend; [2]The *P* for heterogeneity; [3]Monthly family income in Indian National Rupee (INR). One INR is approximately 0.016 US dollars. In the analysis examining the relation between socioeconomic status and gastric cancer risk, a relative risk was obtained from the following model: $H = H_s \exp(B_i X_i)$, where the background hazard, H_s, was stratified by attained age and calendar time, and X_i denotes categorical variables of the sociodemographic factors shown in the table.

analysis of grouped data was conducted to estimate relative risks (RRs) and 95%CIs using the survival data cross-classified by 5-year categories of attained age (30-84 years), calendar year (1990-1997, 1998-2003, and 2004-2009), and other variables[29].

To examine the relation between GC risk and bidi smoking, the RRs of former smokers (denoted by X_2) and current smokers (denoted by X_3) were estimated using the following model: H_0 (calendar year, attained age, occupation, and education) exp ($\beta_2 X_2 + \beta_3 X_3$), where H_0 denotes the baseline, or background, GC incidence rate (*e.g.*, incidence rate in never smokers). The attained age of each cohort member was calculated at the mid-point of one-year intervals during the follow-up period using the DATAB procedure (EPICURE). The AMFIT procedure of the EPICURE program gave Maximum Likelihood Estimates of β_2 and β_3, after adjusting for calendar year, attained age, occupation, and education. These estimates are the log RRs for the indicator variables X_2 and X_3, respectively, with the reference category X_1[10]. We used similar models for the analysis of all other variables.

The entry date into the cohort was defined as the interview date, from January 1st, 1990 to December 31st, 1997. A cohort member was considered censored when diagnosed with a cancer other than GC or when died of any other cause. Thus, the end of follow-up was defined as the diagnosis date for cancer cases, the date of death for the deceased, the migration date, the date of attaining the age of 85 or the end of the study period (December 31st, 2009).

RESULTS

The present study examined 65553 men aged 30-84 years old. By the end of 2009, 116 cases of GC (ICD9: 151) were identified. Table 1 summarizes the analysis results regarding the association of GC risk with socioeconomic status. No significant association between GC risk and religion, family income or education was found. In contrast, statistically significant heterogeneity in GC risk was found among occupational groups (*P* = 0.008).

Table 2 summarizes the risk analysis results with respect to tobacco use and alcohol drinking. Bidi smoking was significantly associated with GC risk (RR = 1.6; 95%CI: 1.0-2.5; *P* = 0.042). Bidi smoking increased the risk of GC among never cigarette smokers (RR = 2.2; 95%CI: 1.3-4.0) more evidently. The number of only bidi smokers was 7592, and the number of bidi smokers who also smoked cigarettes was 17740. By restricting our further analyses to only bidi smokers, we would have omitted more than two-thirds of the subjects. Therefore, we decided to include all bidi smokers in the further analyses (Table 3), regardless of whether they were cigarette smokers. Former cigarette smokers showed a 40% increase in GC risk (RR = 1.4; 95%CI: 0.8-2.4), which was not statistically significant. Tobacco chewing was not related to GC risk. Current alcohol drinkers had a 30% increase in GC risk compared to never alcohol drinkers (RR = 1.3; 95%CI: 0.9-2.0); however, this association was not statistically significant either.

Table 2 Gastric cancer risk in relation to tobacco use and alcohol drinking among men

	n	Person-years	Gastric cancer cases	RR	95%CI	*P* value[1]
Total	65553	900721				
Bidi smoking						0.042
Never	31277	441290	35	1.0	Reference	
Former	5830	70584	15	1.3	0.7-2.5	
Current	25403	347383	62	1.6	1.0-2.5	
Unknown	3043	41464	4	1.0	0.4-3.0	
Cigarette smoking						0.265
Never	29205	398841	57	1.0	Reference	
Former	5603	71488	18	1.4	0.8-2.4	
Current	27835	390298	39	0.8	0.5-1.2	
Unknown	2910	40093	2	0.4	0.1-1.7	
Tobacco chewing						> 0.5
Never	42190	582656	73	1.0	Reference	
Former	4383	54094	9	0.8	0.4-1.6	
Current	18568	258317	34	0.9	0.6-1.4	
Unknown	412	5653	0			
Alcohol drinking						0.175
Never	33296	454553	51	1.0	Reference	
Former	7857	98248	16	0.9	0.5-1.7	
Current	24399	347905	49	1.3	0.9-2.0	
Unknown	1	14	0			

[1]The *P* for trend (those in unknown categories were excluded). The model used for statistical analysis to obtain the RRs associated with tobacco and alcohol use was as follows: $H = H_s \exp(B_i X_i)$, where the background hazard, Hs, was stratified by attained age, calendar time, occupation and education, and X_i denotes categorical variables related to tobacco and alcohol use. Similar models were used in the analysis that produced the results presented in Tables 3-5.

Table 3 Gastric cancer risk in relation to bidi smoking among men

	Person-years	Gastric cancer cases	RR	95%CI	*P* value[1]
Bidis smoked per day					0.012
Never	441290	35	1.0	Reference	
Former	70584	15	1.3	0.7-2.5	
1-4	40768	4	1.0	0.4-2.8	
5-14	131494	20	1.4	0.8-2.5	
15-24	105359	23	1.9	1.1-3.4	
⩾ 25	67303	15	1.7	0.9-3.2	
Unknown	43924	4	1.0	0.3-2.8	
Duration of bidi smoking					0.036
Never	441290	35	1.0	Reference	
1-14	141330	10	1.3	0.6-2.8	
15-29	123435	14	1.1	0.6-2.1	
30-44	84545	30	2.0	1.2-3.5	
⩾ 45	68393	23	1.6	0.9-3.0	
Unknown	41728	4	1.0	0.4-3.0	
Age at start of bidi smoking					0.161
< 18	64800	14	2.0	1.0-3.9	
18-22	172714	32	1.8	1.1-2.9	
⩾ 23	109709	16	1.3	0.7-2.5	
Never	441290	35	1.0	Reference	
Unknown	41624	4	0.0		
Years since quitting bidi smoking					0.424
Current smokers	347383	62	1.0	Reference	
< 5	28435	6	0.9	0.4-2.0	
5-9	18058	5	1.1	0.5-2.9	
⩾ 10	23055	4	0.6	0.2-1.7	
Never	441290	35	0.6	0.4-1.0	
Unknown	42500	4	0.6	0.2-1.7	

[1]The *P* for trend (those in unknown category were excluded).

As shown in Table 3, where the results of more detailed analysis regarding bidi smoking are summarized, GC risk increased with the number of bidis smoked daily ($P = 0.012$) and with the duration of bidi smoking ($P = 0.036$). Those individuals who started bidi smoking at 22 years old or younger had an elevated GC risk. The cumulative amount of bidi smoking was calculated as the product of the number

Table 4 Gastric cancer risk in relation to cigarette smoking among men

	Person-years	Gastric cancer cases	RR	95%CI	*P* value[1]
Cigarettes smoked per day					0.212
Never	398841	57	1.0	Reference	
Former	71488	18	1.4	0.8-2.3	
≤ 14	357740	36	0.8	0.5-1.2	
≥ 15	43325	3	0.6	0.2-2.1	
Unknown	29327	2	0.6	0.1-2.5	
Duration of cigarette smoking					> 0.5
Never	398841	57	1.0	Reference	
1-14	212691	12	0.9	0.5-1.8	
15-29	134583	14	0.8	0.4-1.5	
30-44	72749	18	1.0	0.6-1.7	
≥ 45	41727	13	0.9	0.5-1.8	
Unknown	40129	2	0.4	0.1-1.7	
Age at start of cigarette smoking					> 0.5
< 18	53786	6	0.8	0.3-1.9	
18-22	193597	20	0.8	0.5-1.4	
≥ 23	143035	13	0.7	0.4-1.3	
Never	398841	57	1.0	Reference	
Unknown	39974	2	0.0		
Years since quitting cigarette smoking					0.109
Current smoker	390298	39	1.0	Reference	
< 5	27180	7	2.0	0.9-4.3	
5-9	18804	6	2.4	1.0-5.6	
≥ 10	24089	5	1.4	0.5-3.5	
Never	398841	57	1.3	0.9-2.0	
Unknown	41508	2	0.5	0.1-2.0	

[1]The *P* for trend (those in unknown categories were excluded).

of bidis smoked per day and the number of years of smoking (bidi-year). GC risk increased with bidi-year (P = 0.017). The RRs for those individuals with 400-799 and 800+ bidi-years were 1.7 (95%CI: 1.0-2.9) and 1.8 (95%CI: 1.0-33), respectively.

As summarized in Table 4, further analysis regarding the effect of cigarette smoking and GC risk showed that the increase in GC risk in former smokers was more evident among those who had quit smoking during the 10 years before the survey. The increase in risk was statistically significant for the group who had quit smoking 5-9 years before the survey (RR = 2.4; 95%CI: 1.0-5.6). The cumulative amount of cigarette smoking, which was calculated as the product of the number of cigarettes smoked daily and the number of years of smoking, was not associated with GC risk.

Further analysis regarding tobacco chewing showed no significant association between GC risk and the amount or duration of the habit (data not shown).

As shown in Table 5, GC risk was not associated with the amount or duration of alcohol drinking. We observed elevated RRs for all types of alcoholic beverages, with the highest RR observed for toddy (RR = 2.3; 95%CI: 0.7-7.3), followed by arrack (RR = 1.7; 95%CI: 0.9-3.3); however, none of the results were statistically significant.

Regarding drinking, we did not have sufficient information to calculate the accurate amount of alcohol consumed per day for each of a wide variety of alcohol types. Therefore, we limited the analyses regarding the amount of alcohol consumption to only arrack drinkers because arrack is the most common liquor (with high alcohol content) among our study population. No association between arrack drinking and GC risk was found in our study population. The RRs for former (n = 862) and current (n = 4139) arrack drinkers versus never alcohol drinkers were 1.5 (95%CI: 0.5-4.9) and 1.7 (95%CI: 0.9-3.2), respectively. In the analysis of daily arrack consumption, the RRs for daily consumption of less than 70 mL per day and 70 mL or more per day were 1.3 (95%CI: 0.3-5.3) and 1.6 (95%CI: 0.8-3.5), respectively. Although GC risk increased with the amount of daily arrack consumption, P for the trend was not statistically significant (P = 0.098). The cumulative amount of arrack drinking was calculated as the product of the amount of daily arrack consumption in ml and the duration of the habit in years (mL-year). No significant increase in GC risk was associated with the ml-year of arrack consumption (P = 0.377).

DISCUSSION

The present study showed that bidi smoking was associated with a higher risk of GC. GC risk increased with the increased number of bidis smoked daily (P = 0.012) and with a longer duration of bidi smoking (P = 0.036). Bidi smoking that started at 22 years old or younger was shown to be significantly associated with a higher risk of GC. Cigarette smoking or tobacco chewing was not significantly associated with GC risk. Alcohol drinking was not significantly associated with

Table 5 Gastric cancer risk in relation to alcohol drinking among men

	Person-years	Gastric cancer cases	RR	95%CI	*P* value
Type of alcohol					
Never drinker	454152	51	1.0	Reference	> 0.5[1]
Former drinker	98248	16	0.9	0.5-1.7	
Toddy	10404	3	2.3	0.7-7.3	
Arrack	60956	12	1.7	0.9-3.3	
Foreign	63835	7	1.4	0.6-3.3	
Combination	209740	27	1.2	0.7-1.9	
Other	3384	0	0.0		
Duration of alcohol consumption					> 0.5[2]
Never	454553	51	1.0	Reference	
1-14	148811	14	1.8	1.0-3.3	
15-29	176222	19	1.0	0.5-1.6	
30-44	71460	26	1.4	0.9-2.3	
⩾ 45	11079	1	0.3	0.0-2.5	
Unknown	38596	5	1.3	0.5-3.2	
Age at start of alcohol drinking					0.179[2]
< 25	146010	15	1.0	0.5-1.7	
⩾ 25	166508	29	1.6	1.0-2.6	
Never	454553	51	1.0	Reference	
Unknown	35400	5	1.5	0.6-3.7	

[1]The *P* for heterogeneity; [2]The *P* for trend (those in unknown categories were excluded).

GC risk either.

Three previous Indian studies examined the relation between bidi smoking and GC risk[11-13]. Among them, two studies (a case-control study and a cohort study) found no association[12,13]. However, the case-control study compared the risk among current smokers with the risk among never smokers and former smokers[12]. In the cohort study[13], we cannot deny the possibility that non-bidi smokers included a significant number of cigarette smokers.

In the present study, we did not see an increase in GC risk in current cigarette smokers; however, an increase in risk, although non-significant, was observed among former smokers. Notably, an increase in GC incidence was observed in those individuals who had quit smoking during the 10 years before the survey. The risk of developing GC for those individuals who stopped smoking less than 5 years before the survey was 2 times the risk for current smokers (RR = 2.0; 95%CI: 0.9-4.3), and the RR increased to 2.4 (95%CI: 1.0-5.6) for those individuals who had stopped smoking between 5-9 years before the survey. This excess risk could be a result of quitting smoking due to experiencing symptoms of chronic atrophic gastritis, intestinal metaplasia or gastro-duodenal ulcers. The same phenomenon has been mentioned as the reason for the decrease in the RR estimates often observed in studies in the highest exposure category possibly due to a lower tolerance of people suffering from symptoms such as chronic indigestion due to the above-mentioned conditions[30].

In the present study, GC risk increased among alcohol drinkers, but the association was not statistically significant. Among the types of alcoholic beverages used, toddy had the largest RR. However, the analysis was based on only three GC cases with a toddy drinking habit. Three previous Indian case-control studies were conducted in Madras, Mumbai and Trivandrum. None of these studies found a significant association between alcohol drinking and GC risk[11,12,16]. In the case-control study performed by Gajalakshmi *et al*[11] in Madras, the estimated odds ratio (OR) was 0.8 (95%CI: 0.41-1.77) for the comparison between current and never drinkers and was 1.4 (95%CI: 0.54-3.40) for ex-drinkers versus never drinkers. Among the types of alcoholic beverages used, statistically significant increases in risk were observed for arrack (OR = 2.6; 95%CI: 1.49-4.40) and foreign liquors (OR = 3.0; 95%CI: 1.49-5.96) but not for toddy (OR = 0.4; 95%CI: 0.09-2.20)[11]. In this alcohol-type specific analysis, the values for former drinkers and current drinkers were combined. The results obtained in the present study were consistent with those findings except for toddy. Indeed, the present study showed statistically non-significant elevated RRs for arrack drinkers (RR = 1.7) and foreign liquor drinkers (RR = 1.4).

The association between tobacco chewing and GC risk has been studied in epidemiological studies from different regions of India, including Madras, Mumbai, Trivandrum and Mizoram[11,12,15,16]. Among these studies, only the hospital-based case–control study in Mizoram found an evident risk increase among tobacco chewers; this study reported an OR of 2.6 (95%CI: 1.1-4.2) for those individuals chewing tobacco alone and an OR of 2.0 for those individuals chewing tobacco with betel nuts (95%CI: 1.3-5.3)[15]. Although the cohort study in Mumbai showed an association of GC risk with smokeless tobacco use, the relation was not statistically significant, and the observed RR was not

large (RR = 1.28; 95%CI: 0.68-2.43)[13].

A recent meta-analysis of 36 studies on socioeconomic position (SEP) and GC risk cited in PubMed and EMBASE from 1966 to 2013 observed a significant increase in GC risk among the lowest SEP categories in occupation and education[31]. The present study showed significant heterogeneity in GC risk among occupational groups (P = 0.008). Those findings most likely indicate that SES factors are related to dietary habits, which are known to be related to GC[32-34].

Helicobacter pylori (*H. pylori*) infection is the most important risk factor of GC and is known to trigger a consequence of pathological changes leading to GC[35]. Risk factors such as tobacco use and alcohol drinking can modify the risk of GC induced by *H. pylori*. The evidence showing that tobacco smoking remains a risk factor among individuals infected with *H. pylori* supports this notion[36]. However, studies in India have not shown a strong association between GC risk and *H. pylori* infection, although its prevalence is high. The high prevalence of *H. pylori* infection in India, despite relatively low GC incidence, is known as the Indian enigma[37]. Taken together, in the results of the present study suggests that bidi smoking increases the risk of *H. pylori*-related GC. However, we cannot deny the possibility that bidi smoking also increases *H. pylori*-unrelated GC risk because we do not have any information on *H. pylori* infection in the present study population.

We do not have any information regarding GC pathology; therefore, we could not distinguish the intestinal and diffuse types. However, the diffuse type is considered only weakly related to lifestyle-related factors such as smoking and dietary habits[38]. Thus, the relations between bidi smoking and GC observed in this study were primarily from the associations with the intestinal type. However, we cannot tell whether bidi smoking increased the risk of both subtypes, although to different magnitudes, or only increased the risk of the intestinal type.

Another limitation of this study is the lack of data regarding dietary habits. A case-control study by Mathew *et al*[16] conducted at Regional Cancer Centre, Trivandrum, South India, found that GC risk was not associated with the consumption of dried fish, which is the primary food item with a high concentration of salt in our study area. Moreover, the consumption of dried fish was not common in the study population. Therefore, we believe that our study results regarding the association between bidi smoking and GC risk is unlikely to be substantially affected by salt intake, which was not considered in the present study.

Mathew *et al* also found that GC risk was related to more frequent rice intake (OR = 3.9; 95%CI: 1.6-10.0 for daily users), hot chili consumption (OR = 7.4; 95%CI: 4.0-13.5) and high-temperature food use (OR = 7.0; 95%CI: 3.7-12.9). The ORs for hot chili and high temperature foods are relatively large in this study, but their CIs are wide. In addition, those habits are less common in the study area; therefore, the percentage of stomach cancer cases related to those habits is expected to be small although the ORs are relatively high. Notably, the association of those factors with bidi smoking is unlikely to be large enough to be able to explain the association between bidi smoking and GC completely. However, the weak associations of cigarette smoking and alcohol drinking with GC risk may be explained by the associations with dietary habits. Regarding rice eating, this habit is so common that everybody eats it; the amount of rice consumption is unlikely to be strongly associated with bidi smoking.

To summarize, in the present cohort study, bidi smoking emerged as a risk factor of GC with a positive dose-response relation with the number and duration of bidi smoking.

COMMENTS

Background

In south Asia, bidi smoking is a popular form of tobacco smoking. Bidi smoking has been shown to cause various cancers. However, the association between bidi smoking and the risk of cancers in the lower digestive tract remains unclear.

Research frontiers

Bidi smoking has been shown to cause cancers of the respiratory tract and upper digestive tract by several epidemiological studies, including the Karunagappally cohort study, one of the most important cohort studies in south Asia. To date, only a few case-control studies have examined the relation between bidi smoking and gastric cancer (GC) risk.

Innovations and breakthroughs

This study is the first cohort study in India to investigate the association of bidi smoking with GC incidence, which has not been shown to date.

Applications

From a public health viewpoint, revealing the associations of bidi smoking with all the major cancers in Asia is important for establishing effective and efficient preventive measures. The information obtained by this study will be useful for this purpose.

Terminology

Bidi: Bidi smoking is a common form of tobacco use in South Asia. A bidi consists of 0.15-0.25 g of sun-dried tobacco flakes hand-wrapped in a temburni leaf. Arrack: Arrack is an alcoholic beverage that is common in South Asia. This distilled beverage is made from paddy, wheat, or palm sap and contains approximately 25%-45% alcohol. Toddy: Toddy is an alcoholic beverage that is common in India. This beverage is prepared locally from the sap of coconut flowers or palm trees and has an alcohol content of approximately 5%-10%.

Peer-review

In the present retrospective cohort study, authors analyzed tobacco smoking and alcohol intake as risk factors for GC. The main limitation is that it has been conducted on a population with a wide variety in socio-economic conditions. Moreover, the type of smoking (bidi) and alcoholic beverage (toddy and arrack) are widespread only in India, and their preparation may change according to the city where the recipe was formulated.

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P- Reviewer: Ierardi E **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12686

World J Gastroenterol 2015 November 28; 21(44): 12686-12695
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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SYSTEMATIC REVIEWS

Transanal total mesorectal excision: Towards standardization of technique

Albert M Wolthuis, Gabriele Bislenghi, Anthony de Buck van Overstraeten, André D'Hoore

Albert M Wolthuis, Gabriele Bislenghi, Anthony de Buck van Overstraeten, André D'Hoore, Department of Abdominal Surgery, University Hospital Leuven, 3000 Leuven, Belgium

Author contributions: Wolthuis AM and D'Hoore A designed the study; Wolthuis AM, Bislenghi G and de Buck van Overstraeten A performed the study; Wolthuis AM and Bislenghi G analysed the data; Wolthuis AM, Bislenghi G, de Buck van Overstraeten A and D'Hoore A wrote the paper.

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

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Correspondence to: Albert M Wolthuis, MD, Department of Abdominal Surgery, University Hospital Leuven, Herestraat 49, 3000 Leuven, Belgium. albert.wolthuis@uzleuven.be
Telephone: +32-16-344265
Fax: +32-16-344832

Received: May 16, 2015
Peer-review started: May 20, 2015
First decision: July 14, 2015
Revised: August 1, 2015
Accepted: October 23, 2015
Article in press: October 26, 2015
Published online: November 28, 2015

Abstract

AIM: To describe the role of Transanal total mesorectal excision (TaTME) in minimally invasive rectal cancer surgery, to examine the differences in patient selection and in reported surgical techniques and their impacts on postoperative outcomes and to discuss the future of TaTME.

METHODS: MEDLINE (PubMed), EMBASE, and The Cochrane Library were systematically searched through the 1st of March 2015 using a predefined search strategy.

RESULTS: A total of 20 studies with 323 patients were included. Most studies were single-arm prospective studies with fewer than 100 patients. Multiple transanal access platforms were used, and the laparoscopic approach was either multi- or single port. The procedure was initiated transanally or transabdominally. If a simultaneous approach with 2 operating surgeons was chosen, the operative time was significantly reduced.

CONCLUSION: TaTME was also associated with better TME specimens and a longer distal resection margin. TaTME is thus feasible in expert hands, but the learning curve and safety profile are not well defined. Long-term follow-up regarding anal function and oncological outcomes should be performed in the future.

Key words: Laparoscopy; Colorectal surgery; Rectal cancer; Total mesorectal excision; Transanal total mesorectal excision; Natural orifice specimen extraction; Transanal; Transanal minimally invasive surgery; Reverse total mesorectal excision

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Core tip: Transanal total mesorectal excision (TaTME) is a result of recent developments in transanal endoscopic microsurgery, transanal minimally invasive surgery, natural orifice specimen extraction, natural orifice transluminal endoscopic surgery, transanal abdominal transanal proctosigmoidectomy, and laparoscopic total mesorectal excision. TaTME is an exciting convergence

of various existing surgical techniques that represents the future of rectal cancer surgery. A substantial number of patients, and especially obese males with a narrow pelvis, will benefit from this minimally invasive approach. This systematic review addresses all aspects of TaTME and discusses the advantages and disadvantages of this technique. Different surgical approaches are used, but it is clear that experience with TaTME is increasing worldwide. Standardization of the technique and reporting of outcomes is required.

Wolthuis AM, Bislenghi G, de Buck van Overstraeten A, D'Hoore A. Transanal total mesorectal excision: Towards standardization of technique. *World J Gastroenterol* 2015; 21(44): 12686-12695 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12686.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12686

INTRODUCTION

Total mesorectal excision (TME) was first described by Heald *et al*[1] in 1982 and is the gold standard for the treatment of rectal cancer. This technique results in larger negative circumferential resection margins, with subsequent reductions in locoregional recurrence and improved oncological outcomes[2]. A minimally invasive approach to TME has additionally been developed to optimize postoperative short-term outcomes. Laparoscopic TME exhibits oncological outcomes similar to those of open TME[3-6] and is also associated with better postoperative recovery, including lower postoperative morbidity and shorter hospital stays[7,8]. However, a steep learning curve is associated with laparoscopic TME, making the implementation of this technique a long process[9]. The technical challenges of laparoscopic TME are linked to operating in a deep and often narrow pelvis, with difficulties in obtaining adequate exposure. Laparoscopic ultra-low TME requires substantial retraction, which hampers visualization of the most distal part of the rectum. This critical step may lead to both breaches in the mesorectal fascia and incorrect identification of the distal resection margin, compromising oncological outcomes. Moreover, distal rectal transection using currently available laparoscopic staplers can be difficult. A suboptimal angle in the deep bony pelvis[10] often requires different staple firings for rectal transection, and the need for more than 2 linear stapling firings is associated with increased postoperative morbidity and anastomotic leakage[11]. The abovementioned technical challenges of operating in a confined space result in considerable rates of conversion from laparoscopic to open TME. Conversion rates as high as 34%[3,4,12-15] have been reported, and these conversions are linked to increased morbidity and worse oncological outcomes[13,16]. The risk of conversion is higher in males and obese patients[17].

A recent transanal approach was introduced to facilitate mobilization of the most distal rectum and to overcome the inherent shortcomings of laparoscopic TME[18,19]. In particular, transanal TME (TaTME) is a new minimally invasive procedure that basically merges different concepts of transanal surgery. TaTME was developed as a result of combined experience in transanal endoscopic microsurgery (TEM)[20], transanal abdominal transanal proctosigmoidectomy (TATA)[21], transanal minimally invasive surgery (TAMIS)[22], natural orifice specimen extraction (NOSE)[23], and natural orifice transluminal endoscopic surgery (NOTES)[24,25]. However, although TaTME appears to be an attractive option for improving postoperative outcomes, the technique has not been extensively investigated. The aims of this systematic review were to describe the role of TaTME in minimally invasive rectal cancer surgery, to examine the differences in patient selection and in reported surgical techniques and their impacts on postoperative outcomes and to discuss the future of TaTME.

MATERIALS AND METHODS

MEDLINE (PubMed), EMBASE, and The Cochrane Library were systematically searched through the 1st of March 2015. Boolean AND/OR operators were used to combine keywords and subject headings. The following search criteria were used: (total mesorectal excision or TME) and (transanal or transanal minimally invasive surgery or TAMIS or transanal specimen extraction or natural orifice specimen extraction or NOSE or natural orifice transluminal endoscopic surgery or NOTES). Search results were supplemented with subject headings for Medline. The reference lists of retrieved articles were also hand searched for additional publications. Cross-referencing was continued until no further relevant publications were identified. Randomized controlled clinical trials as well as observational cohort studies (excluding case reports) that described a technique to mobilize the most distal rectum transanally using endoscopic instruments were considered for inclusion. Studies of paediatric surgery were excluded. Studies using cadaveric and animal series were also excluded. First, the titles were screened, and appropriate studies were selected. Second, the full text of these studies was acquired. There was no language restriction. The quality of the included studies was assessed using the Newcastle-Ottawa Scale. This scale assesses the quality of non-randomized clinical trials and evaluates patient selection, the comparability of study groups and outcome assessment. A maximum of 9 stars can be achieved[26,27]. Relevant data from the included studies were extracted using a standard fillable form of predefined parameters and were entered into an Excel database. The following data were extracted: publication year, study type, inclusion and exclusion criteria, sample size, patient

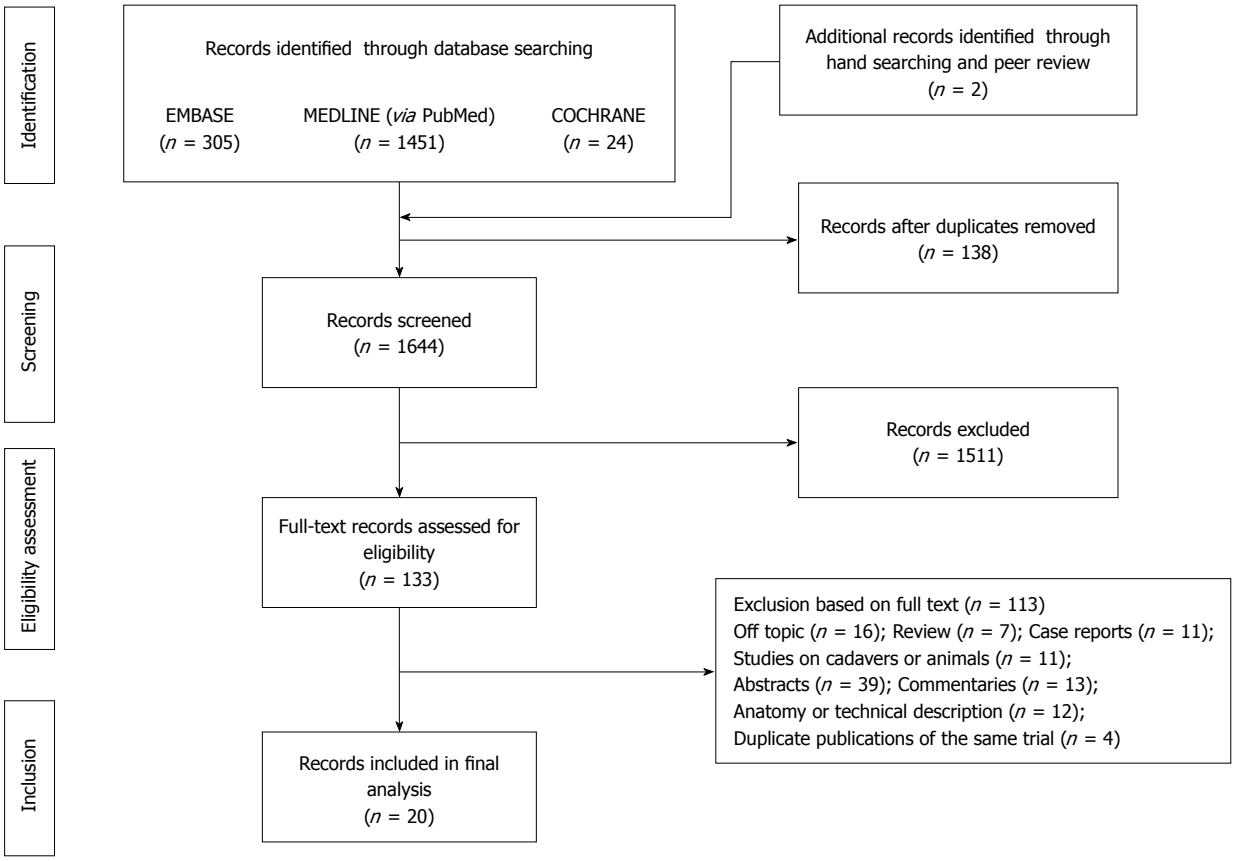

Figure 1 PRISMA flow diagram of the systematic literature review.

characteristics (age, gender, and body mass index (BMI)), neoadjuvant treatment, tumour characteristics (clinical stage and distance from anal verge or dentate line), surgical technique (approach, transanal platform used, specimen extraction, anastomotic technique, and defunctioning stoma), and operative outcomes (duration of surgery, estimated blood loss, postoperative complications, length of hospital stay, and follow-up). This systematic review was conducted in compliance with the PRISMA guidelines[28].

RESULTS

The predefined search strategy returned 1644 non-duplicated references (Figure 1). Publication titles and abstracts were screened, and 133 publications were retrieved for full-text review. Subsequently, 113 articles were excluded for the following reasons after a detailed review of the studies: 16 studies were off topic, 11 studies were case reports, 7 studies were reviews, 11 studies reported on cadaveric or animal series, 39 studies were abstracts, 14 studies were expert commentaries, 11 studies described the surgical technique or the anatomy involved in TaTME, and 4 studies were duplicate publications of the same clinical series. The article with the most comprehensive data was used in the last case. In total, 20 publications with a total of 323 patients were included[29-48]. Seventeen studies were prospective studies, 1 study was a case-control study, 1 study was a comparative study, and 1 study was a retrospective cohort study.

Patient selection

Only small studies, with fewer than 100 patients, were found (Table 1). Patients were predominantly male (male:female = 2:1), with a reported median age of approximately 65 years. The median BMI ranged from 22-31 kg/m^2. Eighteen of the 20 studies reported the tumour distance from either the anal verge or the dentate line. This distance ranged from 0-15 cm, with reported median distances of between 1.7 and 9.7 cm. Most patients in studies reporting on neoadjuvant therapy received induction chemoradiotherapy (231 of 296 patients). Patient and/or tumour characteristics played a role in patient selection. Specifically, patients were selected according to age (> 18 years old)[31,43], BMI (BMI > 30 kg/m^2, BMI < 40 kg/m^2)[31,33,42], or pelvic anatomy (pubococcygeal diameter < 10 cm)[29,33,42]; in 15 studies, tumour characteristics determined patient selection. Here, patients were selected if they were diagnosed with tumours located anteriorly in 1 study[42], with low (< 5 cm) rectal cancer in 8 studies[29,30,34,36,39,40,44,45], or with tumours within 12 cm from the anal verge in 3 studies[31,41,42]. Only T1-T3 tumours, as staged by magnetic resonance imaging, were included in 1 study[43], and tumours that

Table 1 Types of studies and patient selection for transanal total mesorectal excision

Author, year	Type of study	No. of patients	Median age (yr)	Gender ratio (M:F)	Median BMI (kg/m²)	Neoadjuvant therapy	Median distance from AV/DL (cm)
Zorron, 2010	Prospective	9	63 (range, 52-81)	5:4	NR	4/9	AV 7.5 (range, 4-12)
Dumont, 2012	Prospective	4	67 (range, 70-66)	4:0	23 (range, 22-25)	NR	AV 5.3 (range, 4-7)
de Lacy, 2013	Prospective	20	65 ± 10.2[1] (range, 44-77)	11:9	25 ± 3.8 (range, 19-33)	14/20	DL 6.5 ± 3.3[1] (range, 2-15)
Lacy, 2013	Prospective	3	73 (range, 71-75)	1:2	22 (range, 16-25)	2/3	9.7 (range, 9-10)
Rouanet, 2013	Prospective	30	65 (range, 43-82)	30:0	26 (range, 21-32)	29/30	AV Low rectum (0-5 cm) 20/30; Middle rectum (5-10 cm) 10/30; Upper rectum (10-15 cm) 0/30
Sylla, 2013	Prospective	5	49 ± 9.8[1]	3:2	26 ± 2.3	2/5	AV 5.7 (range, 4-10)
Atallah, 2014	Prospective	20	57 (range, 36-73)	14:6	24 (range 18-41)	17/20	AV 5 (range 1-9)
Chen, 2014	Prospective	20	58 ± 10.1[1]	11:9	25 ± 3	11/20	AV 5.9 ± 1.7 (range, 2-8)
Chouillard, 2014	Prospective	16	58 (range, 34-81)	6:10	28 (range, 21-38)	Yes	NR
Fernandez-Hevia, 2014	Prospective Comparative	37	65 ± 11.8[1]	24:13	24 ± 3.6 (range, 18-31)	27/37	AV Middle rectum 8.1 ± 1.7[1]; Low rectum 3.5 cm ± 1.2[1]
Kneist, 2014	Prospective	6	56 (range, 45-65)	5:1	25 (range, 23-28)	4/6	DL 1.7 (range, 0-3)
Meng, 2014	Prospective	3	80 (range, 76-82)	2:1	NR	1/3	AV 4.3 (range, 4-5)
Tuech, 2014	Prospective	56	65 (range, 39-83)	41:15	27 (range, 20-42)	47/56	AV 4 (range, 0-5)
Velthuis, 2014	Prospective Case-Matched	25	64 (range, 49-86)	18:7	25 (range, 20-36)	25/25	AV 8 (range, 0-16)
Wolthuis, 2014	Prospective	7	65 (range, 38-87)	6:1	25 (range, 17-32)	NR	NR
Atallah, 2015	Retrospective	4	45 (range, 26-59)	3:1	31 (range, 21-38)	3/4	AV 3.3 (range, 1-4)
Gomez Ruiz, 2015	Prospective	5	53 (range, 38-67)	4:1	26 (range, 22-31)	4/5	AV 5 (range, 4-6)
Knol, 2015	Prospective	10	61 (range 36-70)	8:2	27 (range 22-34)	10/10	DL 2.9 ± 1.21
Muratore, 2015	Prospective	26	66 (range, 38-84)	16:10	26 (range, 17-38)	19/26	4.4 (range, 3-6)
Prochazka, 2015	Prospective	17	68 (range, 49-81)	11:6	28 (range, 22-32)	12/17	AV 6 (range, 3-8)

[1]Values reported as the mean ± SD. AV: Anal verge; DL: Dentate line; NR: Not reported; M: Male; F: Female; BMI: Body mass index.

responded well to neoadjuvant therapy were included in another study[36]. cT4 tumours were excluded in 4 studies[33,37,40,45], and Rullier type Ⅱ/Ⅲ[49] tumours were excluded in 1 study[40].

Operative technique

TaTME was initiated transanally in 7 studies[31,33,40,41,44,46,47], and the abdominal phase was performed first in 7 other studies (Table 2)[29,30,35-37,42,45]. TaTME was performed simultaneously with the presence of laparoscopic and perineal operative teams[32,34,38,39,43,48]. This simultaneous approach was taken to reduce the operative time. Seven different transanal access platforms were used: the GelPOINT Path Transanal Access Platform (Applied Medical, Inc., Rancho Santa Margarita, CA, United States), SILS Port (Covidien, Mansfield, MA, United States), TriPort (Olympus Medical Europe Holding GmbH, Germany), TEO proctoscope (Karl Storz, Tuttlingen, Germany), TEM proctoscope (Richard-Wolf, Knittlingen, Germany), Endorec Trocar (Aspide Medical, La Talaudiere, France), and PAT transanal access port (Developia-humV, Santander, Spain). The tumour distance, as measured from the anorectal junction, determined the type of anastomosis (stapled or hand-sewn) before insertion of the transanal access platform. Therefore, the tumour height dictated the type of dissection. Intersphincteric dissection was required using an open approach under direct vision if the tumour was located at the anorectal junction. A Lone Star Retractor (Lone Star Medical Products Inc., Houston, TX, United States) was first inserted. Circumferential sleeve mucosectomy was then performed at the dentate line to safeguard the internal sphincter, and the rectum was closed using a purse-string suture. In particular, the rectal lumen was occluded with a purse string if the distal tumour margin (at least 1 cm below the tumour) allowed for a stapled coloanal anastomosis. Subsequently, the transanal access platform was placed into the anal muscular cuff, and insufflation was initiated with CO_2 to a pressure of 8-15 mmHg using a conventional CO_2 insufflator. The TME plane was identified in a reverse manner, beginning at the top of the puborectal muscle. The posterior TME plane was developed under direct vision using conventional laparoscopic instruments *via* incision of the endopelvic fascia and dissection in front of the presacral fascia to preserve the mesorectal envelope. Anterior dissection was performed in the rectovaginal septum or Denonvilliers' fascia (rectoprostatic plane) as cephalad as possible until the pouch of Douglas could be opened. Lateral dissection involved division of the middle rectal artery and connection of the anterior and posterior planes bilaterally. Only one study reported pure NOTES TaTME[31], whereas TaTME was performed with laparoscopic assistance (Hybrid TaTME) in most studies. Laparoscopy was performed using multiport laparoscopy (3-5 ports)[32,34-40,43,44,46,47] or single-port access[31,33,44,45,48]. The single port was placed in the future ileostomy site. High ligation

Table 2 Operative techniques reported for transanal total mesorectal excision

Author, year	Transanal first	Transanal platform	Pure NOTES	Number of laparoscopic ports	Splenic flexure mobilization	Specimen extraction	Anastomosis	Defunctioning stoma (loop ileostomy)
Zorron, 2010	Yes	TriPort	No	3	Yes	Transanal 7; transabdominal 2	Hand-sewn 1/9, hand-sewn J-pouch 3/9, stapled 5/9	Yes
Dumont, 2012	Yes	GelPOINT Path	No	GelPOINT	Yes	Transabdominal	Hand-sewn	Yes
de Lacy, 2013	Simultaneous	GelPOINT Path	No	3.2 ± 0.8[1] (range, 3-4)	NR	Transanal	Hand-sewn 13/20, stapled 7/20	16/20
Lacy, 2013	Simultaneous	GelPOINT Path	No	4	2/3	Transanal	Stapled	2/3
Rouanet, 2013	Not systematically	TEO proctoscope	No	NR	Yes	Transanal or transabdominal	Hand-sewn straight 12/30, hand-sewn J-pouch 18/30	Yes
Sylla, 2013	Simultaneous	TEO proctoscope	No	4 or 5	Yes	Transanal	Hand-sewn	Yes
Atallah, 2014	No	SILS™ Port or GelPOINT path	No	NR	Yes	NR	Hand-sewn straight 11/20, stapled 4/20	14/20
Chen, 2014	No or simultaneous	GelPOINT Path	No	GelPOINT + 1	4/20	Transanal or transabdominal	Hand-sewn 6/20, stapled 14/20	Loop colostomy 12/20, loop ileostomy 5/20
Chouillard, 2014	Yes	SILS™ Port or GelPOINT path	Yes (10/16)	GelPOINT	NR	NR	Hand-sewn	Loop ileostomy 4/16, permanent ileostomy 1/16, permanent colostomy 1/16
Fernandez-Hevia, 2014	Simultaneous	GelPOINT Path	No	4 or 5	14/37	Transanal 36; transabdominal 1	Hand-sewn 16/37, stapled 21/37	32/37
Kneist, 2014	No	SILS™ Port or GelPOINT path	No	3-5	Yes	Transanal	Hand-sewn J-pouch 2/6, hand-sewn E-E 2/6, hand-sewn S-E 1/6, stapled J-pouch 1/6	Yes
Meng, 2014	Simultaneous	TEM platform	No	5	Yes	Transabdominal	Stapled	NR
Tuech, 2014	Yes	SILS™ Port or GelPOINT path or Endorec®	No	4 or single port in future ileostomy site	NR	Transanal	Hand-sewn J-pouch 4/56, hand-sewn S-E 29/56, hand-sewn straight 13/56	Permanent colostomy 4/56, loop ileostomy 44/56
Velthuis, 2014	5/25	SILS™ Port or GelPOINT path	No	SILS™ Port	Yes	Transanal	Hand-sewn or stapled	Loop ileostomy 19/25, permanent colostomy 6/25
Wolthuis, 2014	Yes	GelPOINT Path	No	3	Yes	Transanal	Hand-sewn	Yes
Atallah, 2015	No	GelPOINT Path	No	NR	Yes	Transabdominal	Hand-sewn 3/4	Loop ileostomy 3/4, permanent ileostomy 1/4
Gomez Ruiz, 2015	No	Transanal access port proctoscope	No	4	Yes	Transanal	Hand-sewn straight 2/5, stapled 3/5	Yes
Knol, 2015	No	GelPOINT Path	No	4 or 5	8/10	Transanal 1; transabdominal 9	Hand-sewn 3/10, stapled 7/10	Yes
Muratore, 2015	Yes	SILS™ Port	No	3	Yes	Transanal	Hand-sewn straight 17/26, hand-sewn J-pouch 8/26	Yes
Prochazka, 2015	Yes	SILS™ Port or GelPOINT path or Endorec®	No	NR	Yes	Transanal	Hand-sewn	Yes

[1]Values reported as the mean ± SD. E-E: End-to-end; NR: Not reported; S-E: Side-to-end.

Table 3 Operative outcomes after transanal total mesorectal excision

Author, year	Median duration of surgery (min)	Median blood loss (mL)	Postoperative morbidity	Median length of stay (d)	Median follow-up (mo)	NOS
Zorron, 2010	311 (range, 200-420)	96 (range, 20-250)	Anastomotic leakage 1/9	7 (range, 4-27)	NR	3
Dumont, 2012	360 (range, 270-460)	175 (range, 50-300)	Anastomotic leakage 1/4	13 (range, 10-21)	4.3 (range, 3-9)	4
de Lacy, 2013	234.7 ± 56[1] (range, 150-325)	45 ± 151 (range, 10-110)	Urinary retention 2/20, POI 1/20, high-output ileostomy 1/20	6.5 ± 3.1[1]	NR	6
Lacy, 2013	143 (range, 125-155)	65 (range, 15-30)	High-output ileostomy 1/3	5 (range, 4-5)	NR	4
Rouanet, 2013	304 (range, 120-432)	NR	POI 2/30, transient urinary dysfunction 2/30	14 (range, 9-25)	21 (range, 10-41)	6
Sylla, 2013	274.6 ± 85.4[1]	166 (range, 80-300)	POI 1/5, transient urinary dysfunction 2/5	5.2 ± 2.6[1]	5.4 ± 2.3[1]	5
Atallah, 2014	243 (range, 140-495)	153 (range 30-500)	SSI 2/20, Pelvic abscess 4/20, POI 4/20, Anastomotic leakage 1/20	4.5 (range, 3-24)	6 (range, 1-24)	6
Chen, 2014	200.8 ± 47.7[1]	68 ± 106[1]	Urinary retention 3/20, pelvic abscess 2/20	8.85 ± 2.5[1]	NR	6
Chouillard, 2014	265 (range, 155-440)	225 (range, 50-600)	SBO 2/14, pelvic abscess 1/16	10 (range, 4-29)	9 (range, 3-29)	6
Fernandez-Hevia, 2014	215 ± 60[1] (range, 120-360)	NR	Anastomotic leakage 2/37, haemorrhage 1/37, urinary retention 1/37, POI 4/37	6 (range, 3-17)	NR	8
Kneist, 2014	NR	NR	NR	NR	NR	3
Meng, 2014	NR	NR	No	NR	NR	2
Tuech, 2014	270 (range, 150-495)	NR	Anastomotic leakage 3/56, pelvic sepsis 3/56, transient urinary dysfunction 5/56	10 (range, 6-21)	29 (range, 18-52)	6
Velthuis, 2014	NR	NR	NR	NR	NR	8
Wolthuis, 2014	148 (range, 85-250)	49 (range, 0-150)	Pelvic haematoma 1/7	9 (range, 3-14)	6 (range, 2-14)	6
Atallah, 2015	376 (range, 40-409)	200 (range, 50-300)	High-output ileostomy 1/20, SSI 1/20	4 (range, 4-5)	9 (range, 6-12)	4
Gomez Ruiz, 2015	375 (range, 270-450)	76 (range, 25-120)	Anastomotic leakage 1/5	6 (range, 5-7)	NR	4
Knol, 2015	235 (range, 150-290)	220 (range, 65-480)	POI 1/10	6 (range, 5-9)	NR	4
Muratore, 2015	241 (range, 150-360)	NR	Urinary retention 1/26	7 (range 3-25)	18 (range, 16-30)	6
Prochazka, 2015	280 (range, 212-375)	200 (range, 40-900)	Anastomotic leakage 2/17, POI 2/17, UTI 1/17, SSI 1/17	9 (range, 6-30)	NR	4

[1]Values reported as the mean ± SD. NR: Not reported; POI: Postoperative ileus; SBO: Small-bowel obstruction; SSI: Surgical site infection; UTI: Urinary tract infection.

of the inferior mesenteric artery and vein was performed. In 12 studies, after full mobilization of the left and sigmoid colon and connection to the TME plane were performed, the specimen was extracted transanally[32,34-36,38,40,41,43-47]. This extraction was only performed if the specimen was not too bulky. Adequate length was obtained *via* mobilization of the splenic flexure. Nine studies described hand-sewn coloanal anastomoses[30,31,33,40-44,46], and only stapled anastomoses were performed in 2 studies[38,39]. Both hand-sewn and stapled coloanal anastomoses were created in another 9 studies[29,32,34-37,45,47,48]. Additionally, a diverting-loop stoma (loop ileostomy or loop colostomy) was created in nearly every case.

Outcomes

The median total surgery duration ranged from 143-375 min (Table 3). This time included the transabdominal and transanal operative times. The operative times were significantly reduced when TaTME was performed using a 2-team approach compared with laparoscopic TME[34]. The reported median blood loss ranged from 45-225 mL. Overall, rendezvous was not possible using laparoscopy in 9 patients, so conversion to laparotomy was performed. The reasons for conversion included small-bowel adhesions (3 patients)[44,46], obesity (2 patients: 1 male and 1 female)[44], a posteriorly fixed tumour (2 patients)[42], a bulky tumour (1 patient)[47], and uncontrolled bleeding from the presacral plane (1 patient)[48]. Seventeen studies also reported postoperative morbidity. The anastomotic leak rate in the included studies was calculated to be 3.8%. There were also 3.4% pelvic abscesses. Other postoperative complications noted in the studies were (prolonged) postoperative ileus (15 patients)[29,32,34,37,41-43], transient urinary dysfunction (9 patients)[42-44], urinary retention (7 patients)[32,34,40,48], surgical site infection (4 patients)[29,30,41], high-output ileostomy (3 patients)[30,32,38], adhesive small-bowel obstruction (2 patients)[31], haemorrhage/pelvic haematoma (2 patients)[34,46], and urinary tract infection (1 patient)[41]. The median length of hospital stay ranged from 4-14 d, and there was no 30-day mortality.

DISCUSSION

This systematic review of TaTME for rectal cancer demonstrated that TaTME is feasible in select patients. The abdominoperineal rectum amputation rate is approximately 21% for low rectal cancer, but most patients are candidates for reconstruction after TME to avoid permanent colostomy[49]. Acceptance of a shorter distal resection margin (1 cm)[50], an increased interval after neoadjuvant chemoradiotherapy[51] and (partial) intersphincteric dissection increase the rates of sphincter-saving surgery in patients with distal rectal cancer[49]. The recent introduction of TaTME suggests that every patient who is selected for sphincter-saving surgery would undergo a minimally invasive approach, without conversion to laparotomy. This review clearly demonstrated that TaTME is currently performed in a non-standardized manner, which reflects surgeons exploring the technical boundaries of ultra-low rectal cancer surgery. Heterogeneity in patient selection and operative techniques leads to differences in surgical, oncological, and functional outcomes, which in turn hinder inter-study comparisons. Operative techniques specifically differed among studies in the present analysis, with use of different numbers of ports, different transanal platforms and different methods of performing TaTME. The procedure can be initiated either transabdominally or transanally, and the extent of dissection from either side can be tailored to each individual patient. The additional use of Airseal technology leads to a stable workspace (pneumopelvis), which avoids any "flapping" of the specimen and facilitates pneumodissection[52].

TaTME has certain advantages over laparoscopic TME, but there are still issues that must be addressed. TaTME is advocated in the case of a narrow male pelvis, so most studies have selected male patients. Only a few studies considered BMI or pelvic anatomy for patient selection. Less than half of the studies included here exclusively selected low (*i.e.*, < 5 cm from the anal verge) rectal tumours for TaTME. TaTME is an attractive alternative to laparoscopic TME because of several benefits, including determination of the distal resection margin, creation of a single stapled anastomosis, and avoidance of abdominal wall incision for specimen retrieval. If TaTME is first initiated in the transanal phase, then the distal resection margin and the level of the future anastomosis can be chosen under direct vision. A significantly longer distal resection margin has been reported using TaTME compared with conventional laparoscopic TME[34]. TaTME also results in better TME specimens[45]. Furthermore, this review demonstrated that a hand-sewn anastomosis was performed in approximately half of the studies, which may reflect the selection of patients with ultra-low rectal cancers. A stapled coloanal anastomosis using the double purse-string technique results in a single stapled anastomosis. This technique may eventually lead to a decreased anastomotic leak rate, but whether this technique improves functional outcomes is not clear. Moreover, TaTME will ultimately be performed as a pure NOTES procedure, which may be its greatest advantage. In this way, the diseased target organ can be reached transanally, so future developments should focus on pure NOTES TaTME. If laparoscopy can be omitted in this setting, then true NOTES may become possible in a consecutive series of patients. Mobilization and extraction of the specimen can presently be performed *via* the anus if the splenic flexure is mobilized using laparoscopic assistance. Therefore, TaTME is a NOSE technique that shares all of the advantages of NOSE. The avoidance of abdominal wall incision that is tailored to specimen and tumour sizes is important because the extraction site carries a morbidity risk. A wound infection rate of 9% has been documented, albeit generally, with only local septic complications in particular[53]. This review demonstrated that specimens were extracted transanally in 12 studies. Differences between transanal NOSE techniques involving laparoscopic TME using the anus as the extraction site and techniques involving transanal TME in the literature must be highlighted. Both procedures are transanal NOSE techniques, but transanal TME is performed in a reverse manner. TaTME may offer several advantages over laparoscopic and open TME, but it also has limitations. One major perioperative complication that is specific to TaTME is urethral injury. For example, Rouanet *et al*[42] described two urethral injuries that were sutured transanally. Moreover, the impact of TaTME on the anal sphincter is not known, and therefore, functional outcomes after TaTME are of interest. Additionally, this technique is in its infancy, so the learning curve is ill defined. Further prospective studies will therefore be required to describe the safety profile of and learning curve for TaTME. However, it is clear that a reverse approach to the mesorectum forces surgeons to recognize new anatomical landmarks and to perform the fundamental steps of TaTME[54,55]. Most studies have only reported short-term outcomes, which reflects the novelty of TaTME. Whether this new approach exhibits similar oncological outcomes in terms of local recurrence, disease-free survival and cancer-specific survival will require further study in prospective trials that compare TaTME with conventional laparoscopic or robotic TME over substantial follow-up periods.

In conclusion, this state-of-the-art narrative review presents recent developments in the TaTME technique. The technical possibilities and shortcomings of TaTME are also described. A new era of further optimization of distal rectal cancer surgery has dawned: standardization of surgical technique and implementation in daily practice are the steps required to take TaTME to the next level. In addition, large prospective studies should focus on safety and functional and oncological outcomes, and the presumed benefits of TaTME must be studied in controlled trials.

COMMENTS

Background

Laparoscopic ultra-low total mesorectal excision remains cumbersome because it is technically difficult to mobilize the most distal part of the rectum, especially in obese male patients with a narrow pelvis. Transanal total mesorectal excision (TaTME) may resolve all issues related to pelvic exposure, cross-stapling, and specimen quality. TaTME seems to be an attractive new methodology in rectal cancer surgery, but this approach has not been extensively investigated.

Research frontiers

The aims of this systematic review were to describe the role of TaTME in minimally invasive rectal cancer surgery, to examine the differences in patient selection and in reported surgical techniques and their impacts on postoperative outcomes and to discuss the future of TaTME.

Innovations and breakthroughs

A total of 20 studies with 323 patients were included. Most studies were single-arm prospective studies with fewer than 100 patients. Multiple transanal access platforms were used, and the laparoscopic approach was either multi- or single port. The procedure was initiated transanally or transabdominally. If a simultaneous approach with 2 operating surgeons was chosen, the operative time was significantly reduced.

Applications

TaTME was also associated with better TME specimens and a longer distal resection margin. TaTME is thus feasible in expert hands, but the learning curve and safety profile are not well defined.

Peer-review

Well written paper, well conducted review.

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P- Reviewer: Piccinni G **S- Editor**: Yu J **L- Editor**: A
E- Editor: Ma S

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i44.12696

World J Gastroenterol 2015 November 28; 21(44): 12696-12708
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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SYSTEMATIC REVIEWS

Probe based confocal laser endomicroscopy of the pancreatobiliary system

Majid A Almadi, Helmut Neumann

Majid A Almadi, Division of Gastroenterology, Department of Medicine, King Saud University Medical City, King Saud University, Riyadh 11461, Saudi Arabia

Majid A Almadi, Division of Gastroenterology, The McGill University Health Center, Montreal General Hospital, McGill University, Montreal H3G 1A4, Canada

Helmut Neumann, Department of Medicine I, University of Erlangen-Nuremberg, Ulmenweg 18, 91054 Erlangen, Germany

Author contributions: Almadi MA contributed to the concept of the manuscript, study design and acquisition of data, interpretation of data, drafted the manuscript, final revisions and made the final approval; and Neumann H contributed to concept of the manuscript, study design, interpretation of data, made revisions and made the final approval.

Supported by Deanship of Scientific Research at King Saud University through the Research Group Project number RGP-VPP-279.

Conflict-of-interest statement: Majid A Almadi: "No relevant conflicts of interest exist"; Helmut Neumann: Consultant for Pentax, Mauna Kea Technologies, SpectraScience; Funding from Pentax, SpectraScience, Olympus, Siemens, Smart Medical, Fujifilm.

Data sharing statement: No additional data are available.

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Correspondence to: Majid A Almadi, MBBS, FRCPC, MSc, Assistant Professor, Division of Gastroenterology, Department of Medicine, King Saud University Medical City, King Saud University, PO Box 2925 (59), Riyadh 11461, Saudi Arabia. maalmadi@ksu.edu.sa

Telephone: +966-11-4679167
Fax: +966-11-4671217

Received: March 28, 2015
Peer-review started: March 29, 2015
First decision: June 19, 2015
Revised: July 8, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: November 28, 2015

Abstract

AIM: To review applications of confocal laser endomicroscopy (CLE) in pancreatobiliary lesions and studies that assessed training and interpretation of images.

METHODS: A computerized literature search was performed using OVID MEDLINE, EMBASE, Cochrane library, and the ISI Web of Knowledge from 1980 to October 2014. We also searched abstracts from major meetings that included the Digestive Disease Week, Canadian Digestive Disease Week and the United European Gastroenterology Week using a combination of controlled vocabulary and text words related to pCLE, confocal, endomicroscopy, probe-based confocal laser endomicroscopy, and bile duct to identify reports of trials. In addition, recursive searches and cross-referencing was performed, and manual searches of articles identified after the initial search was also completed. We included fully published articles and those in abstract form. Given the relatively recent introduction of CLE we included randomized trials and cohort studies.

RESULTS: In the evaluation of indeterminate pancreatobiliary strictures CLE with ERCP compared to ERCP alone can increase the detection of cancerous strictures with a sensitivity of (98% *vs* 45%) and

has a negative predictive value (97% *vs* 69%), but decreased the specificity (67% *vs* 100%) and the positive predictive value (71% *vs* 100%) when compared to index pathology. Modifications in the classification systems in indeterminate biliary strictures have increased the specificity of pCLE from 67% to 73%. In pancreatic cystic lesions there is a need to develop similar systems to interpret and characterize lesions based on CLE images obtained. The presence of superficial vascular network predicts serous cystadenomas accurately. Also training in acquiring and interpretation of images is feasible in those without any prior knowledge in CLE in a relatively simple manner and computer-aided diagnosis software is a promising innovation.

CONCLUSION: The role of pCLE in the evaluation of pancreatobiliary disorders might be better suited for those with an intermediate and low probability.

Key words: Probe based confocal laser endomicroscopy; Confocal; Endomicroscopy; Probe-based confocal laser endomicroscopy; Bile duct; Pancreatobiliary; Stricture; Endoscopic retrograde cholangiopancreatography; Cholangioscopy; Endoscopic ultrasound; Systematic review

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Core tip: Current endoscopic evaluation of biliary and pancreatic duct strictures and pancreatic lesions using standard methods are suboptimal. Confocal laser endomicroscopy (CLE) is starting to establish a role in such cases with multiple studies suggesting that image interpretation is not as difficult as initially perceived. Furthermore the diagnostic discriminatory value of images obtained by CLE could decrease the need for repeated and invasive investigations, as the case in the serous cystadenomas. Although classification systems have been developed and improved with regards to the performance of CLE in biliary strictures they are still evolving for pancreatic lesions and require further validation.

Almadi MA, Neumann H. Probe based confocal laser endomicroscopy of the pancreatobiliary system. *World J Gastroenterol* 2015; 21(44): 12696-12708 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12696.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12696

INTRODUCTION

Despite the technological developments in the field of imaging as well as available options for endoscopic evaluation whether through endoscopic retrograde cholangiopancreatography (ERCP), cholangioscopy, or endoscopic ultrasound (EUS) the diagnostic yield of these tests is still suboptimal with regards to pancreatobiliary disorders, mainly biliary and pancreatic duct strictures as well as pancreatic cystic or solid lesions. The applications of confocal laser endomicroscopy (CLE) have expanded beyond luminal applications to direct tissue imaging including the pancreas[1] and the liver[2]. Thus, CLE has permitted real time *in-vivo* histological evaluation of areas of suspected neoplasia. With this new technology it has become possible to detect neoplasia and subsequently acquiring targeted biopsies as well as the confirmation of non-diseased tissue and the decreased need of random biopsies[3].

In CLE a low-power laser light is focused on a single point in a microscopic field of view. The light emanating from that point is focused through a pinhole to a detector and thus the point of illumination and the pinhole are focused onto the same point and are said to be "confocal" with each other[4], this process decreases the effect of scattered light thus permitting a higher spatial resolution. The beam focused spot traverses a line rapidly from left to right, and is swept top to bottom that would cover the area of interest and then the detected signal is digitized, resulting in the construction of a two-dimensional grey-scale image (Supplement figure 1).

As the experience with CLE is relatively new we sought to systematically review the literature for the available evidence with regards to the benefits of CLE in patients with pancreatobiliary disorders not overlooking abstracts at major congresses.

MATERIALS AND METHODS

A computerized literature search was performed using OVID MEDLINE, EMBASE, Cochrane library, and the ISI Web of Knowledge from 1980 to October 2014. We also searched abstracts from major meetings that included the Digestive Disease Week, Canadian Digestive Disease Week and the United European Gastroenterology Week. We used a combination of controlled vocabulary and text words related to pCLE, confocal, endomicroscopy, probe-based confocal laser endomicroscopy, and bile duct to identify reports of trials (Appendix 1). In addition, recursive searches and cross-referencing were performed, and manual searches of articles identified after the initial search was also completed.

Study selection

We included all clinical studies, both fully published and those to date having appeared only in abstract form that assessed the use of probe based CLE (pCLE) whether it was used through a needle (nCLE), for pancreatic lesions, or through a cholangioscope or catheter for the evaluation of pancreatobiliary disorders. We included all adult human studies published in any language.

Table 1 Interpretation of findings on confocal endomicrosopy and findings on confocal laser endomicroscopy for specific pancreatic lesions

Findings on CLE	Interpretation
Normal	
Reticular pattern seen in normal tissue and smaller than blood vessels[6]	Lymphatics
Thick white bands[52]	Angiogenesis
Reticular arrangement of dark-gray bands on a light-gray background and normal vessels (thin and regular) and no visible glands[9]	Normal common bile duct chorion
Black clumps[69]	Areas of decreased fluorescein uptake
Large white streaks/bands[69]	Dilated, tortuous blood vessels
Loss of mucosal architecture[6,69]	Fibrosis
Multiple thin white bands[15]	Vascular congestion
Thin dark bands forming a reticular pattern (diameter < 20 μm)	Submucosal collagen network
Thin white bands (diameter < 20 μm)	Small caliber blood vessels
Light grey background	Lymphatic sinuses
Abnormal	
Thick collagen bundles	Desmoplasia (growth of fibrous and connective tissue)
Thick white bands (diameter > 20 μm)	Malignant bile duct stricture
Multiple white bands	Inflammatory bile duct stricture
Dark clumps and epithelium	Malignancy
Type of pancreatic lesion	Characteristics on nCLE
Exocrine adenocarcinomas	Dark cells aggregates with pseudo-glandular aspects Straight hyperdense elements more or less thick corresponding to tumoral fibrosis
Tumors with acini cells and neuroendocrine tumors	Very dense network of small vessels on a dark background
Serous cystadenoma	Highly dense and dynamic network of blood capillaries, present in the superficial layer of the cyst wall, superficial vascular network, (pathognomonic)
Intraductal papillary mucinous neoplasm	Papillae characterized by two epithelial borders surrounding a bright vascular flow
Mucinous cystadenoma neoplasm	An epithelial border lined the cyst wall, with or without deep blood vessels, and without papillary organization

CLE: Confocal laser endomicroscopy; nCLE: Needle CLE.

RESULTS

Contrast agent used in pancreatobiliary CLE

To enhance the image quality of CLE certain contrast agents are used. Intravenous fluorescein sodium in a concentration of 10% is the most widely used contrast agent in CLE, a portion of it is bound to albumin while the remainder diffuses into the capillaries and causes enhancement of the extracellular matrix of tissue[5]. The use of fluorescein has been found safe with only mild adverse events in 1.4% of 2272 procedures performed[3], these included nausea/vomiting, transient hypotension without shock, injection site erythema, diffuse rash and mild epigastric pain. Fluorescein is usually administered about 2 to 3 min prior to acquiring images[6].

Technical aspects

Confocal miniprobes used in pancreatobiliary disorders: The CholangioFlex miniprobe (Mauna Kea Technologies, Paris, France) is usually used for confocal imaging of the pancreatobiliarysystem. The probe requires a working channel of at least 1.2 mm and has a working length of 4 m. Lateral resolution of the probe is 3.5 μm with a field of view of 325 μm.

For cystic or mass lesions, the nCLE system (Mauna Kea Technologies, Paris, France) is used. The miniprobe is passed through a 19-gauge needle and has a lateral resolution of 3.5 μm and a field of view of 300 μm.

Interpretation of the findings of CLE

The initial evaluation of pancreatic cells in *ex-vivo* animal studies suggested that microvessel structures and connective tissue structure were better visualized by CLE when compared to conventional histology[7] and CLE images have also been correlated very well with histology in animal models[8]. The findings on CLE and there interpretation are displayed in (Table 1) (Figure 1), of note during the imaging of the biliary system due to the depth of penetration of the CLE probe the normal mucosa of the CBD, that is very thin, cannot be seen on CLE images that are obtained[9]. One of the key features of neoplastic lesions is disorganized angiogenesis[10].

Classification systems

The Miami classification was an attempt to identify as well as standardize the interpretation of finding on pCLE of the biliary system in cases on indeterminate biliary strictures[11,12]. The highest sensitivity was found when there was the combination of thick white bands or dark clumps or epithelial structures, with a sensitivity of 94% and specificity of 46%[11]. While

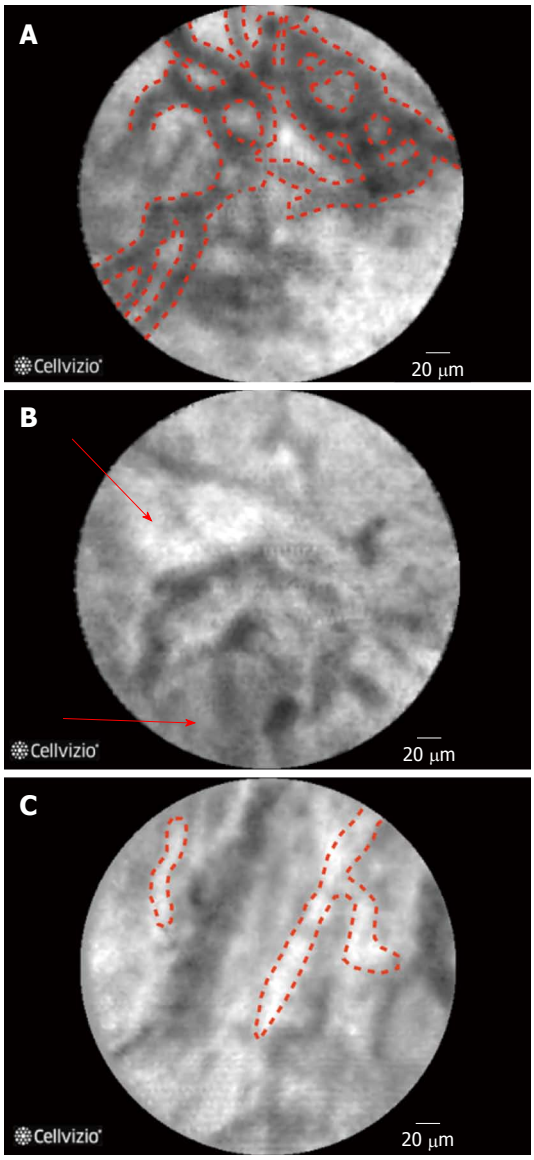

Figure 1 Confocal laser endomicroscopy images for a normal bile duct. A: Reticular network of thin dark branching bands (< 20 μm); B: Light grey background; C: Normal blood vessels (< 20 μm).

the highest specificity was found when there was a combination of white bands or thick white bands or fluorescein leakage or dark clumps, with a sensitivity of 61% and a specificity of 100%[11].

The interobserver agreement on the findings of pCLE was found to be poor to fair when evaluating videos sent to 6 observers in 5 centers even when the Miami classification was used to standardize image interpretation[13].

A refinement of the Miami classification[12,14], was recently made and was coined the Paris classification[15]. The aim of the Paris classification was to decrease the number of false positive results when evaluating indeterminate strictures of the biliary system as in inflammatory strictures. Sixty cases from a prospective registry were reviewed and four criteria for benign inflammatory strictures were described: vascular congestion, dark granular patterns with scales, increased inter-glandular space, and thickened reticular structure[15]. In a validation study for the Paris classification it was found to increase the specificity to 73% compared to 67% when using the Miami criteria[16], a similar finding was obtained in a second study[17].

Giovannini *et al*[18] attempted to identify CLE findings for EUS nCLE in patients in pancreatic lesions whether they were cystic or solid as well as lymph nodes in the celiac area and mediastinum. Although the number of cases in that series was small (11 cases in total), nonetheless the authors found that in benign intraductal papillary mucinous neoplasms (IPMNs) there were finger-like projections corresponding to the villous changes seen in IPMN (Figure 2) while in pancreatic adenocarcinomas, there was vascular leakage with irregular vessels and leakage of fluorescein into the tumor as well as large dark clumps that corresponds to humps of malignant cells[18]. The *in vivo* nCLE study in the pancreas with endosonography of cystic tumors (INSPECT) group had also attempted to develop image criteria and a classification of nCLE findings in pancreatic cysts[19]. All these proposed criteria will require further validation in future studies.

Learning the technique and interpretation of images

In one recent study by Meining *et al*[20] the investigators that performed the procedures had no prior experience with the use or interpretation of pCLE, except for one. Each endoscopist had to complete a standard training module of 20 pCLE videos and then had to complete a review of another 20 pCLE video cases and provide a correct diagnosis of benign versus malignant in each video with a minimum score of 90%[20].

Furthermore, when comparing the first 45 cases of the same cohort to the remainder of cases there was an increase in the specificity of the diagnosis reached by the endoscopists from 55% to 71%, but this did not reach statistical significance[20]. Ease of obtaining pCLE video sequences and image interpretation slightly improved but the ease of tissue sampling was the same[20]. Although these results are encouraging it should be noted that the endoscopists were not blinded to the pre-procedure evaluation and these results might be biased but at the same time they do represent real-life experience.

A study involving gastroenterology fellows with no prior experience with pCLE underwent training with a set of ten video images that were repeated till the trainees were comfortable then they were asked to interpret 20 videos per week over three weeks, at the last session the accuracy by all beginners was 83% with an interobserver agreement of 0.63 which is substantial[21]. This study is encouraging with regards to the interpretation of images obtained but does not address the point of the technical skills required for obtaining these images. Another study

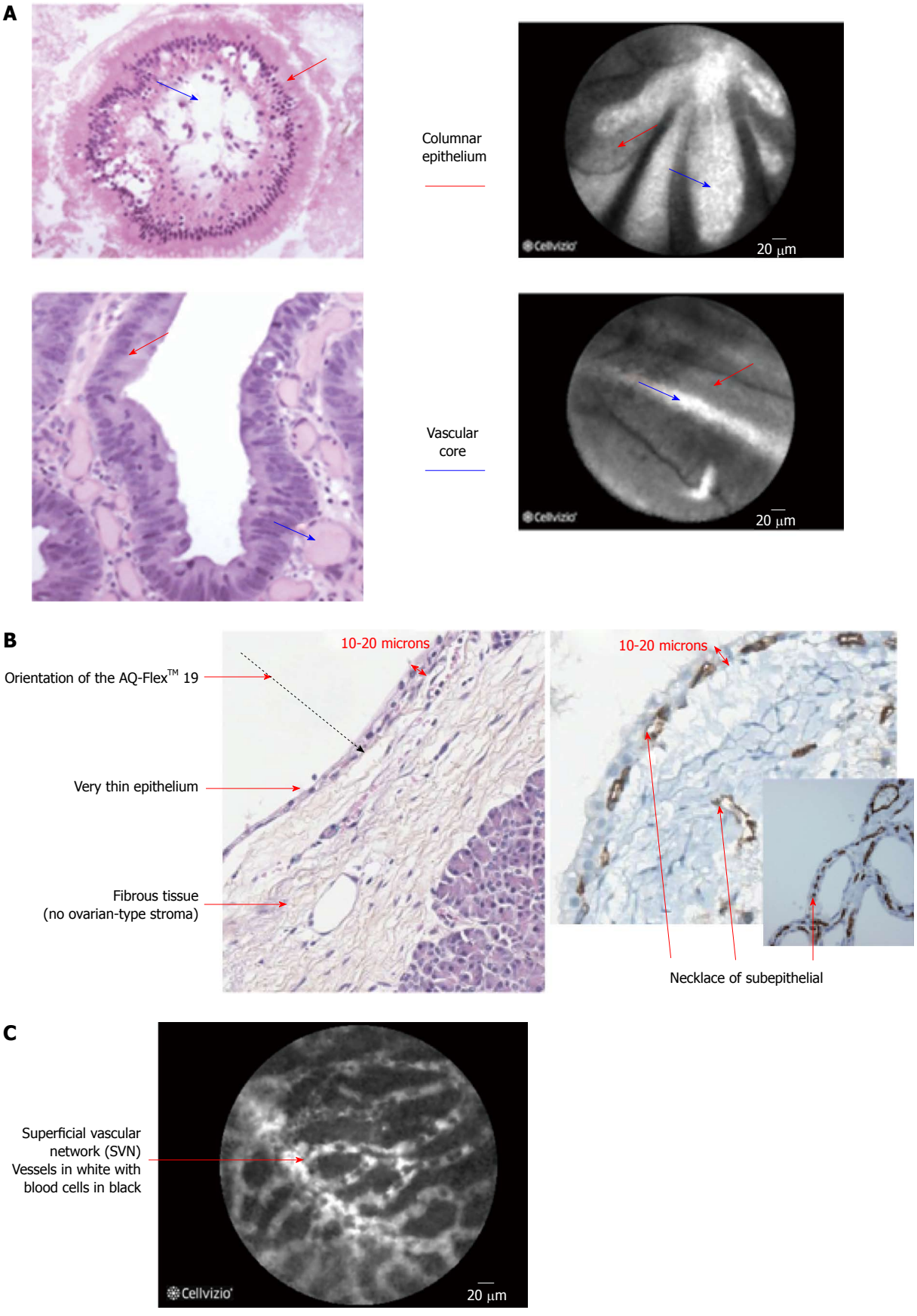

Figure 2 Wall of the intraductal papillary mucinous neoplasms is composed of villous structures consisting of columnar epithelium and a vascular core (A); Histological specimen of a serous cystadenoma that is composed of a thin epithelium lining and underlying fibrous tissue and is characterized by the presence of a necklace of subepithelial vessels all around the cyst (B); Confocal laser endomicroscopy image of a serous cystadenoma (C).

involving five practicing gastroenterologists and the majority with less than 10 cases of experience with pCLE demonstrated that when receiving formal training by an expert (experience with more than 50 procedures) with educational videos pre- and post-training improved the diagnostic accuracy as well as the interobserver agreement[22].

A computer-aided diagnosis software (Smart Atlas; Mauna Kea Technologies, Paris, France) that assist endoscopists interpreting pCLE sequences and automatically discriminates between benign and malignant strictures[23] as well as cystic lesions of the pancreas[24]. Preliminary results of the performance characteristics of this software are encouraging and the results of prospective trials evaluating the new technique are highly anticipated.

Application in different organs and diseases

nCLE of solid and cystic pancreatic lesions: A number of studies reported the use of nCLE *in-vivo* for pancreatic lesions[25-27]. A feasibility study on the use of nCLE through a 19-gauge needle inserted into pancreatic lesions, mainly cysts, was technically successful in the majority of the cases (17/18 cases) and the image quality was good to very good in 10/18 cases. Two patients in the study cohort developed pancreatitis[1]. To overcome this an investigational prototype pCLE (AQFlex) has been developed that is compatible with a 19-gauge FNA needle The probe was used through a transgastric as well as a transduodenal approach for a total of 66 patients with cystic lesions in the pancreas[28]. In this series the 2 patients had pancreatitis while one developed transient abdominal pain, and three developed intracystic bleeding without need for any intervention[28]. Also in the DETECT study the use of nCLE alone had a sensitivity of 75% in differentiating mucinous from non-mucinous cysts while when combined with cystoscopy it had a sensitivity of 100%[29]. Although the procedure is safe and feasible, standard criteria for interpretation of obtained images are still to be established[27], some characteristics of pancreatic lesions are displayed in Table 1. Preliminary data from the Clinical evaluation Of nCLE in The lymph nodes Along with masses and Cystic Tumors of the pancreas (CONTACT) demonstrated that the normal pancreatic tissue had an image resembling "coffee beans", histologically corresponding to acini. Exocrine adenocarcinomas had two signs on nCLE; (1) dark cells aggregates with pseudo-glandular aspects; and (2) straight hyperdense elements more or less thick corresponding to tumoral fibrosis. Both these signs were absent in tumors with acinarcells and neuroendocrine tumors, instead these tumors demonstrated a very dense network of small vessels on a dark background[30]. The same study described findings in cystic lesions of the pancreas[31,32]. One particular finding is that serous cystadenomas have a superficial vascular network, which can be highlighted by nCLE (Figure 2). The specificity and positive predictive value (PPV) of this sign was found in a preliminary series to be 100%. This is of importance as its presence could avoid unnecessary surgery[33,34].

Pancreatic duct strictures

The first case report of the use of pCLE in the pancreatic duct was by Meining *et al*[35] in 2009. Evaluation of pancreatic duct strictures involves insertion of the pCLE through a cholangioscope[35]. A case series of 5 patients who had pancreatic duct strictures underwent pCLE during ERCP and the findings were compared to cytology and when available histology. pCLE was able to identify main duct intraductal papillary mucinous neoplasm with severe dysplasia and adenocarcinoma as well as benign strictures accurately[36]. A second series similarly identified IPMN with dysplasia but in this series two pancreatic duct strictures had positive Miami classification criteria although on histology they were proven to be inflammatory[37]. Another case series of 18 patients with indeterminate pancreatic duct strictures demonstrate almost perfect agreement between cyto/histopathology and pCLE (kappa coefficient = 0.8, $P \leqslant 0.001$)[38].

Molecular imaging of the pancreas

In a pilot study by Nakai *et al*[39] *in vivo* detection of epidermal growth factor receptor (EGF-R) and survivin were possible using a EUS-guided fine needle imaging technique that incorporated nCLE after injection of FITC-labeled specific antibodies against EGF-R and survivin. The EGF-R antibodies were localized to ductal-lining cells and many acinar cells while survivin was confined to acinar cells[39].

Ampullary lesions

Bakhru *et al*[40] assessed the interobserver agreement for five variables for 12 pCLE videos these variables were the presence of an epithelial outer border with irregular thickness, dark epithelium without discernable individual cells, heterogeneously distributed elongated crypts, reduced number of goblet cells, neovascularization, and the final diagnosis. The study included 6 gastroenterologists from five centers and the interobserver agreement was poor to slight for all variables except for the presence of an epithelial outer border with irregular thickness that was fair[40]. The results did not differ much when the gastroenterologists were stratified by their level of experience[40].

Biliary strictures

The evaluation of biliary strictures, whether in the context of primary sclerosing cholangitis (PSC) or not, can be a challenge for clinicians, and the current approaches suffer from a low sensitivity. The application of pCLE for the differentiation between

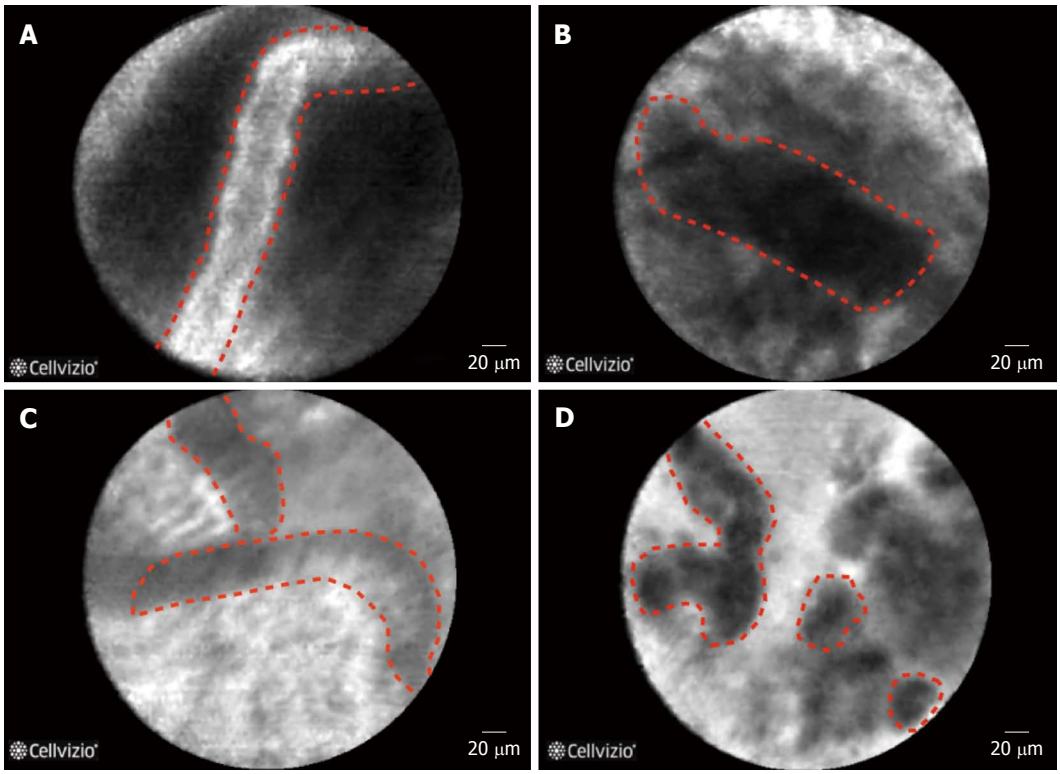

Figure 3 Features of malignant bile duct strictures on confocal laser endomicroscopy. A: Thick white bands (> 20 μm); B: Thick dark bands (> 40 μm); C: Epithelium; D: Dark clumps.

neoplastic and non-neoplastic strictures have yielded very promising results[41,42] (Figure 3). The advantage of pCLE is its higher sensitivity in the detection of neoplastic lesions and an earlier detection of disease at a potentially resectable stage[15]. Furthermore, the more confident the diagnosis the lesser the need for repeated investigations and thus reducing the costs as well as morbidity associated with repeated manipulation of the biliary system[15]. The performance characteristics of CLE in pancreatobiliary strictures are displayed in Table 2 as well as in specific organs in supplement Table 1.

Different probes have been used for pCLE of the biliary system, GastroFlex (UHD; Mauna Kea Technologies, Paris, France) miniprobe[43] and CholangioFlex probes. The drawback of the GastroFlex probe is its larger diameter and thus it might be difficult to negotiate through strictures although a case series demonstrated successful insertion of this probe in 10/11 patients with various indications for pCLE with one patient developing pancreatitis after the procedure. Although the GastroFlex probe has a higher lateral resolution when compared to the CholangioFlex probe (1 μm *vs* 3.5 μm)[44] there is no clear advantage of the use of one over the other[45].

The pCLE probe has been delivered successfully through various cholangioscopes as well as catheters[20,46]. When using a cholangioscope pCLE had a sensitivity of 96% (95%CI: 84% to 100%) and a specificity of 76% (95%CI: 53% to 91%), while when using a catheter the sensitivity was 100% (95%CI: 83% to 100%) and the specificity was 62% (95%CI: 45% to 78%)[46] but there was no statistical difference in the accuracy between these delivery techniques[46] but the operators confidence about the diagnosis was much higher when using cholangioscopy when compared to a catheter based approach for pCLE of biliary strictures (43.2% *vs* 9.8%, respectively)[46]. In a randomized trial for comparison between catheter-guided (Fluoroscopy only) pCLE and cholangioscopy-guided pCLE the accuracy of cholangioscopy-guided pCLE was 82% compared to 78% for catheter-guided pCLE. Of note, the sample size of the study was small[47].

The addition of pCLE with ERCP in the evaluation of indeterminate pancreatobilary strictures can increase the detection of cancerous strictures[9,48] with a sensitivity of (98% *vs* 45%) and a negative predictive value (NPV) (97% *vs* 69%), although it decreased the specificity (67% *vs* 100%) and the PPV (71% *vs* 100%) when compared to index pathology[48]. Similar findings were found in other studies[6,37,42,49-54].

Although conventionally the use of pCLE for the evaluation of biliary strictures is through a side viewing duodenoscope, a case series showed pCLE through direct peroral cholangioscopy in 22 out of 24 patients with biliary strictures[54]. An interesting approach for this case series is that they classified patients based on the pre pCLE evaluation for the probability of a malignant etiology for biliary stricture into a range

Table 2 Performance of characteristics of findings on confocal laser endomicroscopy for pancreatobiliary strictures

Criterion	Author and the description of the examined area	Sensitivity	Specificity	PPV	NPV
	Meining *et al*[41,53]				
Malignant biliary strictures	Loss of reticular pattern of epithelial bands of less than 20 μm Irregular epithelial lining, villi, or gland-like structures Tortuous, dilated, and saccular vessels with inconsistent branching Presence of "black areas" of more than 60 to 80 μm (focally decreased uptake of fluorescein)	83%	88%	NR	NR
	Meining *et al*[20]				
Suggestive of malignancy	Thick, dark bands (> 40 μm) Thick, white bands (> 20 μm) Dark clumps Epithelium visualized (villi, glands) Fluorescein leakage	98%	67%	71%	97%
Suggestive of benign strictures	Thin, dark (branching) bands Thin, white bands				
	Meining *et al*[14]				
Normal bile ducts	Reticular network of thin dark branching bands (< 20 μm) Light gray background Vessels < 20 μm	97%	33%	80%	80%
Malignant biliary strictures	Thick white bands (> 20 μm) Thick dark bands (> 40 μm), Dark clumps Epithelial structures Normal vessels (thin and regular) No visible glands				
	Giovannini *et al*[18]				
Benign IPMN	Finger-like projections corresponding to villous changes of intestinal IPMN type	NR	NR	NR	NR
Pancreatic adenocarcinoma	Vascular leakage with irregular vessels with leakage of fluorescein into the tumor Large dark clumps which correspond to humps of malignant cells				
Benign Lymph nodes	Diffuse small cells into a homogeneous stroma with normal vascularization				
Malignant lymph nodes	Glandular structures with dark cells, large dark clumps and neo-vascularization with huge leakage of fluorescein				
	Filoche *et al*[56]				
Benign inflammatory strictures of the common bile duct	Vascular congestion but still regular Roughness aspect Increased inter-glandular space Thickened reticular structure	NR	NR	NR	NR
	Caillol *et al*[15]				
Inflammatory stenosis	Multiple thin white bands (Vascular congestion) Roughness aspect Increased inter-glandular space Thickened reticular structure	96.3%	63.6%	68.4%	95.5%

IPMNs: Intraductal papillary mucinous neoplasms; NR: Not reported; PPV: Positive predictive value; NPV: Negative predictive value.

from very unlikely to certainly based on the clinical evaluation as well as imaging[54], pCLE was found to be complementary to peroral cholangioscopy and ERCP in cases where a malignant etiology was suspected and did not effect the management decision but it might be sufficient for confirmation of a malignant etiology when tissue acquisition is not required[54].

The use of pCLE in hilar strictures has also been proven to be of use in a series of 19 patients with the correct identification of all cases with neoplasia but one false positive case was reported[55].

Filoche *et al*[56] identified four characteristics on biliary pCLE that were associated with benign inflammatory strictures; vascular congestion, roughness aspect, increased inter-glandular space and thickened reticular structure (Figure 4). In this study the authors sought to explain the false positive cases in 60 cases that were enrolled in a registry and found that pCLE diagnosis was either influenced by the ERCP impression or the presence of less than 3 malignant Miami classification criteria[56].

Giovannini *et al*[57] evaluated the effect of biliary stenting in 54 patients with indeterminate biliary stenosis and found that biliary stenting decreased the accuracy of pCLE when using the Miami criteria, similar findings were replicated where a decrease in the sensitivity from 88% to 75% and specificity from 83% to 71% was found in those who had cholangitis

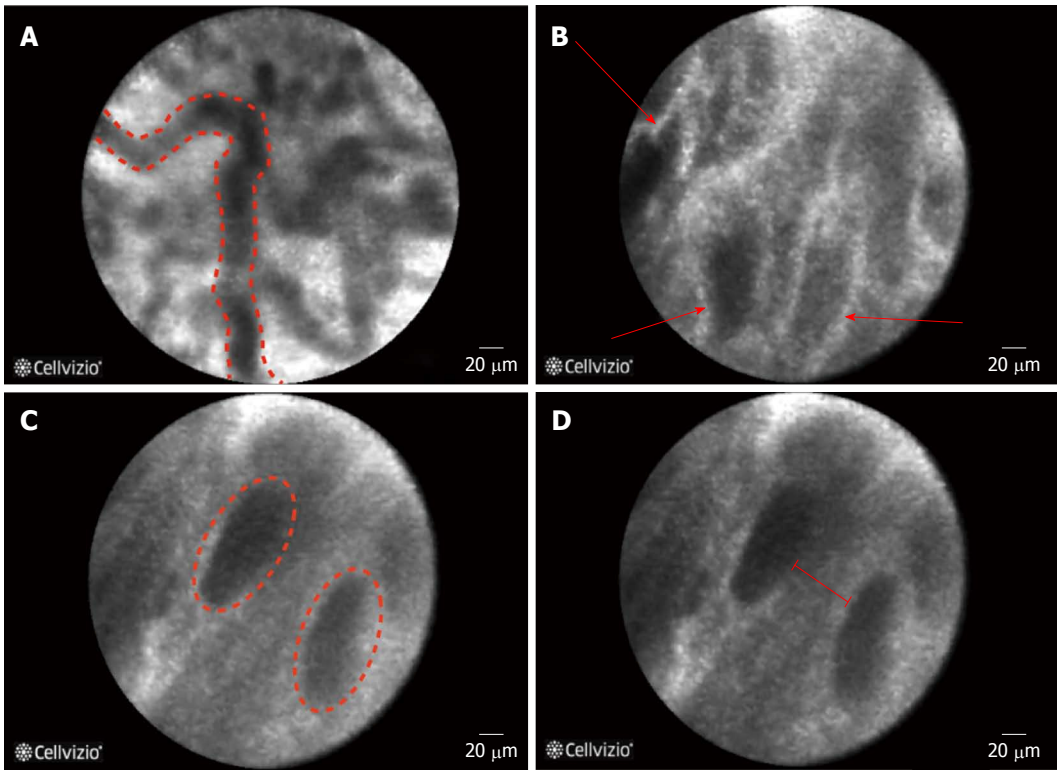

Figure 4 Features of inflammatory bile duct strictures on confocal laser endomicroscopy. A: Thickened reticular structures; B: Multiple white bands; C: Dark granular pattern in scales; D: Increased spaces between scales.

or a stent inserted prior to pCLE imaging[58]. Although this requires validation in other series but it might be prudent to perform pCLE prior to biliary stenting in cases with biliary strictures of unknown etiology. Also, of note, in the study by Caillol *et al*[15] they noted that stricture dilation could induce fluorescein leakage thus giving the impression of a malignant stricture while it was subsequently found to be benign.

A recent consensus report by 16 physicians validated seven statements with regards to the use of pCLE in biliary strictures; (1) CLE can be used to evaluate biliary strictures, and the probe can be delivered via a catheter or a cholangioscope; (2) CLE is more accurate than ERCP with brush cytology and/or forceps biopsy in determining malignant or benign strictures, using established criteria; (3) The accuracy of CLE in indeterminate biliary strictures may be decreased by prior presence of plastic stent; (4) The NPV of CLE is very high; (5) The use of CLE can assist clinical decision-making such as excluding malignancy; (6) CLE should be cited as a valuable tool for an increased diagnostic yield in official guidelines; and (7) The «black bands» that can be seen in pCLE images have been shown to be collagen fibrils that predictably increase in pathologic tissue[59].

A preliminary analysis of the multicenter multinational FOCUS trial demonstrated that the clinical impression of physicians and pCLE during workup of biliary strictures outperform tissue sampling where the combination of brush cytology and biopsy would have missed 5 malignant strictures out of 36 patients[60]. While the addition of histology/cytology to pCLE resulted in a marginal increase in sensitivity (from 89% to 93%) but did not change the specificity (79%) compared to the addition of pCLE alone[61].

Primary sclerosing cholangitis

The work up of patients with PSC who develop strictures can be a challenge. The management will depend on the etiology of these strictures whether they are neoplastic or just inflammatory. In a series of 15 patients with 19 strictures[62], both extra and intrahepatic, were evaluated by ERCP and pCLE. Due to the inflammatory nature of PSC the authors used a scoring system based on the Miami classification when there were 2 of 5 malignant criteria the lesion was classified as "suspicious" and 1 criterion as "reactive" and the finding of a reticular pattern was deemed as "benign"[62] the finding on pCLE were compared to ERCP, cholangioscopy, histology/cytology, liver explants, fluorescence in-situ hybridization (FISH), or 12 mo of follow-up. Visualization was successful in 95% of the procedures, pCLE was found to have a sensitivity of 100% (95%CI: 40% to 100%), specificity of 50% (95%CI: 9% to 90%), PPV of 67% (95%CI: 24.5% to 94%) and a NPV of 100% (95%CI: 20% to 100%)[63]. The authors suggested the high negative predictive value of pCLE could guide in the interval of surveillance in patients with dominant biliary strictures[62].

DISCUSSION

The role of pCLE in the evaluation of pancreatobiliary disorders might not be of high value in cases where the pretest probability of a malignant etiology is high but might be better suited for those with an intermediate and low probability. Furthermore it might guide tissue sampling to increase the yield when evaluating pancreatobiliary disorders. It does not seem, at least for now, that pCLE will eliminate the need for tissue acquisition and a histological diagnosis but it does supplement the evaluation of these patients. The field is evolving and as more centers acquire this technology and studies are conducted[50,63-68] the definite role of pCLE will be better defined.

The diagnostic criteria might need to be refined to be applicable to clinical practices outside centers of expert endoscopists and might need to be tailored to specific underlying disease like PSC and patients with chronic pancreatitis where discriminating normal from abnormal histology might be more challenging.

ACKNOWLEDGMENTS

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project number RGP-VPP-279. Also we would like to thank Mauna Kea Technologies for providing images from their e-learning website: www.cellvizio.net.

COMMENTS

Background

Confocal laser endomicroscopy (CLE) is used for real time *in-vivo* histological evaluation of areas of suspected neoplasia allowing targeted biopsies. The applications of CLE in the pancreatobiliary system are relatively new and the added value of this image enhancing technology is evolving.

Research frontiers

When evaluating indeterminate strictures of the biliary system, a refinement of the Miami classification (the Paris classification) has decreased the number of false positive results. While in the case of pancreatic lesions a classification system is still lacking but some of the imaging features appear to be very specific and might alter the management of how the authors survey patients with pancreatic cystic lesions. Furthermore, computer-aided diagnosis softwares that assist in interpreting CLE sequences are a promising development.

Innovations and breakthroughs

In this systematic review the authors attempted to review the most recent literature which describes potential applications of CLE in the area of diagnostic evaluation of lesions that are particularly difficult to reach using our current imaging and endoscopic armamentarium. There has been a great deal of advancements in the field but there still remains more to be achieved specifically in the fields of training as well as dissemination of this technology.

Applications

Probe based CLE is useful in cases of indeterminate biliary strictures where the pretest probability of malignant etiology are intermediate or low while it might not be the case when the pretest probability is high. As for needle based CLE in solid as well as cystic pancreatic lesions the role is still evolving but is very promising.

Peer-review

Immediate endoscopic optical histology without samples in accessible tissue surfaces is a new tool providing important advances in lesion recognition. CLE is used in assessing histological structure without samples in endoscopy using specially designed scopes but can also be used as thin probes which are able to reach and assess tissue structure in ducts, cysts and accessible tumors. The present paper is an extensive and complete, up-to-date review of the literature available since 2001; nevertheless most the papers were published from 2007 to 2015. This review describes some important aspects of CLE image evaluation, including specificity, sensitivity and positive predictive values, and also the learning curves in image interpretation which all are important for introducing a new device in clinical practice. The paper is long but is a well-designed systematic review and includes one part devoted to technical aspects, another on image interpretation and a third on its application in different localizations.

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P- Reviewer: Aktas S, Bordas JM **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12709

World J Gastroenterol 2015 November 28; 21(44): 12709-12712
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Perforated duodenal ulcer: An unusual manifestation of allergic eosinophilic gastroenteritis

Kevin M Riggle, Ghassan Wahbeh, Elizabeth M Williams, Kimberly J Riehle

Kevin M Riggle, Kimberly J Riehle, Department of General Surgery, University of Washington, Seattle, WA 98195, United States

Ghassan Wahbeh, Division of Gastroenterology, Seattle Children's Hospital, Seattle, WA 98195, United States

Elizabeth M Williams, Division of Pathology, Seattle Children's Hospital, Seattle, WA 98195, United States

Kimberly J Riehle, Division of Pediatric General and Thoracic Surgery, Seattle Children's Hospital, Seattle, WA 98195, United States

Author contributions: Riggle KM, Wahbeh G, Williams EM, and Riehle KJ contributed equally to this work.

Institutional review board statement: This study was reviewed and approved by the Seattle Children's Hospital Institutional Review Board.

Informed consent statement: Informed consent was waived as approved by the Seattle Children's Hospital Institutional Review Board.

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

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Correspondence to: Kimberly J Riehle, MD, Assistant Professor, Department of Surgery, University of Washington, Box 356410, Seattle, WA 98195,
United States. kimberly.riehle@seattlechildrens.org
Telephone: +1+206-9872794
Fax: +1+206-9873925

Received: March 26, 2015
Peer-review started: March 28, 2015
First decision: May 18, 2015
Revised: June 4, 2015
Accepted: September 2, 2015
Article in press: September 2, 2015
Published online: November 28, 2015

Abstract

Spontaneous perforation of a duodenal ulcer secondary to allergic eosinophilic gastroenteritis (EGE) has not been previously reported. We present such a case in a teenager who presented with peritonitis. After exploration and operative repair of his ulcer, he continued to experience intermittent abdominal pain, and further evaluation revealed eosinophilic gastroenteritis in the setting of multiple food allergies. His EGE resolved after adhering to a restrictive diet. Both duodenal ulcers and EGE are very rarely seen in pediatric patients. EGE has a variable presentation depending on the layer(s) of bowel wall affected and the segment of the gastrointestinal tract that is involved. Once diagnosed, it may respond to dietary changes in patients with recognized food allergies, or to steroids in patients in whom an underlying cause is not identified. Our case highlights the need to keep EGE in the differential diagnosis when treating pediatric patients with duodenal ulcers. The epidemiology, pathophysiology, and treatment of EGE are also discussed, along with a review of the current literature.

Key words: Pediatric; Duodenal ulcer; Eosinophilic; Gastroenteritis; Food allergies

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Core tip: We report a case of a perforated duodenal ulcer secondary to allergic eosinophilc gastroenteritis

in a pediatric patient. To our knowledge this is the only reported case of spontaneous duodenal ulcer perforation in this patient population. Herein we discuss the details of our case, highlighting the need for increased suspicion of eosinophilic gastroenteritis (EGE) in pediatric patients with gastrointestinal ulcers. Further, we discuss the epidemiology, pathophysiology, and treatments of EGE, along with a review of the current literature.

Riggle KM, Wahbeh G, Williams EM, Riehle KJ. Perforated duodenal ulcer: An unusual manifestation of allergic eosinophilic gastroenteritis. *World J Gastroenterol* 2015; 21(44): 12709-12712 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12709.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12709

INTRODUCTION

Eosinophilic gastroenteritis (EGE) is a rare disorder characterized by intestinal inflammation with eosinophilic infiltration of the wall of the gastrointestinal tract, with or without peripheral eosinophilia[1,2]. This disease occurs in the absence of most known causes of eosinophilia such as parasitic infection, malignancy, or drug reactions. EGE can involve one or more layers of the bowel wall, resulting in a wide range of presenting symptoms that typically include nausea, abdominal pain, and diarrhea[1,2]. Herein we report an unusual case of EGE, which presented as a perforated duodenal ulcer in a teenager.

CASE REPORT

A sixteen-year-old boy presented with acute epigastric abdominal pain and focal right-sided peritonitis on physical exam. He had no significant medical history, and no history of non-steroidal anti-inflammatory drug (NSAID) or cocaine use. Initial labs demonstrated leukocytosis with a normal eosinophil count, a computed tomography (CT) scan showed a small volume of free air, and he was taken to the operating room for exploratory laparoscopy. He was found to have a perforated ulcer in the 1st portion of the duodenum (Figure 1), which was repaired using a Graham patch.

He was treated presumptively for *Helicobacter pylori* (*H. pylori*), and was discharged home after an uneventful postoperative course on a regular diet. Interestingly, his serological analysis was negative for *H. pylori*, and he continued to have diffuse, non-specific abdominal pain despite ongoing treatment with proton pump inhibitors. Two months after his operation he underwent esophagogastroduodenoscopy (EGD), which demonstrated significant esophageal edema, gastric erythema, and a small residual area of duodenal ulceration at the site of his repair (Figure 2).

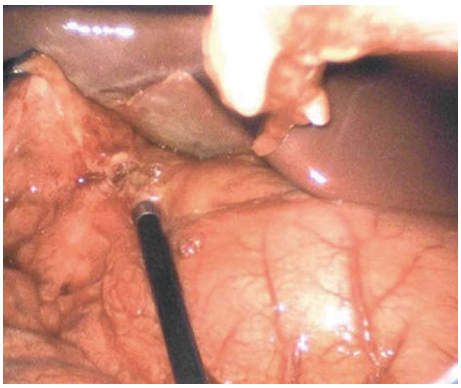

Figure 1 Intraoperative photo depicting the perforated duodenal ulcer in the 1st portion of the duodenum.

Histologic analysis of these grossly abnormal areas revealed eosinophilic infiltration of the esophagus, stomach, and duodenum (Figure 3A-C). Given an absence of other known causes for eosinophilia he underwent allergy testing, and was found to have allergies to multiple foods, including dairy, eggs, and peanuts. Five months after initiation of a restrictive diet, his abdominal pain subsided, and repeat endoscopy with biopsies confirmed that his esophageal and gastric inflammation had significantly improved, with resolution of the duodenal inflammation (data not shown). After three more months he re-introduced milk and peanuts into his diet, leading to recurrent abdominal pain. EGD with biopsies at that time confirmed recurrent severe esophagitis, prompting him to resume his restricted diet.

Interestingly, we have treated two other patients at our institution with duodenal ulcers secondary to EGE, one of which was found to have milk allergies as the presumed etiology. After that patient adopted a lactose free diet, the EGE resolved. The third patient had resolution of the EGE following corticosteroid treatment. All three patients were *H. pylori* negative and presented with abdominal pain as their primary symptom.

DISCUSSION

EGE is a rare benign disorder known to cause inflammation in all locations and layers of the gastrointestinal (GI) tract, resulting in highly variable presenting symptoms[1,2]. It is more commonly seen in adults, so may not be considered in pediatric patients with abdominal complaints[3]. EGE should be a part of the differential diagnosis in patients with unexplained GI symptoms, especially in those with peripheral eosinophilia or a history of allergies. The primary diagnostic criteria for EGE include GI symptoms, biopsies showing an eosinophilic infiltrate in one or more layers of the gastrointestinal wall, and the absence of other diseases that cause eosinophilia, such as drug reactions or

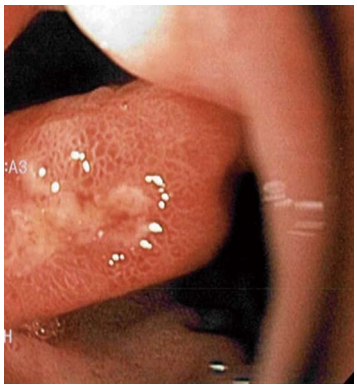

Figure 2 Initial endoscopy showing residual duodenal ulceration after repair.

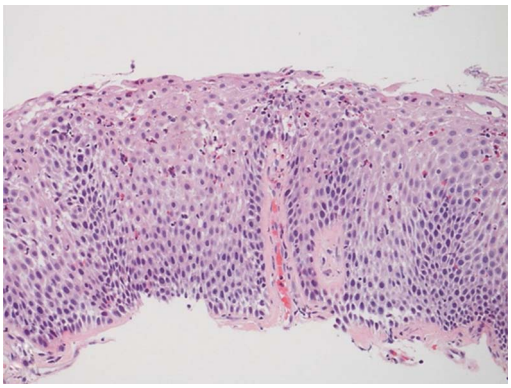

Figure 3 Esophageal (A), gastric (B), and duodenal (C) biopsies showing moderate to severe eosinophilic infiltration of the lamina propria and epithelium. The duodenal biopsy also shows gastric metaplasia. Hematoxylin-eosin staining, magnification × 200.

parasitic infections. Peripheral eosinophilia is seen in up to 80% of cases, but is not mandatory for diagnosis. EGE may be classified as predominantly mucosal (60%), muscular (30%), or serosal (10%), and can occur in any segment of the GI tract[4].

Gastroduodenal ulcers are similarly uncommon in pediatric patients, and spontaneous perforation of an ulcer in a child is extremely rare[5]. Most pediatric duodenal ulcers are secondary to *H. pylori* infection, NSAIDS, or Zollinger-Ellison syndrome. To our knowledge, there are only a handful of gastric or duodenal ulcers secondary to EGE that have been reported in pediatric patients[6-10]. Only two of these were duodenal ulcers, and there are no previous reports of EGE presenting as spontaneous duodenal ulcer perforation. The only other case of EGE presenting with perforated duodenal ulcer occurred after blunt abdominal trauma[6].

The precise pathophysiology of EGE is poorly understood, but is presumed to involve either IgE-dependent or independent eosinophil recruitment and activation, followed by T-cell mediated chemokine production by eosinophils[1,2]. In support of IgE-mediated mechanisms driving this disease, it has been reported that up to 75% of patients with EGE have a personal or family history of food, medication, or pollen allergies[11]. In patients with EGE and food allergies, adherence to a restrictive diet will often result in remission of the disease[11,12]. In those who do not respond to allergen avoidance, up to 90% will respond to corticosteroid therapy[3]. Accordingly, our patient had resolution of his symptoms and pathologic findings after initiation of a restrictive diet. Furthermore, he had recurrence of his gastrointestinal inflammation following liberalization of his dietary intake, supporting the notion that food allergies and EGE caused his perforated duodenal ulcer.

In conclusion, EGE is a rare condition that has a highly variable presentation depending on the layer(s) of bowel wall affected and the segment of the gastrointestinal tract that is involved. Once diagnosed, it may respond well to dietary changes in patients with recognized food allergies or to corticosteroid treatment.

Our series of three patients highlights the need to keep EGE in the differential diagnosis when faced with duodenal ulcers in pediatric patients, particularly in the setting of negative *H. pylori* testing.

COMMENTS

Case characteristics

A sixteen year-old boy presented with intermittent, non-specific epigastric pain for two months following repair of a perforated duodenal ulcer.

Clinical diagnosis

On physical exam he demonstrated mild epigastric tenderness.

Differential diagnosis

Helicobacter pylori (*H. pylori*) infection, non-steroidal anti-inflammatory drug-induced peptic ulcers, Zollinger-Ellinson syndrome, antral gastritis.

Laboratory diagnosis

Initial labs demonstrated leukocytosis with a normal eosinophil count, allergy testing revealed multiple food allergies.

Imaging diagnosis

Esophagogastroduodenoscopy showed edema/erythema of distal esophagus, stomach, and duodenum.

Pathological diagnosis

Histologic analysis of gastric biopsies revealed eosinophilic infiltration of the esophagus, stomach, and duodenum.

Treatment

Adherence to a restrictive diet resulted in resolution of his symptoms and histologic inflammation.

Related reports

Few cases of eosinophilic gastroenteritis (EGE) have been reported in the pediatric population, and duodenal ulceration is an extremely rare presentation of EGE. In reports of EGE caused by food allergies, adherence to a restrictive diet is often curative.

Term explanation

Eosinophilic gastroenteritis is a rare disease known to cause inflammation in one or more layers of the bowel wall.

Experiences and lessons

This case report highlights the need to keep EGE in the differential diagnosis when faced with duodenal ulcers in pediatric patients, particularly in the setting of negative *H. pylori* testing.

Peer-review

The authors present a rare and interesting case of allergic eosinophilic gastroenteritis with associated duodenal ulcer perforation. It highlights the clinical characteristics of this rare disease, and describes resolution of the inflammation after elimination of the inciting allergens.

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P- Reviewer: Huang TY **S- Editor**: Yu J **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12713

World J Gastroenterol 2015 November 28; 21(44): 12713-12721
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Characteristic clinical features of *Aspergillus* appendicitis: Case report and literature review

Mihajlo Gjeorgjievski, Mitual B Amin, Mitchell S Cappell

Mihajlo Gjeorgjievski, Mitchell S Cappell, Division of Gastroenterology and Hepatology, William Beaumont Hospital, Royal Oak, MI 48073, United States

Mitual B Amin, Anatomic Pathology, William Beaumont Hospital, Royal Oak, MI 48073, United States

Mitual B Amin, Mitchell S Cappell, Oakland University William Beaumont School of Medicine, Royal Oak, MI 48073, United States

Author contributions: Gjeorgjievski M and Cappell MS contributed equally to the paper; Gjeorgjievski M and Cappell MS are responsible for all the clinical aspects of the paper; Amin MB contributed solely to the pathologic aspects of this paper and is responsible for this section of the paper.

Institutional review board statement: Case report exempted/approved by William Beaumont Hospital IRB on 12/01/2014.

Conflict-of-interest statement: None for all authors. This paper does not discuss any confidential pharmaceutical industry data reviewed by Dr. Cappell as a consultant for the United States Food and Drug Administration Advisory Committee on Gastrointestinal Drugs. Dr. Cappell is a member of the speaker's bureau for AstraZeneca. This paper does not discuss any drug manufactured or marketed by AstraZeneca.

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Correspondence to: Mitchell S Cappell, MD, PhD, Chief, Division of Gastroenterology and Hepatology, William Beaumont Hospital, MOB #602, 3535 West Thirteen Mile Road, Royal Oak, MI 48073, United States. mscappell@yahoo.com
Telephone: +1-248-5511227
Fax: +1-248-5517581

Received: June 1, 2015
Peer-review started: June 2, 2015
First decision: June 19, 2015
Revised: July 13, 2015
Accepted: August 30, 2015
Article in press: August 31, 2015
Published online: November 28, 2015

Abstract

This work aims to facilitate diagnosing *Aspergillus* appendicitis, which can be missed clinically due to its rarity, by proposing a clinical pentad for *Aspergillus* appendicitis based on literature review and one new case. The currently reported case of pathologically-proven *Aspergillus* appendicitis was identified by computerized search of pathology database at William Beaumont Hospital, 1999-2014. Prior cases were identified by computerized literature search. Among 10980 pathology reports of pathologically-proven appendicitis, one case of *Aspergillus* appendicitis was identified (rate = 0.01%). A young boy with profound neutropenia, recent chemotherapy, and acute myelogenous leukemia presented with right lower quadrant pain, pyrexia, and generalized malaise. Abdominal computed tomography scan showed a thickened appendiceal wall and periappendiceal inflammation, suggesting appendicitis. Emergent laparotomy showed an inflamed, thickened appendix, which was resected. The patient did poorly post-operatively with low-grade-fevers while receiving antibacterial therapy, but rapidly improved after initiating amphotericin therapy. Microscopic examination of a silver stain of the appendectomy specimen revealed fungi with characteristic *Aspergillus* morphology, findings confirmed by immunohistochemistry. Primary *Aspergillus* appendicitis is exceptionally rare, with only 3 previously reported cases. All three cases presented with (1)-neutropenia, (2)-recent chemotherapy, (3)-acute leukemia, and (4)-suspected appendicitis;

(5)-the two prior cases initially treated with antibacterial therapy, fared poorly before instituting anti-*Aspergillus* therapy. The current patient satisfied all these five criteria. Based on these four cases, a clinical pentad is proposed for *Aspergillus* appendicitis: clinically-suspected appendicitis, neutropenia, recent chemotherapy, acute leukemia, and poor clinical response if treated solely by antibacterial/anti-candidial therapy. Patients presenting with this proposed pentad may benefit from testing for *Aspergillus* infection by silver-stains/immunohistochemistry and considering empirical anti-*Aspergillus* therapy pending a tissue diagnosis.

Key words: Aspergillosis; *Aspergillus* appendicitis; Fungal appendicitis; Appendicitis; Neutropenia; Chemotherapy; Acute myelocytic leukemia

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Core tip: This work reports the fourth reported case of isolated *Aspergillus* appendicitis, further characterizes this syndrome, and identifies a clinical pentad associated with this syndrome: clinically-suspected appendicitis, neutropenia, recent chemotherapy, acute leukemia, and poor clinical course if treated solely with antibacterial or anti-Candidial antibiotics. These risk factors are biologically reasonable. Immunosuppression from neutropenia and acute leukemia may promote *Aspergillus* appendicitis. Local gastrointestinal ulcers from recent chemotherapy provides a nidus for fungal colonization. In patients presenting with this proposed pentad, *Aspergillus* appendicitis should be considered in the differential diagnosis, special silver stains should be performed to evaluate for this infection, and empiric anti-*Aspergillus* therapy may be considered pending tissue diagnosis.

Gjeorgjievski M, Amin MB, Cappell MS. Characteristic clinical features of *Aspergillus* appendicitis: Case report and literature review. *World J Gastroenterol* 2015; 21(44): 12713-12721 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12713.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12713

INTRODUCTION

The diagnosis of isolated (primary) *Aspergillus* appendicitis may be clinically overlooked or delayed because of its exceptional rarity, as shown by a review of the English literature revealing only three cases, and its association with profound immunosuppression (Table 1)[1-3] that can delay or mask the usual clinical features of appendicitis[4,5]. Yet, delayed diagnosis and therapy for *Aspergillus* appendicitis may have dire patient consequences, as occurred in two of the three previously reported cases[1,2]. A new patient is reported of isolated *Aspergillus* appendicitis who presented with five striking clinical findings, and a novel syndromic pentad is proposed as a diagnostic tool, based on this new case report and review of the three previously reported cases that demonstrated nearly identical findings.

Computerized search using the words "appendicitis" and "*Aspergillus*" of the pathology database at William Beaumont Hospital, Royal Oak, July 1999-December 2014, revealed only 1 case of *Aspergillus* appendicitis (0.01% rate) among 10980 pathology reports of appendicitis proven by pathologic examination of the appendectomy specimen. Published reports of *Aspergillus* appendicitis were identified by computerized literature search using PubMed; and review of general textbooks and specialized monographs in gastroenterology, pathology, and infectious diseases. One case report of isolated *Aspergillus* appendicitis written in German was professionally translated[6]. Case reports of small intestinal Aspergillosis without known or documented appendiceal involvement were excluded[7]. This study received exemption/approval by William Beaumont Hospital IRB on December 1, 2014.

CASE REPORT

An 8-year-old boy with acute myelogenous leukemia (AML), diagnosed one month earlier, and with profound neutropenia, after receiving two cycles of IV chemotherapy with daunorubicin, cytarabine, thioguanine, etoposide and dexamethasone, presented with low-grade pyrexia and chills for one day. Physical examination revealed temperature = 36.8 ℃, pulse = 111 beats/min, blood pressure = 94/63 mmHg, and mild right lower quadrant (RLQ) tenderness without rebound tenderness. The leukocyte count = 200 cells/mm^3 (normal 4800-10100 cells/mm^3), neutrophil count < 100 cells/mm^3 (normal 1600-7200 cells/mm^3), hematocrit = 36.3% (normal in male boy, age 6-9 years: 33.6%-43.4%), and platelet count = 90000/mm^3 (normal 150000-400000/mm^3). Abdominal ultrasound was unrevealing. Abdomino-pelvic computed tomography (CT) showed a mildly enlarged appendix without an appendicolith or periappendiceal inflammation, findings possibly consistent with early appendicitis. The patient was rehydrated with normal saline and administered IV antibiotic therapy with amoxicillin, gentamicin and metronidazole, but developed increasing RLQ abdominal pain and several episodes of vomiting during the ensuing 24 h. Repeat abdomino-pelvic CT scan revealed a dilated appendiceal lumen, thickened appendiceal wall, and periappendiceal fat stranding, findings consistent with acute appendicitis (Figure 1).

Emergency laparotomy revealed an inflamed, edematous, dusky appendix without appendiceal perforation but with thickened periappendiceal mesentery. The appendix was resected. Microscopic examination of hematoxylin and eosin stains of

Table 1 Case reports of isolated *Aspergillus* appendicitis without other known *Aspergillus* infection

Age and sex [reference]	Underlying condition	Chemotherapy received prior to appendicitis	Symptom appearance after initiating chemotherapy	Neutropenia at presentation	Abdominal imaging	Pathologic findings in resected appendix	Therapy and outcome
8-yr-old male[1]	ALL	Initial therapy: Vincristine, prednisolone, L-asparaginase, intrathecal methotrexate. Chemotherapy during relapse: not reported	14 mo after therapy restarted for a relapse	Yes	USD: enlarged appendix, cecal wall thickened, and small periappendiceal fluid collection	Necrosis and inflammation in mucosa and nearby muscle consistent with acute appendicitis. Many septated fungal hyphae showing acute-angle branching, characteristic of *Aspergillus*	Fared poorly for 10 d while treated with conventional antibiotic therapy of ceftazdime and amikacin before undergoing appendectomy. Did well after appendectomy. Discharged in stable condition at postoperative day 10 (not mentioned whether received antifungal therapy after appendectomy)
41-yr-old male[2]	ALL (B-cell type with BCR-ABL translocation)	Induction therapy: cyclophosphamide, daunorubicin, vincristine, prednisone, L-asparaginase, and dasatinib; Maintenance therapy: 6-mercaptopurine and dasatinib; Received stem cell transplantation	12 d after transplantation; 5 mo after initiation of chemotherapy	Yes	CT: cecal wall thickened, thickened retrocecal appendix, and periappendiceal inflammatory changes; small amount of free fluid present	Full-thickness invasion of appendiceal wall including serosa; fungal angioinvasion with vessels occluded by hyphal forms; positive methenamine-silver stain	Fared extremely poorly for 6 d with conventional antibiotic therapy plus acyclovir and fluconazole. Improved after appendectomy and after receiving liposomal amphotericin B and micafungin (switched on day 3 to voriconazole and micafungin due to acute renal injury). Discharged 46 d after appendectomy. Clinically stable 12 mo after hospitalization, without further aspergillus complications
21-yr-old male[3]	AML- M1	Mitozantrone and cytarabine. Later treatment with daunorubicin and cytarabine	30 d after diagnosis	Yes. WBC = 500/mm^3 (no neutrophils seen)	USD: dilated bowel loops and right hydronephrosis. CT scan: right hydroureter extending down into pelvis with loss of fat planes in the region, consistent with inflammatory process around distal ureter	Coagulative necrosis of appendiceal tip with septate fungal hyphae with dichotomous branching pattern, permeating and occluding arterial branches. Immunoperoxidase stain demonstrated *Aspergillus* flavus	Amphotericin B Second laparotomy 6 d after first showed 3 small bowel perforations. Died at day 49 from bleeding from *Aspergillus* invasion of iliac vein
[1]39-yr-old, sex not stated[6]	Not reported	NA	NA	Not reported	Not reported (case published in 1959 before abdominal CT or USD became available)	At surgery: inflamed, enlarged, gangrenous appendix with severe surrounding inflammation. Microscopic pathology not reported	Did poorly postoperatively with high spiking fevers, overwhelming sepsis, and progressive jaundice while receiving streptomycin and 2 other antibacterial antibiotics. On day 9 therapy with antimycotic trichomycin initiated after *Aspergillus* nidulans isolated from appendiceal culture. Died 3 d later from progressive organ failure
8-yr-old male [Current Case Report]	AML-M5	Daunorubicin, cytarabine, thioguanine, etopiside, and dexamethasone	30 d	Yes. WBC= 200/mm^3	USD: no evident right lower quadrant abscess or free fluid. CT: inflammatory changes in right lower quadrant with thick-walled appendix and dilated appendiceal lumen	Branched, septate fungal hyphae invading full thickness of appendiceal wall without discrete perforation	Underwent appendectomy: Did poorly initially postoperatively while receiving antibacterial antibiotics. Recovered after receiving amphotericin B and discharged after 21 d of this therapy. No signs of disseminated Aspergillosis during 8 mo of follow up while receiving prophylaxis with itraconazole

[1]Case excluded from analysis in this paper because this case report was published in 1959 before modern imaging tests became available and this case report lacks critical clinical details due to its brevity. AML: Acute myelogenous leukemia; ALL: Acute lymphocytic leukemia; CT: Computerized tomography; USD: Ultrasound; DIC: Disseminated intravascular coagulation; WBC: White blood cell; NA: Not available.

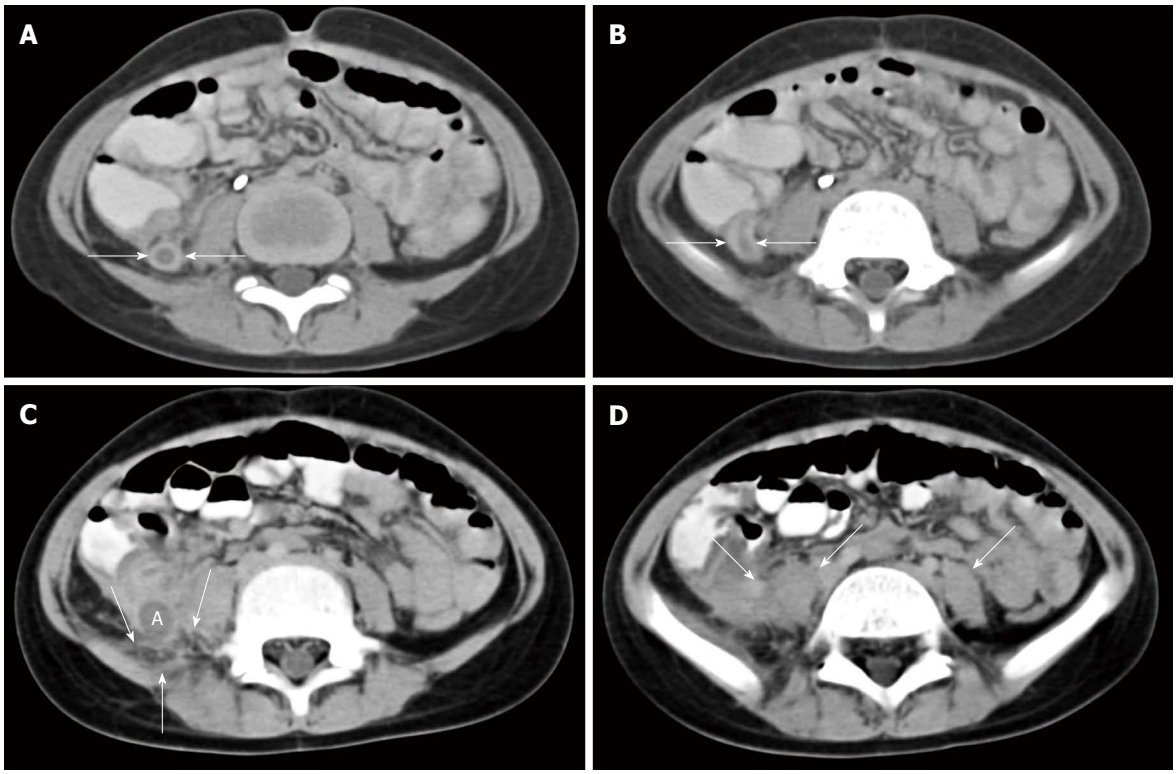

Figure 1 Computed tomography images. Computed tomography images of abdomen using IV non-ionic contrast and standard oral contrast demonstrates dilated appendiceal ostium and thickened appendiceal wall in cross-section (arrows in A); dilated lumen and thickened wall of vermiform appendix in longitudinal section (arrows in B); periappendiceal fat stranding (arrows in C) and an enlarged right psoas muscle with indistinct margins from local extension of the appendiceal inflammation (enlarged right psoas muscle with indistinct margins identified in D by 2 arrows, as compared to normal-sized left psoas muscle with distinct margins identified by 1 arrow). The appendix measures approximately 11 mm in diameter from outer wall to outer wall. All these findings are consistent with acute appendicitis. There is no evident appendicolith or typhlitis.

histologic sections of the resected appendix showed an acute necrotizing appendicitis, with scattered questionable fungal hyphal forms. Postoperatively, the patient continued to be ill with persistent moderate pyrexia for 12 d, while receiving IV ampicillin, gentamicin, and metronidazole antibiotic therapy and filgrastim (granulocyte colony stimulating factor). Due to this poor clinical response, evident risk factors for fungal appendicitis, and questionable findings of fungal hyphae on a conventional hematologic stain, a Grocott-Gomori methenamine-silver nitrate stain was performed on the resected specimen which revealed numerous fungal hyphae that showed features characteristic of *Aspergillus* of septation and acutely angled branching, and which was confirmed as *Aspergillus* by immunohistochemistry (Figure 2A and B). The specific species causing the *Aspergillus* infection could not be determined in the formalin fixed tissue. On day 13 IV amphotericin-B was added to the antibiotic regimen and the patient rapidly improved clinically, with defervescence and gradual recovery of the leukocyte count to 3400 cells/mm^3. He was discharged after 21 d of IV amphotericin therapy to receive oral itraconazole as an outpatient. During 8 mo of follow up, the patient had 2 relapses of AML, but no evident *Aspergillus* recurrence, while continuing oral antifungal prophylaxis with itraconazole.

DISCUSSION

Aspergillus, a widespread fungus in the environment, usually enters the human body by airborne transmission and colonizes the nasal cavities or facial sinuses. Invasive pulmonary aspergillosis accounts for 90%-98% of invasive *Aspergillus* infections, but hematogenous spread may cause disseminated infection[8-12], or rarely isolated infections of other organs, including central nervous system, heart, liver, kidneys, and gastrointestinal (GI) tract[13-17]. Based on a literature review of more than 3000 cases of *Aspergillus* infection, Denning *et al* hypothesized that isolated GI aspergillosis generally arises from ingestion of food contaminated with *Aspergillus*, and colonization by *Aspergillus* of GI ulcers which can arise from antecedent chemotherapy[10,18].

Aspergillus fumigatus is the most frequent species that causes human infections, followed by A. flavus, A. terreus, A. niger, and A. nidulans[19]. The risk of pulmonary infection and hematogenous spread greatly increases with immunosuppression, including profound neutropenia, glucocorticoid therapy, and neutrophilic or phagocytic dysfunction from acute leukemia[20,21], chronic granulomatous disease, and advanced human immunodeficiency virus infection[7,22-24].

GI aspergillosis is rare[7,22,25], isolated GI aspergillosis

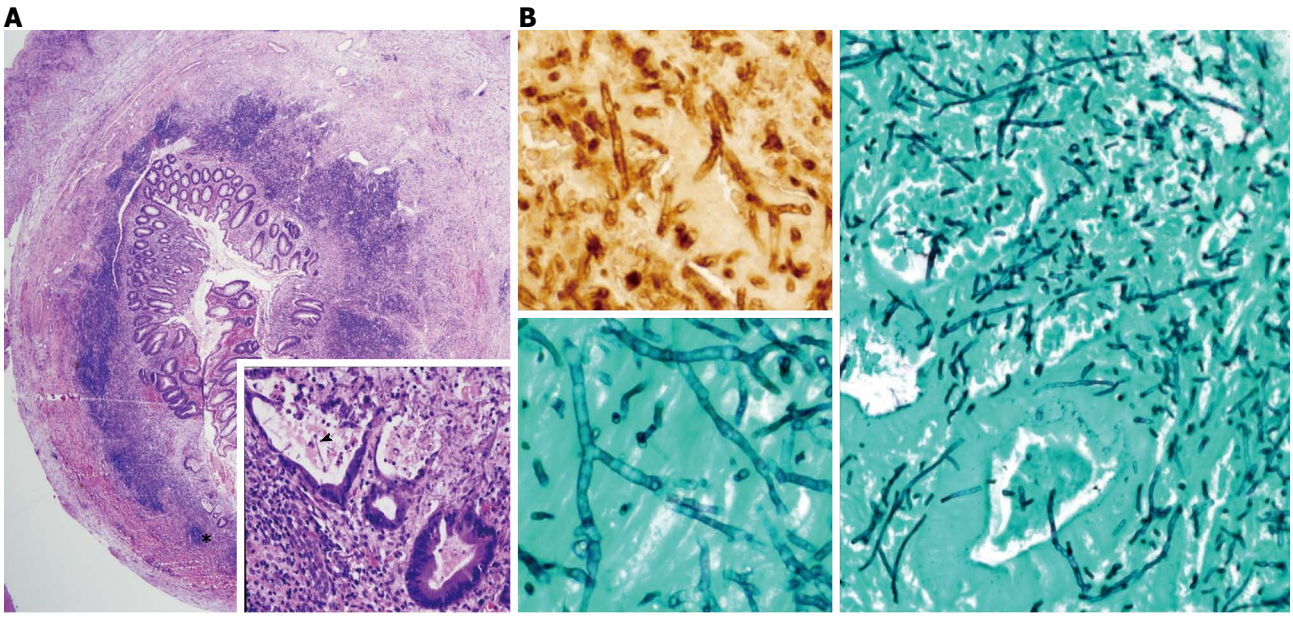

Figure 2 Photomicrograph. A: Photomicrograph of a hematoxylin-eosin stained, full thickness, cross-section of the resected appendiceal specimen shows a thickened appendiceal wall due to a severe mixed inflammatory infiltrate (A, low power). The high power view (A-inset) of an area within the low-power view, shows one questionable fungal hyphae (arrowhead) within a gland partially destroyed by the necrotizing inflammation; B: Photomicrograph of a Grocott-Gomori methenamine-silver (GMS) nitrate stain reveals invasive, septate, hyphal forms with acutely angled branches, characteristic of *Aspergilus* species (B-right side-low power, B-left lower inset-high power). The hyphae are confirmed as *Aspergillus* species by immunohistochemistry (B-left upper inset-high power).

is even rarer, and isolated *Aspergillus* appendicitis is exceedingly rare. For example, in a review of 1538 patients with aspergillosis, only 85 (5.5%) had GI aspergillosis, and only 14 (0.8%) patients had isolated GI aspergillosis[7]. The currently reported case of isolated appendiceal aspergillosis represents only the fourth reported case in the English literature (Table 1). A fifth case published in German in 1959[6] is detailed in Table 1, but not presently analyzed because (1) it was published before the advent of modern laboratory and imaging tests for appendicitis, such as abdominal ultrasound or CT; and (2) the case report lacked critical clinical details, such as microscopic findings, due to brevity of the report.

All four analyzed cases, including the current case, presented with a distinct syndrome of clinically-suspected appendicitis, acute leukemia [either AML or acute lymphocytic leukemia (ALL)], recently administered chemotherapy, and neutropenia (Table 1)[1-3]; and three of the patients, including the current case, initially did poorly when receiving antibacterial therapy without anti-*Aspergillus* therapy[1,2]. The fourth case received anti-*Aspergillus* therapy promptly without delay. Four other cases of appendiceal aspergillosis were reported with other GI involvement, but without clinically apparent extraintestinal spread (Table 2)[26-29], of which one had disseminated aspergillosis demonstrated by autopsy[29]. All four of these cases, like the four cases of isolated *Aspergillus* appendicitis, had underlying AML or ALL, neutropenia, and recent chemotherapy.

To promote early diagnosis and appropriate treatment, a clinical pentad is proposed for suspected *Aspergillus* appendicitis of: (1) clinically-suspected appendicitis; (2) neutropenia; (3) recent chemotherapy; (4) underlying acute leukemia; and (5) poor response if administered antibacterial antibiotics or anti-Candidial therapy without anti-*Aspergillus* therapy. The hypothesized pathophysiology of the proposed syndromic pentad is *Aspergillus*, presumably ingested in contaminated food (1) colonizes GI ulcers or areas of mucositis induced by chemotherapy. Mucosal ulcers commonly occur after chemotherapy with daunorubicine, which was administered in 3 of the 4 reported cases[2,3,current report], or after chemotherapy with cytarabine[30], which was administered in 2 of the 4 reported cases[3,current report], (fourth patient received unspecified chemotherapy[1]). *Aspergillus* then invades the appendiceal wall due to immunosuppression from (2) neutropenia and (3) acute leukemia[31]. The patients, despite immunosuppression, still present clinically (4) with RLQ pain and fever suggestive of appendicitis; and (5) the patients with *Aspergillus* infection should not respond to conventional antibacterial or anti-Candidial antibiotics.

Patients satisfying this pentad should be: (1) evaluated for *Aspergillus* by microscopic examination with special stains, such as Grocott-Gomori methenamine silver or periodic acid-Schiff (PAS)-diastase stains, supplemented by immunohistochemistry as necessary because the fungal elements may not be readily visible on routine hematoxylin and eosin stained slides; (2) should have the entire resected appendix submitted for histologic examination if the standardly reviewed one or two representative sections of the appendix lack identifiable fungal structures; and (3)

Table 2 Reported cases of *Aspergillus* appendicitis with additional gastrointestinal involvement

Age and sex. areas of aspergillus infection [reference]	Underlying condition	Chemotherapy received prior to appendicitis	Presentation with symptoms after initiation of chemotherapy	Neutropenia at time of developing symptoms	Abdominal imaging	Pathologic findings in resected appendix	Antifungal therapy: Outcome
11-yr-old male. Extensive GI involvement including appendix and cecum (typhlitis)[28]	AML	Cytarabine, daunorubicin, and etoposide	Day 12 after initiating chemotherapy	Yes	USD: thickened intestinal walls with indistinct hypoechogenic area reaching from cecal pole to mesenteric root	Performed cecal resection and appendectomy. Chronic, partially hemorrhagic inflammation of intestine infiltrated by *Aspergillus*. Fungal hyphae also demonstrated within blood vessels	Amphotericin B and fluorocytosine: Patient succumbed to septic shock while on persistent antifungal therapy 6 wk after admission. Autopsy demonstrated disseminated Aspergillosis
38-yr-old male. Only appendix and cecum infected[26]	ALL	Vincristine and prednisone and intrathecal methotrexate. Later changed to cytoxan and adriamycin	Hospital day 7	Yes, WBC = 100/mm^3	Gallium scan: increased uptake in midabdomen and pelvis consistent with infectious process. CT: increased density in right lower quadrant consistent with an abscess or fluid-filled cecum	Laparotomy: appendix not found (apparently due to destruction), but cecal perforation with surrounding abscess with multiple coloenteric fistulas found. Resected specimen showed *Aspergillus* hyphae in necrotic area of bowel wall invading peritoneal surfaces	Amphotericin B: Stable at 6 mo follow-up, with right lung infiltrate that identified on previous X-ray, being stable in size
62-yr-old female. Appendix, cecum, ascending colon and ileum infected[27]	AML M6	Induction therapy: cytarabine for 7 d and idarubicin for 3 d	Day 16 after initiating chemotherapy	Yes, WBC = 600/mm^3, no neutrophils	CT: inflammatory changes and fat stranding surrounding dilated appendix. Small amount of adjacent free fluid in pelvis	Resected 2.5 cm segment of small bowel and 60 cm segment of cecum and ascending colon. Microscopic evaluation of sections of bowel and appendix showed transmural intestinal infarction with hemorrhagic plugs within intestine blood vessels and fungal hyphae with septation and acute branching angles. Fungal stain revealed morphology consistent with *Aspergillus*	Voriconazole started empirically 20 d after admission, before surgery: Patient expired from cardiac arrest 26 d after admission
5-yr-old female. Appendix involved with widespread GI infection[29]	AML and diffuse large B-cell lymphoma	6 cycles of ThaiPOG protocol	Not specified	Yes	CT: early abscess formation in distal ileum and appendix	Pathological confirmation of appendicitis caused by invasive aspergillosis	Amphotericin B, metronidazole and piperacillin with tazobactam: Died 1 d later from septicemia with DIC; Autopsy disclosed fungal infection disseminated throughout body

GI: Gastrointestinal; AML: Acute myelogenous leukemia; ALL: Acute lymphocytic leukemia; NA: Not applicable; WBC: White blood cell; CT: Computerized tomography; USD: Ultrasound; DIC: Disseminated intravascular coagulation. ThaiPOG protocol: Thailand Pediatric Oncology Group protocol as described in: Seksarn P, Wiangnon S, Veerakul G, Chotsampancharoen T, Kanjanapongkul S, Chainansamit SO. Outcome of childhood acute lymphoblastic leukemia treated using the Thai National Protocols. *Asian Pac J Cancer Prev* 2015; **16** (11): 4609-4614.

should be considered for empiric anti-*Aspergillus* therapy if doing poorly on conventional antibiotic therapy pending a tissue diagnosis. The importance of early anti-*Aspergillus* therapy is emphasized by two of the four reported cases of isolated *Aspergillus* appendicitis improving dramatically after instituting anti-*Aspergillus* therapy and appendectomy. Of note, the prognosis of isolated *Aspergillus* appendicitis appears to be better than that of *Aspergillus* appendicitis combined with *Aspergillus* enterocolitis.

This work illustrates that neutropenia after chemotherapy is an important risk factor for *Aspergillus* appendicitis/enterocolitis, and emphasizes the importance of filgrastim therapy to decrease the severity or duration of neutropenia after chemotherapy. Filgrastim helps prevent febrile neutropenia from *Aspergillus* or other opportunistic infections in experiments in mice[32,33], or in clinical trials[34,35], and is likely important in treating *Aspergillus* appendicitis or enterocolitis occurring in the setting of

neutropenia.

The proposed pentad is subject to criticism. First, the data are limited to four cases of isolated *Aspergillus* appendicitis and four cases of *Aspergillus* appendicitis with other GI involvement, and therefore require further confirmation. However, all four case reports generally satisfied the proposed pentad. Second, the reviewed cases may lack clinical details because of their retrospective nature. Third, cases obtained from the literature, particularly individual case reports, are subject to reporting bias. For example, clinicians might selectively report and journal editors might selectively publish only dramatic cases of poor response to antibacterial therapy and rapid recovery after instituting anti-*Aspergillus* therapy. Fourth, although the proposed syndromic pentad may be highly sensitive for *Aspergillus* appendicitis, the specificity is unstudied and might only be moderate. The differential diagnosis in patients satisfying this pentad also includes (bacterial) neutropenic typhlitis, with or without bacterial appendicitis[36,37], and appendicitis caused by other fungi[38], including Candida[29], Mucor[39], and Histoplasma[40,41]. Therefore, this pentad should not be viewed as diagnostic, but merely as clinically useful to raise a suspicion of potential *Aspergillus* appendicitis. All five criteria appear to be typical for *Aspergillus* appendicitis, but the first four criteria may also occur with (bacterial) neutropenic typhlitis/enterocolitis and only the fifth criterion of poor response to conventional antibacterial/anti-Candidial antibiotic therapy is relatively specific for *Aspergillus* appendicitis.

This clinical pentad may become increasingly important clinically because of an increasing incidence of invasive *Aspergillus*[42], and an increasing incidence of severe neutropenia from more potent immunosuppressive chemotherapy and more frequent stem cell transplantation[14]. It is important to recognize neutropenic patients with invasive *Aspergillus* because they may fare poorly with solely antibacterial or anti-Candidial therapy (*e.g.,* fluconazole) without specific anti-*Aspergillus* therapy (*e.g.,* voriconazole or amphotericin). Addition of another factor to the pentad of persistently positive galactomannan antigenemia in patients with suspected appendicitis might be useful diagnostically but is not warranted at this point because of a lack of data on the galactomannan antigen status in the reported cases of appendiceal aspergillosis[43].

COMMENTS

Case characteristics

An 8-year-old boy with acute myelogenous leukemia, diagnosed one month earlier, and with profound neutropenia, after receiving two cycles of IV chemotherapy with daunorubicin, cytarabine, thioguanine, etoposide and dexamethasone, presented with low-grade pyrexia and chills for one day.

Clinical diagnosis

Physical examination revealed temperature = 36.8 ℃, pulse = 111 beats/min, blood pressure = 94/63 mmHg. and mild right lower quadrant tenderness without rebound tenderness. The leukocyte count = 200 cells/mm^3 (normal 4800-10100 cells/mm^3), neutrophil count < 100 cells/mm^3 (normal 1600-7200 cells/mm^3), hematocrit = 36.3% (normal in male boy, age 6-9 years: 33.6%-43.4%), and platelet count = 90000/mm^3 (normal 150000-400000/mm^3).

Differential diagnosis

Clinical presentation is an episode of febrile neutropenia. The differential diagnosis includes neutropenic typhlitis and/or appendicitis, due to local opportunistic infections including bacterial infections, Candida, Mucor, Histoplasma, or *Aspergillus*.

Laboratory diagnosis

The leukocyte count = 200 cells/mm^3 (normal 4800-10100 cells/mm^3), and neutrophil count < 100 cells/mm^3 (normal 1600-7200 cells/mm^3). These findings demonstrate profound neutropenia.

Imaging diagnosis

Abdomino-pelvic computed tomography scan revealed a dilated appendiceal lumen, thickened appendiceal wall, and periappendiceal fat stranding, findings consistent with appendicitis.

Pathological diagnosis

Microscopic examination of hematoxylin and eosin stains of histologic sections of the resected appendix showed an acute necrotizing appendicitis, with scattered questionable fungal hyphal forms. A Grocott-Gomori methenamine-silver nitrate stain performed on the resected specimen revealed numerous fungal hyphae that showed features characteristic of *Aspergillus* of septation and acutely angled branching, and which was confirmed as *Aspergillus* by immunohistochemistry.

Treatment

The patient underwent appendectomy for the appendicitis and was administered IV filgrastim (granulocyte colony stimulating factor) for the profound neutropenia. On postoperative day 13 IV amphotericin-B therapy was added when *Aspergillus* appendicitis was identified by special stains performed on the resected appendix.

Related reports

Review of the modern literature revealed only 3 other reported cases of isolated *Aspergillus* appendicitis, aside from one case very briefly reported in German in 1959.

Experiences and lessons

Analysis of the current case and review of the three prior case reported in the modern literature suggest that patients with *Aspergillus* appendicitis may clinically present with a pentad of: clinically-suspected appendicitis, neutropenia, recent chemotherapy, acute leukemia, and poor clinical response if treated solely by antibacterial/anti-candidial therapy.

Peer-review

The strengths of the article include: reporting a well-documented case of isolated *Aspergillus* appendicitis, thorough review of the three previously reported cases as identified by review of the modern literature, and the proposal of a clinical pentad to facilitate clinical recognition of *Aspergillus* appendicitis.

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P- Reviewer: Charfi S, Kim MY **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i44.12722

World J Gastroenterol 2015 November 28; 21(44): 12722-12728
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Successful resection of metachronous para-aortic, Virchow lymph node and liver metastatic recurrence of rectal cancer

Nobuyoshi Takeshita, Toru Fukunaga, Masayuki Kimura, Yuji Sugamoto, Kentaro Tasaki, Isamu Hoshino, Takumi Ota, Tetsuro Maruyama, Tomohide Tamachi, Takashi Hosokawa, Yo Asai, Hisahiro Matsubara

Nobuyoshi Takeshita, Toru Fukunaga, Masayuki Kimura, Yuji Sugamoto, Kentaro Tasaki, Isamu Hoshino, Takumi Ota, Tetsuro Maruyama, Tomohide Tamachi, Takashi Hosokawa, Yo Asai, Department of Surgery, Numazu City Hospital, Shizuoka 410-0302, Japan

Hisahiro Matsubara, Department of Frontier Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

Author contributions: Takeshita N and Matsubara H planned the report; Takeshita N, Fukunaga T, Kimura M, Sugamoto Y, Tasaki K, Hoshino I, Ota T, Maruyama T, Tamachi T, Hosokawa T and Asai Y performed the treatment for this case; and Takeshita N wrote the paper.

Institutional review board statement: The case report was reviewed and approved by Numazu City Hospital Institutional Review Board.

Informed consent statement: The patient provided informed written consent prior to treatment and publication.

Conflict-of-interest statement: We have no financial relationships to disclose.

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Correspondence to: Nobuyoshi Takeshita MD, PhD, Department of Surgery, Numazu City Hospital, Sizuoka, Shizuoka 410-0302, Japan. ntakeshita1225@gmail.com
Telephone: +81-55-9245100
Fax: +81-55-9245133

Received: March 19, 2015
Peer-review started: March 20, 2015
First decision: July 17, 2015
Revised: August 17, 2015
Accepted: September 13, 2015
Article in press: September 14, 2015
Published online: November 28, 2015

Abstract

A 66-year-old female presented with the main complaint of defecation trouble and abdominal distention. With diagnosis of rectal cancer, cSS, cN0, cH0, cP0, cM0 cStage Ⅱ, Hartmann's operation with D3 lymph node dissection was performed and a para-aortic lymph node and a disseminated node near the primary tumor were resected. Histological examination showed moderately differentiated adenocarcinoma, pSS, pN3, pH0, pP1, pM1 (para-aortic lymph node, dissemination) fStage Ⅳ. After the operation, the patient received chemotherapy with FOLFIRI regimen. After 12 cycles of FOLFIRI regimen, computed tomography (CT) detected an 11 mm of liver metastasis in the postero-inferior segment of right hepatic lobe. With diagnosis of liver metastatic recurrence, we performed partial hepatectomy. Histological examination revealed moderately differentiated adenocarcinoma as a metastatic rectal cancer with cut end microscopically positive. After the second operation, the patient received chemotherapy with TS1 alone for 2 years. Ten months after the break, CT detected a 20 mm of para-aortic lymph node metastasis and a 10 mm of lymph node metastasis at the hepato-duodenal ligament. With diagnosis of lymph node metastatic recurrences, we performed lymph node dissection. Histological examination revealed moderately differentiated adenocarcinoma as metastatic rectal cancer in para-aortic and hepato-duodenal ligament areas. After the third operation, we started chemotherapy with modified FOLFOX6 regimen. After 2 cycles of modified FOLFOX6 regimen, due to the onset of neutropenia and liver dysfunction, we switched to capecitabine alone

and continued it for 6 mo and then stopped. Eleven months after the break, CT detected two swelling 12 mm of lymph nodes at the left supraclavicular region. With diagnosis of Virchow lymph node metastatic recurrence, we started chemotherapy with capecitabine plus bevacizumab regimen. Due to the onset of neutropenia and hand foot syndrome (Grade 3), we managed to continue capecitabine administration with extension of interval period and dose reduction. After 2 years and 2 mo from starting capecitabine plus bevacizumab regimen, Virchow lymph nodes had slowly grown up to 17 mm. Because no recurrence had been detected besides Virchow lymph nodes for this follow up period, considering the side effects and quality of life, surgical resection was selected. We performed left supraclavicular lymph node dissection. Histological examination revealed moderately differentiated adenocarcinoma as a metastatic rectal cancer. After the fourth operation, the patient selected follow up without chemotherapy. Now we follow up her without recurrence and keep her quality of life high.

Key words: Rectal cancer; Surgical resection; Virchow lymph node metastasis; Para-aortic lymph node metastasis; Liver metastasis; Peritoneal carcinomatosis; Long-term survival

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Core tip: A 66-year-old female who had para-aortic lymph node metastasis and peritoneal dissemination of rectal cancer underwent Hartmann's operation. Beginning from stage Ⅳ, liver metastasis, para-aortic and hepato-duodenal ligament lymph node and Virchow lymph node recurrence were detected during follow up period. According to the previous reports, the resection of these severe recurrences is controversial. We conducted four operations during 8 years for Stage Ⅳ rectal cancer and its recurrences. Finally, there is no recurrence radiologically. It should be considered that surgical resection may bring longer term survival especially in cases with difficulty in management of chemotherapy.

Takeshita N, Fukunaga T, Kimura M, Sugamoto Y, Tasaki K, Hoshino I, Ota T, Maruyama T, Tamachi T, Hosokawa T, Asai Y, Matsubara H. Successful resection of metachronous para-aortic, Virchow lymph node and liver metastatic recurrence of rectal cancer. *World J Gastroenterol* 2015; 21(44): 12722-12728 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12722.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12722

INTRODUCTION

In recent decades, great progress has been achieved in the management of colorectal cancer, especially in the development of new chemotherapeutic agents and surgical technique. The prognosis of patients with stage Ⅳ colorectal cancer and its recurrence used to be very poor, but depending on metastatic site, surgical resection combined with chemotherapy may bring longer term survival nowadays. Now, we herein report a case of four times successful resections during 8 years for Stage Ⅳ rectal cancer and its liver, para-aortic, hepato-duodenal ligament and Virchow lymph node metastases.

CASE REPORT

A 66-year-old female presented with the main complaint of defecation trouble and abdominal distention. Abdominal X-ray showed much of colon gas and computed tomography (CT) showed hypertrophy and obstruction of rectum and severe colonic dilatation (Figure 1A). No metastasis was revealed in the liver and lungs. Radiographical examination revealed rectosigmoid obstruction and a decompressive tube couldn't pass the stenosis (Figure 1B). Mild dehydration was shown, but other laboratory tests and serum levels of carcino-embryonic antigen and carbohydrate antigen 19-9 were all within normal limits. The preoperative diagnosis was rectal cancer, cSS, cN0, cH0, cP0, cM0 cStage Ⅱ by the Japanese Classification of Colorectal Carcinoma (JCCC)[1]. Hartmann's operation with D3 lymph node dissection was performed and a swelling para-aortic lymph node and a disseminated node near the primary tumor were resected. R0 (microscopically negative margin) surgery was achieved. Histological examination showed moderately differentiated adenocarcinoma, pSS, pN3, pH0, pP1, pM1 (para-aortic lymph node, dissemination) fStage Ⅳ by JCCC (1) (Figure 1C and D). After the operation, the patient received chemotherapy with FOLFIRI regimen (starting doses: irinotecan at 150 mg/m^2, folinic acid at 200 mg/m^2, fluorouracil (5-FU) bolus at 400 mg/m^2 on day 1 and 5-FU continuous intravenous infusion at 2400 mg/m^2 on days 1-2, every two weeks). Between 12 cycles of FOLFIRI regimen, due to the onset of neutropenia (Grade 3), we managed to continue FOLFIRI regimen with extension of interval period and dose reduction. After 12 cycles of FOLFIRI regimen (one year after first operation), CT detected an 11 mm of liver metastasis in the postero-inferior segment of right hepatic lobe (Figure 2A and B). With diagnosis of liver metastatic recurrence, we performed partial hepatectomy. Histological examination revealed moderately differentiated adenocarcinoma as a metastatic rectal cancer with cut end microscopically positive (R1) (Figure 2C). After the second operation, the patient received chemotherapy with TS1 alone for 2 years. For 2 years, due to the onset of neutropenia (Grade 3), we managed to continue TS1 administration with extension of interval period and dose reduction. Finally, because of no recurrence, TS1 administration was stopped by mutual consultation between the patient

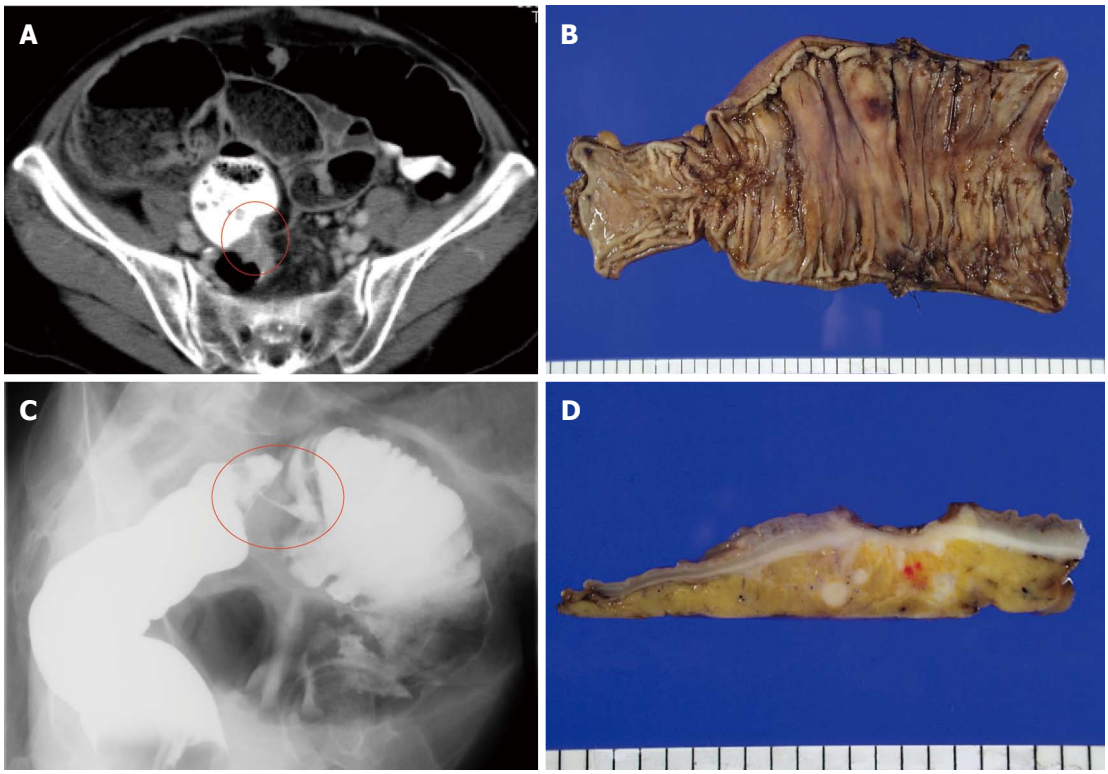

Figure 1 Computed tomography showed hypertrophy and obstruction of rectum and severe colonic dilatation (A); Radiographical examination revealed rectosigmoid obstruction (B); and Histological examination showed moderately differentiated adenocarcinoma, pSS, pN3, pH0, pP1, pM1 (para-aortic lymph node, dissemination) fStage Ⅳ (C and D).

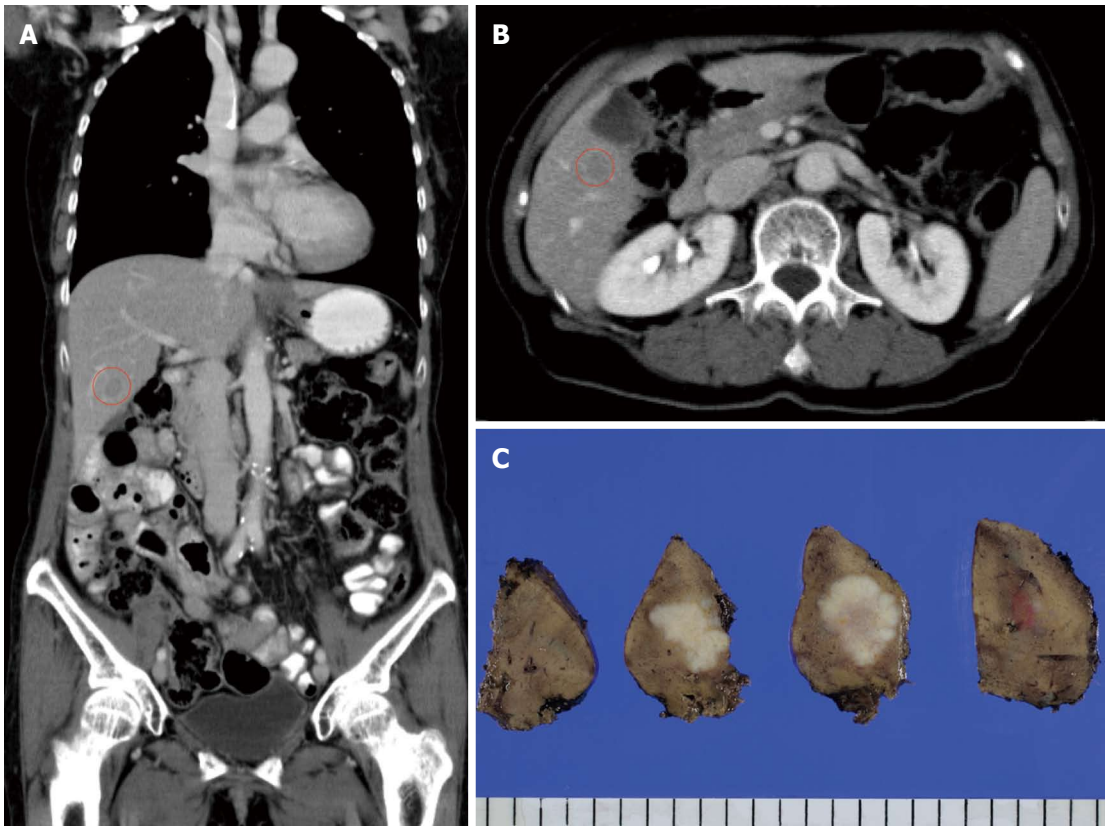

Figure 2 Computed tomography detected an 11 mm of liver metastasis in the posteroinferior segment of right hematic lobe (A and B); and Histological examination revealed moderately differentiated adenocarcinoma as a metastatic rectal cancer with cut end microscopically positive (C).

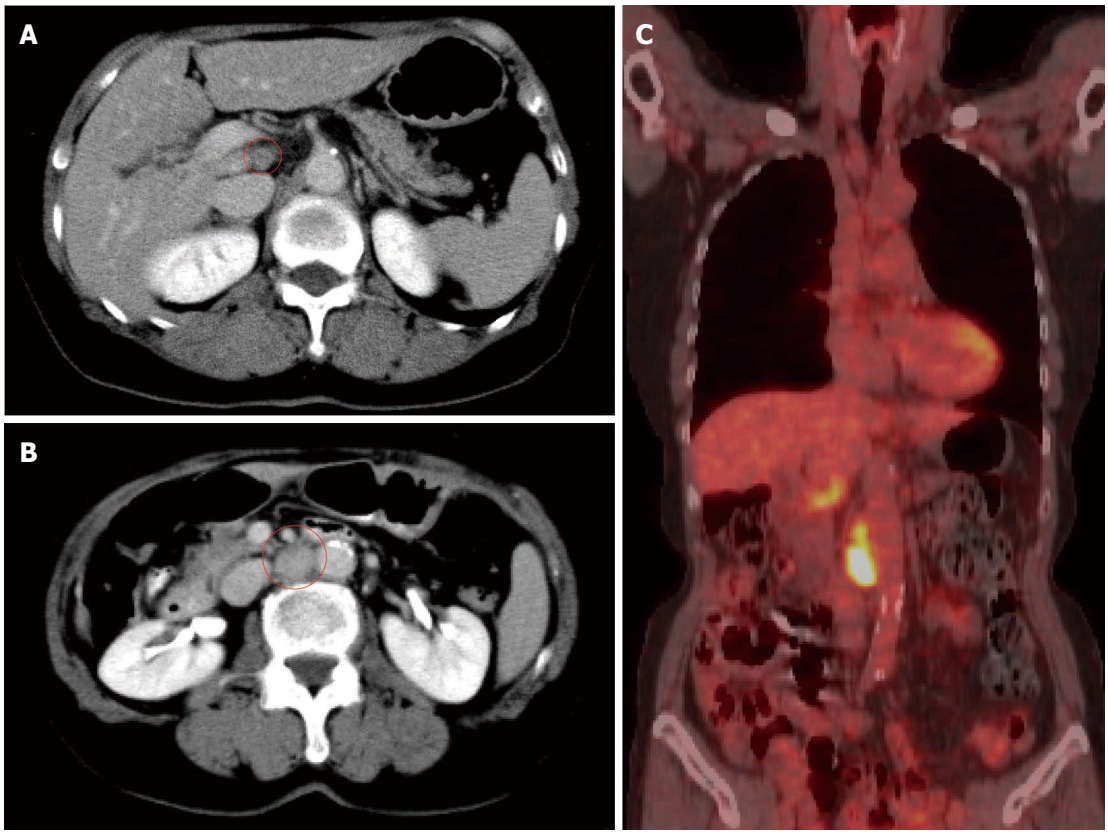

Figure 3 Computed tomography detected a 20 mm of para-aortic lymph node metastasis and a 10 mm of lymph node metastasis at the hepato-duodenal ligament (A and B); and Positron emission tomography was performed and detected the recurrences at the same lesions (C).

and us. Ten months after the break, CT detected a 20 mm of para-aortic lymph node metastasis and a 10 mm of lymph node metastasis at the hepato-duodenal ligament (Figure 3A and B). Positron emission tomography was performed and detected the recurrences at the same lesions (Figure 3C). With diagnosis of lymph node metastatic recurrences, we performed lymph node dissection (4 years after first operation). R0 surgery was achieved. Histological examination revealed moderately differentiated adenocarcinoma as metastatic rectal cancer in para-aortic and hepato-duodenal ligament areas. After the third operation, we started chemotherapy with modified FOLFOX6 regimen (starting doses: oxaliplatin at 85 mg/m^2, intravenous infusion folinic acid at 200 mg/m^2, bolus 5-FU at 400 mg/m^2 on day 1 and continuous intravenous infusion of 5-FU at 2400 mg/m^2 on days 1-2, every two weeks). After 2 cycles of modified FOLFOX6 regimen, due to the onset of neutropenia and liver dysfunction, we switched to capecitabine alone and continued it for 6 mo and then stopped. Eleven months after the break, CT detected two swelling 12 mm of lymph nodes at the left supraclavicular (Figure 4A). With diagnosis of Virchow lymph node metastatic recurrence, we started chemotherapy with capecitabine plus bevacizumab regimen (5 years and 6 mo after first operation). Due to the onset of neutropenia and hand foot syndrome (Grade 3), we managed to continue capecitabine administration with extension of interval period and dose reduction. After 2 years and 2 mo from starting capecitabine plus bevacizumab regimen, Virchow lymph nodes had slowly grown up to 17 mm (Figure 4B). Because no recurrence had been detected besides Virchow lymph nodes for this follow up period, considering the side effects and quality of life, surgical resection was selected by mutual consultation between the patient and us. We performed left supraclavicular lymph node dissection (8 years after first operation). R0 surgery was achieved. Histological examination revealed moderately differentiated adenocarcinoma as a metastatic rectal cancer. After the fourth operation, the patient selected follow up without chemotherapy. Now we follow up her without recurrence and keep her quality of life high. Figure 5 shows the clinical course.

DISCUSSION

As a result of the significant advances in the clinically available chemotherapeutic agents, such as 5-FU, leucovorin and oxaliplatin combination chemotherapy (FOLFOX) and 5-FU, leucovorin and irinotecan combination chemotherapy (FOLFIRI), the median survival time of patients with metastatic colorectal cancer improved to > 20 mo in a recently published report[2]. Furthermore, recently, bevacizumab and anti-

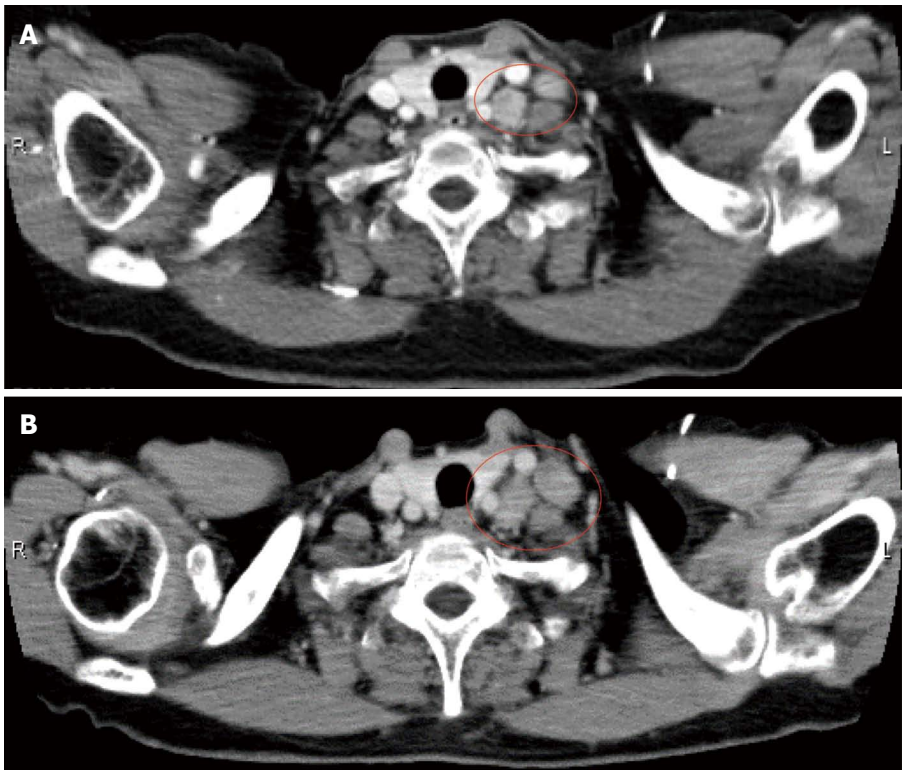

Figure 4 Computed tomography detected two swelling 12 mm of lymph nodes at the left supraclavicular (A); and Virchow lymph nodes had slowly grown up to 17 mm (B).

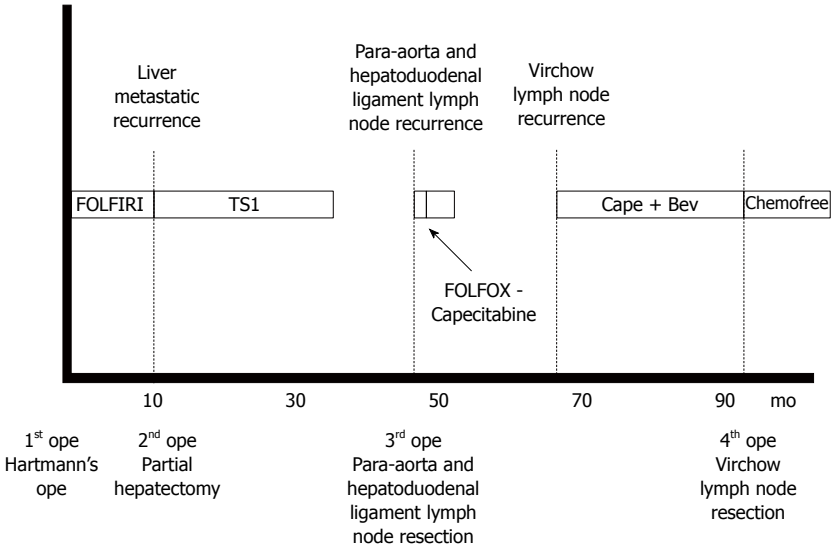

Figure 5 Clinical course of this case.

EGFR monoclonal antibody have been reported to improve the survival times of patients[3,4]. Many kinds of chemotherapeutic agents are available, but it is important to manage side effects and keep quality of life high[5].

In colorectal cancer, up to about 20% of all patients present with stage Ⅳ disease at initial diagnosis[6,7]. Liver, lung and peritoneum are the most frequently observed metastatic site. It was reported that sites of metastasis were liver 85%, lung 4% and peritoneum 10%[7]. For patients with resectable metastatic lesion, R0 surgery was thought to lead to improvement of prognosis. In the cases of liver or lung metastasis, this policy has been well acceptable and 5 year survival rates were reported to be above 60%[8,9]. Peritoneal carcinomatosis is thought to result from peritoneal spread of cancer cells or seeding of the peritoneum during surgery, and it has been associated with a median survival time of 7 mo[10]. Recently, the combination of multimodality therapy

and cytoreduction surgery has been accepted and using this approach, median survival exceeding 60 mo has been reported[11]. Although in the limited cases, surgical resection was acceptable option for the patient with peritoneal carcinomatosis from colorectal cancer.

Extra-mesenteric lymph node metastasis such as para-aortic lymph node and lateral pelvic lymph node metastasis are thought to be poor prognostic risk factors and the indication of surgical resection for these conditions is controversial[12]. On the other hand, para-aortic lymph node recurrence after curative surgery for colorectal cancer is more rare condition. It was reported that isolated para-aortic lymph node recurrence was identified in 1.3% of the patients who underwent curative surgery for colorectal cancer, and the median survival time from para-aortic lymph node recurrence for the resected patients was 34 mo, whereas it was 12 mo for those who did not undergo resection[13]. Lymph node metastasis outside the mesenteric lesion may represent metastasis from metastasis. Traditionally, metastatic disease in the hilar or para-aortic lymph nodes has been considered a strong relative contraindication to surgery due to poor prognosis. However, more recently, with more effective chemotherapy, the role of resection of these extra hepatic lymph node metastasis has been reconsidered. The patients of colorectal liver metastasis with lymph node metastasis in hepato-duodenal ligament and retro-pancreatic area had a 5-year survival greater than 30%. These data may have important implications to help select patients for surgical resection. On the other hand, these patients with lymph node metastasis in para-aortic area had median survival of only 17 mo and every patient experienced a recurrence. On the basis of these data, surgical resection for the cases with para-aortic lymph node metastasis should be more controversial[14]. Left supraclavicular lymph node is often called Virchow lymph node. The lymphatic vessels including the thoracic duct arrive at the Virchow lymph node, and this is recognized as being "final destination" of lymph nodes[15]. The metastasis to this lesion is thought to be extremely poor prognostic risk factor and the evidence of the treatment option for this is insufficient. There are some reports about synchronous or metachronous metastasis of Virchow lymph node which were treated by chemotherapy or chemoradiotherapy and achieved good prognosis[16,17].

In this case, para-aortic lymph node metastasis and peritoneal dissemination of rectal cancer were revealed in first operation. Beginning from stage Ⅳ, three times operations were done for liver metastatic recurrence, para-aortic and hepato-duodenal ligament lymph node recurrence and Virchow lymph node recurrence. According to previous reports, the resection of liver metastatic recurrence was acceptable, but the indication of the extrahepatic disease, such as para-aortic, hepato-duodenal ligament and Virchow lymph node is controversial. Furthermore, para-aortic and Virchow lymph node recurrences may be thought to be extremely poor prognostic factors. Meanwhile, this patient had difficulty with the management of chemotherapeutic side effects. In fact, extension of interval period and dose reduction of chemotherapy was necessary and her quality of life was low due to the onset of neutropenia and other side effects. As to Virchow lymph node metastasis, during the period of chemotherapy which had been continued for two years, other new metastatic lesion had not revealed and the target lesion had been slowly increasing. Although we could change the regimen and continue other chemotherapy, the patient didn't wish to suffer new side effect and preferred surgical resection. It was not easy to propose the option of surgical resection, but we thought that the option of surgery could be acceptable when adequate informed consent about the risks, benefits and other therapeutic options are obtained from the patient. After the R0 surgery of Virchow lymph node recurrence, the necessity of chemotherapy may be controversial. The patient understood the risk of recurrence and preferred the follow up without chemotherapy with keeping her quality of life high. In this case, we conducted four times operations during 8 years for Stage Ⅳ rectal cancer and its recurrences. As a result, now, there is no recurrence lesion radiologically.

Although surgery alone for sever metastatic recurrence is rarely selected due to the advance of multimodality therapy, it should be considered that surgical resection may bring longer term survival especially in cases with difficulty in management of side effects or necessity of dose reduction chemotherapy. In the recurrent lesion without enough data about long term prognosis, such as para-aortic and Virchow lymph node, we hope that the reports like our approach will be accumulate and make use in decision of therapeutic options in future.

COMMENTS

Case characteristics

A female patient underwent four times operations during 8 years for Stage Ⅳ rectal cancer and its liver, para-aortic, hepatoduodenal ligament and Virchow lymph node metastasis.

Clinical diagnosis

Rectal cancer and its recurrence.

Imaging diagnosis

Computed tomography showed liver, para-aortic, hepato-duodenal ligament and Virchow lymph node metastatic recurrence.

Pathological diagnosis

Histological examination showed moderately differentiated adenocarcinoma.

Treatment

Surgical resection and chemotherapy were performed.

Related reports

According to previous reports, the resection of liver metastatic recurrence was acceptable, but the indication of the extrahepatic disease, such as para-aortic, hepato-duodenal ligament and Virchow lymph node is controversial. There are some reports about synchronous or metachronous metastasis of Virchow lymph node which were treated by chemotherapy or chemoradiotherapy (not by surgical resection) and achieved good prognosis.

Explanation of terms

Left supraclavicular lymph node is often called Virchow lymph node. The lymphatic vessels including the thoracic duct arrive at the Virchow lymph node, and this is recognized as being the end of lymph nodes.

Experiences and lessons

Although surgery alone for sever metastatic recurrence is rarely selected due to the advance of multimodality therapy, it should be considered that surgical resection may bring longer term survival especially in cases with difficulty in management of side effects or necessity of dose reduction chemotherapy.

Peer-review

This paper is good.

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P- Reviewer: Brajuskovic GN, Marickar YMF **S- Editor**: Ma YJ
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DOI: 10.3748/wjg.v21.i44.12729

World J Gastroenterol 2015 November 28; 21(44): 12729-12734
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Management of early hepatic artery occlusion after liver transplantation with failed rescue

Chih-Yang Hsiao, Cheng-Maw Ho, Yao-Ming Wu, Ming-Chih Ho, Rey-Heng Hu, Po-Huang Lee

Chih-Yang Hsiao, Cheng-Maw Ho, Yao-Ming Wu, Ming-Chih Ho, Rey-Heng Hu, Po-Huang Lee, Department of Surgery, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei 100, Taiwan

Author contributions: Hsiao CY, Ho CM and Hu RH contributed to data collection, interpretation of data, and drafted the manuscript; Ho MC and Hu RH contributed to study concept and design; Hsiao CY, Ho CM, Wu YM, Ho MC, Hu RH and Lee PH contributed to critical revision of the manuscript.

Conflict-of-interest statement: All authors declare no conflicts of interest.

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Correspondence to: Ming-Chih Ho, MD, Attending Surgeon, Department of Surgery, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei 100, Taiwan. mcho1215@ntu.edu.tw
Telephone: +886-2-23123456
Fax: +886-2-23934358

Received: May 20, 2015
Peer-review started: May 22, 2015
First decision: June 2, 2015
Revised: June 23, 2015
Accepted: August 30, 2015
Article in press: August 31, 2015
Published online: November 28, 2015

Abstract

Hepatic artery thrombosis is a serious complication after liver transplantation which often results in biliary complications, early graft loss, and patient death. It is generally thought that early hepatic artery thrombosis without urgent re-vascularization or re-transplantation almost always leads to mortality, especially if the hepatic artery thrombosis occurs within a few days after transplantation. This series presents 3 cases of early hepatic artery thrombosis after living donor liver transplantation, in which surgical or endovascular attempts at arterial re-vascularization failed. Unexpectedly, these 3 patients survived with acceptable graft function after 32 mo, 11 mo, and 4 mo follow-up, respectively. The literatures on factors affecting this devastating complication were reviewed from an anatomical perspective. The collective evidence from survivors indicated that modified nonsurgical management after liver transplantation with failed revascularization may be sufficient to prevent mortality from early hepatic artery occlusion. Re-transplantation may be reserved for selected patients with unrecovered graft function.

Key words: Complication; Hepatic artery; Thrombosis; Liver transplantation; Revascularization

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Core tip: We present 3 cases of early hepatic artery thrombosis after living donor liver transplantation, in which surgical or endovascular attempts at arterial re-vascularization failed. Unexpectedly, these 3 patients survived with acceptable graft function after 32 mo, 11 mo, and 4 mo follow-up, respectively. The literatures on factors affecting this devastating complication were reviewed from an anatomical perspective. Our three cases raise the possibility that a modified nonsurgical management strategy may be sufficient for recovery from early hepatic artery thrombosis after liver transplantation with failed revascularization procedures.

Hsiao CY, Ho CM, Wu YM, Ho MC, Hu RH, Lee PH. Management of early hepatic artery occlusion after liver transplantation with failed rescue. *World J Gastroenterol* 2015; 21(44): 12729-12734 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i44/12729.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i44.12729

INTRODUCTION

Hepatic artery thrombosis (HAT) is a serious complication after liver transplantation (LT) which often results in biliary complications, early graft loss, and patient death[1-3]. HAT is defined according to the time of onset, with early HAT occurring 30 d or less after LT and late HAT occurring more than 30 d after LT[2]. In a large study of 21822 patients who underwent LT[3], the overall incidence of early HAT was 4.4%, higher in children than in adults (8.3% *vs* 2.9%), and diagnosed at a median of postoperative day (POD) 7. Early HAT resulted in an overall re-transplantation rate of 53.1% (children higher than adults, 62% *vs* 50%) and an overall mortality rate of 33.3% (adults higher than children, 34.3% *vs* 25%)[3]. It is generally thought that early HAT (especially within the first few days after transplantation) without urgent re-vascularization or re-transplantation almost always leads to mortality. However, in this case series, 3 patients with early HAT after living donor LT with failed arterial re-vascularization survived with acceptable graft function after 32 mo, 11 mo, and 4 mo follow-up, respectively (Table 1). Possible explanations were discussed from an anatomical perspective. Our three cases raise the possibility that a modified nonsurgical management strategy may be sufficient for recovery from early HAT after LT with failed revascularization procedures.

CASE REPORT

Case 1

A 60-year-old woman with recurrent hepatocellular carcinoma (HCC) and hepatitis C virus-related liver cirrhosis underwent living donor LT (right lobe from her 31-year-old son). Hepatic artery anastomosis was performed smoothly under an operating microscope. Extubation was performed on POD 2. However, she had abrupt elevation of liver enzymes and hyperbilirubinemia on POD 7 [alanine transaminase (ALT) 1183 U/L from 211 U/L on POD 6, total bilirubin (T-bil) 4.25 mg/dL from 1.78 mg/dL]. Doppler ultrasonography showed no HA blood flow. CT angiography further disclosed the absence of intrahepatic arterial flow and a hypodense lesion suspected to be an infarction at S7-8 of the liver. Angiography confirmed the diagnosis of thrombosis at the proper HA anastomosis (Figure 1), and the angiographic micro-catheter failed to pass through the occlusion site. She then underwent urgent laparotomy. Re-anastomosis of the HA failed and the HA graft was not suitable for another attempt at re-anastomosis. Under supportive treatment, the patient's liver function recovered gradually (POD 31: ALT 160 U/L, T-bil 2.02 mg/dL) and she was discharged 31 d after liver transplantation. The patient took aspirin 100 mg daily thereafter. She had 3 episodes of biliary tract infection (BTI) on the 2nd, 4th, and 5th month after LT, which required hospitalization for intravenous antibiotics treatment. CT scan at the post-operative 5th month showed no remarkable intrahepatic duct dilatation or infarction of the liver parenchyma. She has remained well with normal graft function after 32 mo of follow-up.

Case 2

A 49-year-old man with alcoholic liver cirrhosis underwent living donor LT (right lobe from her 23-year-old son). The HA was anastomosed end-to-end under an operating microscope. Extubation was performed on POD 1. Elevation of liver enzymes and hyperbilirubinemia were found on POD 1 (ALT 456 U/L from 17 U/L, T-bil 7.78 mg/dL from 1.73 mg/dL). Doppler ultrasonography showed no HA flow. CT angiography disclosed total occlusion of the grafted HA. An attempt to place an intra-arterial catheter for endovascular management on POD 1 resulted in extravasation distal to the anastomosis site. On POD3, an intra-arterial catheter was placed at the anastomosis site for endovascular thrombolysis, but still no arterial flow was noted. On POD7, the intra-arterial catheter was re-implanted for urokinase infusion (60000 IU/h for 4 h), but angiography on POD8 showed persistent thrombosis at the anastomosis site (Figure 2A). The patient's liver function improved gradually under supportive treatment (POD 17: AST 53 U/L, ALT 169 U/L, T-bil 2.98 mg/dL, D-bil 1.51 mg/dL) and he was discharged on POD 17 after LT. The patient took aspirin 100 mg daily after discharge. In the following 9 mo, he had one episode of BTI at the 7th month after LT which required hospitalization for intravenous antibiotics treatment. Magnetic resonance angiography on the 8th month after LT showed recanalization of the intrahepatic artery *via* another artery (possibly the right inferior phrenic artery; Figure 2B). He has remained well with normal graft function after 11 mo of follow-up.

Case 3

A 13-year-old boy with primary sclerosing cholangitis (after common bile duct excision and Reux-en-Y hepaticojejunostomy at age 11) underwent living donor LT (left lobe from his 44-year-old mother). Hepatic artery anastomosis had been performed twice because of donor artery intima dissection. He had elevated liver enzymes on POD1 (ALT 699 U/L from 61 U/L, T-bil 8.21 mg/dL from 1.77 mg/dL). CT on POD 2 showed occlusion of the proper HA at the anastomosis

Table 1 Demographic and clinical data of patients with early hepatic artery thrombosis after living donor liver transplantation

	Case 1	Case 2	Case 3
Age (yr)	60	49	13
Gender	Female	Male	Male
Indication of LT	HCV/HCC	Alcoholic cirrhosis	PSC
Donor age (yr)/gender	31/male	23/male	44/female
Graft	Right lobe	Right lobe	Left lobe
HA anastomosis condition	9-0, once	9-0, twice	8-0, twice
	82 min	36 + 43 min	30 + 99 min
Time of diagnosis of HAT	POD 7	POD 1	POD 2
Management	Surgical reanastomosis, failed	Angiographic procedure, failed	Angiographic procedure, failed
Discharge time	POD 31	POD 17	POD 36
Follow-ups	32 mo	11 mo	4 mo
	(BTI x 3)	(BTI x 1)	(no BTI)

LT: Liver transplantation; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis; POD: Postoperative day; BTI: Biliary tract infection.

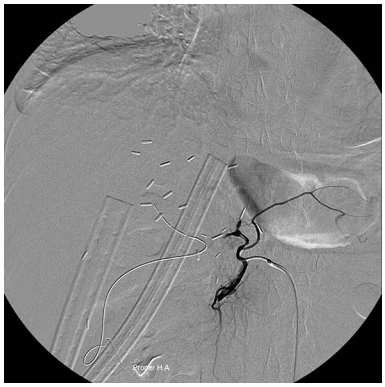

Figure 1 Angiography in a 60-year-old woman 9 d after living donor liver transplantation shows thrombosis at the proper hepatic artery anastomosis, in which endovascular intervention failed to restore blood flow through the hepatic artery occlusion.

site. Angiography showed total occlusion of the proper HA (Figure 3), prompting urokinase 20000 IU injection through a microcatheter with its tip inserted within the thrombosed proper HA, but in vain. The patient's liver function improved gradually with no further treatment of the thrombosed HA (POD 36: ALT 23 U/L, T-bil 0.69 mg/dL). He was discharged on POD 36 after LT and prolonged ascites drainage. He has remained well with normal graft function 4 mo after LT without any BTI episode during follow-up.

DISCUSSION

HAT is a serious complication after LT which often results in patient death and is the 2nd leading cause of early graft failure after primary nonfunction[1]. The etiology of early HAT is thought to be related not only to surgical factors such as vessel kinking, stenotic anastomosis, and intimal dissection, but also to non-surgical factors such as elderly donors, hypercoagulable state, and rejection episodes[4]. Risk factors of early HAT reported in literature include cytomegalovirus mismatch, re-transplantation, use of an arterial conduit, prolonged operation time, low recipient body weight, variant arterial anatomy, lower volume LT hospital[3], and delay in arterial reperfusion[4]. Besides, use of continuous rather than interrupted sutures to anastomose the ends of an hepatic artery was associated with higher incidence of HAT[5]. The most frequent clinical presentation (30%) of early HAT is acute fulminant hepatic failure[1], and the diagnosis of early HAT is often confirmed by thrombo/embolic occlusion of the hepatic artery on Doppler ultrasonography and/or CT/angiography. Routine Doppler ultrasonography in the first 3 d after LT allows early detection of HAT, and makes rescue interventions before liver damage possible[6]. Introduction of microvascular reconstruction has significantly decreased the incidence of HAT[7].

Therapeutic options for HAT include arterial revascularization and re-transplantation. Revascularization could be surgical re-anastomosis (or thrombectomy) or endovascular treatments such as intra-arterial thrombolysis and percutaneous transluminal angioplasty with/without stent placement or balloon dilatation. Although re-transplantation is traditionally the gold standard of therapy for HAT, in areas with organ shortage such as Asia, timely re-transplantation may not be feasible. Thus endovascular or surgical revascularization is often the first line treatment for patients with HAT. Arterial revascularization *via* endovascular or surgical procedures may reduce graft loss and improve outcome in both adult[8] and pediatric[9] LT recipients with early HAT. Most studies suggest the use of endovascular urokinase or heparin for HAT after LT[10], which has a 68% success rate (with internal bleeding as its most common complication)[10]. Several studies reported that antiplatelet prophylaxis can reduce the incidence of HAT in selected adult patients after LT, with no increase in the incidence of bleeding events and wound complications[11,12].

HAT causes ischemic injury of the biliary system and liver parenchyma leading to biliary necrosis

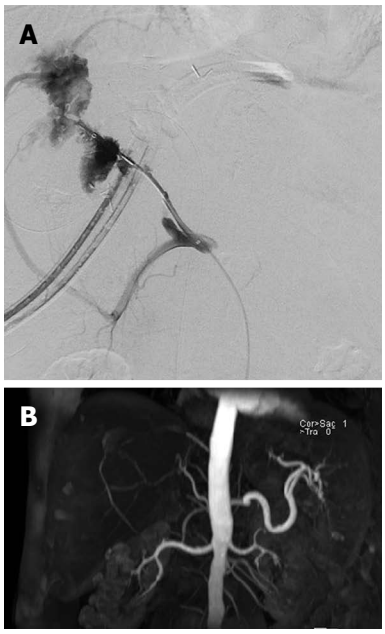

Figure 2 Angiography in a 49-year-old man 8 d after living donor liver transplantation shows persistent thrombosis at the anastomosis site of hepatic artery (A), however magnetic resonance angiography at the 8th month after liver transplantation shows recanalization of intrahepatic artery *via* right inferior phrenic artery (B).

and the formation of liver abscesses[1,2]. In our 3 cases, endovascular or surgical revascularization was performed but failed to restore blood flow. However, the grafts functioned well, without the need for re-transplantation. Recently, Boleslawski *et al*[13] recommended HA ligation as a reasonable option for HA ruptures following LT. Of the seven patients who received HA ligation for HA rupture, six survived well with functional grafts during follow-up. It is unclear why these patients did not develop severe ischemic cholangiopathy. Looking at this problem from an anatomical perspective, the biliary system has three zones with different blood supplies: the intrahepatic bile ducts, hilar ducts, and extrahepatic bile ducts. The intrahepatic biliary ducts are surrounded by a rich microvascular network (the peribiliary plexus)[14]. Some experimental studies have supported the suggestion of arterioportal communication through the peribiliary plexus[15]. The peribiliary plexus represents a collateral source of arterial blood to the liver when the hepatic artery is occluded[16]. Therefore, biliary ischemia of the distal bile ducts usually occurs because of injury to the arteries supplying the distal extrahepatic ducts but is not always inevitable because of the existence of arterioportal collaterals[17]. If the hepatic ducts of a liver graft were transected at a proximal level during the operation to remove the living donor's liver, the risk of devastating biliary ischemia after HA occlusion would be expected to be lower because proximal hepatic ducts behave more like the intrahepatic ducts. In 2 of our 3 cases, the bile ducts of the liver grafts were proximally transected, supporting our hypothesis.

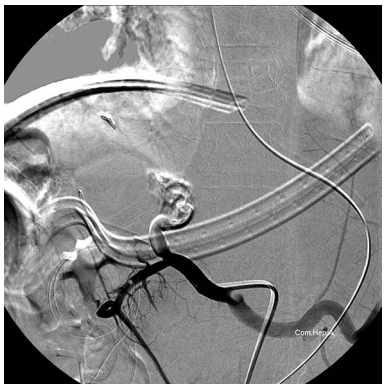

Figure 3 Angiography in a 13-year-old boy 2 dafter living donor liver transplantation shows total occlusion of the proper hepatic artery despite injection of 10000 IU of urokinase through a microcatheter with tip inserted into the thrombosed proper hepatic artery.

In case 1, the bile ducts in the right lobe liver graft had two orifices, and in case 3, left donor hepatectomy was performed. Moreover, in a rat model of liver transplantation, arterial reconstruction was regarded as unnecessary and thus not routinely performed[18]. Recent studies reported the same survival rate in LT rats with HA reconstruction and LT rats without HA reconstruction (though the latter are associated with more graft parenchymal damage in the early postoperative period)[15]. The detail mechanism of hepatic arterial collateral formation *via* inferior phrenic artery is not clear, however, arterial collaterals through the bare area of liver in recurrent hepatocellular carcinoma after transarterial chemoembolization is not uncommon.

Development of HAT within 7 d of LT is an indication of emergency transplant status (UNOS status 1)[19]. However, emergency LT and especially emergency re-transplantation are often associated with lower patient and graft survival as compared with non-emergency LT and elective re-transplantation[20-23]. Considering that the real outcome of early HAT may not be as bad as once thought, patients may not need to undergo high risk emergency re-transplantation routinely. As a result, we recommend a short-term "wait and see" policy when patients developed early HAT even if it happens within 7 d of LT, as long as their biochemical and hemodynamic status remains relatively stable. Re-transplantation may be reserved for select patients who present with unrecovered graft function after early HAT and fail to be rescued by endovascular or surgical intervention. Besides, mesenteric arteriovenous shunt (partial portal arterialization) had been reported effective in preventing hepatic failure caused by interruption of hepatic artery flow, and might also be an option to gain time until collateral arterial vessels develop or re-transplant[24]. In summary, our cases illustrate that very early HAT without successful urgent re-vascularization or urgent re-transplantation may not always lead to mortality. In patients with very early HAT, urgent endovascular intervention or surgical

exploration for re-vascularization is recommended. However, when re-vascularization fails, observation with supportive care such as maintaining relatively high blood pressure and arterial patency by anti-platelet therapy may be a feasible strategy. Re-transplantation may be preserved for selected cases such as those who fail to recover graft function.

COMMENTS

Case characteristics

Three patients presented with symptoms of liver dysfunction within one week after living-donor liver transplantations.

Clinical diagnosis

Hepatic artery thrombosis after living donor liver transplantations.

Differential diagnosis

Primary graft nonfunction, rejection, portal vein thrombosis.

Laboratory diagnosis

The three patients had various degree of elevation of liver enzymes and hyperbilirubinemia.

Imaging diagnosis

For three cases, computed tomographic angiography scan showed occlusion of hepatic artery.

Pathological diagnosis

No pathological diagnosis in these three cases.

Treatment

The first patient underwent urgent surgical re-anastomosis of the hepatic artery but failed. All of the three patients underwent endovascular treatment but failed.

Related reports

One recent study recommended hepatic artery ligation as a reasonable option for hepatic artery ruptures following liver transplantations.

Term explanation

Hepatic artery thrombosis is a serious complication after liver transplantation which often results in biliary complications, early graft loss, and patient death.

Experiences and lessons

These three cases raise the possibility that a modified nonsurgical management strategy may be sufficient for recovery from early hepatic artery thrombosis after liver transplantation with failed revascularization procedures.

Peer-review

The authors have described three cases of early hepatic artery thrombosis after living donor liver transplantation, in which surgical or endovascular attempts at arterial re-vascularization failed. Unexpectedly, these 3 patients survived with acceptable graft function after 32 mo, 11 mo, and 4 mo follow-up, respectively. These cases outlines that a modified nonsurgical management strategy may be sufficient for recovery from early hepatic artery thrombosis.

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P- Reviewer: Hashimoto N **S- Editor**: Ma YJ **L- Editor**: A
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ISSN 1007-9327

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