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

World Journal of *Gastroenterology*

World J Gastroenterol 2017 November 21; 23(43): 7653-7812

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

Contents

Weekly Volume 23 Number 43 November 21, 2017

Contents

World Journal of Gastroenterology
Volume 23 Number 43 November 21, 2017

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Ze-Mao Gong, Vice Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
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PUBLICATION DATE
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World J Gastroenterol 2017 November 21; 23(43): 7653-7656

DOI: 10.3748/wjg.v23.i43.7653

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

EDITORIAL

Noncoding RNAs as drivers of the phenotypic plasticity of oesophageal mucosa

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Author contributions: Facchin S, Munari G, Fanelli GN, Lorenzon G reviewed the literature and wrote the first draft of the paper; Fassan M and Savarino E conceived the idea, reviewed the literature and contributed to writing the paper and edited it extensively.

Conflict-of-interest statement: No conflict of interest related to this publication to be declared.

Manuscript source: Invited manuscript

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Received: August 22, 2017
Peer-review started: August 23, 2017
First decision: September 28, 2017
Revised: October 18, 2017
Accepted: October 26, 2017
Article in press: October 26, 2017
Published online: November 21, 2017

Abstract

The histological commitment of the lower oesophageal mucosa largely depends on a complex molecular landscape. After extended inflammatory insult due to gastroesophageal reflux disease, squamous oesophageal mucosa may differentiate into columnar metaplastic mucosa. In this setting, the presence of intestinal metaplasia is considered the starting point of Barrett's carcinogenetic cascade. Aside from secondary prevention strategies for Barrett's mucosa (BM) patients, there are multiple endoscopic ablative therapies available for BM eradication and for the replacement of metaplastic epithelia with a neosquamous mucosa. However, BM frequently recurs in a few years, which supports the notable phenotypic plasticity of the oesophageal mucosa. In recent years, several reports pinpointed a class of small noncoding RNAs, the microRNAs (miRNAs), as principal effectors and regulators of oesophageal mucosa metaplastic (and neoplastic) transformation. Because of miRNAs notable stability in fixed archival diagnostic specimens, expression profiling of miRNAs represent an innovative diagnostic, prognostic and predictive tool in the stratification of phenotypic alterations in the oesophageal mucosa.

Key words: Barrett's mucosa; Biomarkers; Noncoding RNAs; MicroRNAs; Metaplasia

Core tip: Recent advances in understanding the molecular role of noncoding RNAs in Barrett's carcinogenesis have significantly contributed to the identification of novel and alternative molecular pathways involved in this carcinogenetic setting. In the future, these data may significantly influence the planning of secondary prevention strategies for Barrett's mucosa patients and help to select new therapies.

Fassan M, Facchin S, Munari G, Fanelli GN, Lorenzon G, Savarino E. Noncoding RNAs as drivers of the phenotypic plasticity of oesophageal mucosa. *World J Gastroenterol* 2017; 23(43): 7653-7656 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7653.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7653

INTRODUCTION

The phenotypic commitment of gastro-oesophageal junction mucosa suffers from the "original sin" of its extreme morphological plasticity during intrauterine development. Specifically, it corresponds to a hybrid epithelium that can differentiate towards divergent mucosal phenotypes[1].

In the adult, the squamous-differentiated mucosa conserves the ability to metaplasticly reverse its phenotype under the stimulus of long-lasting inflammatory insults, resulting in differentiation into columnar metaplastic mucosa[2]. Among the different columnar phenotypes, however, only the histological finding of intestinal metaplasia is considered by most gastroenterology societies to be a prerequisite for a diagnosis of Barrett's mucosa (BM), the cancerization field in which a significant number of oesophageal adenocarcinomas develop[2]. In fact, BM represents the initial phenotypic shift of a multistep carcinogenetic process known as Barrett's carcinogenesis[3].

Aside from secondary prevention strategies mainly based on endoscopic (and bioptic) surveillance protocols, there are multiple endoscopic therapies available for BM eradication. These include radiofrequency ablation, cryoablation and photodynamic therapy[2]. These eradicative therapies rely of the replacement of BM with neosquamous epithelium, which further supports the extraordinary phenotypic plasticity of the oesophageal mucosa. However, both the stability and functional characteristics of this neosquamous mucosa have not yet completely comprehended. More importantly, it has been shown that BM frequently recurs in a few years, which suggests that the transforming characteristics of the neosquamous oesophageal mucosa do not change after treatment.

Only fragmentary information is available on the molecular changes driving the phenotypic shift from native squamous oesophageal epithelium to metaplastic Barrett's. Most studies have focused on the dysregulation of the Homeobox gene family, which is involved in keeping the squamous commitment of the oesophageal mucosa. In recent years, several reports pinpointed a class of small noncoding RNAs (ncRNAs), the microRNAs (miRNAs), as principal effectors and regulators of oesophageal mucosa plasticity[4].

AFFINITY OF MIRNA ANALYSIS FOR GASTROINTESTINAL BIOPSIES

The great dichotomy observed between the well-established histopathological characterization and classification of the phenotypic lesions occurring in the gastrointestinal tract (both inflammatory and neoplastic) in comparison to their poor molecular typing, is mainly due to the incompatibility of comprehensive molecular testing on formalin-fixed paraffin-embedded (FFPE) tissues. Notably, FFPE specimens currently represent the largest proportion of routine diagnostic gastrointestinal samples.

The introduction of innovative technologies such as targeted next-generation sequencing (NGS) has allowed for feasible, accurate and comprehensive molecular characterization of FFPE neoplastic specimens. However, the molecular landscape of early preneoplastic lesions, such as oesophageal mucosa metaplasia, is mainly characterized by the dysregulation of complex epigenetic pathways rather than the accumulation of genetic alterations. This significantly downgrades the efficacy of NGS applications in the study of (oesophageal) preneoplastic lesions.

Among the different tested biomarkers, miRNAs emerged because of their notable in structural stability, which allows them to be assayed in FFPE tissue samples. The excellent reproducibility and accuracy of miRNA expression profiling in archived specimens has been broadly demonstrated, and the introduction of FFPE-compatible high-throughput miRNA detection technologies, such as microarray profiling, allowed the extensive study of miRNA dysregulation in many gastrointestinal settings. Another important miRNA-related tool is the visualization of miRNA expression at cellular/subcellular level by *in situ* hybridization (ISH), which enabled the discovery of the cellular source of the miRNA's dysregulation (*i.e.*, epithelial *vs* inflammatory commitment).

The expression of miRNAs can be either up-regulated or down-regulated in pathological tissue samples. They can also act as tumour-suppressor genes or oncogenes based on their miRNA-specific downstream target or targets. Notably, different molecular mechanisms, including chromosomal alterations of the miRNA genes, point mutations, epigenetics mechanisms or alterations in the machinery responsible for miRNA production, have been described.

Most miRNA studies in gastrointestinal pathology have consisted of high-throughput profiling to investigate global patterns of miRNA dysregulation.

The so-called "miRNA fingerprints" have been largely demonstrated to discriminate among pre-neoplastic, inflammatory conditions and malignancies, which is an important attribute in the gastrointestinal diagnostic setting.

NONCODING RNA DYSREGULATION DURING BARRETT'S CARCINOGENESIS

Several reports have used comprehensive miRNA expression profiling to demonstrate a clear involvement of miRNA dysregulation in oesophageal Barrett's carcinogenesis[5]. Oesophageal epithelial miRNAs may be used to diagnose BM and possibly monitor its progression to adenocarcinoma.

A recent meta-analysis on this topic revealed that, compared to normal squamous mucosa, BM is characterized by up-regulated miR-192, miR-194 and miR-215 and down-regulated miR-203 and miR-205[6]. In the same analysis, the authors demonstrated that, compared to normal squamous mucosa, Barrett's adenocarcinoma had a higher expression of miR-21, miR-192, miR-194 and miR-215 and a reduced expression of let-7c, miR-203, miR-205 and miR-944[6].

Among the most dysregulated miRNAs, the "oncomiR" miR-21 emerged as one of the most highly up-regulated during Barrett's carcinogenesis, being up-regulated in both high-grade dysplastic and adenocarcinoma samples. Notably, this miRNA is exerting its oncogenic function by targeting of several tumour-suppressor genes, such as *PTEN*, *PDCD4*, *RECK* and *TPM1*[5].

Another significantly up-regulated miRNA that did not emerge from the meta-analytic study, is the miR-196a, which targets *ANXA1*, *SPRR2C*, and *S100A9*[5]. Interestingly, the expression of the related proteins of these three targeted genes is characteristically decreased or lost during the neoplastic transformation of oesophageal tissue. Moreover, miR-196a expression, in combination with three other miRNAs profiles (*i.e.*, miR-192, miR-194, and miR-196b), can adequately stratify BM patients according to their risk of disease progression over a course of 5 years[5].

Some miRNAs are organized as a cluster of genes expressed by a single transcription unit, called a polycistron. The miR-106b-25 polycistron on chromosome 7q22.1, which contains miR-25, miR-93 and miR-106b, has been found to be increasingly activated in successive stages of Barrett's carcinogenesis, with potential *in vitro* proliferative, antiapoptotic, and cell cycle promoting effects and *in vivo* tumourigenic effects by targeting p21 and Bim *et al*[5].

Aside from oncogenic miRNAs, other important down-regulated miRNAs during Barrett's carcinogenesis are miR-31 and miR-375, which have been proposed to be specifically associated with early- and late-stage malignant progression, respectively[5].

As stated above, one of the most important goals in the study of Barrett's pathology is to find adequate predictive biomarkers of BM recurrence after endoscopic ablative therapy and formation of the neosquamous epithelium. Dijckmeester and colleagues found that miR-143 expression was significantly higher in neosquamous and normal squamous epithelium from BM patients before and after ablative therapy compared to normal squamous epithelium from control subjects[7]. It is worth adding that miR-143 is highly expressed in normal colon tissues, and it has a significant role in suppressing colorectal cancer cell growth by inhibiting *KRAS* translation. Overall, these data suggest that neosquamous epithelium is an unsteady "flexible" phenotype prone to reversion to BM.

In the current issue of World Journal of Gastroenterology, Sreedharan and colleagues supported the phenotypical fragility of the neosquamous epithelium investigating miRNA expression profiles using high-throughput screening. They found that neosquamous mucosa arising after ablation of BM is characterized by miRNA dysregulation that may contribute to a decreased barrier function that leads to an increased susceptibility to reflux-induced disease (and therefore a faster metaplastic transformation)[8]. Notably, these data may have clinical implications since they open the field to a more tailored medical management of BM patients. Specifically, they suggest the need for more aggressive therapy in ablated patients with a specific miRNA dysregulation who are at higher risk of intestinal metaplasia recurrence.

The recent characterization of the functional relevance of the "noncoding genome" has demonstrated that miRNAs represent just the tip of an iceberg of ncRNA families. This iceberg includes transcribed ultraconserved regions (T-UCRs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lincRNAs) and, overall, the heterogeneous group of long non-coding RNAs (lncRNAs)[9].

Among the others, the actin filament associated protein 1-antisense RNA 1 (AFAP1-AS1) lncRNA is overexpressed in both BM and adenocarcinoma compared to matched normal samples. This finding supports an oncogenic function during oesophageal mucosa transformation[9].

Our group investigated the expression profiles of T-UCRs during Barrett's carcinogenesis using microarray analysis. We found that a 9 T-UCR signature was associated with BM but not with normal squamous mucosa[10]. T-UCRs were discovered in 2004 after bioinformatic comparisons drawn between mouse, rat, and human genomes. They are absolutely conserved (100% identity with no insertions or deletions) between the three vertebrate species. Interestingly, we observed that a peculiar T-UCRs expression profile was

associated with similar histological lesions in humans and in two murine models of Barrett's carcinogenesis, which supports T-UCRs as novel diagnostic tools for the biological profiling of BM-associated lesions.

CONCLUSION

As in other carcinogenetic settings, advances in the understanding of the molecular role of ncRNAs in Barrett's carcinogenesis are significantly contributing to the identification of novel and alternative molecular pathways. These data are starting to influence the planning of secondary prevention strategies and the selection of new therapies[6,9]. In fact, dysregulated expression of miRNAs has been readily detected in a variety of biological fluids obtained from patients with gastrointestinal cancer, highlighting the high molecular stability of miRNAs in these biofluids and providing a biological rationale for developing them as liquid biopsy biomarkers[11]. In comparison to traditional secondary prevention strategies, the liquid biopsy approach is mini-invasive and allows an overall molecular comprehension of the disease, not suffering from the presence of intratumoral molecular heterogeneity.

From a therapeutic perspective, preclinical models have consistently underlined the feasibility and efficacy of ncRNA-based therapies, which have been successfully translated into clinical trials. Of note, in just the past 5 years, over 100 miRNAs antisense oligonucleotide-based therapies have been tested in phase Ⅰ clinical trials, a quarter of which have reached phase Ⅱ/Ⅲ [12]. Overall, these data are highlighting the clinical impact of miRNAs' dysregulation during esophageal carcinogenesis. The next step will be the definitive introduction of the ncRNA world into clinical practice.

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P- Reviewer: Mavridis K, Yu XJ **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Ma YJ

World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7657-7665

DOI: 10.3748/wjg.v23.i43.7657

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

MINIREVIEWS

Functional interaction of endoplasmic reticulum stress and hepatitis B virus in the pathogenesis of liver diseases

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Author contributions: Kim SY and Kyaw YY prepared the manuscript; Cheong J designed and revised the manuscript.

Supported by the National Research Foundation of South Korea, No. NRF-2009-0093195; and Basic Science Research Program through the NRF funded by the Ministry of Education, No. NRF-2013R1A1A2057634.

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

Manuscript source: Invited manuscript

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Received: April 21, 2017
Peer-review started: April 25, 2017
First decision: June 22, 2017
Revised: September 1, 2017
Accepted: November 1, 2017
Article in press: November 1, 2017

Published online: November 21, 2017

Abstract

Hepatitis B virus (HBV) is a non-cytopathic virus that causes acute and chronic inflammatory liver diseases, often leading to the pathogenesis of hepatocellular carcinoma (HCC). Although many studies for the roles of HBV on pathogenesis of the liver diseases, such as non-alcoholic fatty liver disease (NAFLD), hepatic inflammation, cirrhosis, and HCC, have been reported, the mechanisms are not fully understood. Endoplasmic reticulum (ER) and mitochondria have the protective mechanisms to restore their damaged function by intrinsic or extrinsic stresses, but their chronic dysfunctions are associated with the pathogenesis of the various diseases. Furthermore, HBV can affect intra- or extracellular homeostasis through induction of ER and mitochondrial dysfunctions, leading to liver injury. Therefore, the mechanism by which HBV induces ER or mitochondrial stresses may be a therapeutic target for treatment of liver diseases.

Key words: Liver disease; Hepatitis B virus; Hepatitis B virus X protein; Endoplasmic reticulum stress; Unfolded protein response

Core tip: Endoplasmic reticulum (ER) is the major site of protein folding and calcium storage. Beside the role of ER in protein homeostasis, it controls the cholesterol production and lipid-membrane biosynthesis as well as surviving and cell death signaling mechanisms in the cell. It is well-documented that abnormal metabolic regulation induces adverse effects in liver disorders, such as non-alcoholic steatosis hepatitis,

fibrosis, cirrhosis, and hepatocellular carcinoma which are associated with hepatitis B virus (HBV) infection. Recent animal model and human studies have showed ER stress as an emerging factors involved in the development of metabolic and liver diseases. In this review, we will summarize the crucial effects of ER stress response in the pathogenesis of HBV-induced liver diseases.

Kim SY, Kyaw YY, Cheong J. Functional interaction of endoplasmic reticulum stress and hepatitis B virus in the pathogenesis of liver diseases. *World J Gastroenterol* 2017; 23(43): 7657-7665 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7657.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7657

INTRODUCTION

Hepatitis B virus (HBV), a prototype member of the *Hepadnavirus* family, is a small enveloped DNA virus with a virion diameter of 42 nm. The HBV genome is a relaxed circular, partially double-stranded DNA molecule encoding four overlapping open reading frames (ORFs), named C, S, P, and X coding for core protein, surface proteins (pre-S1, pre-S2, and S), DNA polymerase, and X protein, respectively[1]. Of the HBV-encoded proteins, the function of hepatitis B virus X protein (HBx) is not clearly understood, but it may function as a multifunctional transactivator in HBV replication and host gene transcription through interaction with host proteins[2]. HBV primarily infects hepatocytes and causes acute and chronic liver diseases. In particular, chronic HBV infection can lead to cirrhosis of the liver, liver failure, liver cancer, and even death. According to the report of World Health Organization (WHO), there are more than 350 million people worldwide who have chronic HBV infections and more than 780 thousand people die every year due to the acute or chronic HBV infection[3]. To date, many studies have been reported on the molecular mechanisms for relation between HBV infection and pathogenesis of hepatic diseases, but the mechanisms are still not fully understood.

Cellular organelle is a specialized compartment enclosed by lipid bilayers within a cell and has specific functions. It is classified into major organelle such as endoplasmic reticulum (ER), Golgi apparatus, mitochondria, vacuole, and nucleus and minor organelle such as autophagosome, lysosome, peroxisome, and vesicle. Damage or dysfunction of cellular organelles by intra- or extra-cellular stress is associated with the pathogenesis of various diseases. For examples, mitochondrial dysfunction induces the diseases such as myopathy, diabetes, and multiple endocrinopathy[4] and ER dysfunction induces the diseases such as obesity, diabetes, atherosclerosis, and cancer[5]. Here, we review relation between the HBV-encoded proteins and damage of cellular organelles and the influence on pathogenesis of hepatic diseases.

HBV AND INTRACELLULAR ORGANELLE

HBV-mitochondria

Mitochondria are the double membrane-bound structure and consist of five compartments with specialized functions including the outer mitochondrial membrane, the inter membrane space, the inner mitochondrial membrane, the cristae space, and matrix. Mitochondria have their own independent genome which is a single circular DNA molecule encoding 37 genes[6]. Division and genome of mitochondria are similar to those of bacterial cell. Mitochondria play critical roles in production of cellular energy, calcium and redox homeostasis, cellular signaling, regulation of cellular metabolism and cell death, and heat production[7-9]. Of the functions of mitochondria, the most prominent function is to synthesize cellular energy, adenosine triphosphate (ATP), which is used as a source of chemical energy for metabolism and a substrate in signaling pathways[10].

Mitochondria are very dynamic and continually fuse and divide in response to physiological conditions. Moreover, mitochondria are fragmented as the consequence of enhanced fission during apoptosis[11] and elongated to maintain ATP production during starvation[12]. Many mitochondrial protein complexes are composed of nuclear or mitochondrial DNA-encoded proteins. Any imbalance in the complex assembly can lead to accumulation or aggregation of unassembled or unfolded proteins[13]. In order to cope with the accumulation of unassembled or unfolded proteins within mitochondria, mitochondria activates the mitochondrial unfolded protein response (UPR) that up-regulates the expression of mitochondrial chaperones and proteases like ER stress response[14]. Although the environmental conditions inducing mitochondrial stress are still not clearly understood, the accumulated evidences suggest that high levels of reactive oxygen species (ROS) or inhibition of mitochondrial genome replication and transcription can induce mitochondrial UPR[15-17]. Unfolded proteins accumulated in matrix activate mitogen-activated protein kinase kinase (MEK)/c-Jun N-terminal protein kinase 2 (JNK2)/c-Jun pathway and protein kinase R (PKR). Activated c-Jun increases the transcription of transcription factors C/EBP homologous protein (CHOP) and CCAAT/enhancer-binding protein β (C/EBPβ), and then the heterodimer of CHOP and C/EBPβ activate the transcription of mitochondrial proteases and chaperons[18]. Activated PKR phosphorylates eukaryotic translational initiation factor 2α (eIF2α), leading to attenuating translation similar with the protein kinase RNA-like ER kinase (PERK) pathway of ER stress response[19]. In addition, unfolded proteins accumulated

in the intermembrane space (IMS) activate estrogen receptor and NAD-dependent deacetylase sirtuin-3 (SIRT3). Activated estrogen receptor up-regulates the transcription of nuclear respiratory factor 1 (NRF1) and mitochondrial serine protease, high-temperature requirement A2 (HTRA2), and SIRT3 pathway induces anti-oxidant machinery and mitophagy to alleviate mitochondrial stress[20,21].

Accumulated evidences have suggested that HBx protein is associated with mitochondrial aggregation or damage. HBx protein induces an abnormal aggregation of mitochondrial structures at the periphery of nucleus, which may be eventually connected with cell death[22]. In HBx-expressing cells, the abnormal aggregation of mitochondria is induced by the increase of microtubule-dependent dynein activity through HBx-induced p38 mitogen-activated protein kinase (MAPK) activation[23]. Siddiqui group showed that HBx protein is associated with mitochondrial damage through interaction with voltage-dependent anion channel (HVDAC3) [24], which is known as mitochondrial porins and form pores in the outer membranes of mitochondria[25]. The interaction induces the alteration of mitochondrial transmembrane potential leading to generation of ROS, resulting in activation of transcription factors signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NFκB)[24,26]. The ability of HBx protein to transactivate AP-1 and NFB is abolished by positioning HBx protein in the nucleus artificially[27]. These evidences represent the ability of HBx protein as a transactivator, which is to induce gene expression through cytoplasmic factors but not nucleus.

HBx protein can affect mitochondrial functions or cell fate by regulating gene expression or translocating a series of proteins to mitochondria, respectively. HBx down-regulates the expression of mitochondrial encoded subunit proteins of electron transport in oxidative phosphorylation, resulting in a high level of cellular ROS by impairment of electron transport[28]. Besides, HBx down-regulates the expression of nuclear encoded genes involved in mitochondrial β-oxidation of fatty acids, resulting in a low level of cellular ATP by deficiency of energy sources[29]. HBx translocates Raf-1 kinases involved in the Ras-induced MAPK pathway or apoptosis regulator bcl-2-associated X protein (BAX) to mitochondria, leading to hepatic cell proliferation or apoptosis, respectively[30,31]. These evidences suggest that mitochondria dysfunction by HBx contributes to the HBV-induced pathogenesis of hepatocellular carcinoma such as proliferation, metastasis, chemoresistance and other aspects of tumorigenesis.

To date a lot of researches have reported for relation between HBx protein and mitochondrial damage. However, the research for HBx protein and mitochondrial UPR has not yet been reported. Therefore, the research to clarify correlation between HBx protein or HBx-induced mitochondrial damage and mitochondrial UPR needs to be performed in future. In addition, it will be a worthy to research for association between HBx protein and the proteins activated by mitochondrial UPR such as SIRT3, estrogen receptor, CHOP, or C/EBPβ.

HBV-ER

The endoplasmic reticulum (ER) is dynamic tubular structure and forms an interconnected network with almost every membrane-bound organelles, including mitochondria, the Golgi apparatus, endosome, peroxisome, and plasma membrane through contact sites[32,33]. The ER acts as a sensor for intra- or extracellular stimuli and is essential for cell homeostasis. The ER is classified into rough ER and smooth ER, which are externally distinguished by ribosome, a molecular machine synthesizing biological protein. The rough ER has the ribosome binding sites, named translocon on the ER outer membrane and is involved in synthesis, folding, and glycosylation of secretary or integral membrane proteins[34]. Therefore rough ER is well-developed in specialized secretary cells such as hepatocytes, pancreatic islet cells and immune cells. The smooth ER lacks ribosome and involved in several metabolic processes including synthesis of lipids (phospholipids and steroids), metabolism of carbohydrates, regulation of calcium concentration, detoxification of drugs[35]. The smooth ER plays also a fundamental role in the assembly of very low density lipoprotein (VLDL) particles in liver. In addition to the above-mentioned functions, ER also participate in the following processes through contact sites with other organelles: *e.g.*, ER-mitochondria: mitochondria biogenesis, lipid exchange during biosynthesis, and Ca^{2+} transfer from ER to mitochondria[32,35]; ER-Golgi: transport of secretory proteins and non-vesicular lipid transport[36]; ER-endosome: regulation of the intracellular distribution of endosomes[37]; ER-peroxisome: non-vesicular lipid transport[38]; ER-plasma membrane: regulation of phosphatidyl inositol metabolism and non-vesicular sterol transfer[39,40].

Of the ER functions, the proper folding and modification of proteins are the most important and best characterized function of the ER, and are processed under strict quality-control process (QCR)[41]. QCR means that only correctly matured proteins are exported to the Golgi complex and misfolded proteins are left in the ER to complete the process or to degrade the proteins[42]. Many ER-resident proteins such as chaperones, foldases, and lectins are involved in the QCR. Most ER chaperones are Ca^{2+} dependent and have ATPase activities. N-linked glycosylation and disulfide bond formation by foldases also play significant roles in protein maturation[43]. Viral infection induces the synthesis of a vast amount of viral proteins, leading to protein overload in ER. Therefore, the QCR is inhibited by the various stimuli such as Ca^{2+} output, nutrient deficiency or overload, hypoxia, and viral infection. The unfolded or misfolded proteins induced by the stimuli are accumulated and aggregated in ER, leading

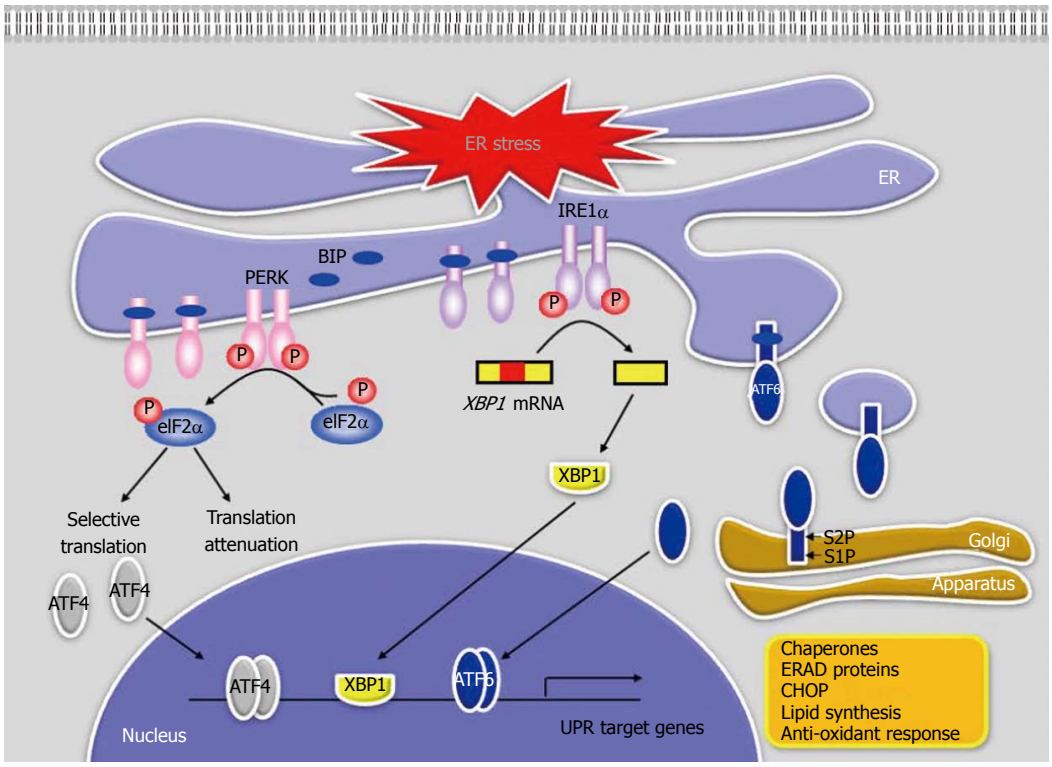

Figure 1 Endoplasmic reticulum stress and unfolded protein response signaling pathways. ER: Endoplasmic reticulum; BIP: Binding immunoglobulin protein; PERK: Protein kinase RNA-like ER kinase; eIF2α: Eukaryotic translational initiation factor 2α; ATF4: Activating transcription factor 4; IRE1: Inositol-requiring protein 1; XBP1: X-box binding protein 1; ATF6: Activating transcription factor 6; S1P: Site 1 protease; S2P: Site 2 protease; UPR: Unfolded protein response; ERAD: ER associated degradation; CHOP: C/EBP homologous protein; P: Phosphate.

to ER stress. Fortunately, the ER has four adaptive mechanisms, named UPR, to alleviate ER stress and restore ER to its normal physiological conditions[5,41]: (1) translational attenuation for reduction of protein load, (2) induction of the expression of ER chaperones and foldases for enhancement of folding capacity, (3) induction of the expression of ER associated degradation (ERAD) proteins for clearance of unfolded proteins, (4) induction of apoptosis for removal of cells impaired by ER stress. In mammalian cells, these four responses are regulated by the regulatory pathways as described below (Figure 1).

PERK pathway: PERK is a type Ⅰ transmembrane protein located in the ER. Under a normal condition, PERK exists as a monomer, an inactive state, by binding with ER chaperone, 78kD glucose-regulated protein (GRP78)/binding immunoglobulin protein (BiP)[44]. In response to ER stress, BiP is dissociated from PERK, which is activated through oligomerization and trans-autophosphorylation[45]. Activated PERK phosphorylates eIF2α, leading to translational attenuation[45]. Interestingly, phosphorylated eIF2α allows the translation of activating transcription factor 4 (ATF4) mRNA[46] and ATF4 induces the expression of UPR target genes involved in amino acid metabolism, antioxidant response, and ER-stress-induced apoptosis[47].

IRE1 pathway: Inositol-requiring protein 1 (IRE1) is a type I transmembrane protein that has a RNase domain. Under a normal condition, IRE1 exists as a monomer by binding with BiP like PERK[44]. In response to ER stress, BiP is dissociated from IRE1, which is activated through oligomerization and trans-autophosphorylation. Activated IRE1 triggers its RNase activity, which catalyses unconventional splicing of x-box binding protein 1 (XBP1) pre-mRNA to synthesize the active transcription factor spliced XBP1 (XBP1s)[48]. XBP1s induces the expression of UPR target genes involved in ERAD and lipid synthesis as well as the expression of ER chaperones[49,50].

ATF6 pathway: Unlike PERK and IRE1, activating transcription factor 6 (ATF6) is a type Ⅱ transmembrane protein and a basic leucine zipper transcription factor[51]. Under a normal condition, translocation of ATF6 to the Golgi apparatus is inhibited because Golgi-localization signal of ATF6 is covered by BiP[52]. In response to ER stress, ATF6 is released from BiP and translocates to the Golgi apparatus[53]. In the Golgi apparatus, ATF6 is cleaved by site 1 protease (S1P) and S2P, and then a functional fragment of ATF6 is released into the cytosol[54]. This fragment translocates to the nucleus and induces the expression of ER chaperone, ERAD components, and XBP1[55].

Hepatocytes have well-developed ER because liver is a highly active organ for protein and lipid synthesis. The UPR could alleviate and restore ER damaged

by extraordinary task as a protein synthesis factory. In addition to protein synthesis, ER has a variety of functions, which also are affected by physiological or pathological stress. The UPR activated by rhythmic or transient physiological conditions (*e.g.*, feeding-fasting cycles) is sufficient to restore ER stress[56]. However, the UPR activated by irreversible or chronic stress (*e.g.*, viral infection and obesity) is not sufficient to restore ER stress[56] and causes hepatic dysfunction, leading to the pathogenesis of the liver diseases including non-alcoholic fatty liver disease (NAFLD), cholestatic liver disease, insulin resistance, diabetes, viral hepatitis, and liver cancer[57-59].

In HBV-infected cells, a vast amount of HBV surface proteins is synthesized and folded in ER during its productive life-cycle, often leading to perturbation of the ER homeostasis, resulting in ER stress. This becomes known by identifying mutant surface proteins (preS1 and preS2 mutants) accumulated in the ER of the cells, termed ground glass hepatocyte (GGH) showing a hypertrophy of ER[60,61]. ER stress activated by HBV can lead to the expression of ER degradation enhancer, mannosidase alpha-like 1 (EDEM1) involved in ERAD pathway through the activation of IRE1/XBP1 pathway[62]. The activated ERAD pathway can limits the amount of surface proteins to alleviate ER stress and to protect the cells. Therefore, ER stress is essential for proper viral protein folding and HBV replication, enabling the chronic HBV infection. In general, autophagy is highly regulated catabolic process that removes the damaged organelles or intracellular microbial pathogens through the formation of double-membrane-bound structure called the autophagosome[63]. However, HBV can induce autophagy *via* ER stress or HBx protein for viral replication and envelopment[64-66]. Moreover, HBV can strategically protect itself from autophagic degradation through accumulation of immature lysosomes induced by HBx protein[67].

In vivo it seems insufficient to induction of ER stress by only surface proteins except productive life-cycle. ER stress can be induced by the change of intracellular conditions through other viral proteins. For example, HBx protein can generate ROS and decrease mitochondrial membrane potential and cellular ATP/ADP ratio through mitochondrial damage[29]. Although the molecular mechanisms by which HBx induces ER stress are not clearly understood, these changes by HBx protein may synergistically contribute to induction of ER stress in conjunction with surface proteins. In fact, some researchers reported that UPR or ERAD pathway are activated by HBx protein alone[62,68]. We also reported that ER stress is induced by low intracellular glucose or ATP levels as well as HBx protein[29].

As mentioned previously, rhythmic or transient ER stress is a protective mechanism for cell survival. However, chronic ER stress under the pathological conditions such as chronic HBV infection can cause various liver diseases. We showed that HBx up-regulates the expression of cyclo-oxygenase 2 (COX2) and stromal cell-derived factor-1 (SDF1) through PERK-eIF2α-ATF4 pathway and IRE1-XBP1 pathway activated by ER stress, respectively[29,69]. COX2 converts arachidonic acid to prostaglandin (*e.g.*, prostaglandin E2), which is an important mediator of inflammation. SDF1, a small cytokine, is strongly chemotactic for lymphocytes and induces the recruitment of immune cells into liver of HBx transgenic mice[69]. These evidences suggest that chronic ER stress induced by HBx protein may contribute to pathogenesis of hepatic inflammation and fibrosis (Figure 2).

Cyclic AMP responsive element-binding protein H (CREBH) is an ER-resident transmembrane bZIP transcription factor and a member of old astrocyte specifically induced substance (OASIS) family which show cell- or tissue-specific expression pattern[70]. CREBH is abandant expressed in liver and its activation mechanism is similar to that of ATF6[70]. Activated CREBH plays critical roles in iron metabolism, triacylglycerol metabolism, hepatic gluconeogenesis and lipogenesis, and inflammation by regulating the expression of various genes as a master gene[71-75]. Considering the published papers, activated CREBH plays essential roles in various hepatic metabolisms under physiological conditions and in hepatic inflammation and cell proliferation under pathological conditions. We showed that CREBH is activated by HBV and HBx protein as well as ER stress inducer, leading to hepatic cell proliferation by inducing the expression of oncogenic genes in cooperation with HBx protein[75]. These evidences suggest that ER stress may be closely associated with pathogenesis of HCC in patients with chronic hepatitis B.

THERAPEUTIC IMPLICATIONS

To date, the various drugs against HBV are developed and used to treat HBV patients, *e.g.* lamivudine, adefovir, entecavir, and tenofovir[76]. All of the drugs are nucleoside/nucleotide analogues that target reverses-transcriptase (RT) domain of HBV polymerase which play the essential role on viral replication[76,77]. Although the drugs can repress the viral replication efficiently, existing virus and covalently closed circular DNA (cccDNA) are not eliminated by the drugs from the infected cells. Since HBV polymerase lacks a proofreading exonuclease activity, misincorporated bases can't be removed from newly synthesized viral genome, leading to the mutations in the progeny DNA. Therefore, the resistance for the drugs often occurs in HBV patients during long-term therapy. Besides, there are the adverse effects of the drugs, including myopathy induced by depletion of mitochondrial DNA and myonecrosis, and nephrotoxicity induced by inhibition of kidney function[78].

In addition to nucleoside/nucleotide analogues, the non-nucleoside agents that target viral entry, protein, or replication are developing. The development of agents that target viral entry was available due to

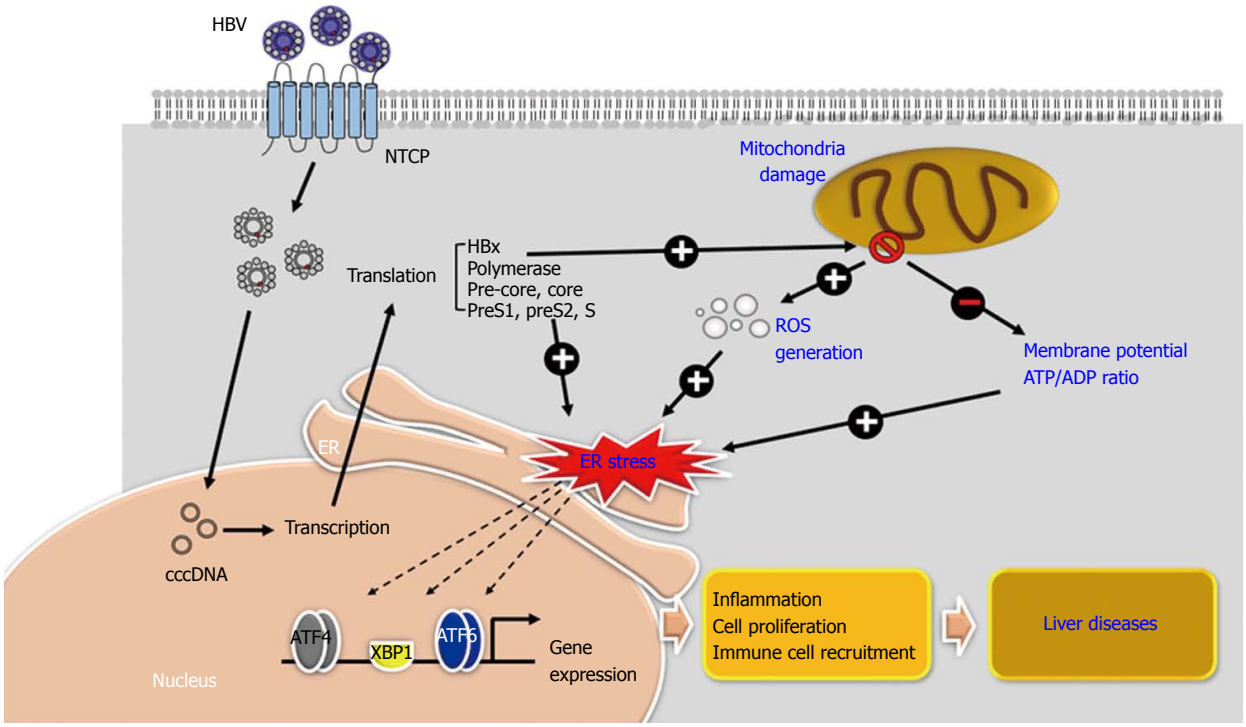

Figure 2 Mechanisms for the pathogenesis of hepatitis B virus-induced liver diseases *via* mitochondrial damage and endoplasmic reticulum stress. Plus and minus symbols are up- and down-regulated responses, respectively. HBV: Hepatitis B virus; cccDNA: Covalently closed circular DNA; ER: Endoplasmic reticulum; HBx: Hepatitis B virus X protein; ROS: Reactive oxygen species; ATF4: Activating transcription factor 4; XBP1: X-box binding protein 1; ATF6: Activating transcription factor 6.

identification of sodium taurocholate cotransporting polypeptide (NTCP) known as a HBV entry receptor[79]. Cyclosporin A and Myrcludex-B strongly inhibit HBV infection into hepatocytes by binding to NTCP on plasma membrane[80,81]. Anti-HBV agents that inhibit the secretion of viral proteins or the interaction between core and surface proteins have been reported[82,83]. Anti-HBV agents that inhibit the viral replication by blocking RNA packing or gene expression also have been reported[84,85]. Sorafenib, anti-liver cancer drug, suppresses the HBV gene expression, but it induces cell death at high-dose treatment[86,87]. On the other hand, since surface protein and HBx proteins can induce ER or mitochondria stress which lead to pathogenesis of liver diseases, the development of the agents which inhibit ER or mitochondria stress is necessary in future. The development of host-targeting anti-HBV agents makes patients expect some advantages, including the low frequency of drug resistance, the synergic effect with currently available anti-HBV agents, and supply an alternative therapy.

CONCLUSION

Under normal physiological conditions, our body has adaptive system to maintain homeostasis from various stresses. Even though faced with pathological conditions, our body can be protected from the conditions by removing and restoring the damaged cells and tissues through innate and adaptive immune system. However, HBV has the abilities to escape from the host's immune response and even to utilize autophagy for viral replication and envelopment. The abilities facilitate chronic infection of HBV, leading to chronic ER and mitochondrial stress, resulting in the pathogenesis of various liver diseases including NAFLD, cholestatic liver disease, viral hepatitis, and liver cancer. Here we indicated the mechanisms by which HBV proteins induce the dysfunction of cellular organelles and the hepatic diseases developed by the expression of UPR target genes or by disturbance of cellular signaling pathway. From a therapeutic perspective, it will be important to understand how HBV induce ER or mitochondrial dysfunctions and understanding the mechanisms will provide new treatment options to chronic HBV patients.

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P- Reviewer: Chen YJ, Sazci A **S- Editor**: Gong ZM **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7666-7677

DOI: 10.3748/wjg.v23.i43.7666

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

MINIREVIEWS

Pathological process of liver sinusoidal endothelial cells in liver diseases

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Author contributions: Ni Y designed the review and wrote the article; Li JM, Liu MK, Zhang TT, Wang DP, Zhou WH and Hu LZ designed the review, wrote the article and made critical revisions; Lv WL contributed to the critical revision of the manuscript for important intellectual content.

Supported by the Young Elite Scientists Sponsorship Program by CAST, No. 2016QNRC001 and Beijing Natural Science Foundation, No. 7172187.

Conflict-of-interest statement: The authors declare no conflict of interests related to this article.

Manuscript source: Unsolicited manuscript

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Received: July 22, 2017
Peer-review started: July 24, 2017
First decision: August 28, 2017
Revised: September 13, 2017
Accepted: September 28, 2017
Article in press: September 28, 2017
Published online: November 21, 2017

Abstract

Cirrhosis develops from liver fibrosis and is the severe pathological stage of all chronic liver injury. Cirrhosis caused by hepatitis B virus and hepatitis C virus infection is especially common. Liver fibrosis and cirrhosis involve excess production of extracellular matrix, which is closely related to liver sinusoidal endothelial cells (LSECs). Damaged LSECs can synthesize transforming growth factor-beta and platelet-derived growth factor, which activate hepatic stellate cells and facilitate the synthesis of extracellular matrix. Herein, we highlight the angiogenic cytokines of LSECs related to liver fibrosis and cirrhosis at different stages and focus on the formation and development of liver fibrosis and cirrhosis. Inhibition of LSEC angiogenesis and antiangiogenic therapy are described in detail. Targeting LSECs has high therapeutic potential for liver diseases. Further understanding of the mechanism of action will provide stronger evidence for the development of anti-LSEC drugs and new directions for diagnosis and treatment of liver diseases.

Key words: Sinusoidal endothelial cells; Hepatitis; Fibrosis; Cirrhosis; Liver disease

Core tip: Liver sinusoidal endothelial cells (LSECs) comprise the highest proportion of nonparenchymal cells in the liver. Their fenestrae and basement membrane structure, and high endocytic clearance ability play an indispensable role in the physiology and pathology of the liver. LSECs mainly participate in the regulation of liver pathology, such as hepatitis, liver fibrosis, cirrhosis and liver regeneration, by exerting anti-inflammatory activity, endocytosis, secretion, synthesis of angiogenesis signaling molecules and maintaining the hepatic stellate cell phenotype. It is important to elucidate the mechanism of action of LSECs, which will provide important information for future targeted therapy and clinical diagnosis.

Ni Y, Li JM, Liu MK, Zhang TT, Wang DP, Zhou WH, Hu LZ, Lv WL. Pathological process of liver sinusoidal endothelial cells in liver diseases. *World J Gastroenterol* 2017; 23(43): 7666-7677 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7666.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7666

INTRODUCTION

Liver sinusoidal endothelial cells (LSECs) form the wall of the hepatic sinusoids and have unique morphology and function. These cells contain many fenestrae with uniform diameters of 100-150 nm, thus creating open channels for the exchange of substances between the blood and liver parenchyma[1]. However, the diameter and number of fenestrae are influenced by a variety of agents and liver disease. LSECs are reported to play a key role in triggering liver regeneration and contribute to hepatic complications, such as hepatitis, liver fibrosis and cirrhosis[2].

Hepatotropic viruses usually pass through the protective filter constructed by LSECs to gain access to the liver parenchyma. For example, hepatitis B virus (HBV) crosses the liver endothelium *via* transcytosis[3]. LSECs contribute to the clearance of HBV and hepatitis C virus (HCV) from the bloodstream and control HCV replication[4,5]. Meanwhile, LSECs produce large amounts of anti-inflammatory cytokines, such as transforming growth factor-beta (TGF-β)[6]. LSECs constitutively express major-histocompatibility complex I-restricted antigens and co-stimulatory molecules, which shift the hepatic immune balance toward tolerance[7,8]. Since many patients with chronic hepatitis often have liver fibrosis[9], it is important to know the changes in LSECs during development of liver diseases.

Liver fibrosis is characterized by excess deposition of extracellular matrix (ECM), which may lead to progression of chronic liver diseases like viral hepatitis[10]. Hyaluronic acid, a marker of ECM deposition, is synthesized by hepatic stellate cells (HSCs) and degraded by LSECs. So, liver fibrosis is closely related to LSECs[11]. When liver fibrosis happens, the LSECs lose their fenestrae and form a basement membrane, which is accompanied by production and release of soluble factors that affect the phenotype of neighboring cells[12]. For example, LSECs with defenestration cannot suppress activation of HSCs[13]. Early-stage fibrosis regresses to a near-normal level after successful treatment, but advanced fibrosis can cause irreversible damage. The capillarization of LSECs plays a key role in the pathogenesis and progression of this process[14].

In cirrhosis, LSECs frequently transform to a vascular type with a basement membrane, which interferes with the bidirectional exchange of molecules. Fibrosis, as a precursor of cirrhosis, is a common pathological process of all chronic liver diseases[15]. Angiogenesis contributes to progression of fibrosis to cirrhosis in patients with chronic liver diseases[16]. Many growth factors, such as vascular endothelial growth factor (VEGF), promote transformation of LSECs into the vascular type. Cellular crosstalk among LSECs, HSCs and hepatocytes plays an important role in the angiogenesis process during development of cirrhosis[17,18]. However, due to poor understanding of the molecular mechanisms leading to cirrhosis, there is still a lack of effective strategies to treat cirrhosis. So, it is important to know the pathogenesis of liver cirrhosis, and the change and function of LSECs and HSCs during the transformation of fibrosis to cirrhosis and liver regeneration.

In this review, we describe the morphological changes of LESCs in different liver diseases, such as hepatitis, liver fibrosis and cirrhosis. We discuss the proangiogenic markers and endothelial cell markers that can influence the angiogenic capability of LSECs. We list several agents that inhibit LESC-induced angiogenesis, such as tetramethylpyrazine and sorafenib plus gadolinium chloride ($GdCl_3$).

LSECs IN NORMAL LIVER

The liver is composed of parenchymal and non-parenchymal cells. LSECs represent a small fraction of total liver cell volume, comprising about 70% of non-parenchymal cells. The LSEC surface is smooth and lacks filopodia, generally. The LSECs are held together through special intercellular junctions and there are no gaps existing between cells[19,20]. Transmission electron microscopy (TEM) shows that the thin attenuated cytoplasm of LSECs contains numerous fenestrae (Figure 1A). Scanning electron microscopy (SEM) shows high porosity of LSECs (Figure 1B)[21]. Atomic force microscopy (AFM) can retain the natural environment of LSECs[22]. AFM shows profound streaking artefacts (Figure 1C and D), which indicates that the AFM images on different kinds of liver sinusoidal cells interact closely with the cell surface. The LSEC cytoplasm contains vesicles and organelles with functions of uptake,

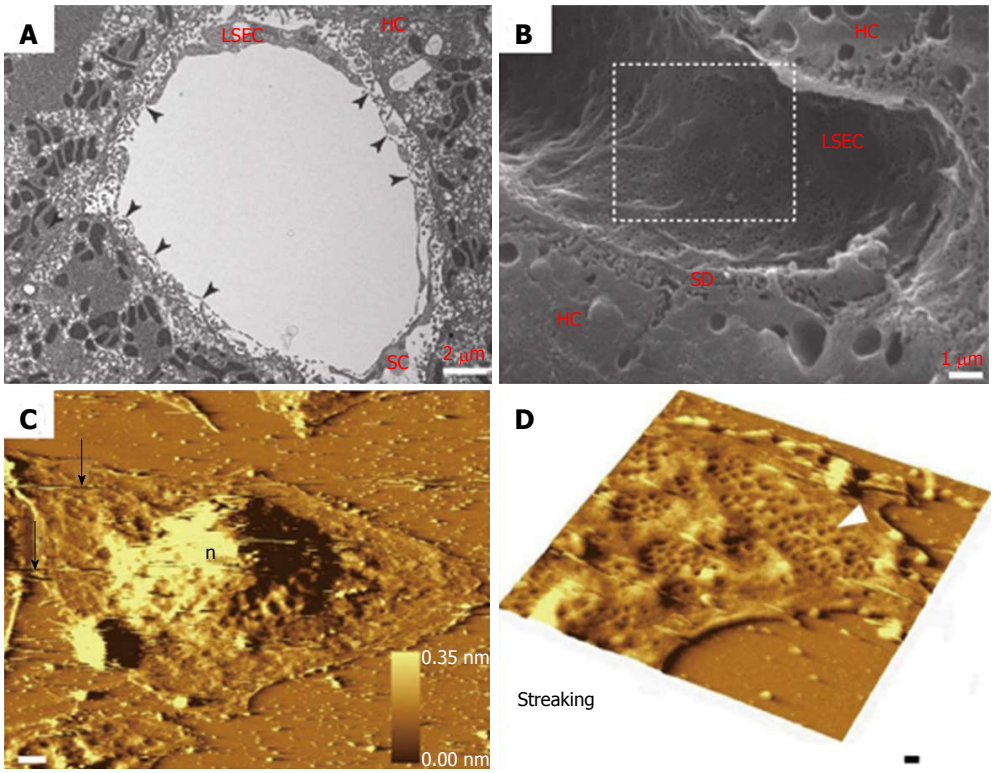


Figure 1 Morphological changes of liver sinusoidal endothelial cells. A: TEM image of rat liver sinusoid after transversal cut; B: SEM image of rat liver sinusoid; C: AFM image of LSECs with a 10 nm gold layer; D: A high scan range of AFM image of LSECs. A-B: ref 21 Copyright @1985 by the American Association for the Study of Liver Diseases; C-D: ref 22 Copyright @ 2012 Elsevier Ltd. AFM: Atomic force microscopy; LSECs: Liver sinusoidal endothelial cells; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy.

transport and degradation of endocytosed material.

The LSECs with fenestrae, absence of diaphragm and lack of basement membrane show high permeability, allowing exchanges of molecules but also active uptake and degradation of molecules[2]. The fenestrae, which comprise many small pores, are organized in clusters termed sieve plates. The number and size of fenestrae vary according to their localization in the liver. Larger but fewer fenestrae per sieve plate are located in the periportal region and smaller but more fenestrae per sieve plate are located in the centrilobular region, which is related to oxygen tension[23,24]. Furthermore, the size and number of fenestrae vary in different physiological and pathological conditions. For example, fasting individuals show fewer but larger fenestrae[25,26]. In normal liver, LSECs act as a selective sieve for soluble molecules, lipoproteins, metabolites, small chylomicron remnants, virus particles and other nanoparticles with a diameter below that of the fenestrae[27]. In addition, the cytoskeleton of LSECs, including its components such as actin, fibronectin, myosin and calmodulin, plays an important role in the modulation of fenestrae. In particular, actin filaments are in the vicinity of the fenestrae, and are involved in the contraction and dilatation of the fenestrae[28].

The maintenance of fenestrae of LSECs needs both paracrine and autocrine cell signaling[29]. VEGF, derived from adjacent hepatocytes and HSCs, is crucial in this regulation and acts through nitric oxide (NO)-dependent and -independent pathways. For example, VEGF stimulates NO release *via* endogenous NO synthase (eNOS) in the LSECs[30].

Meanwhile, the LSECs possess one of the highest endocytic capacities compared with other cells. Thus, LSECs can clear soluble macromolecules and small particles through endocytic receptors, resulting in LSECs having an immunological function. The endocytosis receptors of LSECs include scavenger receptors, mannose receptors and Fcγ receptor IIb2[31]. The scavenger receptors mediate endocytosis of polyanionic molecules, such as oxidized and acetylated low-density lipoproteins, and advanced glycation end products. The mannose receptors bind to glycoproteins and microbial glycans for further use in LSECs. Fcγ receptor IIb2 expressed by LSECs can mediate the clearance of small circulating immune complexes. Moreover, LSEC endocytosis also contributes to the transfer of molecules from the sinusoids to the space of Disse, a process named transcytosis[32]. In addition to expression of scavenger receptors, LSECs also express many pattern recognition receptors. For example, Toll-like receptors (TLRs) respond to low concentrations of lipopolysaccharide, which produces proinflammatory mediators such as interleukin (IL)-6[33]. Furthermore, cytokines, released by LSECs *via* TLRs, serve as antiviral effectors and contribute to liver targeting by

hepatotropic viruses, such as HBV or HCV[4,34,35]. In summary, LSECs not only express scavenger functions in innate immune defense but also contribute to viral infection of the liver.

LSECs IN HEPATITIS

LSECs protect underlying tissues against external agents such as viruses, bacteria and parasites[36-37]. However, some viruses, such as HBV, have developed sophisticated ways to overcome the protective filter formed by LESCs and gain access to the underlying liver parenchyma. For example, HBV crosses the LSEC membrane *via* transcytosis[3]. In hepatitis, the number and size of fenestrae in LSECs decrease and a discontinuous basement membrane appears. These new characteristics become obvious with the aggravation of hepatitis[1]. Moreover, the scavenging LSECs play a key role in the initial uptake of viral pathogens into the liver. In order to observe the process, Breiner *et al*[3] combined duck HBV particles with fluorescent gold particles in test animals, as well as in primary liver cell culture. It is reported that molecules > 12 nm in diameter cannot pass through the LSECs to the hepatocytes[38]. The size of HBV-coated gold particles exceeds 50 nm, which cannot directly access the fenestrae of LSECs. The data support that viruses reach the hepatocytes through active transport across the liver endothelium. Furthermore, viral clearance in hepatitis only happens in hepatocytes so that viruses in LSECs could act as a reservoir for endogenous reinfection[39]. LSECs are unique antigen-presenting cells; for example, LESCs show antigen-specific immune tolerance in $CD4^+$ and $CD8^+$ cells. Thus, they may help viruses escape from the immune system[3].

The LSECs mediate hepatic immune tolerance toward self or foreign antigens by expression of anti-inflammatory mediators under physiological conditions, but they achieve proinflammatory functions upon viral infection[40]. In order to learn more about the role of LSECs in hepatic inflammation during acute viral hepatitis, Bleau *et al*[41] investigated the effects of LSECs infection on their proinflammatory profiles and aggravation of acute hepatitis using a mouse model of murine hepatitis virus type 3 (MHV3) infection. Three different types of MHV3 were injected intraperitoneally into mice: attenuated 51.6-MHV3, YAC-MHV3 and virulent MHV3. The inflammatory foci surrounded necrotic cells of virulent-MHV3-infected mice at 24 h after infection and disappeared at 72 h, while hepatocyte necrosis became extensive. The mice infected with 51.6-MHV3 showed delayed occurrence of inflammatory foci at 48 h after infection, while the mice infected with YAC-MHV3 had a few small inflammatory infiltrates and had no observable hepatic necrosis at 72 h after infection (Figure 2A and B). This demonstrated that the lower tropism of attenuated MHV3 variants for LSECs was associated with less severe damage and less viral replication in the liver. *In vitro* attenuated MHV3 strains induced less fibrinogen-like protein 2, a prothrombinase that promotes vascular thrombosis and hepatic inflammation, which is expressed by LSECs. Virulent MHV3 altered the production of anti-inflammatory cytokines expressed by LSECs; for example, lower induction of caveolin-1, which is a key component of LSEC fenestrae, and lower induction of IL-33, an alarmin secreted mainly by injured LSECs. These results indicate that virulent MHV3 strain promotes greater release of proinflammatory cytokines. Furthermore, specific activation of TLR2 signaling by MHV3 is related to the higher replication and proinflammatory activation in LSECs during acute hepatitis.

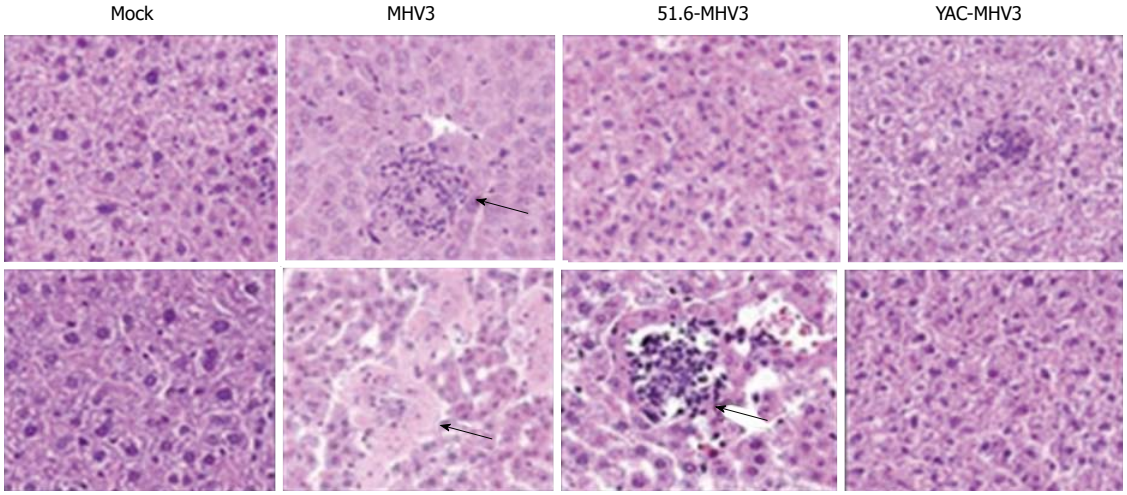
Virus-specific T cells play an important role in the outcome and pathogenesis of viral infections in the liver[3,42]. Liu and co-workers[43] reported that the absence of LSEC lectin led to a high frequency of intrahepatic effector cytotoxic T lymphocytes (CTLs) in a mouse model of HBV infection. LSEC lectin is a cell surface molecule that belongs to the C-type lectin receptors. Compared with wild-type cells, LSEC lectin knockout cells show accelerated liver viral clearance due to a higher frequency of intrahepatic CTLs with higher antiviral activity. Moreover, LSEC lectin expression in the liver is induced by IL-10 or interferon-gamma. And, it also limits immunopathology at the cost of delaying viral clearance. All the results demonstrate that LSEC lectin might facilitate the reduction of liver inflammation.

LSECs IN LIVER FIBROSIS

Liver fibrosis results in disruption of the synthesis and degradation of ECM components, leading to excess connective tissue in the liver[44]. Hyaluronic acid is considered as a marker of ECM deposition. It is a glycosaminoglycan synthesized by HSCs and degraded by LSECs. Damaged LSECs lose their degradative capacity and secrete proinflammatory cytokines, such as TGF-β1, which can stimulate activation of HSCs to synthesize large amounts of ECM[2,11,45].

In the early stage of liver fibrosis, damaged LSECs begin to form capillaries[19]. Defenestration of LSECs is a distinctive structural change during capillarization, and the change can protect the liver from continuing damage by restricting toxins to a specific area. However, defenestration also promotes the formation of a continuous basement membrane of hepatic sinusoids, disrupting the bidirectional exchange of molecules between hepatocytes and hepatic blood sinuses[46]. Disruption of the exchange might induce ischemic atrophy of hepatocytes, which leads to increased fibrogenesis and compensatory hypertrophy of surrounding hepatocytes[14]. Moreover, LSECs with capillarization lose their capacity to inactivate HSCs,

A



B

Virus	Time p.i. (h)	Inflammatory foci	Hepatic necrosis foci
MHV3	24	++	-
	48	++	++
	72	+	++++
51.6-MHV3	24	-	-
	48	+	+
	72	++	+
YAC-MHV3	24	+	-
	48	+	-
	72	+	-

+ (scarce foci); ++ (10% of hepatic tissue); +++ (20%-30% of hepatic tissue); ++++ (up to 40% of hepatic tissue)

Figure 2 Pathological changes of hepatocytes after infection. A: Histopathological analysis was conducted on livers from mock-infected and virus-infected mice from each group at 24 and 72 h after infection; B: Summary of occurrence of necrotic and virus-infected mice at 24, 48 and 72 h after infection. A-B: ref 41 Copyright @ 2016 American Society for Microbiology.

thus promoting fibrogenesis[47].

Defenestration of LSECs is regarded as the main pathological change of liver fibrosis. It is evident in LSECs by shrinking fenestrae and formation of a continuous basement membrane. Healthy LSECs produce a modest amount of macromolecules such as collagen type Ⅳ and fibronectin[48]. The healthy fenestrated LSECs and low density of ECM protein in the space of Disse facilitate transport of cargo from the blood vessel lumen to all kinds of liver cells. However, the quantity of ECM increases many fold in liver fibrosis, contributing to formation of a continuous basement membrane[12]. During the early stages of liver fibrosis, the basement membrane is made up of fibrillary collagen combined with excess collagen type Ⅳ and laminin[49]. Natarajan *et al*[44] reported the relationship between altered fenestral morphology and perisinusoidal matrix, and demonstrated that individual components of the ECM failed to maintain the fenestrae. In liver fibrosis, defenestration is related to accumulation of interstitial collagens in the space of Disse. After appearance of the continuous basement membrane in the liver endothelium, the changes in LSEC phenotype become almost irreversible[50]. So, it is important to inhibit liver fibrosis to control the excess production of ECM in sinusoidal vascular structures[51].

When studying the effects of *Astragalus* polysaccharide (APS), which is the primary effective component of the Chinese herbal medicine *Astragalus membranaceus*, on nanoscale mechanical properties of LSECs in rats, we found that as APS concentration increased, the value of Young's modulus presented an increasing trend, and the surface topography demonstrated that APS was capable of increasing the total area of fenestrae. The observed changes in mechanical properties of LSECs may provide a new therapeutic strategy for mechanistic research of anti-hepatic fibrosis treatments in Chinese medicine[52].

Angiogenesis is defined as the formation of new microvasculature from pre-existing blood vessels and mature endothelial cells. Angiogenesis can occur in many physiological and pathological conditions, such as during liver regeneration and fibrosis[53]. Recent studies have reported that development of liver fibrosis is closely associated with angiogenesis. The inhibition of pathological angiogenesis can effectively alleviate liver fibrosis[10]. So, it is important to know which substances contribute to and which attenuate angiogenesis. Yan *et al*[54] investigated the biological roles of CD147 in LSECs and hepatocytes, and assessed its therapeutic value as a target molecule in a carbon tetrachloride-induced liver fibrosis mouse model. Bioinformatics and experimental data indicate that CD147 promotes liver fibrosis progression *via* VEGF-A/VEGF receptor-2 (VRGFR-2) signaling-mediated crosstalk between hepatocytes and LSECs. Increased expression of VEGFR-2 enhanced the angiogenic capability of LSECs, which induced intrahepatic angiogenesis, leading to liver fibrosis progression. Kantari-Mimoun *et al*[10] pointed out that genetic ablation of VEGF in myeloid cells or pharmacological inhibition of VEGFR-2 signaling could prevent angiogenic response and fibrosis progression. Meanwhile, they reported myeloid cell-derived VEGF as a critical regulator of ECM degradation by LSECs.

In order to attenuate LSEC-induced angiogenesis, Zhao and coworkers[55] evaluated the effect of tetramethylpyrazine (TMP) on angiogenesis and further elucidated the underlying mechanisms. They indicated that TMP alleviated liver fibrosis by hedgehog-dependent inhibition of angiogenesis *in vitro* and *in vivo*. In the mouse fibrotic liver experiment, SEM suggested that the number of fenestrae was decreased in fibrotic liver (Figure 3A and B) but was restored after treatment with TMP and imatinib (Figure 3C and D). Furthermore, expression of proangiogenic markers (VEGF-R2 and VEGF-A) and endothelial cell markers (CD31 and CD34) was up-regulated in fibrotic liver, but TMP and imatinib decreased these markers, indicating that the angiogenic niche involving LSECs was alleviated by treatment with TMP. In addition, *in vitro* experiments also consistently revealed that treatment with TMP inhibited LESC-induced angiogenesis. Furthermore, curcumin and nintedanib attenuated angiogenesis in liver fibrosis *via* inhibiting VEGF expression in HSCs rather than LSECs[13,56].

However, traditional antiangiogenic therapy involves pharmacological inhibition of integrin αvβ3, which promotes fibrosis regression[57]. Liu *et al*[58] analyzed the effects of sorafenib plus $GdCl_3$ on dimethylnitrosamine-induced liver fibrosis in rats and the interactions among LSECs, HSCs and Kupffer cells (KCs) using laser confocal microscopy. Compared with other antiangiogenic therapies, the sorafenib plus $GdCl_3$ attenuated liver fibrosis by inhibiting the expression of angiogenesis-associated cell markers and cytokines, such as CD31 and VEGF. This suppressed the interactions of HSCs, LSECs and KCs, which were assessed by the colocation of α-smooth muscle actin, CD68 and von Willebrand factor. Furthermore, sorafenib plus GdCl3 significantly reduced liver function and hydroxyproline and suppressed collagen accumulation, which indicates that it could be a potential therapeutic strategy for treatment of liver fibrosis. To establish if the release of angiogenic factors from LSECs affect liver regeneration and fibrosis, Manavski *et al*[59] analyzed over-expression of Kruppel-like factor 2 (KLF2) in LSECs and found that KLF2 inhibited hepatocyte proliferation but had no effect on capillary density and liver fibrosis *via* induction of activin A.

Due to the special function of LSECs, the key step in liver regeneration is the replication of LSECs to expand the hepatic sinusoidal vascular network[60]. It is crucial for liver regeneration to replicate LSECs to connect with the existing vascular system. Ding *et al*[61] reported that natural and pharmacological ligands modulate endothelial sphingosine-1-phosphate receptor-1 (S1P1) to stimulate liver regeneration and inhibit fibrosis (Figure 4A). In mice lacking high-density lipoprotein (HDL)-S1P, the liver regenerative responses after partial hepatectomy were significantly inhibited, suggesting that HDL-S1P promotes functional recovery of liver mass after partial hepatectomy. Moreover, the distance between LSECs and hepatocytes was increased, and more perivascular deposition of ECM was observed (Figure 4B), which suggests that lack of HDL-S1P causes liver fibrosis. The data show that HDL-S1P may be a novel therapeutic target for liver fibrosis. All the above research points to the possibility of using antiangiogenic drugs for the treatment of liver fibrosis.

LSECs IN CIRRHOSIS

In cirrhosis, LSECs frequently transform fenestrae to a vascular type with the formation of a true basement membrane. The vascular type of LSECs interfere with the bidirectional exchange of molecules, resulting in decreased sinusoidal compliance with increased resistance to blood flow, which may contribute to development of portal hypertension in cirrhosis[17,18]. Gross hepatic structural disorders also associate with portal hypertension. The modified sinusoids are the main place of resistance to portal blood. The damaged LESCs became more sensitive to endogenous vasoconstrictors, which result in less expression of eNOS and less production of NO, and thus increased resistance for blood supply and oxygen delivery. And, the hypoxia leads more production of proangiogenic and profibrogenic factors. Moreover, impaired paracrine interaction between activated HSCs and LSECs and capillarization play the key role in improving the hepatic vascular resistance to portal blood flow. Meanwhile, they also add structural changes associated

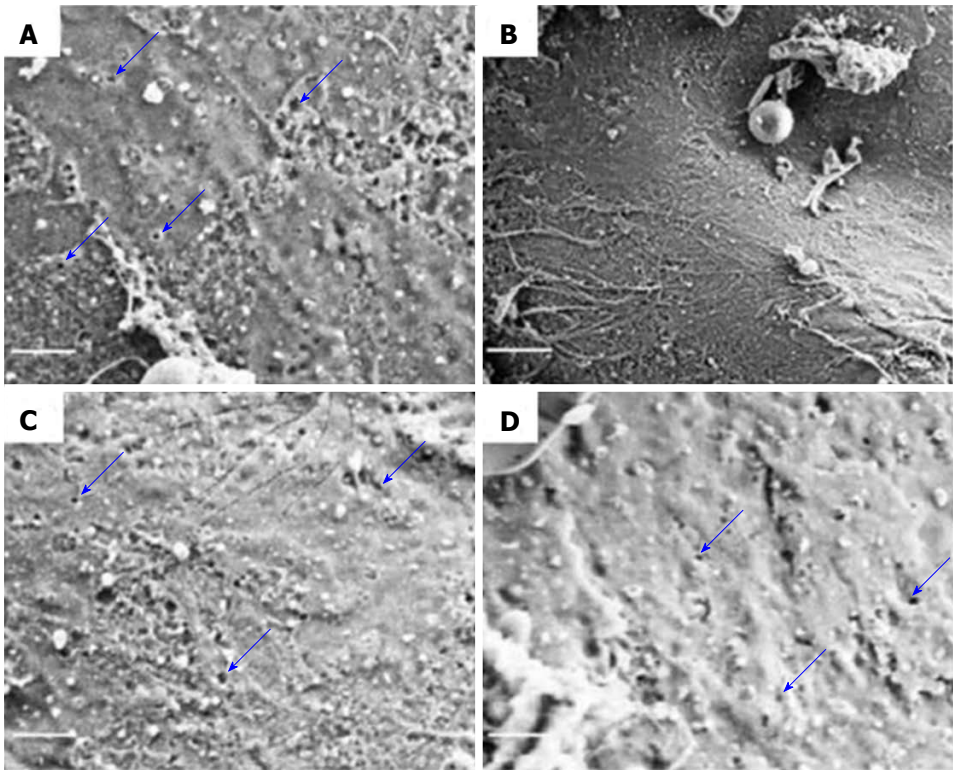


Figure 3 Scanning electron microscopy of liver sinusoidal endothelial cells sieve plates in CCl_4-induced liver fibrosis. A: Control group; B: CCl_4 group; C: TMP- (100 mg/kg) and CCl_4-treated group; D: Imatinib- (10 mg/kg) and CCl_4-treated group. Scale bar = 1 μm. A-D: Ref 54 Copyright @ 2017 International Union of Biochemistry and Molecular Biology. CCl_4: Carbon tetrachloride; TMP: Tetramethylpyrazine.

with diffuse fibrosis and regenerative nodules in liver cirrhosis[62].

Angiogenesis is an important factor that contributes to progression of fibrosis to cirrhosis in patients with chronic liver diseases. VEGF is an important regulator of angiogenesis through eNOS [16,63]. In experimental animal models of liver cirrhosis, HSCs express angiogenic cytokines and related receptors, such as VEGF and angiopoietin-1, which induce migration of HSCs, vessel formation and production of collagen, leading to liver disease progression[64]. Kaur and co-workers[65] focused on numbers and angiogenic functions of circulating endothelial progenitor cells (EPCs) and the interactions of EPCs with LSECs. Compared with controls, the co-culture of LSECs and EPCs from cirrhotic patients showed > 2-fold formation of tube-like closed network circular structures and considerable enhancement of two secretory factors, VEGF and platelet-derived growth factor (PDGF). Moreover, EPCs contribute to the pathogenesis of cirrhosis by stimulating resident LSECs *via* secretion of PDGF and VEGF. Medina *et al*[66] evaluated the cellular infiltrate phenotype and indicated VEGF expression in surrounding LSECs. To know more about hepatic sinusoidal angiogenesis during progression to cirrhosis, Xu *et al*[67] investigated the occurrence of autoantibodies to cell-surface-expressed molecules on LSECs in patients with cirrhosis. They tested the ability of these antibodies to transform LSECs to a vascular type and the reversibility of the transformation process *in vitro*. A high fraction of primary biliary cirrhosis patients had autoantibodies on LSECs compared to normal controls. These antibodies were capable of transforming LSECs into a vascular type. Furthermore, the antibodies induced cell surface expression of CD31, which may play an important role in transformation-inducing signals produced during cirrhosis. During the cirrhotic process, the LSECs with autoantibodies secreted collagen type Ⅳ and laminin, which is a component of basement membrane. In addition, patients with viral hepatitis and non-alcoholic and alcoholic steatohepatitis did not show the presence of these autoantibodies.

Yokomori *et al*[68] investigated the polymerase 1 and transcript release factor (PTRF) and serum deprivation protein response (SDPR) expression and found that these were up-regulated in small angiogenic LSECs with collagen deposition in the perisinusoidal space. Fixation approaches were designed to re-evaluate the precise ultrastructural localizations and changes of PTRF and SDPR expression on LSECs facing the sinusoidal blood flow. PTRF expression was localized primarily to caveola-like structures and vesicles in Child-Pugh class A cirrhotic liver tissues (Figure 5A and B). Several vacuolar components of LSECs, such as pinocytotic vesicles, lysosomes, Golgi apparatus and endosomes, were not observed. SDPR expression was mainly localized on caveola-like structures and

A

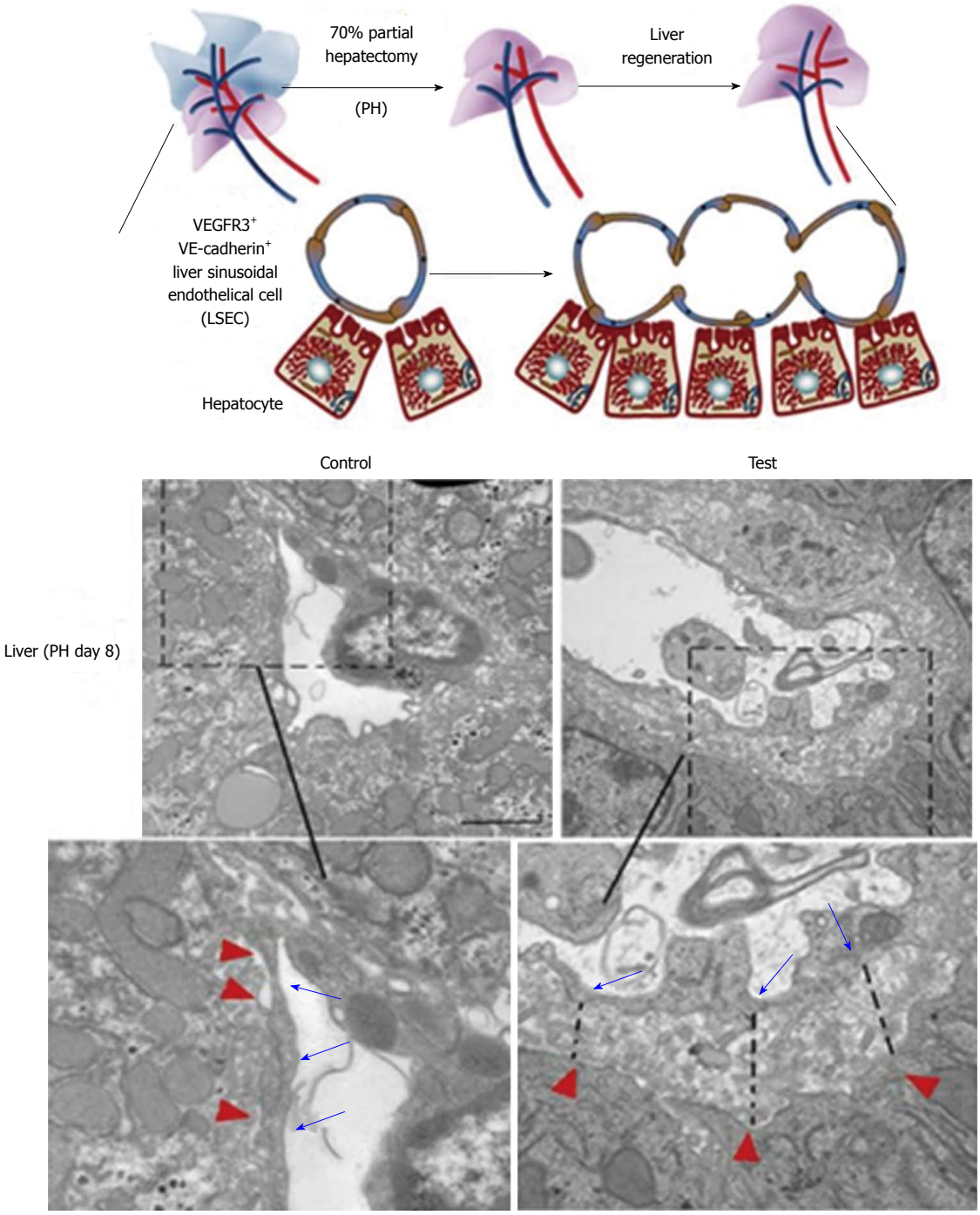


Figure 4 Relationship between liver sinusoidal endothelial cells and liver regeneration. A: Strategy to test liver regeneration in mice lacking HDL-S1P; B: Ultrastructure of LSECs in control and test groups after PH (dashed line represents increased LSEC-hepatocyte distance; inset shows higher level of perivascular matrix protein, black arrows and red arrowheads indicate the borders of LSECs and hepatocytes). Scale bar = 5 μm. A-B: ref 60 Copyright @ 2016 American Society for Clinical Investigation. HDL: High-density lipoprotein; LSECs: Liver sinusoidal endothelial cells; PH: Partial hepatectomy.

to a lesser extent on vesicles of LSECs. Moreover, PTRF played an important role in regulating aspects of caveolin-1 in LSECs, and there was a direct association of PTRF and SDPR with the process of differentiation. Transformation of LSECs inducing hepatic sinusoidal capillarization was related to the progression of cirrhosis.

In order to know more about the development of cirrhosis, Hollenbach and co-workers[69] analyzed the role of glyoxalase (Glo)-I in LSECs and HSCs from rats with early and advanced cirrhosis. Glo-I was the major detoxifying enzyme for methylglyoxal in

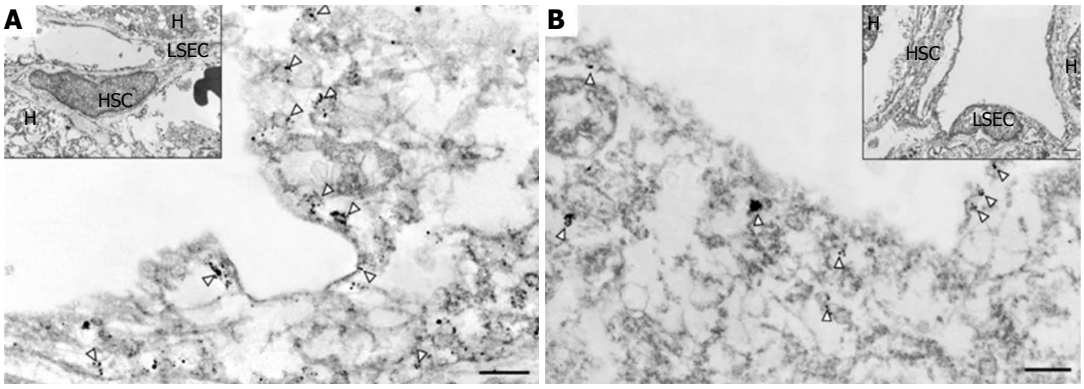


Figure 5 Ultrastructural localization of PTRF and SDPR in cirrhotic tissue. A: Immunogold particles suggesting PTRF expression was observed on caveola-like structures in capillarized, longitudinally cut LSECs; B: Immunogold particles suggesting SDPR expression on caveola-like structures in longitudinally cut LSECs (inset shows lower magnification; white arrowheads indicate caveola-like structures). A-B: ref 66 Copyright @ 2015 Elsevier Ltd. LSECs: Liver sinusoidal endothelial cells; PTRF: Polymerase 1 and transcript release factor; SDPR: Serum deprivation protein response.

cirrhosis. Compared with healthy cells, expression of Glo-I was reduced in early and advanced cirrhosis and methylglyoxal concentration was increased. Rats with advanced cirrhosis had a greater reduction in Glo-I expression than those with early cirrhosis. Protein and mRNA expression of Glo-I in LSECs was less than in HSCs. The reduced expression of Glo-I in cirrhosis leads to increased inflammation, which is more obvious in advanced cirrhosis.

Ex vivo expansion of autologous cells is essential for cell transplantation in patients with cirrhosis. Nakamura *et al*[70] investigated the efficacy of human *ex vivo*-expanded $CD34^+$ cells for treatment of cirrhotic rat liver. Compared with nonexpanded $CD34^+$ cells, the expanded cells had an increase in cell surface markers of vascular endothelial cadherin, VEGFR2 and Tie-2. The increased expression of proangiogenic growth factors and adhesion molecules increased hepatocyte and LSEC proliferative activity. Moreover, the transplanted expanded $CD34^+$ cells enhanced the preventive efficacy of cell transplantation in a cirrhotic model.

CONCLUSION

Most of the recent studies have investigated LSECs at a single pathological stage of liver disease, but we have presented a comprehensive, continuous and dynamic observation of changes in the morphology and function of LSECs in hepatitis, liver fibrosis and cirrhosis in this review. By reviewing previous reports, the following conclusions can be drawn. (1) During hepatitis, the number and size of fenestrae in LSECs decrease and a discontinuous basement membrane appears, and the LSECs express anti-inflammatory cytokines and lectin to reduce the liver inflammation. Viruses such as HBV and HCV reach the hepatocytes through active transport across the LSECs; (2) During liver fibrosis, LSECs lose fenestrae and form a continuous basement membrane. The bidirectional exchange of molecules between hepatocytes and hepatic blood sinuses is disrupted due to the defenestration of LSECs. Meanwhile, damaged LSECs lose their ability to degrade ECM and secrete proinflammatory cytokines. Inhibition of LSEC-induced angiogenesis can reduce intrahepatic angiogenesis, thus alleviating liver fibrosis; and (3) during cirrhosis, LSECs form a vascular type with a true basement membrane, which contributes to the development of cirrhotic portal hypertension. Some secretions are important factors in the progression of angiogenesis, such as VEGF, PDGF and unique autoantibodies. At different stages of cirrhosis, the expression of detoxification enzymes in LSECs is decreased as cirrhosis deteriorates and this constitutes a novel target for antifibrotic therapy. *Ex vivo* expansion of autologous cells enhances the preventive efficacy of cell transplantation in cirrhosis.

As a result of LSECs playing important roles in different liver diseases, drug targeting therapy will be the focus in future research according to the pathological characteristics of LSECs at different stages of liver disease, which may be more effective at improving the fenestrae and basement membrane of LSECs. There are good prospects for new targeted therapy and clinical diagnosis in the area of LSECs for liver diseases.

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P- Reviewer: Doganay L, Garbuzenko DV, Zhu Y **S- Editor**: Qi Y
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7678-7692

DOI: 10.3748/wjg.v23.i43.7678

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

ORIGINAL ARTICLE

Basic Study

Resveratrol modifies biliary secretion of cholephilic compounds in sham-operated and cholestatic rats

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Author contributions: Dolezelova E and Micuda S analyzed the data; Dolezelova E and Micuda S wrote the manuscript; Dolezelova E, Prasnicka A, Cermanova J, Hroch M, Mokry J, Adamcova M, Mrkvicova A and Micuda S performed the in vivo experiments and all molecular analyses; Carazo A, Hyrsova L and Pavek P contributed to the in vitro experiments; All authors participated in the revision and approved the final manuscript.

Supported by Grant Agency of Charles University, No. Progres Q40/05; Specific University Research, No. SVV-2016-260287; and Czech Science Foundation (GA CR), No. 303/12/G163.

Institutional review board statement: The study was reviewed and approved by the Institutional Animal Care and Use Committee of Charles University, Faculty of Medicine in Hradec Kralove.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Charles University in Prague, Faculty of Medicine in Hradec Kralove (3163/2008-30).

Conflict-of-interest statement: The authors declare that they have no conflict of interest related to this study.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: June 30, 2017
Peer-review started: June 30, 2017
First decision: July 25, 2017
Revised: August 15, 2017
Accepted: September 5, 2017
Article in press: September 5, 2017
Published online: November 21, 2017

Abstract

AIM

To investigate the effect of resveratrol on biliary secretion of cholephilic compounds in healthy and bile duct-obstructed rats.

METHODS

Resveratrol (RSV) or saline were administered to rats by daily oral gavage for 28 d after sham operation or reversible bile duct obstruction (BDO). Bile was collected 24 h after the last gavage during an intravenous bolus dose of the Mdr1/Mrp2 substrate azithromycin. Bile acids, glutathione and azithromycin were measured in bile to quantify their level of biliary secretion. Liver expression of enzymes and transporters relevant for bile production and biliary secretion of major bile constituents and drugs were analyzed at the mRNA and protein levels using qRT-PCR and Western blot analysis, respectively. The TR-FRET PXR Competitive Binding Assay kit was used to determine the agonism of RSV at the pregnane X receptor.

RESULTS

RSV increased bile flow in sham-operated rats due to increased biliary secretion of bile acids (BA) and glutathione. This effect was accompanied by the induction of the hepatic rate-limiting transporters for bile acids and glutathione, Bsep and Mrp2, respectively. RSV also induced Cyp7a1, an enzyme that is crucial for bile acid synthesis; Mrp4, a transporter important for BA secretion from hepatocytes to blood; and Mdr1, the major apical transporter for xenobiotics. The findings were supported by increased biliary secretion of azithromycin. The TR-FRET PXR competitive binding assay confirmed RSV as a weak agonist of the human nuclear receptor PXR, which is a transcriptional regulator of Mdr1/Mrp2. RSV demonstrated significant hepatoprotective properties against BDO-induced cirrhosis. RSV also reduced bile flow in BDO rats without any corresponding change in the levels of the transporters and enzymes involved in RSV-mediated hepatoprotection.

CONCLUSION

Resveratrol administration for 28 d has a distinct effect on bile flow and biliary secretion of cholephilic compounds in healthy and bile duct-obstructed rats.

Key words: Resveratrol; Bile production; Bile acids; Pregnane X receptor; Azithromycin

Core tip: For the first time, our results provide information about the ability of resveratrol to increase bile flow in healthy rats by increasing the biliary excretion of bile acids and glutathione *via* posttranscriptional induction of their rate-limiting transporters, Bsep and Mrp2, respectively, and *via* the up-regulation of Cyp7a1, an enzyme that is crucial for bile acid synthesis. Resveratrol simultaneously induced hepatic expression of Mdr1, which was verified by increased biliary excretion of its substrate, azithromycin. Our findings were consistent with an agonistic effect of resveratrol on PXR. Our data therefore imply that oral administration of resveratrol may modify the kinetics of endo- and xenobiotics.

Dolezelova E, Prasnicka A, Cermanova J, Carazo A, Hyrsova L, Hroch M, Mokry J, Adamcova M, Mrkvicova A, Pavek P, Micuda S. Resveratrol modifies biliary secretion of cholephilic compounds in sham-operated and cholestatic rats. *World J Gastroenterol* 2017; 23(43): 7678-7692 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7678.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7678

INTRODUCTION

Bile is the major excretory route for potentially harmful endogenous substrates, such as bilirubin and bile acids (BA), as well as for numerous exogenous lipophilic compounds. The secretion of these substrates into bile is mediated by an interacting network of transporter proteins at the basolateral and apical membranes of hepatocytes. The apical Bsep (Bile salt export pump) and Mrp2 (Multidrug resistance-associated protein 2) are the key rate-limiting transporters for biliary secretion of BA and glutathione that create an osmotic driving force that attracts water to biliary lumen and ensures bile flow. The potential toxicity of BA concentration in the biliary lumen is prevented by the formation of micelles with phospholipids and cholesterol, which are then secreted into the bile by apical Mdr2 (Multidrug resistance protein 2) and Abcg5/g8 (ATP-binding cassette subfamily g5/g8) proteins, respectively[1-3]. Additionally, the apical membrane transporters Mdr1 and Bcrp mainly mediate the excretion of xenobiotics. All these pathways are sensitively regulated and modified by numerous stimuli, including diseases, drugs and food ingredients[4], which may either directly affect the activity of individual transporters or indirectly act by changing their expression. Especially in the second

mechanism, transcriptional regulation of the nuclear farnesoid X receptor (FXR - bile acid sensor) and pregnane X receptor (PXR - xenobiotics sensor) plays a key role in modulating transporter activity. Thus, such events may not only significantly alter bile production but also the kinetics of other substrates, including drugs.

Resveratrol (trans-3,4′,5-trihydroxystilbene) is a natural polyphenol found in grape skin, red wine, peanuts, and now, in food supplements. Numerous reports have documented that this agent mitigates the progression of a wide variety of illnesses, such as malignances, cardiovascular diseases and various ischemic, toxic and inflammatory tissue injuries[5-9]. Further, resveratrol (RSV) showed marked hepatoprotective potential in different situations, such as non-alcoholic fatty liver disease, extrahepatic cholestasis, and α-naphthylisothiocyanate-, acetaminophen-, or carbon tetrachloride-induced hepatotoxicity[10-12]. The majority of these benefits are related to the significant anti-inflammatory and antioxidant properties of resveratrol, as well as to the reduction of hepatic lipid accumulation[10,13]. The molecular mechanism behind these effects originates from the stimulation of the adenosine monophosphate-activated protein kinase/Sirtuin 1 (AMPK-SIRT1) pathway in association with the inhibition of NF-κB-mediated proinflammatory cytokine production, the suppression of p53 with reduction of apoptosis, and the induction of autophagy[11,14,15].

Recent results have also suggested that resveratrol may prevent the impairment of bile formation during α-naphthylisothiocyanate (ANIT)-induced intrahepatic cholestasis[16]. Herein, intraperitoneal administration of RSV (15 and 30 mg/kg BW) to ANIT-induced rats partially recovered bile flow (BF). In accordance, RSV administration to ANIT-induced rats attenuated the impaired biliary excretion (BE) of both BA and glutathione. The authors analyzed the expression of four transporter proteins and attributed this effect to the preserved expression of the Mrp2 transporter as a part of the anti-inflammatory activity of RSV in association with the reduction of BA concentrations in serum[16]. In this study[16], compared to the commonly used oral administration, intraperitoneal administration of RSV overcame its low bioavailability and markedly increased its exposure to the organism. Moreover, the effect of RSV on BF has not been determined in healthy rats or in the general population without cholestatic liver impairment, despite its wide use as a food ingredient.

The aim of the present study was therefore to evaluate the influence of orally administered RSV on multiple mechanisms involved in bile production as well as the biliary secretion of cholephilic compounds in rats with intact liver. The influence of RSV on bile flow and the associated mechanisms was also studied in rats with reversible long-term obstructive cholestasis associated with initiated liver fibrosis.

MATERIALS AND METHODS

Chemicals

Trans-resveratrol was purchased from Sigma-Aldrich (St. Louis, MO, United States). Azithromycin was used in its original formulation for parenteral administration - Sumamed (Pliva-Lachema a.s., Brno, Czech Republic). All other reagents and supplies were obtained from Sigma (St. Louis, MO, United States) and Bio-Rad Laboratories (Hercules, CA, United States), respectively, and were of the highest available purity.

Animals and experimental design

Male Wistar rats (n = 6 in each group) weighing 280 to 320 g (Konarovice, Czech Republic) were used throughout the study. The animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health (NIH publication, 1996). The study protocol (reg. No. 3163/2008-30) was approved by the animal welfare committee of Charles University, Faculty of Medicine in Hradec Kralove. The rats were randomly divided into four groups - two sham-operated (Sham) and two bile duct-obstructed (BDO) groups. Surgery was performed under short pentobarbital anesthesia [50 mg/kg body weight (BW)]. Briefly, sham-operated animals underwent incision of the abdominal wall, manipulation of the bile duct without obstruction and two-layer suturing of the incision. BDO groups underwent bile duct cannulation, where the free end of the cannula was ligated and stitched to the abdominal wall just under the skin. Animals recovered from anesthesia and then received the first dose of either saline (Sham or BDO groups; 0.3 mL/kg BW) or resveratrol (Sham-R and BDO-R groups; 10 mg/kg BW in saline) two hours after surgery by gastric gavage. Next, animals received treatment once a day by gastric gavage for 28 consecutive days in order to initiate biliary cirrhosis in the BDO groups. All animals underwent a clearance study (see below) 24 h after the last gavage of saline or RSV to evaluate bile flow and biliary secretion of cholephilic substrates (bile acids, glutathione, and azithromycin).

The dose of 10 mg/kg resveratrol p.o. was selected on the basis of the minimal effective dose in similar studies in animals[12,17] and according to the recommended dose for resveratrol supplementation in humans (250-2000 mg/d). The dose was tested in a preliminary study.

In vivo clearance study

Under anesthesia induced by pentobarbital (50

mg/kg), the bile duct was either cannulated (sham-operated animals) or its obstruction was released (BDO animals) by cutting the free tip of the biliary cannula. In addition, all rats were cannulated with a polyethylene tube in the right jugular vein for drug administration and continuous infusion of physiological saline (2 mL/h, to replace fluid loss) and the left carotid artery (for blood sampling). Thereafter, the rats received a single intravenous bolus of azithromycin (20 mg/kg), a substrate for Mdr1/Mrp2 transporters. Bile samples were collected in pre-weighed tubes at 30-min intervals for 120 min. The body temperature of the animals was maintained at 37 ℃ by keeping the animals on a heated platform. At the end of the experiment, all rats were sacrificed by exsanguination from the aorta, and the livers were removed and immediately frozen in liquid nitrogen. Plasma samples were obtained from whole blood by centrifugation at 2000 × *g* for 5 min at 4 ℃. Samples were stored at -80 ℃ until analysis. The median lobe of the liver from each animal was used for histological and molecular analyses.

Analytical methods

Bilirubin concentrations in the plasma and ALT, as well as AST activities in the plasma, were measured by routine laboratory methods on a Cobas Integra ® 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Bile acids in the plasma and bile were assayed using a commercial kit (Diazyme, CA, United States). Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were analyzed separately using an HPLC method on a Shimadzu system with fluorescence detection[18]. The total glutathione amount in the bile was calculated as the sum of the reduced and oxidized forms of glutathione. The concentration of azithromycin in bile was determined by a previously described HPLC method[19].

Quantitative real time RT-PCR

Gene expression was examined as previously described[18]. The total RNA from the liver tissue was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO, United States). TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay kits (Table 1) were obtained from Life Technologies (Prague, Czech Republic). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference for normalizing the data.

Western blot analysis

Crude plasma membranes were prepared from rat liver homogenates as previously described[18]. Proteins (100 mg) were separated by SDS-PAGE, then transferred to a PVDF membrane (Millipore, NY, United States) and incubated with the appropriate antibodies (Table 2). The immunoreactive bands on the autoradiography films were quantified using Quantity One imaging software (Bio-Rad Laboratories, Hercules, CA). The equal loading of proteins onto the gel was confirmed by the immunodetection of β-actin.

PXR ligand binding assay

The LanthaScreen® TR-FRET PXR Competitive Binding Assay (Invitrogen/Life Technologies, Carlsbad, CA, United States) was performed to evaluate whether RSV is a potential PXR ligand. This cell-free assay was used since RSV interferes with classical luciferase reporter gene assays. The experiment utilizes human PXR-LBD tagged with glutathione-S-transferase (GST), which was labeled upon binding to a terbium (Tb)-labeled anti-GST antibody. The assay measures the ability of the evaluated compound to replace the fluorescent PXR ligand (Fluormone™ PXR (SXR) Green or "tracer") from the receptor. The competition between the evaluated compound and tracer results in a loss in the fluorescence resonance energy transfer (FRET) signal between the Tb-anti-GST antibody and the tracer, whereby the difference can be measured. The assay was performed according to the manufacturer's instructions. The fluorescence was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, United States) at the recommended settings, except for measuring the emission signal at 528 nm instead of 520 nm. The calculation of TR-FRET ratio was performed by dividing the emission at 528 nm by emission at 495 nm.

Histology

All paraffin-embedded liver tissue sections were stained with Masson's trichrome. For the semi-quantitative analysis, six images were taken of each liver sample, and the area occupied by the biliary ducts and connective tissue was evaluated from each image using ImageJ software (National Institutes of Health, Bethesda, MD; http://rsweb.nih.gov/ij/). The final result for each rat was the mean of the 6 evaluations and was expressed as the percentage of the whole field of view for the images.

Statistical analysis

Experiments were carried out on 6 animals per group. All experimental data are expressed as the mean ± SE. Statistical significance was examined in the groups of control, cirrhotic, and resveratrol-treated animals with one-way ANOVA followed by Newman-Keuls *post hoc* test. For two-group comparisons, Student's *t*-test was employed. All analyses were performed using GraphPad Prism 6.0 software (San Diego, CA, United States). A difference of $P < 0.05$ was considered statistically significant.

RESULTS

Liver biochemical and histological parameters

The administration of RSV to sham rats did not change

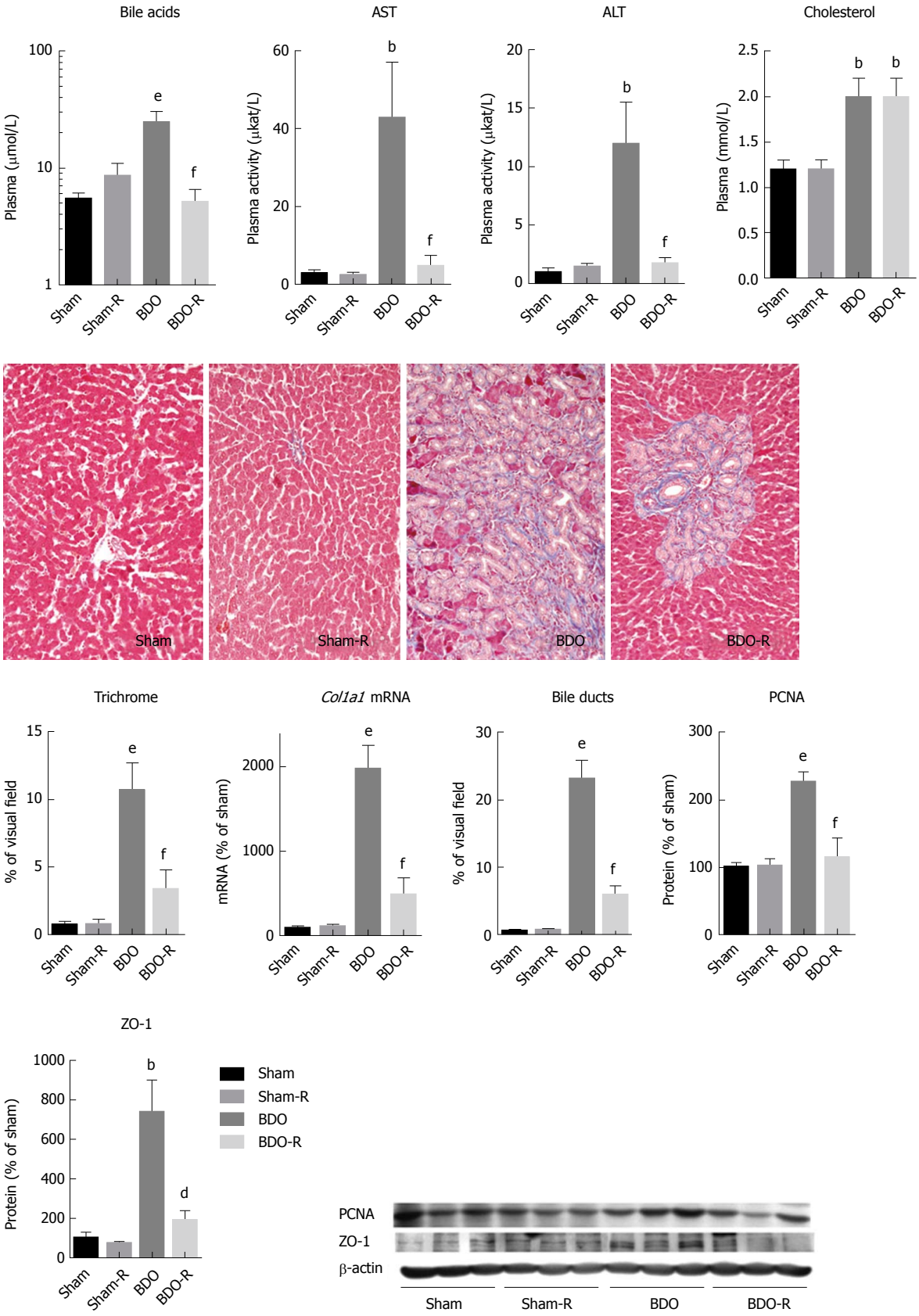

Figure 1 Plasma liver biochemical tests, hepatic histological examination, *Col1a1* mRNA expression, and PCNA and ZO-1 protein expression in sham-operated and bile duct-obstructed rats. Rats were administered either saline or resveratrol (R, 10 mg/kg) daily by oral gavage for 28 d after surgery. Liver sections were stained with Masson's blue trichrome stain for collagen visualization. Sham-operated animals were physiologically normal for the measured parameters; resveratrol administration to Sham animals had no effect. The results of mRNA and protein expression are presented as a percentage of the saline-administered sham group (control). Data are presented as the mean ± SE ($n = 6$); $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* saline-treated sham group; $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* saline-treated BDO group).

Table 1 Pre-designed TaqMan® gene expression assay kits (Life Technologies) used for quantitative real-time RT-PCR

Gene symbol	Transporter/Receptor	Life technologies cat. number
Abcc2	Mrp2	Rn00563231_m1
Abcb11	Bsep	Rn00582179_m1
Abcg2	Bcrp	Rn00710585_m1
Abcb1a	Mdr1a	Rn00591394_ml
Abcb1b	Mdr1b	Rn00561753_ml
Abcb4	Mdr2	Rn00562185_ml
Abcc3	Mrp3	Rn01452854_m1
Abcc4	Mrp4	Rn01465702_m1
Slc10a1	Ntcp	Rn00566894_ml
Slco1a1	Oatp1a1	Rn00755148_m1
Slco1a4	Oatp1a4	Rn00756233_ml
Slc22a7	Oat2	Rn00585513_m1
Slc10a2	Asbt	Rn00691576_m1
Cyp7a1		Rn00564065_m1
Cyp8b1		Rn00579921_m1
NrOb2	Shp	Rn00589173_m1
TNF-α		Rn99999017_m1
IL-6		Rn99999011_ml
Acta2		Rn01759928_g1
TGF-β1		Rn00572010_m1
Col1a1		Rn01463848_m1
Pdgfrβ		Rn00709573_m1
Timp-2		Rn00573232_m1
Nqo1		Rn00566528_m1
GAPDH		4352338E

any of the biochemical parameters of the evaluated plasma samples (Figure 1). Similarly, light microscopy revealed normal liver architecture without pathological processes after sham operation and resveratrol administration, all of which support good tolerance of the compound (Figure 1). In contrast, untreated BDO for 28 d led to a significant increase in plasma indicators of cholestasis, namely, the concentrations of bile acids and cholesterol, as well as indicators of liver cell membrane injury, *i.e.*, in activities of both ALT and AST. Liver histology confirmed changes that were typical for chronic cholestasis, *i.e.*, significant proliferation of bile ducts and fibrosis in the expanded portal areas of the liver acinus. Increased expression of ZO-1 protein (Figure 1), an integral protein of zonula occludens, confirmed the impairment of the blood-biliary barrier integrity during BDO as previously described[20]. The administration of RSV to BDO animals produced significant attenuation of biochemical and histopathological alterations (Figure 1). RSV reduced bile duct proliferation and prevented fibrotic changes as visualized by trichrome staining in histological sections from cholestatic liver. Consistently, RSV markedly reduced protein content of PCNA (proliferating cell nuclear antigen), an indicator of cell proliferation, as well as collagen type 1 alpha 1 (*Col1a1*) expression, as both were significantly increased in BDO rats (Figure 1). BDO rats treated with RSV also demonstrated a reduced expression of ZO-1, indicating the improved integrity of the blood-biliary barrier.

Bile flow and biliary excretion of cholephilic substances

Bile flow was evaluated in rats 24 h after the last oral administration of resveratrol or saline. Resveratrol induced a moderate, but significant, increase in the bile flow of sham-operated rats (Figure 2A). This effect was accompanied by stimulated biliary secretion of bile acids and glutathione (Figure 2C and D), indicating that the choleretic action of long-term RSV administration is both BA-dependent and BA-independent. Moreover, RSV treatment increased biliary secretion of azithromycin, a drug transported by apical multidrug resistance proteins, suggesting the possibility of interaction of RSV with co-administered Mdr1/Mrp2 substrates (Figure 2B). BDO in saline-administered animals decreased biliary excretion of bile acids, glutathione and azithromycin to 49%, 2% and 47%, respectively (Figure 2). In contrast, the same animals demonstrated markedly increased bile flow compared to sham-operated healthy rats, which signified the dominating effect of increased hepatic paracellular permeability (altered function of the blood-biliary barrier) during extrahepatic cholestasis[21]. Oral gavage of RSV significantly reversed the massive choleresis in BDO animals. It accompanied reduced biliary secretion of azithromycin but left biliary excretion of both BA and glutathione unchanged (Figure 2). This discrepancy indicates the protective effect of RSV on the integrity of the blood-biliary barrier.

Interestingly, the liver concentrations of GSH and GSSG did not show any change in the experimental groups (Figure 3). This suggested that the increase in the BE of glutathione in RSV-treated Sham rats was the consequence of enhanced activity of the related Mrp2 transporter but not the increased concentration of glutathione. Additionally, the unchanged GSH/GSSG ratio indicated the absence of marked oxidative stress in cholestatic livers. This result was consistent with unmodified mRNA expression of *NAD(P)H* dehydrogenase quinone 1 *(Nqo1),* a gene activated by oxidative stress (Figure 3).

Gene expression of bile acid, glutathione and azithromycin transporters in the liver

To describe the mechanisms responsible for the observed effects of RSV on bile production and secretion, we analyzed the mRNA levels of 16 genes involved in these processes. However, RSV-treated sham animals demonstrated only the increased mRNA of *Abcb1a* (one of two genes encoding Mdr1 protein) (Figure 4). This indicated a weak influence of RSV on the transcription of these pathways at the given dosage. Untreated long-term obstructive cholestasis down-regulated the expression of most of the uptake transporters localized at the basolateral

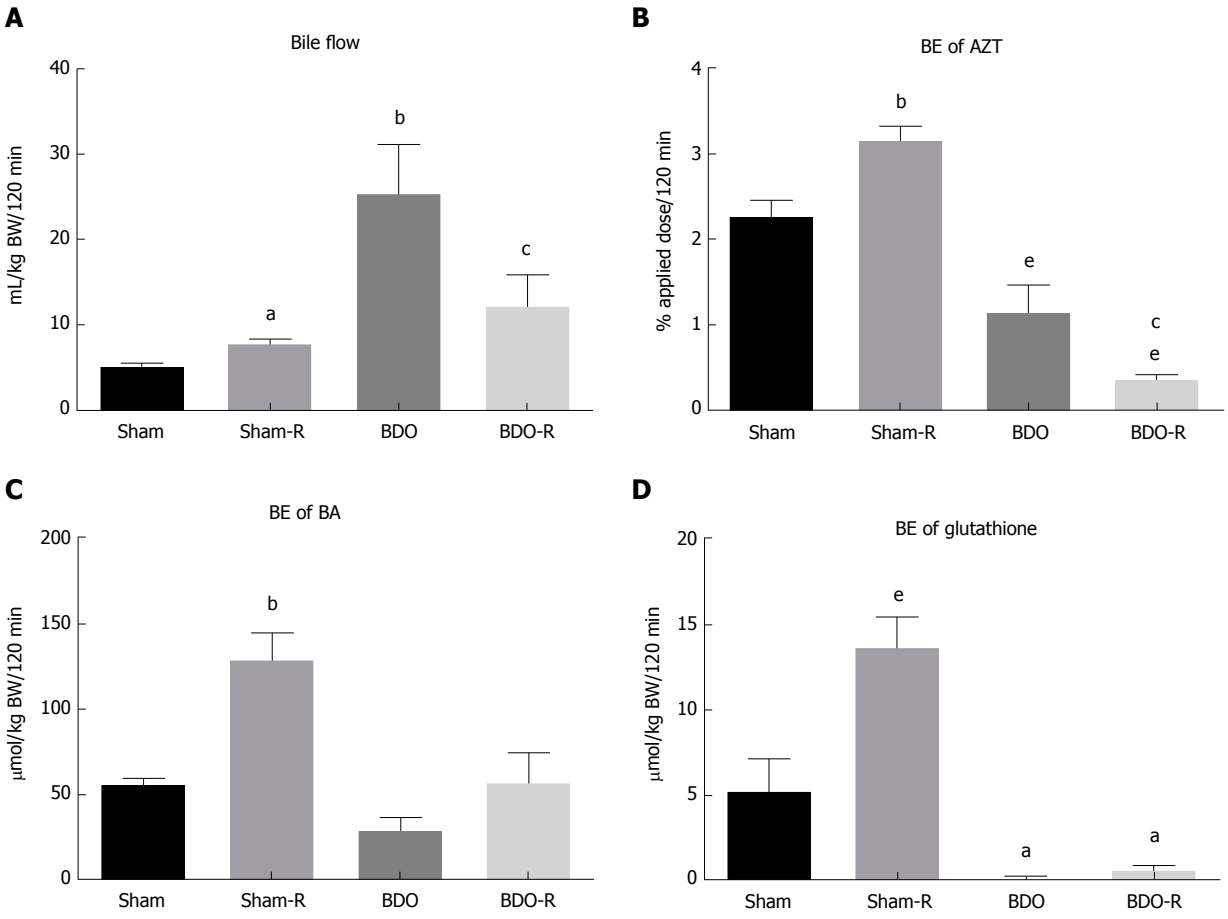

Figure 2 Influence of resveratrol administration and bile duct obstruction on the bile flow rate (A), biliary excretion of azithromycin (B), biliary excretion of bile acids (C), and biliary excretion of glutathione (D). Rats received either saline or resveratrol (R, 10 mg/kg) daily by oral gavage for 28 d after surgical procedure. Data are presented as the mean ± SE (n = 6). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* saline-treated sham group; $^{c}P < 0.05$ *vs* saline-treated BDO group. BE: Biliary excretion; BDO: Bile duct obstruction.

Table 2 Primary and secondary antibodies used in western blot analysis

Protein	Source	Dilution	Secondary antibody dilution
Mrp2	Signet Laboratories	1:500	1:1000
Bsep	Santa Cruz	1:300	1:2000
Bcrp	Signet Laboratories	1:500	1:500
P-gp	Signet Laboratories	1:500	1:1000
Mdr2	Abcam	1:500	1:500
Mrp3	Alexis	1:500	1:500
Mrp4	Abcam	1:1000	1:1000
Ntcp	Santa Cruz	1:300	1:3000
Oatp1a4	Millipore	1:5000	1:5000
Cyp7a1	Thermo Fisher Scientific	1:1000	1:2000
Cyp8b1	Thermo Fisher Scientific	1:500	1:1000
Sirt1	Cell Signaling	1:1000	1:2000
p-AMPKα	Cell Signaling	1:1000	1:2000
PCNA	Sigma-Aldrich	1:800	1:2000
ZO-1	Invitrogen	1:500	1:1000
β-actin	Sigma	1:5000	1:5000

membrane of the hepatocyte such as *Slc10a1* (Ntcp), *Slco1a1* (Oatp1a1), and *Slc22a7* (Oat2). Similarly, the expression of major apical efflux transporters, such as *Abcb11* (Bsep), *Abcc2* (Mrp2), as well as *Cyp8b1* enzyme, were also down-regulated. In contrast, BDO led to a marked induction of *Abcc3* (Mrp3), *Abcb1b* (Mdr1b), and *Cyp7a1* expression. Resveratrol gavage to BDO animals reversed the changes in the expression of the *Slc10a1* (Ntcp), *Slco1a1* (Oatp1a1), and *Abcc3* (Mrp3) transporters, as well as the *Cyp7a1*

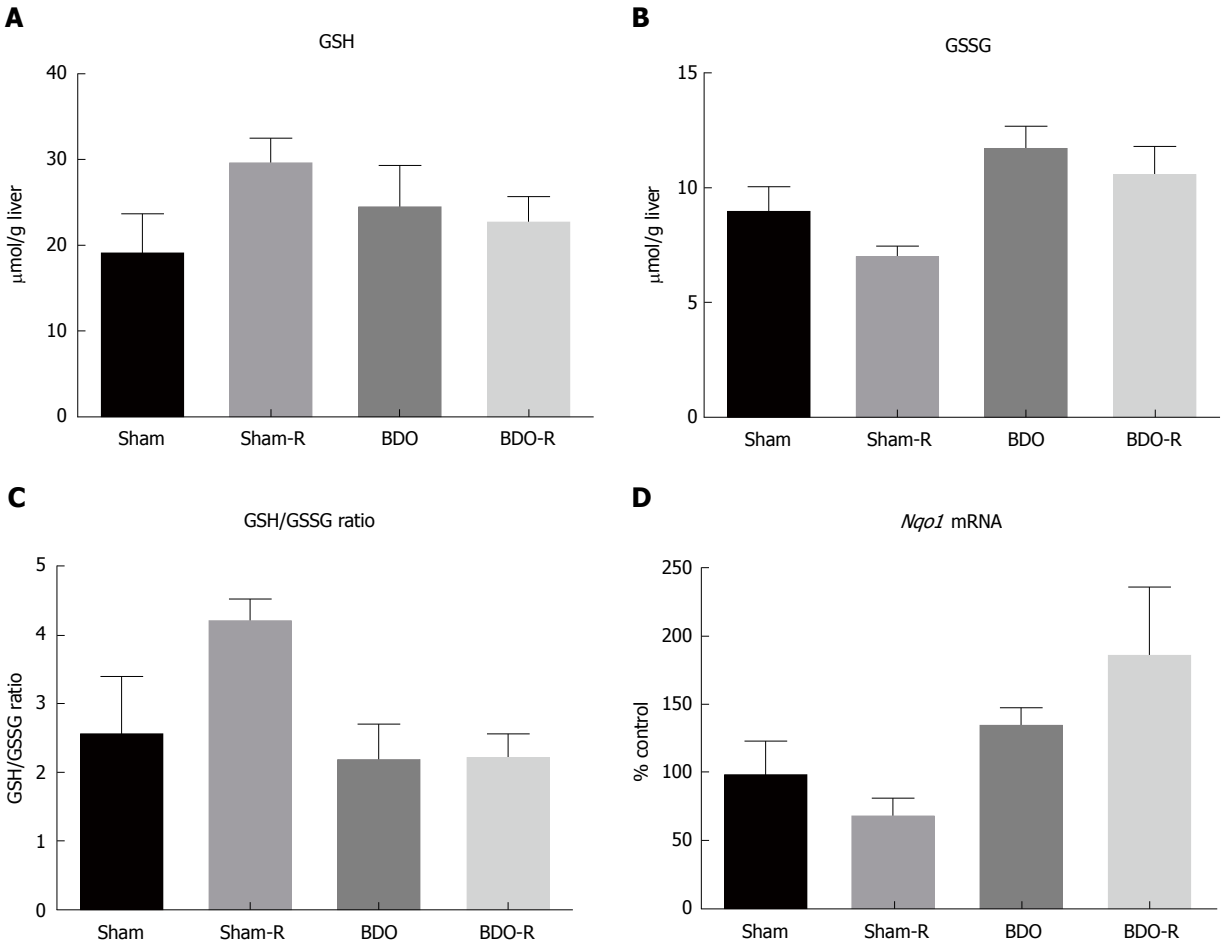

Figure 3 Reduced (GSH) (A) and oxidized (GSSG) (B) glutathione, GSH/GSSG ratio (C), and *Nqo1* mRNA (D) expression in the liver of sham and bile duct-obstructed rats. Rats were administered either saline or resveratrol (R, 10 mg/kg) daily by oral gavage for 28 d after surgery. Data are presented as the mean ± SE (n = 6, in each group). No statistically significant difference was noted between the groups. BDO: Bile duct obstruction.

enzyme.

Protein expression of enzymes and transporter proteins

Resveratrol administration to healthy animals caused a significant increase in the expression of the liver transporter proteins responsible for BA (Bsep, Mrp4), glutathione (Mrp2), and drug (Mdr1/P-gp) export from hepatocytes as well as the increased expression of Cyp7a1, the rate-limiting enzyme for bile acid synthesis from cholesterol (Figure 5). Similarly, to previous reports[21-23], untreated BDO significantly reduced the protein content of Bsep, Mrp2, Mdr2, and Oatp1a4, and increased the protein content of Mrp3, Mrp4, and Mdr1 as a part of the anticholestatic defense response in the liver. Administration of RSV to BDO animals reversed the induction of Mdr1 and Mrp3 expression and the reduction in the expression of the Oatp1a4 transporter (Figure 5).

Resveratrol effect on Sirt1/AMPK pathway

We analyzed Sirt1 and p-AMPKα protein expression to verify the predicted[15] modulation of this pathway by RSV as the major mechanism of its hepatic effects. We found that RSV significantly up-regulated both Sirt1 and p-AMPKα in sham rats but not in BDO animals (Figure 5). Bile duct obstruction alone did not affect p-AMPKα expression. In contrast, the Sirt1 protein level was significantly suppressed in the BDO group. These data indicated that resveratrol activated the Sirt1/AMPK pathway in healthy rats.

Effect of resveratrol on mRNA expression of inflammatory and fibrotic indicators

Due to the significant influence of RSV on the production of major inflammatory cytokines in the liver during various pathologies, we also analyzed these pathways at the level of mRNA (Figure 6). Quantitative RT-PCR analysis revealed no significant changes in the expression of either the acute phase cytokines such as tumor necrosis factor α (*TNF*-α) or *IL-6* in any of the experimental groups. Similarly, RSV did not change the expression of any of the indicators in sham rats. All the obtained data suggested that the modulation of cytokine production is not involved in either the effect of RSV on bile production or in the secretory mechanisms in sham rats. On the other hand, the

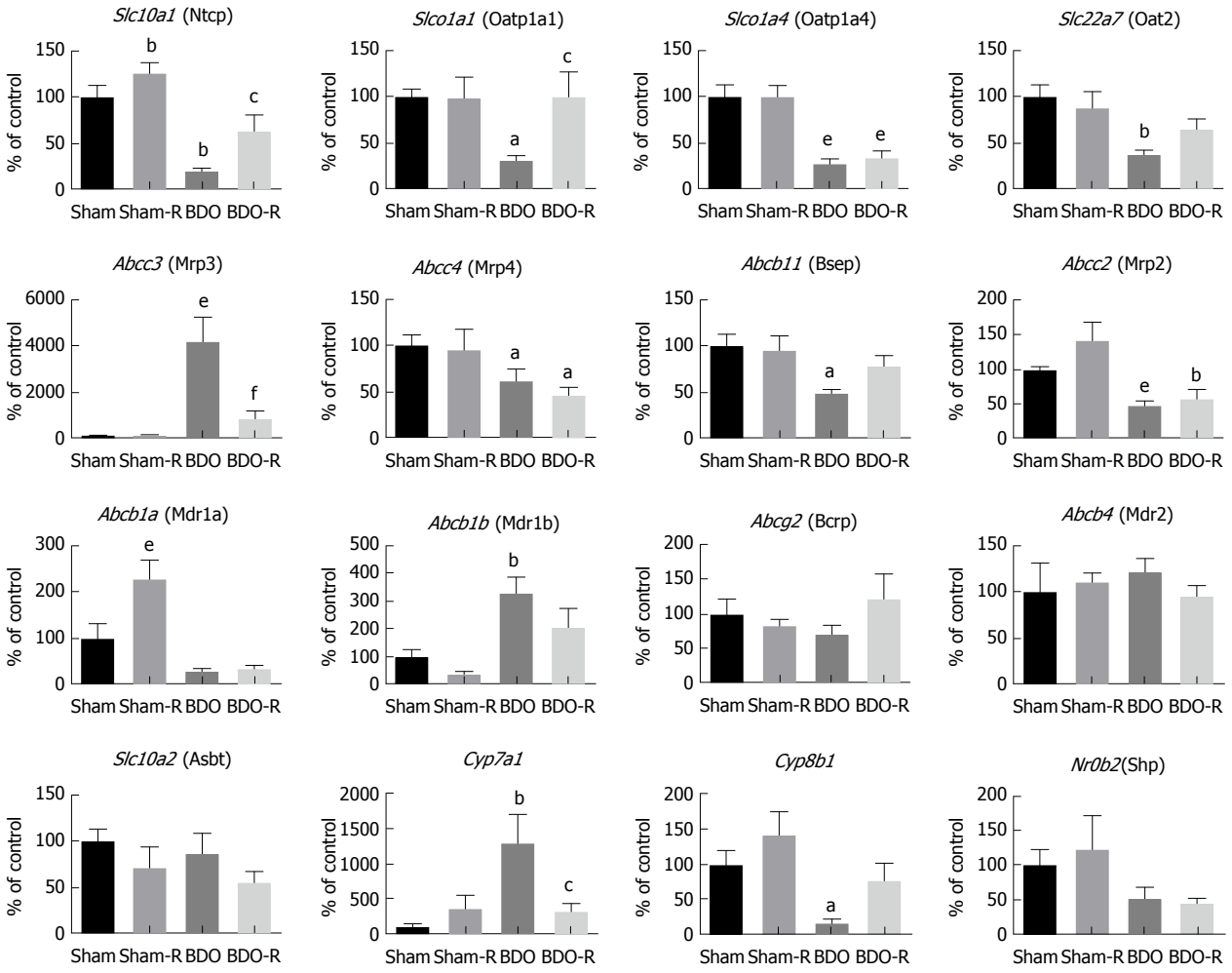

Figure 4 Gene expression of the liver transporters and enzymes involved in bile production and secretion in sham-operated and bile duct-obstructed rats. Rats received saline or resveratrol (R, 10 mg/kg) once daily by oral gavage for 28 d after surgery. The results are presented as a percentage of the saline-administered sham group (control). Data are presented as the mean ± SE (n = 6); $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* saline-treated sham group; $^{c}P < 0.05$, $^{f}P < 0.001$ *vs* saline-treated BDO group. BDO: Bile duct obstruction.

saline treated BDO group showed the induction of mRNA expression of transforming growth factor β1 (*TGF-β1*), platelet-derived growth factor receptor β (*Pdgfrβ*), and tissue inhibitor of metalloproteinase-2 (*Timp-2*). RSV administration to the BDO group led to a significant decline in the gene expression of *Pdgfrβ* (Figure 6).

RSV effect on human PXR

The LanthaScreen® TR-FRET PXR Competitive Binding assay was performed as a cell-free method to determine the ability of PXR to bind to the PXR ligand binding domain (LBD) and replace a fluorescent PXR ligand. We observed a weak, but statistically significant, decrease in fluorescence after administering RSV treatment (5 and 10 μmol/L) (Figure 7). Treatment with SR12813, a model high-affinity PXR agonist, completely suppressed TR-FRET fluorescence in the experiments, indicating the replacement of fluorescent PXR tracer from the PXR cavity. The results demonstrated that RSV is a human PXR activator with lower potency and intrinsic activity.

DISCUSSION

A major finding of our study is the identification of the inducing effect of orally administering RSV at a clinically relevant dosage on bile production and secretion in rats with intact biliary tracts. This effect was based on the increased biliary secretion of the major osmotic constituents of bile, BA and glutathione. The molecular background of RSV-induced choleresis was the induced protein expression of Bsep and Mrp2, the respective rate-limiting transporters for the BA-dependent and BA-independent bile flow at the apical membranes of hepatocytes. These mechanisms provide further support for the reported up-regulation of Mrp2 by RSV in ANIT-induced intrahepatic cholestasis in rats[16]. The increased transport of BA through the bile to the intestine also supports the observed increase in

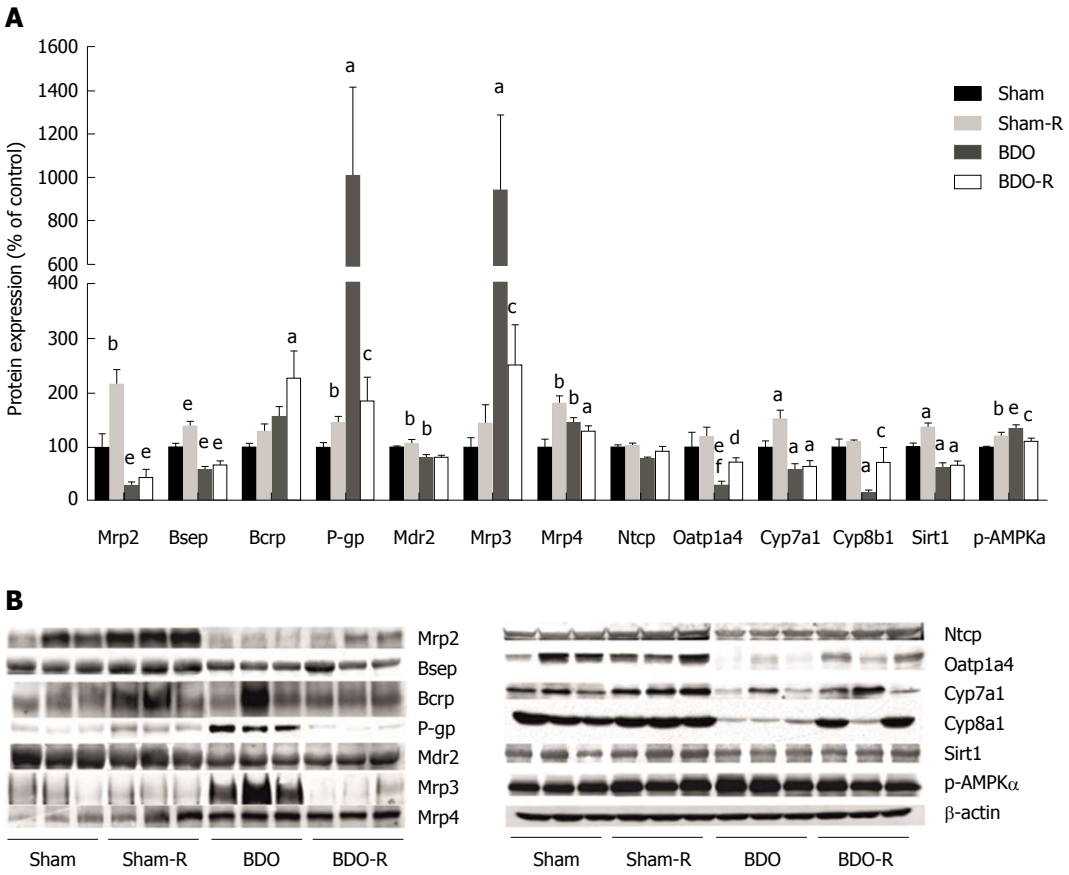

Figure 5 Protein expression of transporters, Cyp7a1, Cyp8b1, Sirt1, and p-AMPKα enzymes in the liver of sham-operated and bile duct-obstructed rats. Rats were administered either saline or resveratrol (R, 10 mg/kg) daily by oral gavage for 28 d after surgery. The results are presented as a percentage of the saline-administered sham (control) group (A). Representative immunoblots (B). Data are presented as the mean ± SE (n = 6); $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* saline-treated sham group; $^{c}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* saline-treated BDO group. BDO: Bile duct obstruction.

fecal excretion of BA in RSV-administered mice[24], as well as in tumor-bearing rats[25]. In addition, RSV administration to sham rats up-regulated hepatic Mrp4, a basolateral efflux transporter for BA. The possibility of increased export of BA back to the blood through Mrp4 is consistent with the unchanged plasma concentrations of BA in RSV-treated sham rats despite the increased BA biliary secretion. Unchanged BA plasma concentrations were also previously reported in RSV-treated mice despite reduced reabsorption of BA in the ileum[24]. These data indicated that RSV induces complex changes in BA transport in the liver of sham rats with intact bile flow.

RSV treatment in our sham rats also increased the protein expression of Cyp7a1, a rate-limiting enzyme for BA synthesis. This was consistent with the recently reported up-regulation of Cyp7a1 by a 0.4% resveratrol diet in mice. Therein, the effect was ascribed to the RSV-mediated reduction of BA reabsorption in the ileum and, consequently, the decreased activation of FXR in the ileum, which was followed by reduced Fgf15 production and disinhibition of Cyp7a1 hepatic mRNA and protein expression[24]. In addition, experiments with RSV-treated HepG2 cells demonstrated a direct induction of *ABCB11* (BSEP) and *CYP7A1* mRNA levels without mediators released from the ileum[26]. We therefore regarded the activation of FXR by RSV as a pivotal transcriptional effect regulating BA metabolism[27]. Importantly, we excluded the direct activation of the human FXR by RSV, as analyzed by a reporter gene assay (data not shown). This result corresponded to the absence of changes in mRNA levels of FXR target genes, such as *Bsep*, *Mrp2*, *Mrp4*, *Shp* or *Cyp7a1* in our RSV-treated sham rats. This indicated the posttranscriptional effect of RSV as one of the major pathways of bile formation. One such mechanism has been described for Bsep and Mrp2 proteins[28]. It is based on the increased positioning and stabilization of Bsep and Mrp2 at the canalicular membrane of hepatocytes, by agents that increase hepatic cyclic adenosine monophosphate (cAMP), with the consequent activation of protein kinase A[29]. RSV increases cAMP by competitive inhibition of degrading phosphodiesterases. The subsequent activation of the cAMP-activated protein kinase A (AMPK)/SIRT signaling pathway is currently considered to be one

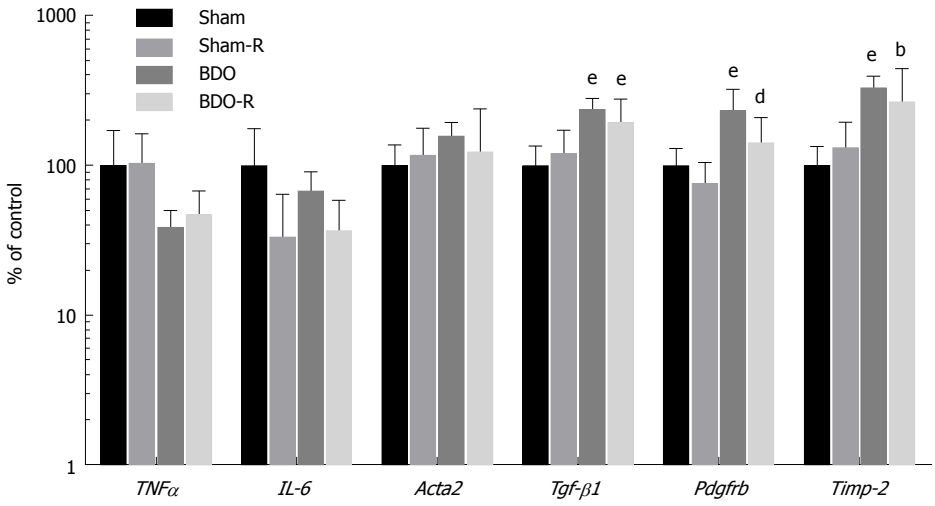

Figure 6 Gene expression of inflammatory and fibrogenic markers in the liver of sham-operated and bile duct-obstructed rats. Rats were administered either saline or resveratrol (R, 10 mg/kg) daily by oral gavage for 28 d after surgery. The results are presented as a percentage of the saline-administered sham group (control). Data are mean ± SE (n = 6); $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* saline-treated sham group; $^{d}P < 0.01$, $^{f}P < 0.001$ *vs* saline-treated BDO group. BDO: Bile duct obstruction.

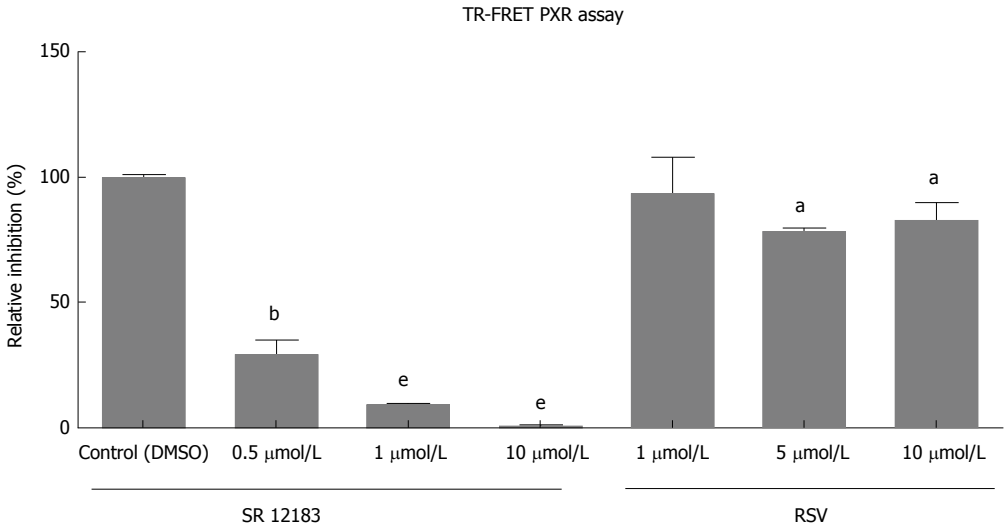

Figure 7 LanthaScreen® TR-FRET PXR competitive binding assay was performed to determine the ability of PXR to bind to the PXR ligand binding domain and replace a fluorescent PXR ligand. SR12813, a model PXR agonist, was used as a high-affinity ligand in the PXR ligand binding domain to validate the method. The data are presented as the mean ± SD from three independent experiments (n = 3) performed in triplicate measurements. Significantly different compared to the control treatment. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$.

of the major hepatoprotective mechanisms of RSV[15]. Indeed, we verified an increase in the expression of p-AMPK and Sirt1 in resveratrol-administered sham animals. It is therefore possible that the activation of this cascade is also responsible for the inducing effect of RSV on Bsep, Mrp2, and Mrp4 in healthy sham rats. The exact posttranscriptional mechanism of RSV in the liver should be further studied.

Choleretic agents stimulate bile production either *via* direct osmotic activity when they are concentrated in the bile, such as bile acids or penicillin G[30], or by transcriptional induction of Bsep or Mrp2 through the stimulation of nuclear receptors, namely, either FXR or PXR[31,32]. The possibility of a direct osmotic effect of resveratrol in our setting may be excluded because bile collection was performed in rats 24 h after receiving the last dose of RSV. Considering the very short half-life of the parent compound, that is approximately 1.31 h[33], the concentrations of unchanged resveratrol in bile should be negligible at the time of the evaluation. Our results also argued for an intensive transcriptional regulation through nuclear receptors. Detailed *in vitro* and *in vivo* experimentation excluded the activation of FXR by RSV. RSV administration to sham rats induced only liver *Abcb1a*, a PXR/Pxr target gene encoding Mdr1, the major canalicular transporter for biliary

excretion of numerous xenobiotics, including drugs[34]. The effect paralleled the induction of Mdr1 protein and increased the BE of the well-known Mdr1 and Mrp2 substrate, azithromycin[34]. This was consistent with the recently reported inducing effect of RSV on the excretion of another Mdr1 substrate, cyclosporine[35]. Thus, we have performed TR-FRET PXR coactivator assay and confirmed that RSV activates PXR (Figure 7). This result indicated a low potency of RSV at the receptor[36]. However, the activation of the PXR-*Abcb1a* axis in RSV-administered sham rats suggests a sufficient concentration of RSV within the liver[37] at clinically relevant dosage. Together with the reported ability of RSV to inhibit the main drug metabolizing enzymes such as CYP3A4 or CYP2C9 with an IC_{50} of 1.1-4.5 μmol/L[38], and the recently described inhibition of Mdr1, Mrp2 and Oat1/3[39], our data suggested the potential of RSV to induce significant variability in the pharmacokinetics of simultaneously administered drugs.

The induction of BA-dependent choleresis during extrahepatic cholestasis is known for the worsening of ongoing liver impairment by raising the intrabiliary pressure and by biliary infarcts with concentrated BA[40]. However, RSV has repeatedly showed hepatoprotection in bile duct-ligated rats[12,17,41]. Therefore, we also examined both the bile production and the liver status in rats with BDO for 28 d. Untreated BDO rats developed typical histological, and biochemical signs upon initiated biliary cirrhosis[42] that were accompanied by increased hepatic gene expression of fibrotic markers such as *TGF-β1, Col1a1, Pdgfrβ* and *Timp-2*. Untreated BDO rats also developed changes in the expression of transporters, which was typical for a spontaneous cholestatic defense response (Figure 2)[22]. Interestingly, BDO rats did not show the mRNA induction of acute inflammatory markers, *TNFα* or *IL-6*, or significant oxidative stress, as measured by glutathione content, GSH to GSSG ratio, and *Nqo1* expression. This discrepancy was produced by the free radical scavenging effect of accumulating bilirubin[43] and impaired biliary secretion of GSH before the release of obstruction. Similar to our previous findings, the release of biliary obstruction resulted in massive choleresis due to the impairment of the blood-biliary barrier[21].

Administration of RSV to BDO rats indeed attenuated histological and biochemical signs of the initiated biliary cirrhosis, including the reduction of BA concentrations in plasma and fibrotic markers in the liver. Hepatoprotection by RSV paralleled the decrease in bile flow, without the corresponding reduction in BE of BA or glutathione. This was consistent with the unchanged hepatic expression of Bsep or Mrp2 in BDO-R animals. In this situation, RSV reversed the BDO-induced up-regulation of ZO-1, an integral protein of tight-junctions, which indicates the protective effect of RSV on the alteration of the permeability of the blood-biliary barrier during obstructive cholestasis. Together with the unchanged expression of the rate-limiting Cyp7a1 enzyme for BA synthesis and the unchanged urinary excretion of bile acids (unpublished observation), these data suggested that the reduced plasma concentrations of BA in BDO-R rats may result from RSV-mediated reduction of intestinal reabsorption as shown in healthy mice[24]. This effect deserves further analysis. However, the marked differences between RSV-mediated changes in BA homeostasis in sham and BDO rats indicated a different regulatory mechanism. As such, we did not detect activation of AMPKα-Sirt1 following RSV treatment in BDO rats. The expression of proinflammatory cytokines was also not modified by RSV treatment in bile duct-obstructed rats. This result was consistent with the previously observed disappearance of the inhibitory effect of RSV on the expression of proinflammatory mediators the seventh day after bile duct ligation[41]. We detected only a reduction in *Pdgfrβ* mRNA expression (Figure 6), which was consistent with the reduced bile duct proliferation in RSV-treated BDO animals. On the other hand, accumulating BA levels are among the main mediators of hepatic damage during obstructive cholestasis that promotes inflammation and fibrosis[44]. Thus, our data indicated that the BA-lowering effect is the primary hepatoprotective mechanism of RSV during BDO in rats.

We noticed the down-regulation of hepatic Mdr1 by RSV in cholestatic animals is supported by the reduced biliary secretion of AZT. This effect resulted from decreased *Mdr1b* gene expression by RSV in BDO rats, perhaps as a consequence of reduced BA levels, which contributes to Mdr1 activation during cholestasis[45]. Although the exact mechanism requires further analysis, our data so far suggest the potential of RSV to alter the elimination of Mdr1 substrates during obstructive cholestasis.

The present study provides insight into the choleretic activity of RSV oral administration in rats with intact liver and biliary tract. This effect was caused by the induction of Bsep, Mrp2 and Cyp7a1 protein expression in the liver. Together with the induction of the expression of the Mdr1 and Mrp4 transporters, the results also indicated the potential of RSV to increase biliary secretion of co-administered substrates, including drugs from the organism. Moreover, a significant hepatoprotective effect of RSV in rats with extrahepatic cholestasis was related to a reduction in the plasma concentrations of BA as well as the prevention of blood-biliary barrier damage. RSV did not induce obvious changes in the expression of BA transporters in BDO rats, which can explain such an observation. Extrahepatic mechanisms of RSV-

mediated reduction of BA plasma concentrations during obstructive cholestasis therefore must be a focus of further studies.

COMMENTS

Background

All pathways responsible for bile secretion, the major excretory route for potentially harmful endogenous substrates such as bilirubin and bile acids (BA) as well as for numerous exogenous lipophilic compounds, are sensitively regulated and modified by numerous stimuli, including diseases, drugs and food ingredients, which may either directly affect the activity of individual transporters or indirectly act by changing their expression. Resveratrol, a natural polyphenol, was shown to have a beneficial effect on a variety of diseases, such as malignancies and toxic and inflammatory tissue injuries, and it was shown to have marked hepatoprotective potential in situations such as non-alcoholic fatty liver disease, extrahepatic cholestasis, and α-naphthylisothiocyanate-, acetaminophen-, or carbon tetrachloride-induced hepatotoxicity.

Research frontiers

Recent results have suggested that intraperitoneally administered resveratrol may prevent the impairment of bile formation during α-naphthylisothiocyanate (ANIT)-induced intrahepatic cholestasis. However, the effect of resveratrol on bile formation and secretion has not been determined in healthy rats or in the general population without cholestatic liver impairment, despite its wide use as a nutraceutical. Moreover, in comparison to the commonly used oral administration, intraperitoneal administration of RSV overcame its low bioavailability and markedly increased its exposure to the organism.

Innovations and breakthroughs

This is the first study to show the ability of orally administered RSV to increase bile flow in healthy rats as a consequence of increased biliary excretion of bile acids and glutathione *via* posttranscriptional induction of their rate-limiting transporters, Bsep and Mrp2, respectively, and by the up-regulation of Cyp7a1, an enzyme crucial for bile acid synthesis. Resveratrol simultaneously induced hepatic expression of Mdr1, which was verified by the increased biliary excretion of its substrate, azithromycin. Our findings were consistent with the agonistic effect of resveratrol on PXR. Moreover, RSV showed significant hepatoprotective potential in rats with long-term extrahepatic cholestasis, which was related to the reduction of plasma concentrations of BA and to the prevention of blood-biliary barrier damage.

Applications

Our data indicated the potential of orally administered resveratrol to increase biliary secretion of co-administered substrates, including drugs from the organism. Moreover, new mechanisms of RSV-mediated hepatoprotection by the reduction of bile acid plasma concentrations during extrahepatic cholestasis supports the safe use of resveratrol in such disorders.

Terminology

Bile is the major excretory route for potentially harmful endogenous substrates (e.g., bilirubin and bile acids), and for numerous exogenous lipophilic compounds. Biliary secretion is an active process mediated by transporting proteins secreting their substrates into the bile canaliculi in the liver. Cholestasis is a pathological condition in which bile cannot flow from the liver to the duodenum.

Peer-review

The authors presented a carefully executed scientific study on the effect of resveratrol on biliary excretion in sham-operated and bile duct-obstructed rats. This study revealed that administration of resveratrol results in an increase in bile flow and the activation of transporters of bile acids and lipophilic compounds in normal rats. The authors showed that resveratrol improves the morphology of the bile duct and disturbed the molecular and biochemical indicators in bile duct-obstructed rats.

ACKNOWLEDGMENTS

We gratefully acknowledge the skillful technical assistance of Jitka Hájková and Hana Laštůvková.

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P- Reviewer: Liaskou E, Reshetnyak VI, Smith RC **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7693-7704

DOI: 10.3748/wjg.v23.i43.7693

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

ORIGINAL ARTICLE

Basic Study

Chitinase 3-like 1 secreted by peritumoral macrophages in esophageal squamous cell carcinoma is a favorable prognostic factor for survival

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Author contributions: Xing S and Zheng X contributed equally to this work; Xing S, Zheng X and Liu WL participated in the experimental design and carried out the experiments; Xing S and Liu WL drafted the manuscript and interpreted the data; Cao YS, Pan KL and Wei C collected the samples; Zeng T and Hou F took part in the acquisition of the data; Zeng MS and Zhong Q provided experimental guidance; and all the authors read and approved the final manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Sun Yat-sen University Cancer Center.

Conflict-of-interest statement: The authors declare no potential conflicts of interest.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: June 30, 2017
Peer-review started: June 30, 2017
First decision: August 15, 2017
Revised: September 13, 2017
Accepted: September 26, 2017
Article in press: September 26, 2017
Published online: November 21, 2017

Abstract

AIM

To identify whether chitinase 3-like 1 (CHI3L1) serves as a suitable biomarker for the prognosis of esophageal squamous cell carcinoma (ESCC) and to analyze this protein's cellular source.

METHODS

An ELISA was conducted to detect the concentration of CHI3L1 in the serum of 150 ESCC patients diagnosed

between January 2001 and February 2005. The prognostic relevance of CHI3L1 was evaluated by a Kaplan-Meier and Cox regression analysis. The immunohistochemistry was reanalyzed, and fluorescent staining was utilized to explore the cellular origins of CHI3L1. We stimulated monocyte-derived macrophages (MDMs) with either IL-6 or the supernatant of the ESCC cell line Eca-109 and later investigated the level of CHI3L1 by qPCR and ELISA.

RESULTS

The level of serum CHI3L1 was higher in older patients (≥ 60) than in patients under the age of 60 (P = 0.001). The patients with higher levels of CHI3L1 had a significantly shorter overall survival, whereas the traditional markers, carcinoembryonic antigen and squamous cell carcinoma antigen, were less effective (P > 0.05). A multivariate Cox analysis (P = 0.001) indicated that CHI3L1 was an independent prognostic factor for ESCC patients. Peritumoral macrophages in ESCC exhibited high levels of CHI3L1. Interleukin-6 (IL-6) and the supernatant of Eca-109 containing IL-6 stimulated MDMs to secrete CHI3L1. The serum concentration of CHI3L1 in the ESCC patients showed a weak correlation with the laboratory inflammatory parameters neutrophil (NEU, P = 0.045), neutrophil/lymphocyte rate (NLR, P = 0.016), and C-reactive protein (CRP, P < 0.001).

CONCLUSION

Our study first established a connection between the pretreated CHI3L1 and patients with ESCC, and the serum CHI3L1 was primarily secreted by ESCC-surrounded macrophages.

Key words: Esophageal squamous cell carcinoma; Prognostic biomarker; Chitinase 3-like 1; Macrophage; Esophageal squamous cell carcinoma

Core tip: The current staging system is inadequate for predicting post-treated survival. Our study first established a connection between pretreated chitinase 3-like 1 (CHI3L1) and patients with esophageal squamous cell carcinoma (ESCC), and serum CHI3L1 was primarily secreted by ESCC-surrounded macrophages, suggesting that CHI3L1 was a simple and inexpensive prognostic factor. This simple, convenient serological testing allows for clinical application. In addition, in our study, the ESCC microenvironment, especially the secretion of IL-6 by esophageal tumor cells, promotes the macrophage production of CHI3L1. These findings might help to identify high-risk patients for treatment decisions and to elucidate the mechanisms of ESCC.

Xing S, Zheng X, Zeng T, Zeng MS, Zhong Q, Cao YS, Pan KL, Wei C, Hou F, Liu WL. Chitinase 3-like 1 secreted by peritumoral macrophages in esophageal squamous cell carcinoma is a favorable prognostic factor for survival. *World J Gastroenterol* 2017; 23(43): 7693-7704 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7693.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7693

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the major histological subtypes of esophageal cancer. ESCC is the fourth most lethal cancer in China[1,2]. Ninety percent of the cases are squamous cell carcinomas[3,4]. With improvements in the diagnosis, staging system and treatment strategies, the overall 5-year survival rate of ESCC patients increased slightly. However, patients at the same stage undergoing similar treatment regimens often have notably different clinical outcomes, which suggests that the current staging system is inadequate for predicting survival. Several recent studies show that clinically used tumor markers, such as squamous cell carcinoma antigen (SCCA) and carcinoembryonic antigen (CEA), play important roles in tumorigenesis and the development of ESCC[5-7]. However, these antigens lack sufficient power and validity to be adopted for prognosis of cancer[8]. Certain studies have identified mRNA and protein biomarkers correlated with the prognosis of ESCC patients[9,10], but few biomarkers have sufficient evidence to be adopted for clinical use. Therefore, the identification of accurate biomarkers for ESCC is necessary to improve the clinical outcome of patients.

Chitinase 3-like 1 (CHI3L1) is a secreted glycoprotein that belongs to a group of mammalian proteins with an amino acid sequence that is similar to the 18-glycosyl hydrolase group of bacterial chitinases[11]. Various human cells, such as synovial, cartilage, endothelial, neutrophil and macrophage cells produce CHI3L1[12]. This protein is associated with the malignant behavior of several types of cancers, and elevated levels of CHI3L1 are notably correlated with a poor prognosis and short survival in ovarian cancer[13], breast cancer[14], lung cancer[15], hepatocellular carcinoma[16] and glioblastoma[17]. However, no information exists regarding whether serum CHI3L1 predicts ESCC patient survival.

Our previous study suggested that CHI3L1 may represent a diagnostic biomarker in ESCC patients[5]. In this study, we examined CHI3L1 expression in the serum from 150 ESCC patient by ELISA and later explored its cellular source. For the first time, we investigated the ability of CHI3L1 to predict ESCC patient survival, as well as the exact origin of CHI3L1 in ESCC. This signature represents a novel biomarker to predict ESCC patient survival more accurately and understand the mechanisms of the genesis and development of ESCC.

MATERIALS AND METHODS

Serum and tissue specimen

In this study, all the selected ESCC patients met the following inclusion criteria: Pathological examination confirmation of primary ESCC by the available biopsy

samples at the Sun Yat-sen University Cancer Center (SYSUCC) and no anticancer treatments having been given previously. The absence of diseases, such as COPD and second primary carcinomas, was assessed by physical examination, clinical history, gastroscopy, colonoscopy and routine laboratory tests (including liver and renal function tests). Follow-up information was available for all of these patients.

The tumors were staged according to the 7th edition of the tumor-node-metastasis (TNM) classification for esophageal carcinoma (UICC, 2009).

Serum from 150 ESCC patients was obtained at the time of diagnosis before treatment from January 2001 to February 2005. The final confirmation date of the patients' condition was in February 2010. Venous blood (3-5 mL) was clotted at room temperature, centrifuged at 3000 r/min for 10 min and stored at -80 ℃ until it was used. A total of 20 formalin-fixed and paraffin-embedded ESCC tumor specimens for immunochemistry and fluorescent staining were obtained from 2012 to 2013 as described before[8].

Prior to the use of these serum and tissues, informed consent was obtained from each participant. This experiment was approved by the Institute Research Ethics Committee of SYSUCC, Guangzhou.

Cell lines

The ESCC cell line Eca-109 (Chinese Academy of Sciences, Shanghai, China) was grown at 37 ℃ in 5% CO_2 in RPMI 1640 (Invitrogen, United States) supplemented with 10% fetal bovine serum. The Eca-109 supernatant was collected after an incubation for 48 h. The cell line was obtained between 2012 and 2014.

ELISA

Serum CHI3L1 levels were determined by a double-antibody sandwich ELISA according to the manufacturer's instructions (R&D systems, United States). Briefly, 96-well microplates were coated with 100 μL/well of the capture antibody (rat anti-human CHI3L1, 2.0 μg/mL) overnight at room temperature. After blocking with 3% BSA for 1 h, 100 μL of the patients' serum (1:100 diluted in blocking buffer) was added and incubated for 2 h at room temperature. Subsequently, 100 μL/well of the detection antibody (biotinylated goat anti-human CHI3L1, 200 ng/mL) was added and incubated for 2 h at room temperature. Next, 100 μL/well of Streptavidin-HRP (1:200) was added and incubated for 20 min at room temperature. Finally, the substrate (tetramethylbenzidine) solution was added, and the reaction was stopped with 2 mol/L H_2SO_4 and read at an OD of 450 nm. Each test included a standard control (CV < 12%).

CEA and SCCA assays

The level of CEA in the serum was assessed using an electrochemiluminescence immunoassay (ECLIA) kit (Roche, German) on a Roche Cobas8000 fully automatic electrochemistry luminescence immunity analyzer (Roche, Germany). The concentration of SCCA in the serum was detected using an ARCHITECT I2000SR immune analyze system (Abbott, United States). Each test included a standard control (CV < 5%).

Immunohistochemistry

IHC was performed in our previous study[8]. The degree of immunostaining was analyzed again by two independent observers.

Fluorescent staining of the paraffin-embedded sections

After rehydrating and blocking, the formalin-fixed, paraffin-embedded ESCC sections incubated with a rabbit polyclonal anti-CHI3L1 antibody (1:100, Bioss, China) and a mouse polyclonal anti-CD68 antibody (1:100, Zhongshan Golden Bridge, China) overnight at 4 ℃. The slides were washed 3 times for 15 min each in PBST wash buffer and were then incubated with a goat anti-rabbit IgG secondary antibody (FITC) and a goat anti-mouse IgG secondary Antibody (PE) for 30 min at room temperature (1:500, Ebioscience, United States). After 3 washes, the tissue sections were incubated by diluted DAPI for 5 min at room temperature, mounted with an anti-fade mounting media and then visualized using a fluorescence microscope by two independent observers.

Cytokines

Cytokines in serum samples were measured with the BD CBA Th1/Th2/Th17 Cytokine Kit (BD Bioscience, United States). The kit was used for the simultaneous detection of interleukin-2 (IL-2), IL-4, IL-6, interferon-γ (IFN-γ) and IL-10 in a single sample. This array kit provides a mixture of capture beads with distinct fluorescence intensities are coated with capture antibodies specific for each cytokine. The operations were performed according to the manufacturer's instructions. The individual cytokine concentrations were indicated by their fluorescent intensities. The concentrations of all the cytokines were reported in pg/mL.

Isolation and culturing of primary monocytes to obtain monocyte-derived macrophages

Human peripheral blood was collected from healthy donors, and monocytes from Ficoll-isolated PBMCs were separated from the lymphocytes by adherence to tissue culture-treated plates[18]. After 48 h of incubation, the non-adherent cells were removed *via* two washes with warm RPMI. The monocytes were differentiated into macrophages (MDMs) by culturing in RPMI medium supplemented with 10% fetal bovine serum for 15 d prior to stimulation. The MDMs were washed with phosphate-buffered saline (PBS), and the culture medium was replaced every 2 d. MDMs were stimulated for 24 h with IL-6 (1 μg/mL) or the Eca-109 cell line supernatant. After 24 h, the RNA was extracted for with the Trizol reagent (Invitrogen, United States), and the

culture medium were collected for ELISA.

Real-time RT-PCR

Total RNA was extracted from the cell lines and frozen ESCC tissues using the Trizol reagent (Invitrogen, United States) according to the manufacturer's instructions. Reverse transcription of the total RNA (2 μg) was derived using SuperScript Ⅱ reverse transcriptase (BioRad, United States). The quantification of the target and reference (GAPDH) genes was performed in triplicate on a LightCycler® 480 Ⅱ (Roche, Applied Science) using a SYBR green-based assay (BioRad, United States). The primers used in the real-time RT-PCR reaction were as follows: CHI3L1, NBCI RefSeq Database entry: NM_001276.2, forward 5′-GAGGATGGAACTTTGGGTCTC-3′ and reverse 5′-TCATTTCCTTGATTAGGGTGGT-3′ and GAPDH, NBCI RefSeq Database entry: NM_001256799.2, forward 5′-GACTCATGACCACAGTCCATGC-3′ and reverse 5′-AGAGGCAGGGATGATGTTCTG-3′. The results were expressed as mean ± SD.

White blood cell, neutrophil, lymphocyte, platelet counts and C-reactive protein assay

White blood cell (WBC), neutrophil (NEU), lymphocyte (LY) and platelet (PLT) were collected from the blood acquired during a routine examination, and the results were detected using a Sysmex XE5000 analyzer (Sysmex, Japan). The NLR was defined as the ratio of NEUs and LYs, and the PLR was defined as the ratio of PLTs and LYs. Serum C-reactive protein (CRP) was determined by a latex-enhanced homogeneous immunoassay on a Hitachi LAS008 analyzer (Hitachi, Japan).

Statistical analysis

The data were analyzed by SPSS standard version 16.0 (SPSS, Chicago, United States). The optimal cutoff level for CHI3L1 was determined as the value with the maximization of the Yuden index by the receiver operating characteristic (ROC) analysis, whereas the cutoff levels for the clinical markers, SCCA and CEA, were the upper limit of normal reference values, which were 5.0 ng/mL for CEA and 1.5 ng/mL for SCCA. The Kaplan-Meier method was used to estimate the overall survival (OS), and the multivariate analysis was performed using the Cox proportional hazards model. The χ^2 test was used to analyze the relationship between CHI3L1 level and the clinicopathological characteristics. The associations of CHI3L1 with inflammatory indexes were analyzed by a Spearman correlation. All of the statistical tests were two-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Patients' characteristics

The clinicopathological and laboratory characteristics of 150 ESCC patients are summarized in Table 1. Among these patients, 113 (75.3%) were males, and 37 (24.7%) were females, exhibiting a median age of 60 (range 30-96). In total, there were 51 (34.0%) early stage (Ⅰ-Ⅱ) ESCC patients and 93 (62.0%) advanced stage (Ⅲ-Ⅳ) patients in the cohort. Of the included patients, 73 patients underwent surgery. Of the 73 patients who underwent surgery, 3 patients received chemotherapy followed by surgical resection, 2 received radiation before surgery and the others did not undergo any additional treatment. Fifty-seven patients received chemotherapy administered concurrently with radiation therapy. Eleven subjects underwent systemic chemotherapy. A variety of chemotherapy regimens were employed, and most commonly, they were cisplatin and 5-Fluorouracil. The relationship between the CHI3L1 and clinicopathological characteristics of the ESCC patients is presented in Table 1. There were no differences of CHI3L1 level in regards to sex (P = 0.713), smoking behavior (P = 0.514), alcohol status (P = 0.984), tumor grade (P = 0.736) T classification (P = 0.886), N classification (P = 0.218), metastasis (P = 0.156), clinical stage (P = 0.712), SCCA level (P = 0.539) CEA level (P = 0.389) or treatment options (P = 0.138). However, the CHI3L1 level was associated with age (P = 0.001). The level of serum CHI3L1 was higher in older patients (≥ 60) than in patients under the age of 60.

Prognostic value of CHI3L1 in ESCC

The median OS was 1.82 years for the entire cohort of patients with a five-year overall survival of 24.67%. The patient survival curves were constructed *via* the Kaplan-Meier method and were compared using the log-rank test. Kaplan-Meier estimates of the OS for patients with CHI3L1 and the levels of the clinically used ESCC markers, SCCA and CEA, are shown in Figure 1. The results showed that patients with elevated CHI3L1 levels were significantly associated with a shorter OS (P = 0.019; Figure 1A). Further stratification of the patient groups based on stage displayed that the correlation of elevated CHI3L1 levels and a shorter OS was statistically significant in Stage Ⅰ-Ⅱ patients with ESCC (P = 0.003; Figure 1B). However, in Stage Ⅲ-Ⅳ, there was no significant association between elevated CHI3L1 levels and a shorter OS (P = 0.089; Figure 1C). The traditional ESCC markers, SCCA and CEA, were not significantly relevant to the OS (SCCA, All: P = 0.087, Figure 1D, Stage Ⅰ-Ⅱ: P = 0.756, Figure 1E, Stage Ⅲ-Ⅳ: P = 0.003, Figure 1F; CEA, All: P = 0.847, Figure 1G, Stage Ⅰ-Ⅱ: P = 0.325, Figure 1H, Stage Ⅲ-Ⅳ: P = 0.835, Figure 1I).

Next, we examined the OS using the Cox proportional hazards model to determine whether the level of CHI3L1 serves as an independent predictor. A series of factors, including age, sex, alcohol intake, G grade, TNM stage, treatment options, SCCA, CEA, and CHI3L1 concentration, were entered into the univariate Cox

Table 1 Levels of chitinase 3-like 1 and clinical and laboratory characteristics of 150 esophageal squamous cell carcinoma patients

Characteristics	No. of patients	Levels of CHI3L1		
		Low	High	*P* value
All	150	97	53	
Age				0.009
Median	60			
Range	30-96			
< 60	81	60	21	
⩾ 60	69	37	32	
Sex				0.713
Male	113	74	39	
Female	37	23	14	
Smoking status				0.514
Smoker	99	66	33	
Non-smoker	49	30	19	
Alcohol intake				0.984
Yes	56	36	20	
No	90	58	32	
Grade				0.736
Grade 1	24	16	8	
Grade 2	57	36	21	
Grade 3	41	29	12	
T status				0.886
pT 1/2	31	21	10	
pT 3/4	113	75	38	
N status				0.154
pN 0	63	46	17	
pN 1/2/3	81	50	31	
M status				0.156
pM 0	112	78	34	
pM 1	32	18	14	
Stage				0.712
Ⅰ-Ⅱ	51	35	16	
Ⅲ-Ⅳ	93	61	32	
SCCA				0.539
⩽ 1.5	101	67	34	
> 1.5	49	30	19	
CEA				0.389
⩽ 5.0	132	87	45	
> 5.0	18	10	8	
Treatment				0.138
Surgery	73	49	24	
Chemoradiotherapy	57	37	20	
Chemotherapy	11	4	7	

CHI3L1: Chitinase 3-like 1; SCCA: Squamous cell carcinoma antigen; CEA: Carcinoembryonic antigen.

regression analysis in Table 2 to assess their impact on the OS of ESCC patients. Sex, smoking status, TNM stage, treatment options, SCCA, and CHI3L1 were significant in the univariate analysis, and therefore, we further analyzed these using a multivariate analysis. The multivariate analysis model revealed that the predominant independent predictors of OS were the CHI3L1 level (HR, 1.004; 95%CI: 1.002-1.005; P < 0.001), sex (HR, 0.346; 95%CI: 0.154-0.776; P = 0.010), TNM stage (HR, 1.755; 95%CI: 1.288-2.390; P < 0.001) and SCCA concentration (HR, 1.056; 95%CI: 1.013-1.100; P = 0.010) as presented in Table 2. Separate analyses of the prognostic effect of CHI3L1 in the subgroup of patients treated with surgery also showed a significant effect for predicting the OS (P = 0.014, not shown).

Tumor surrounded macrophages in ESCC show high expression levels of CHI3L1

In this experiment, we sought to identify the cells in the ESCC tissues secreting CHI3L1. In our previous study[8], we confirmed that CHI3L1 was expressed in 85% of ESCC tissues, among which 7/20 ESCC tissues had a strong stain, and 3/20 did not show any CHI3L1 expression. Additionally, the expression rate of CHI3L1 in the normal esophageal epithelium was only 10%. Through a reanalysis, we found that in 18/20 slices, the esophageal tumor surrounded cells had a high expression of CHI3L1 (Figure 2A). Fluorescence staining was performed to explore the origins of CHI3L1 in the surrounding areas of the tumor cells. As shown in Figure 2B, CD68 protein expression, indicated by the red stain, was found in the same areas as CHI3L1 expression, supporting that CHI3L1 was expressed by macrophages.

Supernatant of the ESCC cell line Eca-109 and IL-6 stimulate Monocyte-derived macrophages to secret CHI3L1

To explore the role of the tumor microenvironment in the secretion of CHI3L1 by macrophages, we used the supernatant of the ESCC cell line Eca-109 to culture the MDMs. Compared with the controls, the stimulation group had a higher level of CHI3L1 expression at both the mRNA level (Figure 3A left, 2.186 ± 1.719 *vs* 0.1982 ± 0.2918, P = 0.0378) and the protein level (Figure 3A right, 59.31 ± 28.31 *vs* 27.28 ± 16.89, P = 0.0337). In order to access which component in the Eca-109 cell supernatant works, we detected the cytokine and CHI3L1 levels in the Eca-109 cell supernatant. As shown in Figure 3B, the IL-6 concentration was the highest (119.21 pg/mL), whereas IL-4, IL-2, IL-10, IFN-γ and TNF-α were rarely detected (< 2.5 pg/mL). To imitate the cell media, we used 2 ng/mL of IL-6 to stimulate healthy MDMs. The results showed that the IL-6 group had a higher level of CHI3L1 expression at both the mRNA level (Figure 3C left, 3.841 ± 3.637 *vs* 1.465 ± 1.813, P = 0.0493) and the protein level (Figure 3C right, 160.9 ± 187.8 *vs* 116.8 ± 194.7, P = 0.0174).

Correlation of CHI3L1 and laboratory inflammatory parameters

Macrophages are the central cell type that directs host inflammatory and immune processes, and inflammation plays a vital role in tumorigenesis. Thus, we further investigated the correlation of CHI3L1 and laboratory inflammatory parameters. The serum concentration of CHI3L1 in the ESCC patients showed a weak correlation with NEUs (R^2 = 0.027, P = 0.045), NLR (R^2 = 0.039, P = 0.016), and CRP (R^2 = 0.093, P < 0.001) but no correlation with WBCs, LYs, PLTs, and PLR when

Table 2 Univariate and multivariate analysis of overall survival

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	0.989	0.970-1.009	0.271	-	-	-
Sex	0.489	0.293-0.815	0.006	0.346	0.154-0.776	0.010
Smoke status	0.745	0.595-0.932	0.010	1.071	0.761-1.507	0.694
Alcohol intake	0.817	0.546-1.223	0.327	-	-	-
G grade	1.131	0.836-1.530	0.533	-	-	-
TNM stage	2.001	1.564-2.560	< 0.001	1.755	1.288-2.390	< 0.001
CHI3L1	1.002	1.001-1.004	0.011	1.004	1.002-1.005	< 0.001
SCCA	1.065	1.028-1.104	< 0.001	1.056	1.013-1.100	0.010
CEA	1.048	0.972-1.130	0.224	-	-	-
Treatment	1.402	1.218-1.613	< 0.001	1.167	0.979-1.391	0.084

Variables: Age, ≥ 60 *vs* < 60; gender, female *vs* male; smoke, smoker *vs* non-smoker; alcohol intake, yes *vs* no; grade, grade 3 *vs* grade 2 *vs* grade 1; stage, Ⅳ *vs* Ⅲ *vs* Ⅱ *vs* treatment, surgery *vs* chemotherapy *vs* chemoradiotherapy; -: Represent "date not available". CHI3L1: Chitinase 3-like 1; SCCA: Squamous cell carcinoma antigen; CEA: Carcinoembryonic antigen.

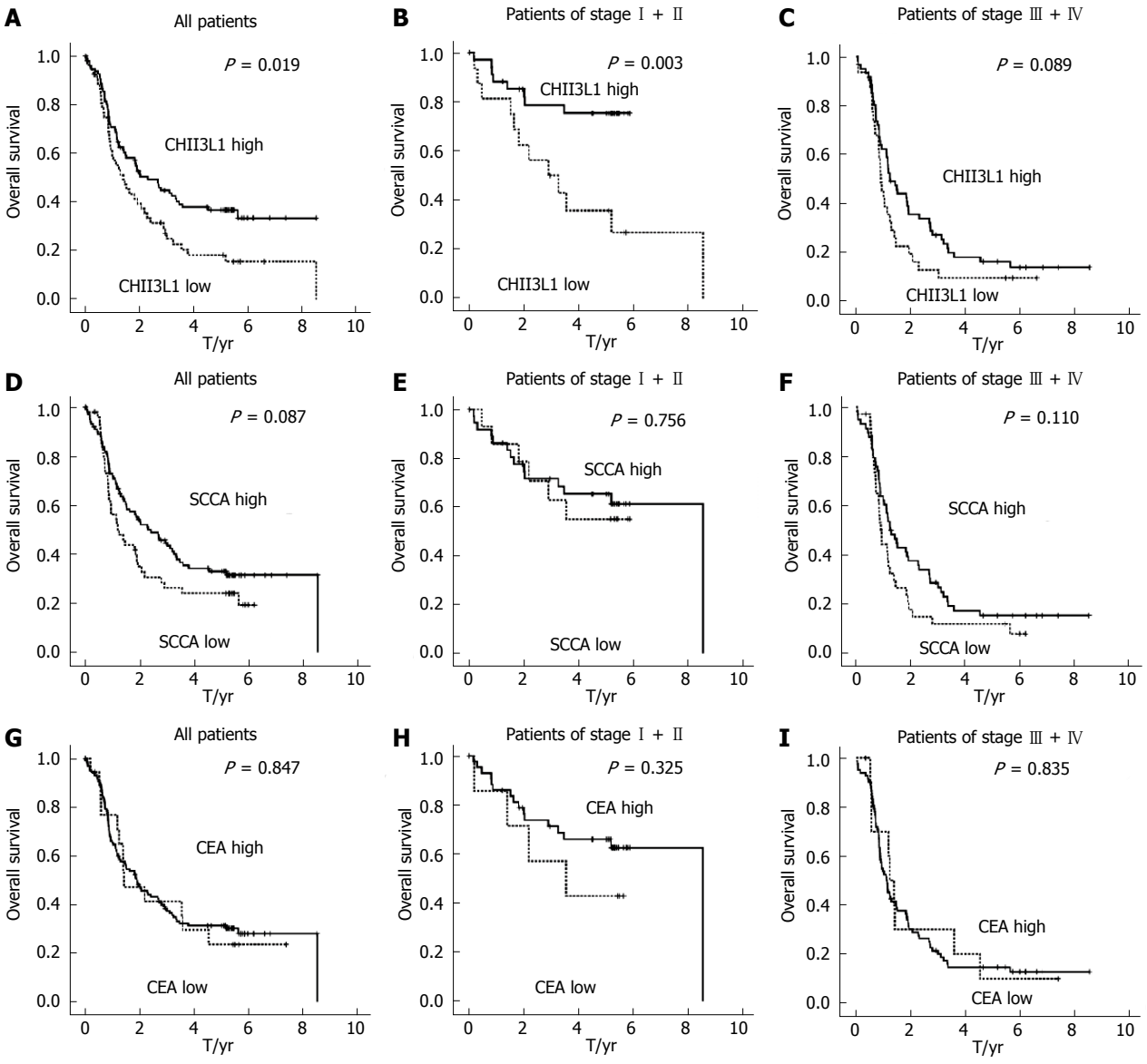

Figure 1 Kaplan-Meier survival curves of esophageal squamous cell carcinoma patients. A: Overall survival of patients with CHI3L1 in all ESCC patients; B: Overall survival of patients with CHI3L1 in patients of Stage Ⅰ-Ⅱ; C: Overall survival of patients with CHI3L1 in patients of Stage Ⅲ-Ⅳ; D: Overall survival of patients with SCCA in all ESCC patients; E: Overall survival of patients with SCCA in patients of Stage Ⅰ-Ⅱ; F: Overall survival of patients with SCCA in patients of Stage Ⅲ-Ⅳ; G: Overall survival of patients with CEA in all ESCC patients; H: Overall survival of patients with CEA in patients of Stage Ⅰ-Ⅱ; I: Overall survival of patients with patients of Stage Ⅲ-Ⅳ. CHI3L1: Chitinase 3-like 1; ESCC: Esophageal squamous cell carcinoma; SCCA: Squamous cell carcinoma antigen.

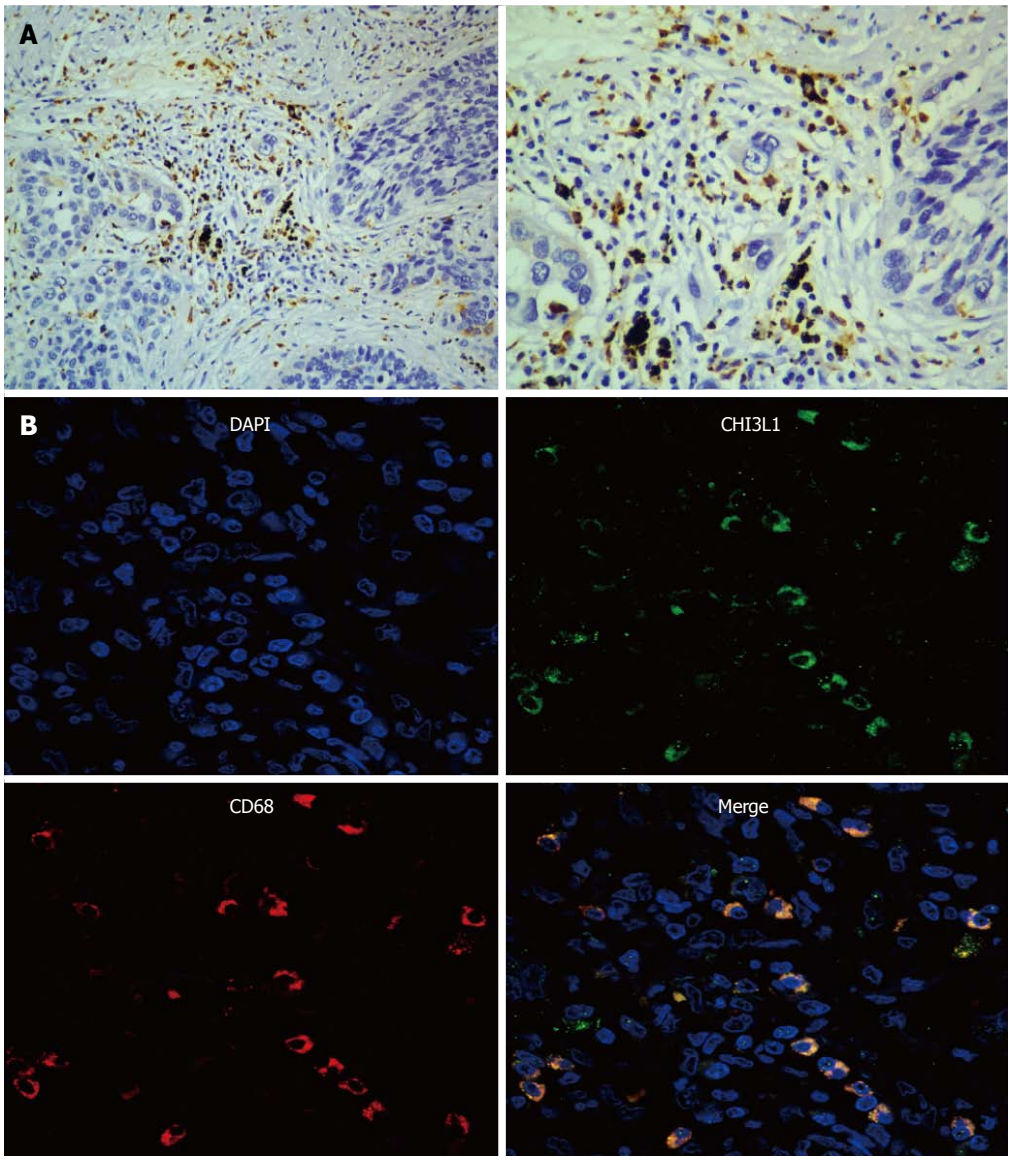

Figure 2 Representative micrographs of esophageal squamous cell carcinoma tissues after immunohistochemistry and immunofluorescence assay. A: Sections stained with a CHI3L1 specific antibody (left- × 200; right- × 40); B: Sections immunofluorescence stained with CHI3L1 (upper left), an antibody against the macrophage specific marker CD68 (upper right), a nuclear counterstain DAPI (lower left) and their merge (lower right). CHI3L1: Chitinase 3-like 1.

measured in the serum of the same patients (Figure 4).

DISCUSSION

To the best of our knowledge, this is the first report that demonstrates serum CHI3L1 could serve as a prognostic predictor for esophageal cancer. ESCC is the eighth most common tumor in the world. Over the past 20 years, with the development of translational medicine and clinical practice, esophageal cancer treatments have made substantial progress. However, the 5-year survival rate of esophageal cancer still varies between 15% and 25%. Thus, an effective biomarker is urgently needed to both screen early ESCC and accurately predict its prognosis.

In our previous study, we observed that CHI3L1 exhibited a higher expression level in esophageal squamous cell carcinoma and served as an ideal tumor marker applied in ESCC screening[8]. In this study, we further explored this protein's prognostic value in ESCC patients. Serum CHI3L1 concentrations were significantly associated with a poor clinical outcome. This was confirmed by Kaplan-Meier survival curves, showing that patients with serum CHI3L1 levels above the cutoff value showed a significantly reduced OS compared to patients with levels below the cutoff value. Moreover, compared to the advanced stages, a stronger correlation between elevated CHI3L1 and short survivals was found at the early stages. Moreover, CHI3L1 performed better than the conventional parameters CEA and SCCA in predicting prognosis. Concordantly, the Cox proportional hazards regressions model analysis illustrated that the level of serum CHI3L1 was an independent prognostic factor, similar to the

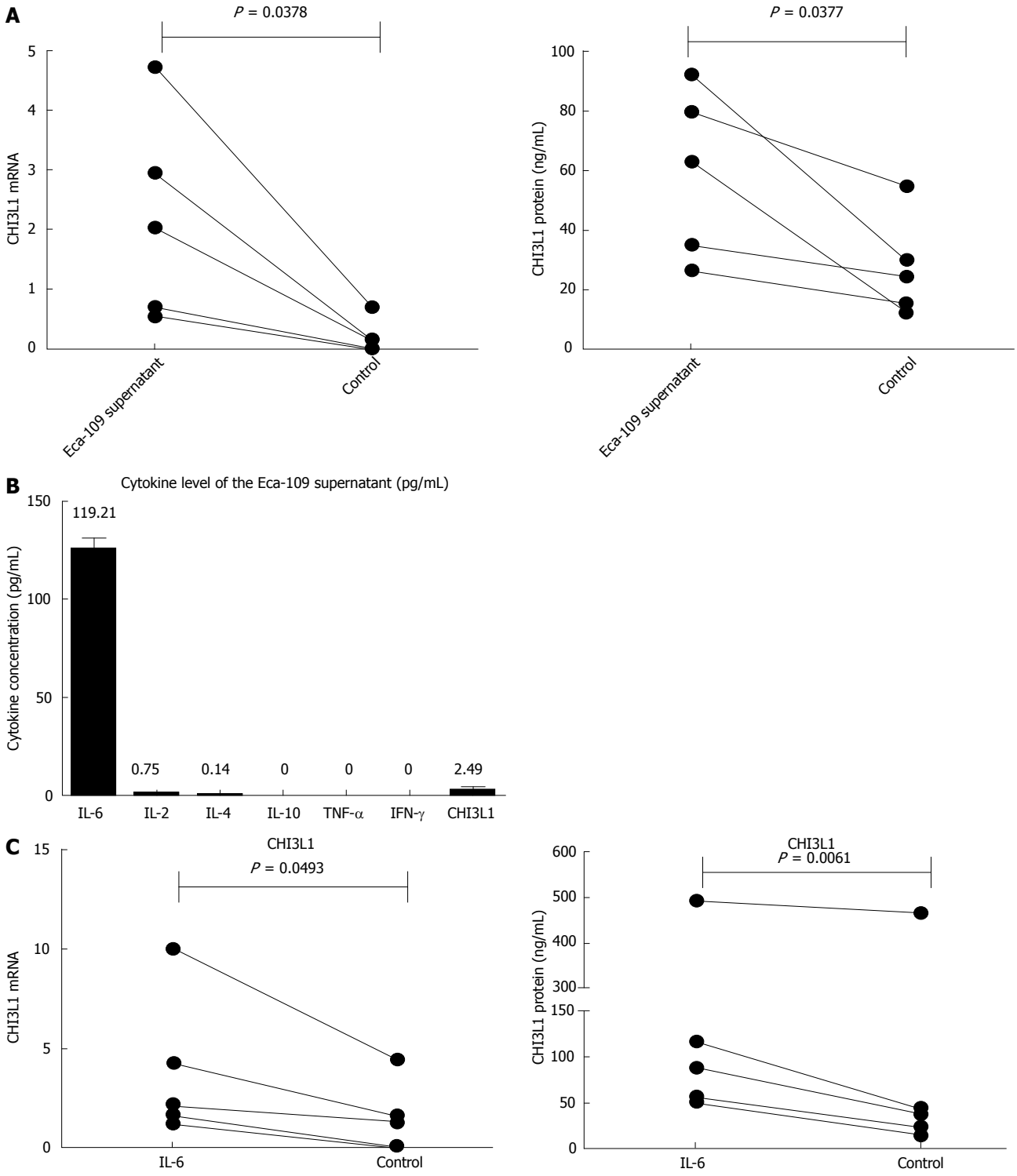

Figure 3 Expression level of chitinase 3-like 1 in monocyte-derived macrophages after stimulation of either supernatant of esophageal squamous cell carcinoma cell line Eca-109 or interleukin-6. A: Expression of CHI3L1 mRNA and protein in monocyte-derived macrophages (upper, mRNA; lower, protein) after stimulation of Eca-109 cell line supernatant was analyzed by real-time PCR and ELISA, respectively; B: Cytokine levels of Eca-109 supernatant were measured with BD CBA Th1/Th2/Th17 Cytokine Kit; C: Level of CHI3L1 mRNA (left) and protein (right) in monocyte-derived macrophages after stimulation of interleukin-6 (IL-6) was analyzed by real-time PCR and ELISA, respectively. Error bars represent standard deviations (SD) calculated from three parallel experiments. CHI3L1: Chitinase 3-like 1.

TMN staging system. Different from the Kaplan-Meier analysis, in the Cox proportional hazards regression model, SCCA was defined as an independent prognostic factor. The statistical approaches may account for some of the differences in error management. Many investigators report similar findings in other types of tumors. Thom *et al*[15] quantified circulating CHI3L1 in the serum of non-small cell lung cancer and found it was an independent predictor of prognosis. This protein's serum level was elevated in patients with

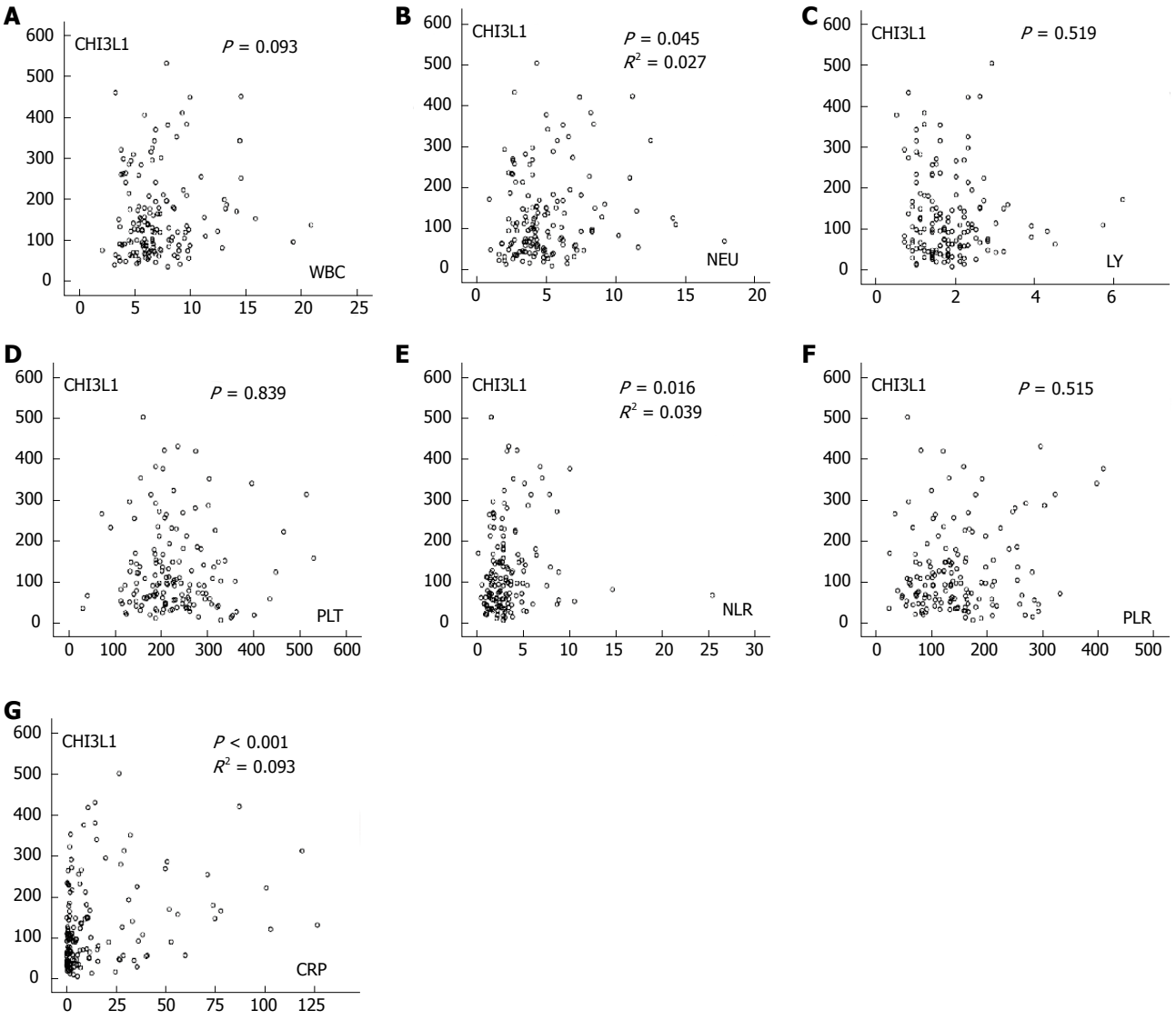

Figure 4 Associations of chitinase 3-like 1 with inflammatory indexes were analyzed by Spearman correlation. A: The associations of chitinase 3-like 1 (CHI3L1) with white blood counts (WBC); B: The associations of CHI3L1 with neutrophil counts (NEU); C: The associations of CHI3L1 with lymphocyte counts (LY); D: The associations of CHI3L1 with platelet counts (PLT); E: The associations of CHI3L1 with NLR (NEU/LY); F: The associations of CHI3L1 with PLR (PLT/LY); G: The associations of CHI3L1 with C-reactive protein (CRP).

poor prognoses. In studies by Zhu *et al*[16], elevated serum CHI3L1 levels also predicted poor prognosis in hepatocellular carcinoma serum. Hogdall EV[19] concluded that high plasma CHI3L1 levels in patients with ovarian cancer stage Ⅲ is related to a shorter survival. Thus, we speculate that serum CHI3L1 might also serve as a prognostic biomarker for ESCC.

Since elevated serum CHI3L1 is found in patients with different types of solid tumors[20,21], the manner in which serum CHI3L1 is released and regulated is essential for the understanding of tumors. Several studies mention the origin of CHI3L1. It is reported that the human osteosarcoma cell line MG-63[22] and the glioblastoma cell line U87 produces CHI3L1, as does the pluripotent myeloid leukemia cell lines HL-60, THP-1 and U937 when they are differentiated into a macrophage-like cell types[23-25]. The location of CHI3L1 expression was explored in several *in vivo* settings. Studies by Roslind A showed the expression of CHI3L1 in malignant HNSCC (squamous cell carcinoma of the head and neck) cells[26]. Junker *et al*[27] concluded that the predominant source of elevated serum CHI3L1 in SCLC is peritumoral macrophages. These studies suggest that CHI3L1 in human tumors is expressed either by cancer cells or stromal cells, such as macrophages, perhaps depending on the cancer type. Appealingly, no observations concerning cell specific expression in ESCC have previously been published. Here, we find pronounced CHI3L1 expression in tumor-surrounded macrophages at the tumor stroma, suggesting that these cells are responsible for the high serum CHI3L1 levels in ESCC patients.

Our observation that ESCC macrophages express CHI3L1 fits well into current knowledge and theories. Serum concentrations of CHI3L1 are often elevated in patients with diseases characterized by extensive

inflammation, the development of fibrosis and remodeling of the extra cellular matrix, such as inflammatory bowel disease[28], active rheumatoid arthritis[29], giant cell arteritis[30], severe bacterial infections[31,32] and development of fibrosis[33,34]. In all the above situations, macrophages are involved. Simultaneously, CHI3L1 expression in macrophages has been confirmed in several *in vivo* settings[24,30,35]. All these phenomena suggest the rationality of the secretion of CHI3L1 by ESCC-associated macrophages. Moreover, CHI3L1 levels were significantly correlated with CRP levels, a systemic marker of inflammation. These findings are consistent with previous studies that reported significant correlations between CHI3L1 levels and CRP[36]. Additionally, the levels of other inflammation markers, such as neutrophil counts (NEU) and the neutrophil/ lymphocyte ratio (NLR), positively correlated with CHI3L1. Therefore, it is possible that CHI3L1 may be involved in the formation and development of tumors by promoting ESCC-related inflammation.

We further explored the release mechanisms of CHI3L1 by peritumoral macrophages. This study shows that CHI3L1 concentrations increase in monocyte-derived macrophages in response to IL-6 stimulation, indicating that a certain increase in IL-6 concentration, as seen in esophageal cancer cell, is needed to stimulate the release of CHI3L1. These findings are consistent with previous studies reporting significant correlations between CHI3L1 levels and IL-6[36,37]. Matsumoto T showed that CHI3L1 was correlated positively with IL-6, and this association seems to be determined by IL-6[36]. Johansen *et al*[38] indicated that CHI3L1 and IL-6 correlate positively in low concentrations of IL-6. Anders R. Nielsen reported that following the IL-6 infusion, the plasma level of CHI3L1 increased from 30 to 57 ng/mL ($P < 0.05$) at 24 h and returned to normal values after 48 h[39]. Therefore, we propose that esophageal cancer cells have the ability to produce IL-6, which promotes the secretion of CHI3L1 by ESCC-associated macrophages.

In conclusion, our study first showed that the level of serum CHI3L1 in patients with ESCC provides a reference mark for evaluating prognosis. This simple, convenient serological testing enables clinical application. In addition, the ESCC microenvironment, in our study, especially the secretion of IL-6 by esophageal tumor cells, promotes the macrophage production of CHI3L1. This provides some guidance for elucidating the pathogenesis of ESCC. Further studies are needed to determine how CHI3L1, produced by ESCC-associated macrophages, influences the development of ESCC. Besides, which type of macrophage, M1 or M2, is responsible for the secretion of CHI3L1 remains to be further explored.

Our study first established a connection between pretreated CHI3L1 and patients with ESCC, and the serum CHI3L1 was primarily secreted by ESCC surrounded macrophages, suggesting that CHI3L1 was a simple, non-invasive and inexpensive prognostic factor. These finding may help to identify high-risk patients for treatment decisions and to understand the mechanisms of ESCC.

ARTICLE HIGHLIGHTS

Research background

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive and lethal human malignancies. With improvements in the diagnosis, staging system and treatment strategies, the overall 5-year survival rate of ESCC patients yielded a slight increase. However, patients at the same stage undergoing similar treatment regimens often have quite different clinical outcomes, which suggests that the current staging system is inadequate for predicting survival. Therefore, the identification of accurate biomarkers for ESCC is necessary to improve the clinical outcome of patients.

Research motivation

Elevated serum chitinase 3-like 1 (CHI3L1) is notably correlated with a poor prognosis and short survival in many malignant tumors. However, no observation concerning the role of CHI3L1 in ESCC prognosis has been reported. In addition, previous studies suggest that CHI3L1 in human tumors are expressed either by cancer cells or stromal cells, such as macrophages. However, no observations concerning the cell specific expression in ESCC were published previously.

Research objectives

To identify whether CHI3L1 serves as a suitable biomarker for the prognosis of ESCC and to analyze this protein's cellular source.

Research methods

ELISA was conducted to detect the concentration of CHI3L1 in serum of 150 ESCC patients. Immunohistochemistry (IHC) was reanalyzed and fluorescent staining utilized to explore the cellular origins of CHI3L1. Cytokines in serum samples were measured with BD CBA Th1/Th2/Th17 Cytokine Kit. We stimulated the monocyte-derived macrophages (MDMs) with either interleukin-6 (IL-6) or the supernatant of ESCC cell line Eca-109 and then investigated the level of CHI3L1 by qPCR and ELISA.

Research results

This study finds that the level of serum CHI3L1 in patients with ESCC provides a reference mark for evaluating prognosis. In addition, the ESCC microenvironment, in our study, especially the secretion of IL-6 by esophageal tumor cells promotes the macrophage production of CHI3L1.

Research conclusions

This study first established a connection between pretreated CHI3L1 and patients with ESCC, and the serum CHI3L1 was primarily secreted by ESCC surrounded macrophages, suggesting that CHI3L1 was a simple, non-invasive and inexpensive prognostic factor. These finding may help to identify high-risk patients for treatment decisions and to understand the mechanisms of ESCC.

Research perspectives

Further studies are needed to determine how CHI3L1, produced by ESCC-associated macrophages, influences the development of ESCC. As CHI3L1 is reported to be associated with the malignant behavior, especially angiogenesis. We will seek whether it plays a role on the development of ESCC through promoting angiogenesis. And which type of macrophage, M1 or M2, is responsible for the secretion of CHI3L1 remains to be further explored.

ACKNOWLEDGMENTS

We would like to thank all of the study participants for agreeing to participate in medical research. We are grateful to Mr. Christopher Lavender, the International Communication Coordinator of the Office of International Collaboration and Public Relations in the Sun Yat-

Sen University Cancer Center, for his assistance in the language editing process.

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P- Reviewer: Eshraghian A, Guo Y **S- Editor**: Ma YJ **L- Editor**: A
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World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7705-7715

DOI: 10.3748/wjg.v23.i43.7705

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

ORIGINAL ARTICLE

Basic Study

Palmitate induces fat accumulation by activating C/EBPβ-mediated G0S2 expression in HepG2 cells

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Author contributions: Zhao NQ and Li XY contributed equally to this work. Zhao NQ and Li XY drafted and designed the study; Wang L, Feng ZL, Li XF, Wen YF and Han JX collected data and performed the majority of experiments; Li XY analyzed the data; Zhao NQ wrote the paper; All the authors participated in revision of the paper, and approved the final version to be published.

Supported by the grants from the Natural Science Foundation of Shanxi Province, China, No. 2014011043-1; and the Social Development Project of Jinzhong, Shanxi Province, No. S1601.

Institutional review board statement: No patients were involved in this study and no specimens were collected clinically, so institutional review board approval is unnecessary.

Conflict-of-interest statement: The authors declare no conflict of interest related to this study.

Data sharing statement: No additional unpublished data are available.

Manuscript source: Unsolicited Manuscript

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Received: August 14, 2017
Peer-review started: August 15, 2017
First decision: August 30, 2017
Revised: September 27, 2017
Accepted: September 28, 2017
Article in press: September 28, 2017
Published online: November 21, 2017

Abstract

AIM

To determine the role of G0/G1 switch gene 2 (G0S2) and its transcriptional regulation in palmitate-induced hepatic lipid accumulation.

METHODS

HepG2 cells were treated with palmitate, or palmitate in combination with CCAAT/enhancer binding protein (C/EBP)β siRNA or G0S2 siRNA. The mRNA expression of C/EBPβ, peroxisome proliferator-activated receptor (PPAR)γ and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) was examined by qPCR. The protein expression of C/EBPβ, PPARγ, and G0S2 was determined by Western blotting. Lipid accumulation was detected with Oil Red O staining and quantified by absorbance value of the extracted Oil Red O dye. Lipolysis was evaluated by measuring the amount of glycerol released into the medium.

RESULTS

Palmitate caused a dose-dependent increase in lipid accumulation and a dose-dependent decrease in lipolysis in HepG2 cells. In addition, palmitate increased

the mRNA expression of C/EBPβ, PPARγ, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) and the protein expression of C/EBPβ, PPARγ, and G0S2 in a dose-dependent manner. Knockdown of C/EBPβ decreased palmitate-induced PPARγ and its target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) mRNA expression and palmitate-induced PPARγ and G0S2 protein expression in HepG2 cells. Knockdown of C/EBPβ also attenuated lipid accumulation and augmented lipolysis in palmitate-treated HepG2 cells. G0S2 knockdown attenuated lipid accumulation and augmented lipolysis, while G0S2 knockdown had no effects on the mRNA expression of C/EBPβ, PPARγ, and PPARγ target genes (*GPR81*, *GPR109A* and *Adipoq*) in palmitate-treated HepG2 cells.

CONCLUSION

Palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression.

Key words: Obesity; Nonalcoholic fatty liver disease; Saturated fatty acid; G0/G1 switch gene 2; CCAAT/enhancer binding protein β; Adipogenesis; Lipolysis; Proliferator-activated receptor γ

Core tip: Obesity-associated nonalcoholic fatty liver disease is characterized by excessive deposition of fat in hepatocytes. The saturated free fatty acid palmitate, the concentration of which is often elevated in obesity, is a major contributor to an increase in intrahepatic triglyceride. G0/G1 switch gene 2 (G0S2) is a critical regulator of hepatic lipid accumulation. However, the role of G0S2 and its transcriptional regulation in palmitate-induced hepatic lipid accumulation is not clear. We found that palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression.

Zhao NQ, Li XY, Wang L, Feng ZL, Li XF, Wen YF, Han JX. Palmitate induces fat accumulation by activating C/EBPβ-mediated G0S2 expression in HepG2 cells. *World J Gastroenterol* 2017; 23(43): 7705-7715 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7705.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7705

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive deposition of fat in hepatocytes in the absence of excessive alcohol intake. It is one of the most common emerging liver diseases throughout the world, coinciding with the global obesity epidemic[1]. Elevated plasma free fatty acid (FFA) levels are a common feature of obesity[2] and play an etiological role in the pathogenesis of NAFLD[3]. In particular, the saturated fatty acid palmitate, which makes up 30%-40% of high plasma FFA concentration[4], is a major contributor to an increase in intrahepatic triglyceride[5]. However, the molecular mechanism by which palmitate contributes to the accumulation of excess triglyceride in hepatocytes is not entirely clear.

Several studies of NAFLD have demonstrated that a decreased rate of triglyceride mobilization promotes triglyceride accumulation in the liver[6,7]. The rate-limiting step of intracellular triacylglycerol mobilization is cleavage of the first ester bond in triglycerides, which is catalyzed by adipose triglyceride lipase (ATGL)[8]. In adipocytes, the protein product of G0/G1 switch gene 2 (*G0S2*) is a dominant inhibitor of ATGL[9]. It binds directly to ATGL and attenuates ATGL-mediated lipolysis *via* inhibiting the triglyceride hydrolase activity of ATGL[9-11]. G0S2 is also abundantly expressed in the liver, suggesting that the regulatory function of G0S2 is not limited to adipose tissue[9]. Notably, *G0S2* overexpression in the liver increases the accumulation of triglycerides and promotes fatty liver formation[12,13]. Conversely, loss of *G0S2* in the liver results in a marked decrease in hepatic triacylglycerol levels and protects against high-fat-diet-induced liver steatosis[13]. These findings implicate an important role for G0S2 as a regulator of triglyceride content in the liver and as a contributor to obesity-associated liver steatosis.

G0S2 expression is regulated by a complex transcriptional mechanism that involves proliferator-activated receptor (PPAR)γ. Transactivation, gel shift and chromatin immunoprecipitation assays have identified *G0S2* as a direct target gene of *PPARγ*[14]. The transcription factor CCAAT/enhancer binding protein (C/EBP)β is involved in adipogenesis and is crucial for inducing initial expression of PPARγ during adipogenesis[15,16]. Importantly, C/EBPβ overexpression increases *PPARγ* mRNA level and triglyceride content in the liver, whereas C/EBPβ RNA interference attenuates palmitate-induced PPARγ expression and triglyceride accumulation in hepatocytes[5].

Based on these observations, we propose the following hypothesis: palmitate stimulates C/EBPβ and its downstream target PPARγ and consequent G0S2 expression, and then G0S2 contributes to palmitate-induced fat accumulation in the liver. In this study, using human HepG2 hepatoma cells, a cellular model of hepatic steatosis[17], we examined lipolysis in hepatocytes, hepatocellular triglyceride accumulation, and the expression of C/EBPβ, *PPARγ* and PPARγ-regulated genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) in response to palmitate treatment. In addition, *via* siRNA-mediated gene knockdown experiments, we investigated the relationship between expression of the aforementioned proteins and hepatocyte lipolysis and

lipid accumulation.

MATERIALS AND METHODS

Cell culture

HepG2 cells (China Center for Type Culture Collection, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, United States) containing 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine. Cells were grown at 37 ℃ in an atmosphere of 5% CO_2/95% air in a cell culture flask. The effect of palmitate was examined by addition of this agent to the cells plated in six-well plates at 2 × 10^5 cells per well.

Preparation of palmitate solution

Palmitate (Sigma, St. Louis, MO, United States) stock solution was prepared by coupling palmitate to bovine serum albumin (BSA; Sigma) as previously described[18]. Palmitate was fully dissolved in pure ethanol for a concentration of 195 mmol/L, ensuring that the final concentration of ethanol in the palmitate stock solution did not exceed 1.5% by volume. This palmitate stock solution was then added to a prewarmed BSA solution (10% w/w, 37 ℃) to achieve a final palmitate concentration of 3 mmol/L. The solution was dissolved by incubating at 37 ℃ in a water bath for a further 10 min. The final molar ratio of palmitate to BSA was 2:1. The control vehicle was prepared using a stock of 10% w/w BSA with an equivalent volume of ethanol added to match that contained in the final palmitate stock. The final concentration of ethanol was < 0.2% by volume in all experiments.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cultured HepG2 cells using TRIzol reagent (Invitrogen), and RNA quality was evaluated *via* electrophoresis. Reverse transcription (RT) was performed using Superscript Ⅱ reverse transcriptase (Invitrogen). The RT conditions for each cDNA amplification were 42 ℃ for 15 min, 85 ℃ for 5 s, and the cDNAs amplified were stored at -20 ℃. Gene expression analysis was performed by quantitative PCR (qPCR) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using SYBR Green as the detection dye. Primer sequences used for the detection of genes were designed as follows: *C/EBPβ* forward primer: 5′-CAAGCACAGCGACGAGTACAAGATCC-3′ and reverse primer: 5′- GCTTGAACAAGTTCCGCAGGGTG -3′; *PPARγ* forward primer: 5′-ACCACTCCCACTCC TTTG-3′ and reverse primer: 5′-GCAGGCTCCACTTT GATT-3′; *G0S2* forward primer: 5′-CCTCTTCGGCG TGGTGCT-3′ and reverse prime: 5′-CTGCTGCTT GCCTTTCTCC-3′; *GPR81* forward primer: 5′-CAGA CAGGCTCGGATGAAGAAG-3′ and reverse prime: 5′-TTGTAGAATTTGGGAAAGGAGGG-3′; *GPR109A* forward primer: 5′-TGGACCTGGCGTTC TTTA-3′ and reverse primer: 5′-GCTCGTGCTGCGGTTATT-3′; *Adipoq* forward primer: 5′-AGGAAAGGAGAACCTGGAGAAG-3′ and reverse prime: 5′-ATAGACTGTGATGTGGTAGGC AAA-3′; *β-actin* forward primer: 5′-TGGCACCCAGCA CAATGAA-3′ and reverse primer: 5′-CTAAGTCATAGT CCGCCTAGAA-3′. The expected size of the amplified products was 194 bp (*C/EBPβ*), 169 bp (*PPARγ*), 160 bp (*G0S2*), 240 bp (*GPR81*), 170 bp (*GPR109A*), 204 bp (*Adipoq*) and 186 bp (*β-actin*). *β-Actin* was used as a control housekeeping gene. Cycling conditions were 94 ℃ for 5 s and 60 ℃ for 30 s, followed by 45 cycles. The predicted size of the PCR products was confirmed by 2% agarose gel electrophoresis stained with ethidium bromide. Melting curve analysis was performed for each sample in direct connection to the PCR, to verify the specificity of the amplified PCR product. The results were stated as the fold difference in expression for each target gene compared to that of β-actin as an internal control in the same sample, using the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in duplicate.

Western blot analysis

To measure the nuclear C/EBPβ protein level, nuclear protein extracts were isolated from HepG2 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, United States). Meanwhile, HepG2 cells were harvested and lysed with ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and proteins were extracted from whole-cell lysates. The protein concentration was quantified using Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, United States). After denaturation by boiling of protein, equal amounts of total protein (40 μg) were loaded and resolved on 10% SDS-PAGE for 2 h at room temperature. The proteins were subsequently transferred to polyvinylidene difluoride membranes (Atto Corporation, Tokyo, Japan). The membranes were blocked with 5% non-fat milk dissolved in Tris-buffered saline/Tween 20 buffer for 2 h and incubated with primary antibodies overnight, and then the secondary antibodies for 1 h. Primary antibodies used were C/EBPβ (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, United States), PPARγ (1:1000; Cell Signaling, NEB, Vienna, Austria) and G0S2 (1:100; Sigma). The β-actin antibody (1:2000;) was used as a loading control. Secondary antibody was goat anti-rabbit IgG-horseradish peroxidase conjugate (1:2000; Bio-Rad Laboratories). The immunoreactive protein bands were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, United States). The density of the band was quantified using ImageJ software (NIH, Bethesda, MD, United States), and the data were transformed

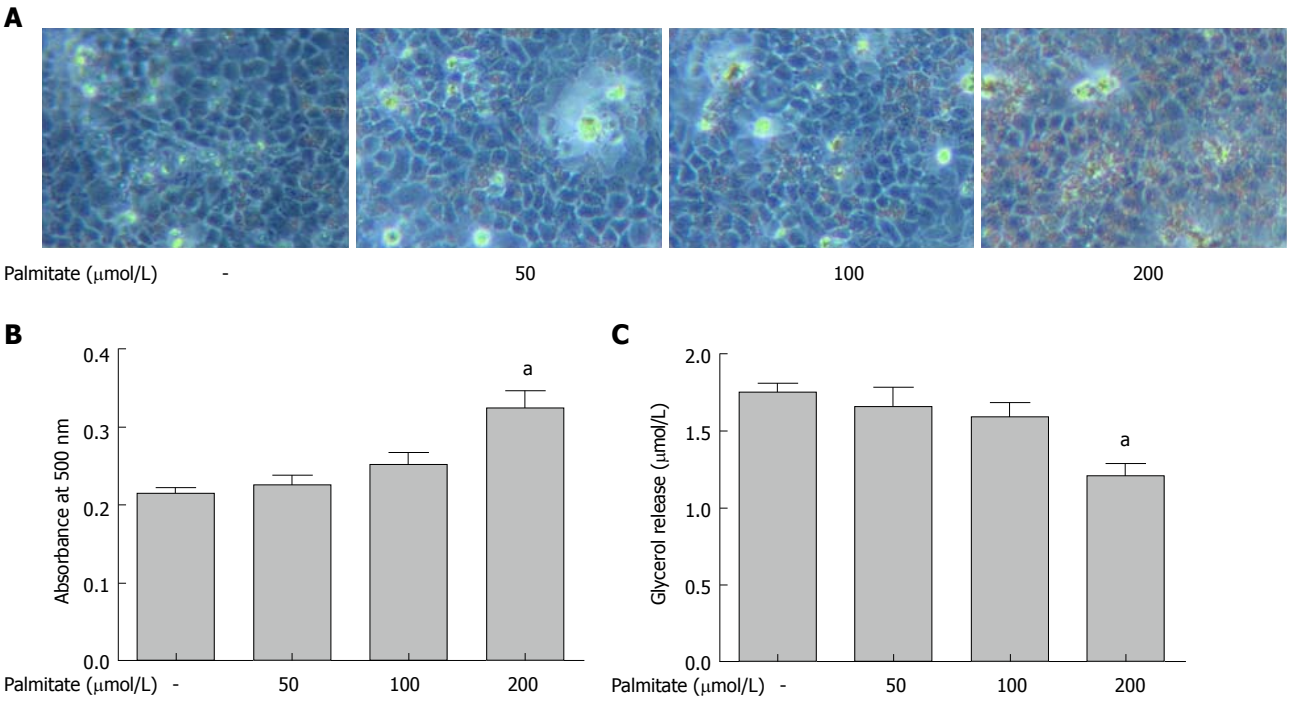


Figure 1 Palmitate-induced lipid accumulation and inhibition of lipolysis in HepG2 cells. A and B: Dose response of lipid accumulation induced by palmitate. HepG2 cells were treated with various concentrations of palmitate for 24 h, and (A) stained with Oil Red O to visualize the intracellular lipid contents (original magnification, × 100); B: Lipid accumulation was quantified by absorbance value of the extracted Oil Red O dye at 500 nm. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ *vs* untreated control. C: Dose response of inhibition of lipolysis induced by palmitate. HepG2 cells were treated with various concentrations of palmitate for 24 h, and lipolysis was assessed by glycerol release into the medium. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ *vs* untreated control.

and normalized relative to β-actin as the integral optical density ratio. All experiments were performed at least three times and representative data were shown.

siRNA-mediated knockdown

RNA oligonucleotides directed against *C/EBPβ* (sense sequence: GCAATCGGTTTAAACATGGCT) and *G0S2* (sense sequence: GCATCCACCAAAGGAGTTTGG) were purchased from GeneChem Co. Ltd. (Shanghai, China) to silence target proteins. A negative control siRNA was also purchased from GeneChem, which had no matches in the human genome. siRNA transfection to HepG2 cells was conducted using Lipofectamine 2000 (Invitrogen). HepG2 cells were seeded into six-well plates and allowed to culture overnight. The aforementioned siRNAs (2.5 μg) and 5 μL Lipofectamine 2000 Reagent were respectively diluted in 250 μL Opti-MEM Medium (Invitrogen) and incubated separately for 10 min at room temperature. After the 10-min incubation, equal volumes of diluted siRNA and Lipofectamine 2000 Reagent were mixed gently and incubated for 10 min at room temperature to form siRNA–lipid complexes. For transfection, the siRNA–lipid complexes were subsequently combined with HepG2 cells in six-well culture plates at 10^6 cells per well and incubated for 24 h. Knockdown efficiency of the siRNAs was determined by Western blotting. Transfected cells were treated with 200 μmol/L palmitate for 24 h before harvesting.

Oil Red O staining

HepG2 cells were grown on six-well plates, washed three times with phosphate-buffered saline, and fixed with 10% formaldehyde for 30 min at room temperature. The fixed cells were washed with deionized distilled water, dipped in 60% isopropanol for 3 min, stained with 2 mg/mL of Oil Red O staining solution (Sigma) for 60 min, and washed with deionized distilled water three times to remove unbound dye. Cell nuclei were counterstained with hematoxylin for 3 min and washed with deionized distilled water. Images were obtained using an Axiovert 40 CFL microscope (Olympus, Tokyo, Japan). After microscopic examination, the Oil-Red-O-based amount of triglyceride was quantified in each well. After washing and drying completely, 200 μL isopropanol extraction solution was added to each staining well and the mixtures were incubated for 10 min, followed by gentle vibration to release Oil Red O for 10 min at room temperature. The extracted dye was removed by gentle pipetting, and its absorbance was measured at 500 nm by microplate reader (Versamax; Molecular Devices, Sunnyvale, CA, United States). All tests were performed in triplicate.

Lipolysis measurement

Lipolysis was evaluated by measuring the amount of glycerol released into the medium. Aliquots of

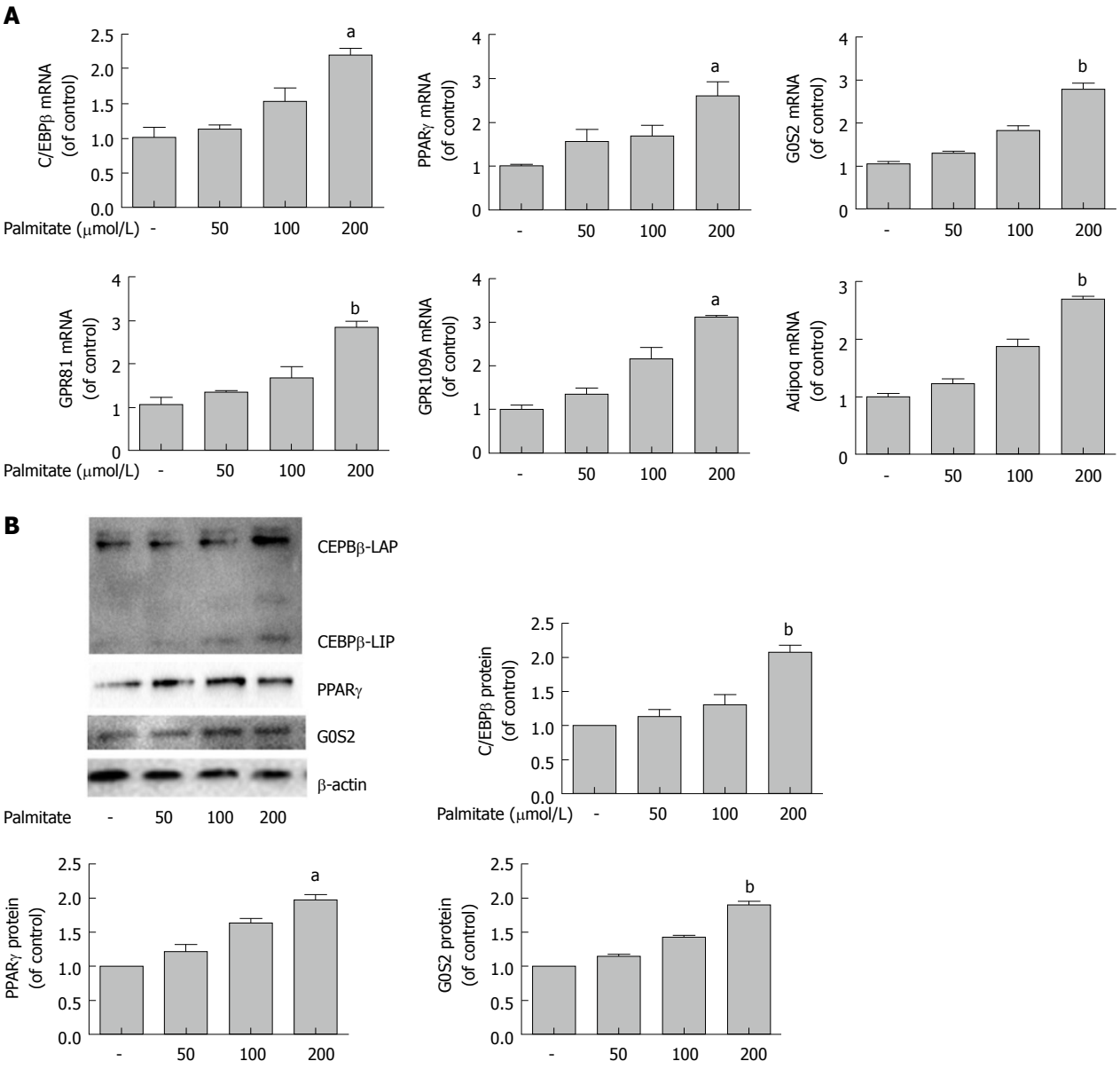


Figure 2 Palmitate-induced expression of C/EBPβ, PPARγ, and PPARγ target genes in HepG2 cells. A: Palmitate increased mRNA expression of C/EBPβ, PPARγ, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) in a dose-dependent manner. mRNA was measured by qPCR. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* untreated control. B: Palmitate increased protein expression of C/EBPβ, PPARγ, and G0S2 in a dose-dependent manner. Protein was examined by western blotting. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* untreated control.

culture medium were centrifuged to remove debris, and directly subjected to glycerol measurement. The amounts of glycerol released were quantified using a glycerol quantification kit (Biovision Inc., Milpitas, CA, United States). Released glycerol was determined using an autoanalyzer (Cobas-Mira; Roche Diagnostics, Basel, Switzerland) to detect the absorbance at 550 nm. All samples were measured in duplicates.

Statistical analysis

All experimental data were expressed as means ± SE. Statistical differences were evaluated by Student's *t* test or one-way analysis of variance where appropriate using SPSS version 18.0 (SPSS, Chicago, IL, United States). Differences were considered as statistically significant when *P* values were < 0.05.

RESULTS

Palmitate induced lipid accumulation and suppressed lipolysis in HepG2 cells

HepG2 cells were incubated with increasing amounts of palmitate for 24 h, and lipid accumulation was examined by Oil Red O staining. Palmitate caused a dose-dependent increase in lipid accumulation in HepG2 cells (Figure 1A and B). HepG2 cells with palmitate also caused a dose-dependent decrease in lipolysis, demonstrated by reduced glycerol release

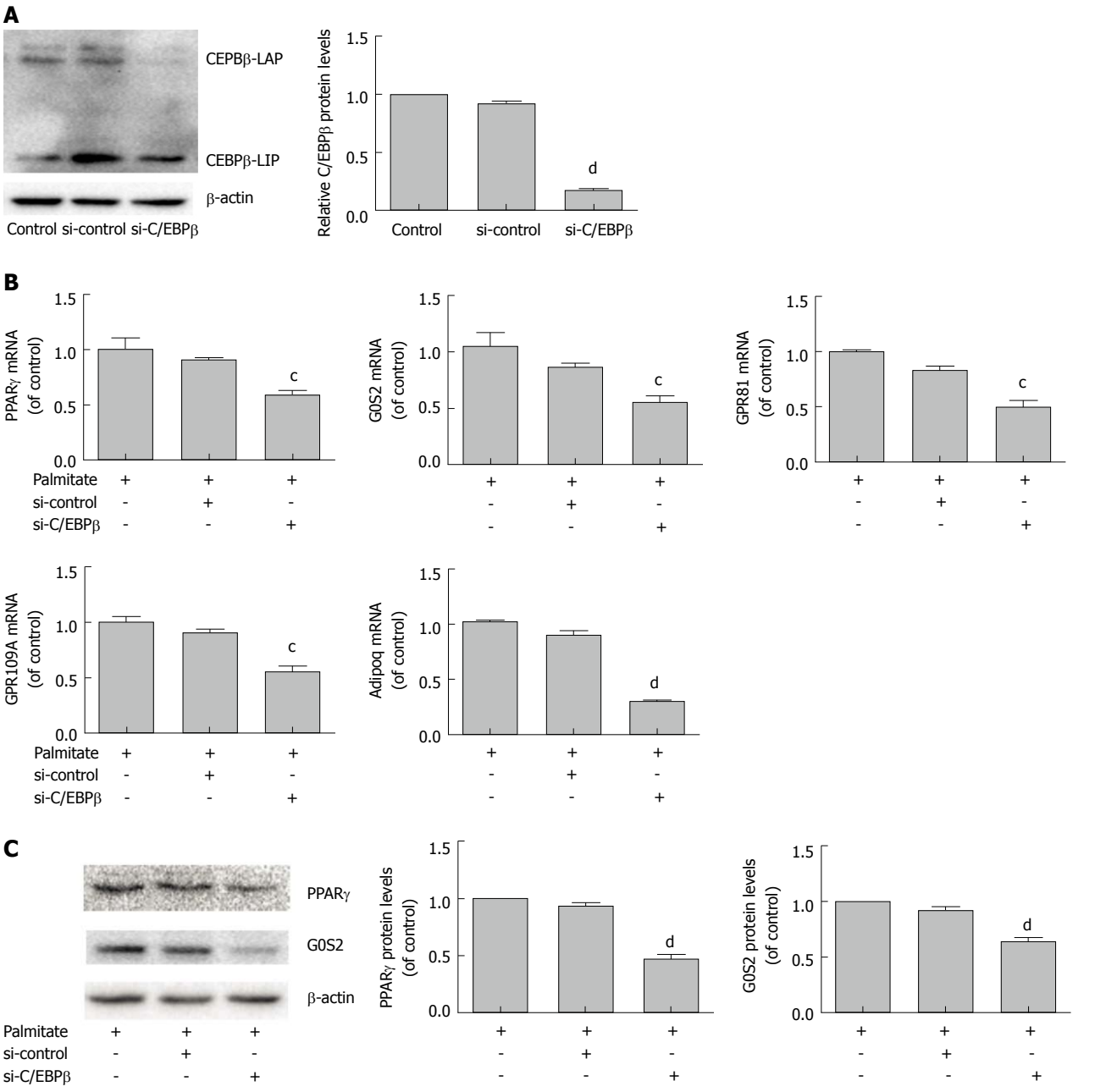


Figure 3 The effects of C/EBPβ knockdown on palmitate-induced *PPARγ* and its target gene expression in HepG2 cells. A: HepG2 cells were transfected with control siRNA or C/EBPβ siRNA, and C/EBPβ expression was measured by Western blotting. At least three independent experiments were conducted. Data are presented as means ± SE. $^{d}P < 0.01$ *vs* control siRNA. B: C/EBPβ knockdown decreased palmitate-induced mRNA expression of *PPARγ* and its target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*). mRNA was measured by qPCR. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* control siRNA. C: C/EBPβ knockdown decreased palmitate-induced protein expression of PPARγ and G0S2. Protein was examined by Western blotting. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{d}P < 0.01$ *vs* control siRNA.

into the medium (Figure 1C). Palmitate at 200 μmol/L, which represents a high physiological level of circulating palmitate in obesity[2], caused a significant increase in lipid accumulation and a significant decrease in lipolysis. Therefore, this concentration of palmitate was used in all the following siRNA knockdown experiments.

Palmitate induced expression of C/EBPβ, PPARγ, and PPARγ target genes in HepG2 cells

Lipid accumulation is controlled by a few key transcription factors, including C/EBPβ and PPARγ. We assessed the effects of palmitate on the expression of C/EBPβ, PPARγ, and several known PPARγ target genes in HepG2 cells. HepG2 cells were incubated with increasing amounts of palmitate for 24 h, and quantitative PCR analysis revealed that palmitate caused a dose-dependent increase in the mRNA expression of *C/EBPβ*, PPARγ, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) (Figure 2A). Western blotting showed that incubation of HepG2 cells with palmitate also caused a dose-dependent increase in the protein expression of C/EBPβ, PPARγ, and G0S2

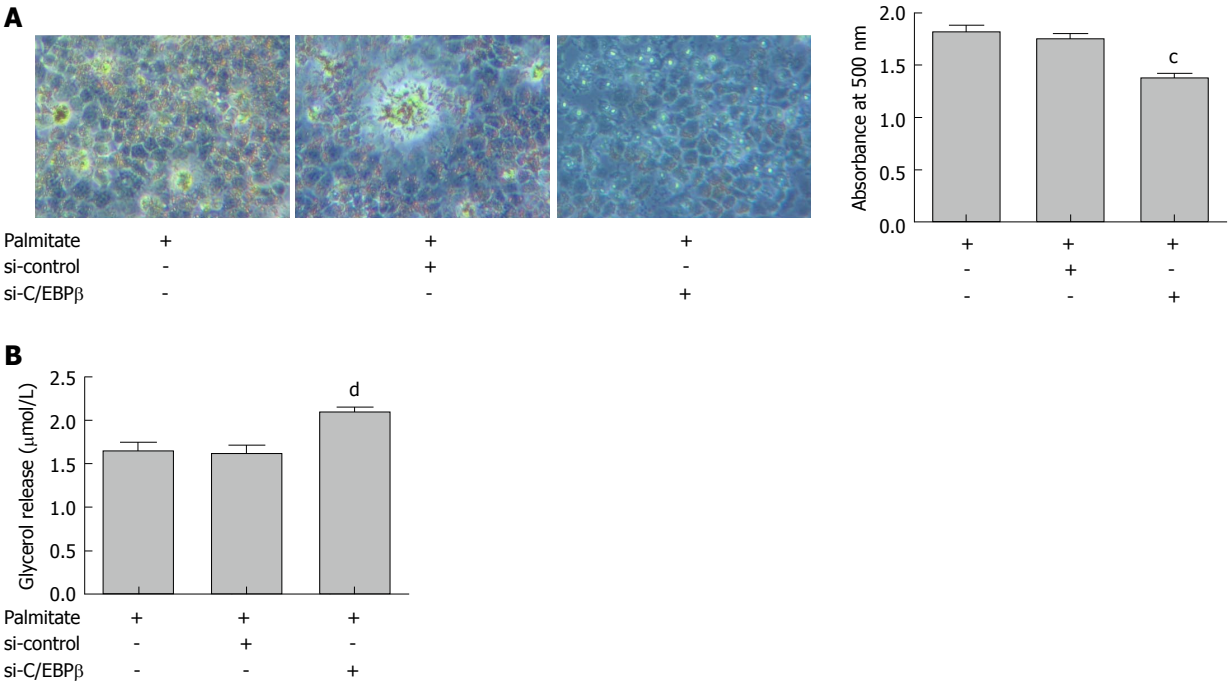


Figure 4 The effects of C/EBPβ knockdown on lipid accumulation and lipolysis in HepG2 cells treated with palmitate. A: C/EBPβ knockdown decreased palmitate-induced lipid accumulation. Lipid accumulation was detected with Oil Red O staining and quantified by absorbance value of the extracted Oil Red O dye at 500 nm. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{c}P < 0.05$ *vs* control siRNA. B: C/EBPβ knockdown increased lipolysis in HepG2 cells treated with palmitate. Lipolysis was assessed by glycerol release into the medium. At least three independent experiments were conducted. Data are presented as means ± SE. $^{d}P < 0.01$ *vs* control siRNA.

(Figure 2B).

C/EBPβ knockdown reduced palmitate-induced PPARγ and its target gene expression in HepG2 cells

We next examined the role of C/EBPβ in palmitate-induced PPARγ and its target gene expression in HepG2 cells. HepG2 cells were transfected with C/EBPβ siRNA and treated with 200 μmol/L palmitate for 24 h. C/EBPβ siRNA efficiently decreased C/EBPβ protein expression (Figure 3A). qPCR analysis revealed that C/EBPβ knockdown significantly decreased palmitate-induced PPARγ and its target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) mRNA expression (Figure 3B). Western blotting showed that C/EBPβ knockdown significantly decreased palmitate-induced PPARγ and G0S2 protein expression (Figure 3C).

C/EBPβ knockdown attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate

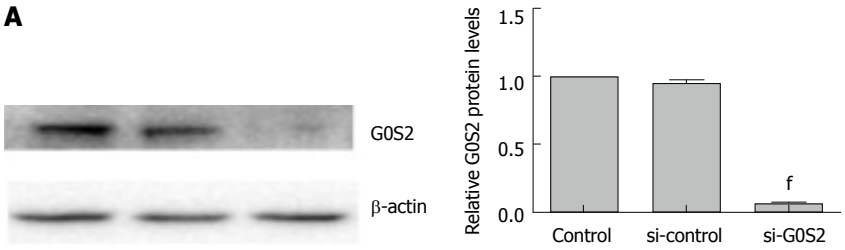
We investigated the effects of C/EBPβ on lipid accumulation and lipolysis in HepG2 cells treated with palmitate. HepG2 cells were transfected with C/EBPβ siRNA and treated with 200 μmol/L palmitate for 24 h. C/EBPβ knockdown significantly attenuated palmitate-induced lipid accumulation in HepG2 cells (Figure 4A). C/EBPβ knockdown significantly augmented lipolysis in HepG2 cells treated with palmitate (Figure 4B).

G0S2 knockdown attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate

G0S2 plays an important role in regulating hepatic lipid accumulation and lipolysis. We explored the effects of G0S2 in lipid accumulation and lipolysis in HepG2 cells treated with palmitate. HepG2 cells were transfected with G0S2 siRNA and treated with 200 μmol/L palmitate for 24 h. G0S2 siRNA efficiently decreased G0S2 protein expression (Figure 5A). G0S2 knockdown significantly attenuated palmitate-induced lipid accumulation in HepG2 cells (Figure 5B). G0S2 knockdown significantly augmented lipolysis in HepG2 cells treated with palmitate (Figure 5C). However, G0S2 knockdown had no effects on palmitate-induced mRNA expression of *C/EBPβ*, PPARγ, and other PPARγ target genes (*GPR81*, *GPR109A* and *Adipoq*) (Figure 5D) and palmitate-induced protein expression of C/EBPβ and PPARγ in HepG2 cells (Figure 5E).

DISCUSSION

Obesity is associated with elevation of circulating FFA due to impaired lipid storage capacity in subcutaneous adipose tissue. The increased FFA supply that occurs as a result leads to lipid accumulation in the liver[19,20]. Previous studies showed that the saturated fatty acid palmitate induces lipid accumulation in HepG2 cells[21,22]. In the present study, we also demonstrated that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G0S2
β-actin
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1.0
0.5
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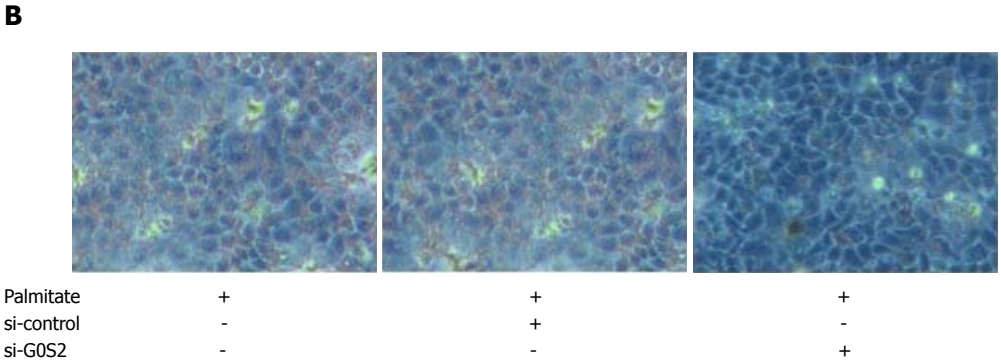
f
Control
si-control
si-G0S2


B
Palmitate + + +
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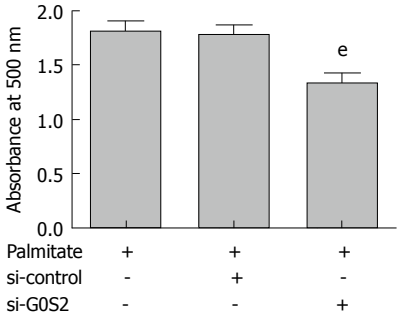
Absorbance at 500 nm
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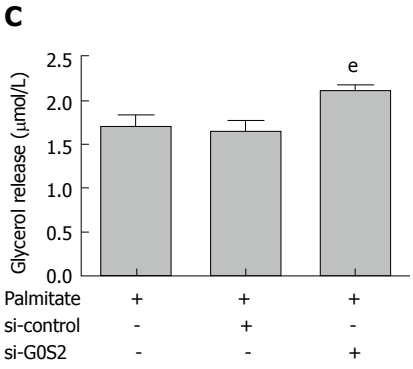
C
Glycerol release (μmol/L)
2.5
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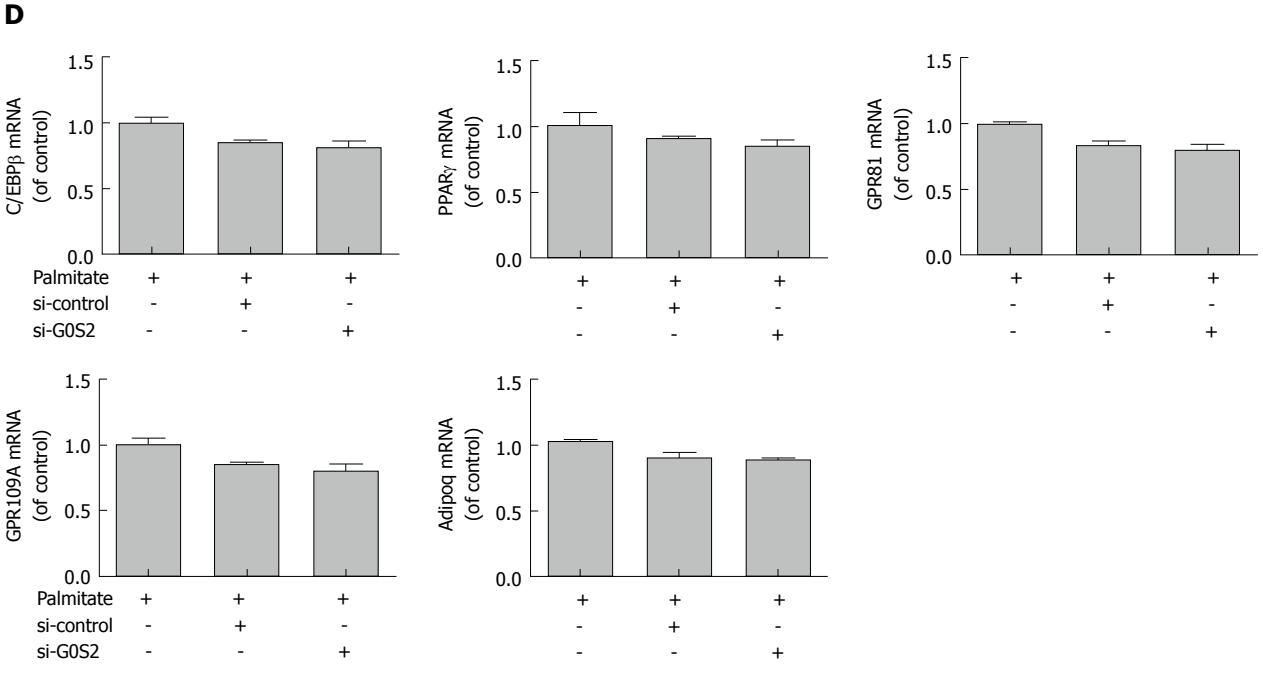
Palmitate + + +
si-control - + -
si-G0S2 - - +


D
C/EBPβ mRNA (of control)
PPARγ mRNA (of control)
GPR81 mRNA (of control)
GPR109A mRNA (of control)
Adipoq mRNA (of control)
1.5
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0.5
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Palmitate + + +
si-control - + -
si-G0S2 - - +

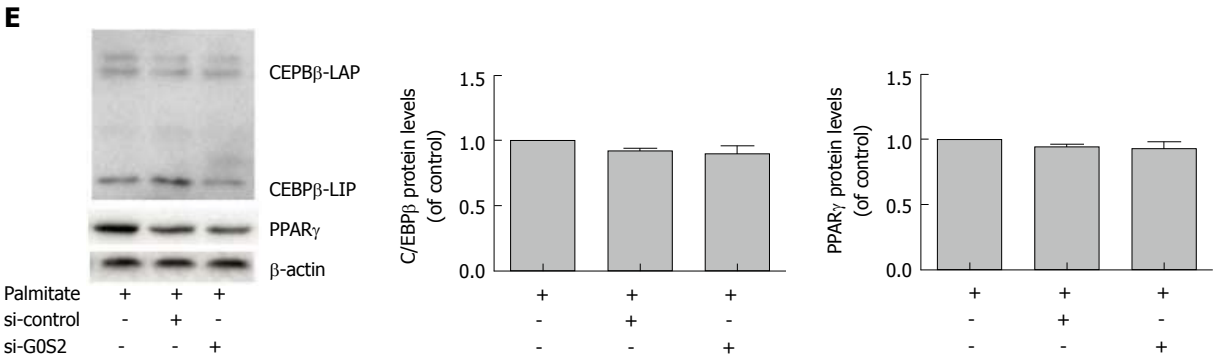

Figure 5 The effects of G0S2 knockdown on *C/EBP*β, *PPAR*γ and PPARγ target genes expression, lipid accumulation, and lipolysis in palmitate-treated HepG2 cells. A: HepG2 cells were transfected with control siRNA or G0S2 siRNA, and G0S2 expression was measured by Western blotting. At least three independent experiments were conducted. Data are presented as means ± SE. $^{f}P < 0.01$ *vs* control siRNA. B: G0S2 knockdown decreased palmitate-induced lipid accumulation. Lipid accumulation was detected with Oil Red O staining and quantified by absorbance value of the extracted Oil Red O dye at 500 nm. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{e}P < 0.05$ *vs* control siRNA. C: G0S2 knockdown increased lipolysis in HepG2 cells treated with palmitate. Lipolysis was assessed by glycerol release into the medium. At least three independent experiments were conducted. Data are presented as means ± SE. $^{e}P < 0.05$ *vs* control siRNA. D: G0S2 knockdown did not affect palmitate-induced *C/EBP*β, *PPAR*γ, and other PPARγ target genes (*GPR81*, *GPR109A*, and *Adipoq*) mRNA expression. mRNA was measured by qPCR. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. E: G0S2 knockdown did not affect palmitate-induced PPARγ and G0S2 protein expression. Protein was examined by Western blotting. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE.

palmitate induced lipid accumulation, and moreover, palmitate suppressed lipolysis in hepatocytes.

C/EBPβ is an important basic leucine zipper transcription factor whose mRNA can produce two C/EBPβ isoforms: liver-enriched activating protein (46 kDa) and liver-enriched inhibitory protein (21 kDa)[23]. C/EBPβ is involved in hepatic lipogenesis[5]. In hepatocytes, palmitate upregulates C/EBPβ expression, which in turn induces PPARγ expression and increases hepatic lipid accumulation[5]. Previous studies showed that C/EBPβ directly binds the PPARγ promoter prior to transcriptional activation during the early phase of adipogenesis[24,25]. PPARγ is a master adipogenic transcription factor, and it is necessary for adipogenesis[16]. *G0S2*, *GPR81*, *GPR109A*, and *Adipoq* are known *PPARγ* downstream target genes involved in lipolysis[26,27]. In the present study, we found that palmitate increased the mRNA expression of *C/EBPβ*, *PPARγ* and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) and the protein expression of C/EBPβ, PPARγ and G0S2 in a dose-dependent manner in hepatocytes. We also found that knockdown of C/EBPβ significantly decreased *PPARγ* and its target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) mRNA expression and PPARγ and G0S2 protein expression in palmitate-treated HepG2 cells. In addition, gene silencing of C/EBPβ attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate. These findings again demonstrate a critical role for C/EBPβ in palmitate-induced hepatic lipid accumulation.

In the above PPARγ target genes, G0S2 acts as an important regulator of triglyceride content in the liver[12,13]. Adipose-tissue-derived fatty acids upregulate fasting G0S2 expression in the liver to induce hepatic triglyceride accumulation[28]. G protein-coupled receptor (GPR)81 functions as a specific receptor for lactate and mediates insulin-induced antilipolytic effects in an autocrine and paracrine manner[27,29]. *GPR109A* is a receptor for the ketone body 3-hydroxybutyric acid and functions as a metabolic sensor that regulates lipolytic activity during starvation to avoid excessive triglyceride degradation[30,31]. Its biological role is related to the ketone body 3-hydroxybutyrate[31]. Adiponectin (Adipoq) is a adipokine that is downregulated in obesity[32]. In the liver, Adipoq can augment the oxidation of fatty acid to alleviate hepatic lipid accumulation[33]. Therefore, G0S2 may play a critical role in C/EBPβ-mediated hepatic lipid accumulation in palmitate-treated HepG2 cells. In this study, we found that knockdown of G0S2 significantly attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate. More importantly, inhibition of the G0S2 expression had no effects on mRNA expression of *C/EBPβ*, *PPARγ*, and PPARγ target genes (*GPR81*, *GPR109A* and *Adipoq*) and protein expression of C/EBPβ and PPARγ in palmitate-treated HepG2 cells. Together, these results indicate that palmitate induces lipid accumulation by activating C/EBPβ-mediated expression of G0S2.

In summary, we observed that palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression. The result provides novel evidence linking G0S2 expression to palmitate-induced hepatic lipogenesis. Considering that liver lipid accumulation is not only a hallmark of NAFLD, but also the first and critical step in the initiation and progression of NAFLD, interfering with G0S2 may represent an effective strategy for the treatment of obesity-related hepatic steatosis.

ARTICLE HIGHLIGHTS

Research background

Obesity-associated nonalcoholic fatty liver disease (NAFLD) is characterized by excessive deposition of fat in hepatocytes. The saturated free fatty acid palmitate, the concentration of which is often elevated in obesity, is a major contributor to an increase in intrahepatic triglyceride. G0/G1 switch gene 2 (G0S2) plays an important role in regulating hepatic lipid metabolism. However, the role of G0S2 and its transcriptional regulation in palmitate-induced hepatic lipid accumulation has remained unclear.

Research motivation

This study was carried out to clarify the molecular mechanism connecting palmitate to obesity-associated NAFLD. As CCAAT/enhancer binding protein beta (C/EBPβ), proliferator-activated receptor gamma (PPARγ) and *G0S2 all* relate to obesity-associated NAFLD, we investigated their roles and interrelationships in palmitate-induced hepatic lipid accumulation. The results lead to important new insights into the molecular mechanism of NAFLD.

Research objectives

The goal of this study was to determine the role of G0S2 and its transcriptional regulation in palmitate-induced hepatic lipid accumulation. The results suggest a previously unknown link between C/EBPβ and G0S2 that contributes to hepatic steatosis.

Research methods

In this study, we examined lipolysis, lipid accumulation, and the expression of C/EBPβ, PPARγ and PPARγ-regulated genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) in response to palmitate treatment in HepG2 cells. Specifically, we investigated the relationships between expression of the aforementioned proteins and hepatocyte lipolysis and lipid accumulation by using siRNA-mediated gene knockdown experiments.

Research results

Palmitate significantly facilitated lipid accumulation and suppressed lipolysis in HepG2 cells. Palmitate also significantly increased the expression of *C/EBPβ*, *PPARγ*, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*). C/EBPβ knockdown significantly reduced palmitate-induced PPARγ and *G0S2* expression. Moreover, C/EBPβ knockdown attenuated lipid accumulation and augmented lipolysis in palmitate-treated HepG2 cells. Importantly, G0S2 knockdown significantly attenuated lipid accumulation and augmented lipolysis in palmitate-treated HepG2 cells, while G0S2 knockdown had no efects on palmitate-induced expression of *C/EBPβ*, *PPARγ*, and PPARγ target genes (*GPR81*, *GPR109A*, and *Adipoq*).

Research conclusions

Palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression. The result provides novel evidence linking G0S2 expression to palmitate-induced hepatic lipogenesis. Considering that liver lipid accumulation is not only a hallmark of NAFLD, but also the first and critical step in the initiation and progression of NAFLD, interfering with G0S2 may represent an effective strategy for the treatment of obesity-related hepatic steatosis.

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P- Reviewer: Kanda T, Tarantino G **S- Editor**: Wei LJ **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7716-7726

DOI: 10.3748/wjg.v23.i43.7716

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Epidemiology and natural history of Wilson's disease in the Chinese: A territory-based study in Hong Kong between 2000 and 2016

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Author contributions: Cheung KS designed and performed the study, were involved in statistical analysis and interpretation of the data, and wrote the manuscript; Seto WK and Mak LY performed the study, statistical analysis and interpretation of the data; Fung J, Lai CL and Yuen MF revised and edited the manuscript.

Institutional review board statement: This study was approved by The Institutional Review Board of The University of Hong Kong/Hospital Authority, Hong Kong West Cluster.

Informed consent statement: The Institutional Review Board of The University of Hong Kong/Hospital Authority waived the need for written informed consent as there were no direct contact with eligible subjects and no additional blood taking.

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

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: July 28, 2017
Peer-review started: July 30, 2017
First decision: August 29, 2017
Revised: September 25, 2017
Accepted: September 28, 2017
Article in press: September 28, 2017
Published online: November 21, 2017

Abstract

AIM

To investigate the epidemiology and natural history of Wilson's disease in the Chinese.

METHODS

Data were retrieved via electronic search of hospital medical registry of the Hong Kong Hospital Authority, which covers all the public healthcare services. We identified cases of Wilson's disease between 2000 and 2016 by the International Classification of Diseases (ICD)-9 code. We analyzed the incidence rate, prevalence and adverse outcomes of Wilson's disease.

RESULTS

We identified 211 patients (male cases 104; female cases 107; median age 27.2 years, IQR: 17.1-38.6 years; duration of follow-up 8.0 years, IQR: 5.0-14.0 years). The average annual incidence rate was 1.44 per million person-years while the prevalence was 17.93 per million. Between 2000 and 2016, there was a decrease in the annual incidence rate from 1.65 to 1.23 per million person-years (P = 0.010), whereas there was an increase in the annual prevalence from 7.80 to 25.20 per million (P < 0.001). Among the 176 cases with hepatic involvement, 38 (21.6%) had cirrhosis, three (1.7%) developed hepatocellular carcinoma, 24 (13.6%) underwent liver transplantations, and 26 (14.8%) died. Seven patients had concomitant chronic viral hepatitis B or C. The 5-year and 10-years rates of overall survival were 92.6% and 89.5%, and for transplant-free survival rates 91.8% and 87.4%, respectively. Cirrhosis and possibly chronic viral hepatitis were associated with poorer overall survival.

CONCLUSION

There was a significant increase in the prevalence of Wilson's disease in Hong Kong. The prognosis was favorable except for those with cirrhosis or concomitant viral hepatitis.

Key words: Hepaticolenticular degeneration; Cirrhosis; Hepatocellular carcinoma; Transplantation

Core tip: There are few studies on the epidemiology and natural history of Wilson's disease in Asia. The present territory-based study was the first to describe both the epidemiology and natural history of Wilson's disease over a long period of time (a span of 17 years from 2000 to 2016) in the Chinese. There was a significant increase in the number of cases of Wilson's disease in Hong Kong. The prognosis was favorable except for those with cirrhosis or concomitant viral hepatitis.

Cheung KS, Seto WK, Fung J, Mak LY, Lai CL, Yuen MF. Epidemiology and natural history of Wilson's disease in the Chinese: A territory-based study in Hong Kong between 2000 and 2016. *World J Gastroenterol* 2017; 23(43): 7716-7726 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7716.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7716

INTRODUCTION

Wilson's disease is an autosomal recessive disease with impairment of hepatic excretion of copper, leading to excessive copper accumulation in various organs and tissues[1]. There is a wide spectrum of clinical manifestations including hepatic, neurological, psychiatric and ophthalmological involvements. Nevertheless, some patients may be asymptomatic, and mutational analysis may be needed for a definitive diagnosis[2]. Untreated patients with hepatic involvement carry significant morbidities including cirrhosis and hepatocellular carcinoma (HCC)[3-5]. Therapies which either chelate the copper (D-penicillamine and trientine) or inhibit its absorption (zinc) are proven to be effective in controlling the disease[6].

There are a few population-based studies on the epidemiology of Wilson's disease in the western population, but the majority were performed before the 1990s. The incidence rate ranges from 17 to 29 per million person-years[7-10], while the prevalence is reported to be 15-30 cases per million population[1,11,12]. Recently, a genetic study of Wilson's disease concluded that prevalence of Wilson's disease may actually be even higher (1 in 72016)[13]. Studies are even more scarce in Asia, except for one population-based study in Taiwan reporting a low incidence (average annual incidence rate of 2.7 per million person-years) and prevalence (ranging from 8.4 to 16.0 per million) of Wilson's disease between 2000 and 2005[14]. However, the study observation period was short, and prognostic risk factors were not determined.

The long-term outcomes of patients with Wilson's disease are reported in the western population but not in the Asians[3,4]. However, these are retrospective studies and not population-based. In addition, the effect of concomitant chronic viral hepatitis B or C infection on the outcomes of patients with Wilson's disease has not been examined. This is an important factor since around 248 million and 150 million people worldwide have chronic hepatitis B (CHB) and C (CHC) infection, respectively[15,16] While metabolic risk factors (diabetes mellitus, obesity, hypertension as well as metabolic syndrome) predispose CHB and CHC patients to an increased risk of fibrosis/cirrhosis as well as HCC[17,18], whether it will lead to a poorer prognosis in patients with Wilson's disease remains unknown.

Therefore, there is an unmet need for an update of the epidemiology of Wilson's disease as well as more comprehensive identification of risk factors for adverse outcomes in the Asians. This will shed light on the better management of affected patients.

The aims of the present study were to determine the epidemiology and the natural history of Wilson's disease in the Chinese population in Hong Kong. In addition, risk factors for liver transplantation requirement and mortality were determined.

MATERIALS AND METHODS

Source of data

We retrieved data from the Clinical Data Analysis and

Reporting System (CDARS), an electronic database managed by the Hong Kong Hospital Authority, which is the sole provider of public healthcare services in Hong Kong with about 90% of the healthcare services being covered[19,20]. Important clinical information are all available in the CDRS, which includes patient demographics, death, diagnoses, admissions, clinic visits, procedures, laboratory results, medication prescription and dispensing history[21]. There has been an increasing number of territory-based studies performed using this electronic database[22-27], with high accuracies of the coding system demonstrated[24,28].

All patients were anonymized in the CDARS, with a unique reference key assigned to each individual. Ethics approval was issued by the Institutional Review Board, The University of Hong Kong and West Cluster of Hospital Authority, Hong Kong.

Study subjects

The study period commenced from 2000 and ended in 2016. Cases of Wilson's disease from 1999 to 2016 were recognized from the CDARS by using the International Classification of Diseases (ICD)-9 code of 275.1.

Demographics (age, sex, nationality, diagnosis date and death) were retrieved. In addition, information on all diagnoses (including various adverse events and comorbidities) of patients were available for data retrieval.

The incidence rate as well as prevalence of Wilsons's disease were calculated. Adverse liver events (cirrhosis, hepatic complications, HCC and liver transplantation), non-liver outcomes, and mortality were also studied. Risk factors (including concomitant chronic viral hepatitis and metabolic factors) associated with overall and transplant-free survival were determined.

Cases with cirrhosis were identified by the ICD-9 code of 571.5 and the presence of portal hypertension, splenomegaly or cirrhotic complications. Cirrhotic complications were defined with the existence of at least one of the following: ascites, spontaneous bacterial peritonitis, gastroesophageal varices, hepatic encephalopathy, or hepatorenal syndrome. Chronic viral hepatitis referred to either CHB or CHC infection. Metabolic factors included diabetes mellitus, hypertension, dyslipidemia, obesity and alcoholism. Obesity was diagnosed with a body mass index of 25 kg/m^2 or more in the Asian population[29]. Dyslipidemias are defined as disorders of lipoprotein metabolism with high levels of triglycerides, low-density or non-high-density lipoprotein cholesterol, or low level of high-density lipoprotein cholesterol. The cutoff value depends on the individual patient's cardiovascular risk profile[30]. Smoking status was not usually entered into the electronic database system. Table 1 illustrates the identification of outcomes and covariates.

Table 1 International classification of diseases-9 code for outcome and covariates

Impaired or abnormal liver function	573.8, 794.8
Hepatitis	573.3, 571.40, 571.41
Chronic liver disease	571.9, 573.9
Acute liver failure	570
Chronic liver failure	571.8
Hepatocellular carcinoma	155.0
Cirrhosis	571.5
Viral hepatitis B carrier	V02.61:0
Viral hepatitis C carrier	V02.62:0
Sequalae of chronic liver disease	572.8
Esophageal varices	456.0, 456.1
Gastric varices	456.8
Ascites	789.5
Spontaneous bacterial peritonitis	567.2
Portal hypertension	572.3
Splenomegaly/Hypersplenism	789.1, 789.2, 789.4
Hepatic encephalopathy	572.2
Hepatorenal syndrome	572.4
Liver transplantation	V42.7
Parkinsonism	332
Tremor	781.0
Dystonic disorder	333.6
Convulsion	780.32
Tics	307.20
Dementia	290
Bipolar affective disorder	296.7
Depression	296.2, 296.3, 311
Anxiety	300.0
Organic affective syndrome	293.83
Psychosis	295, 293.83
Personality disorder	301
Suicide	E950
Hemolytic anemia	283.10
Alcoholism	291, 571.0, 571.1, 571.2, 571.3, 303, 305.0, 980.8, 980.9
Obesity	278.0, 278.1
Diabetes mellitus	249, 250
Hypertension	401-405
Dysplipidemia	272.0-272.4

Validation of data

Diagnosis coding accuracy of the CDARS data was validated by reviewing the eletronic medical records of patients from our center, Queen Mary Hospital. Queen Mary Hospital is a hospital which provides tertiary healthcare services including liver transplantation in Hong Kong.

Statistical analysis

Statistical analyses were done by R version 3.2.3 (R Foundation for Statistical Computing) statistical software. We expressed continuous variables in terms of median with interquartile range (IQR), and used Mann-Whitney *U*-test to assess the difference in continuous variables between two groups. We used χ^2 test or Fisher's exact test for comparing categorical variables. The incidence rate and prevalence of Wilson's disease between 2000 and 2016 were calculated. Poisson regression model was used to evaluate the temporal trends of count data. Variables

Table 2 Clinical characteristics of patients with Wilson's disease in Hong Kong [*n* = 211, *n* (%)]

Variables	Clinical characteristics
Age	27.9 (18.2-39.7)
Male	104 (49.3)
Drug	193 (91.5)
D-penicillamine[1]	129 (61.6)
Trientine[2]	22 (10.4)
Zinc	42 (19.9)
Hepatic disease	176 (83.4)
Cirrhosis	38 (21.6)
Cirrhotic complications	19 (10.8)
Ascites	10 (5.7)
Spontaneous bacterial peritonitis	5 (2.8)
Esophageal varices	10 (5.7)
Gastric varices	5 (2.8)
Hepatic encephalopathy	8 (4.5)
Hepatocellular carcinoma	3 (1.7)
Neurological disease	30 (14.2)
Parkinsonism	15 (50.0)
Tremor	3 (10.0)
Dystonia	4 (13.3)
Tics	1 (3.3)
Dementia	2 (6.7)
Seizure	7 (23.3)
Psychiatric disease	24 (11.4)
Depression	9 (37.5)
Anxiety	2 (8.3)
Bipolar disorder	4 (16.7)
Organic affective syndrome	3 (12.5)
Psychosis	4 (16.7)
Personality disorder	3 (12.5)
Suicide	4 (16.7)
Concomitant hepatic and neurological/ psychiatric diseases	21 (10.0)
Non-immune hemolytic anemia	8 (3.8)
Viral hepatitis	7 (3.3)
Chronic hepatitis B infection	5 (71.4)
Chronic hepatitis C infection	2 (28.6)
Metabolic factors	17 (8.1)
Diabetes mellitus	3 (17.6)
Hypertension	7 (41.2)
Dyslipidemia	4 (23.5)
Obesity	4 (23.5)
Alcoholism	4 (23.5)
Liver transplantation	24 (11.3)
Death	26 (12.3)

[1]Twenty four patients had concomitant zinc therapy during the initial treatment phase or maintenance phase; [2]Eight patients had concomitant zinc therapy during the initial treatment phase or maintenance phase. Age was expressed as median (years) with interquartile range. Categorical variables were expressed as *n* (%).

associated with adverse events were identified by Cox proportional hazards model. The adverse events were analysed by Kaplan-Meier method with statistical significance determined by log-rank test. A two-tailed *P* value < 0.05 was regarded as statistically significant.

RESULTS

Patient characteristics

Two hundred and eleven patients with Wilson's disease were identified. Patient demographics are shown in Table 2. Male sex accounted for 49.3% of the cases (male cases 104; female cases 107). There were 170 newly diagnosed cases (male, 84; female, 86). The median follow-up duration was 8.0 years (IQR 5.0 to 14.0 years), with a total follow-up of 1559.5 person-years. Patients were diagnosed with Wilson's disease at a median age of 27.2 years (IQR 17.1 to 38.6 years). Sixty-five patients (30.8%) were diagnosed before the age of 20. Men were diagnosed with Wilson's disease at a similar age as women [28.5 years (IQR 19.0 to 38.8 years) and 25.0 years (IQR 15.9 to 38.6 years) for men and women, respectively; *P* = 0.326]. On presentation, nine patients had end-stage cirrhosis and nine had acute liver failure, requiring liver transplantation (8.5% of the total cases). One hundred and seventy-six patients (83.4%) had liver involvement. Of these, 21.6% developed cirrhosis (*n* = 38). Forty-nine patients (23.2%) had neurological and/or psychiatric involvement. Thirty patients (14.2%) had neurological involvement, with parkinsonism being the commonest manifestation (*n* = 15; 50.0%). Twenty-four patients (11.4%) had psychiatric manifestation, with depression being most prevalent (*n* = 9; 37.5%). Four patients (16.7%) had history of suicide. Twenty-one patients (10.0%) had both hepatic and neurological or psychiatric involvement. Non-immune mediated hemolytic anemia occurred in eight patients (3.8%). Seven patients (3.3%) had concomitant chronic viral hepatitis (CHB, 5; CHC, 2), and 17 (8.1%) had metabolic factors.

Table 3 The use of different medications in patients with neurological and/or psychiatric involvement

Drugs
Anticonvulsants (carbamazepine and valproate)
Antidepressants (citalopram and fluoxetine)
Anti-parkinson agents (levodopa, bromocriptine, amantadine, ropinorole and benzhexol)
Antipyschotics (clozapine, olanzapine, quetiapine, perphenazine, paliperidone and trifluoperazine)
Baclofen
Benzodiazepines (clonazepam, diazepam and lorazepam)
Lithium
Propranolol
Tetrabenazine

Table 3 shows the different medications used in the patients with neurological and/or psychiatric involvement, including anti-parkinson agents, anti-convulsants, benzodiazepines, propranolol, baclofen, tetrabenazine, antidepressants, antipsychotics and lithium.

When patients were divided into three age strata (< 20, 20 - 39.9, ⩾ 40 years), HCC and mortality were significantly higher in older age groups, while there were no significant differences for other hepatic, neurological or psychiatric manifestations (Table 4).

Incidence rate and prevalence

Between 2000 and 2016, the average annual incidence

Table 4 Clinical characteristics of patients with Wilson's disease according to different age strata [*n* = 211, *n* (%)]

	Age < 20 (*n* = 65)	Age 20-39.9 (*n* = 94)	Age ⩾ 40 (*n* = 52)	*P* value
Hepatic involvement	58 (89.2)	74 (78.7)	44 (84.6)	0.201
Cirrhosis	7 (10.8)	18 (19.1)	13 (25.0)	0.128
Cirrhotic complications	4 (6.2)	9 (9.6)	6 (11.5)	0.584
Hepatocellular carcinoma	0	0	3 (11.5)	0.014
Neurological involvement	9 (13.8)	16 (17.0)	5 (9.6)	0.469
Psychiatric involvement	6 (9.2)	12 (12.8)	6 (11.5)	0.787
Liver transplantation	11 (16.9)	11 (11.7)	2 (3.8)	0.066
Death	4 (6.2)	9 (9.6)	13 (25.0)	0.005

Significant *P* values were bolded.

Table 5 Incidence rate and prevalence of Wilson's disease in Hong Kong

Yr	Total population (million)			New cases			Total cases			Incidence rate (per million person-years)			Prevalence (per million)			Prevalence ratio of M/F
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T	
2000	3.28	3.39	6.67	4	7	11	24	28	52	1.22	2.06	1.65	7.32	8.26	7.80	0.89
2001	3.28	3.43	6.71	6	9	15	28	36	64	1.83	2.62	2.24	8.54	10.5	9.54	0.81
2002	3.28	3.56	6.74	3	8	11	31	44	75	0.91	2.25	1.63	9.45	12.36	11.13	0.76
2003	3.26	3.47	6.73	11	7	18	40	51	91	3.37	2.02	2.67	12.27	14.7	13.52	0.83
2004	3.27	3.52	6.78	9	4	13	47	54	101	2.75	1.14	1.92	14.37	15.34	14.90	0.94
2005	3.26	3.55	6.81	4	5	9	51	57	108	1.23	1.41	1.32	15.64	16.06	15.86	0.97
2006	3.27	3.59	6.86	2	3	5	52	60	112	0.61	0.84	0.73	15.90	16.71	16.33	0.95
2007	3.28	3.63	6.92	2	3	5	54	63	117	0.61	0.83	0.72	16.46	17.36	16.91	0.95
2008	3.29	3.67	6.96	2	2	4	54	63	117	0.61	0.54	0.57	16.41	17.17	16.81	0.96
2009	3.28	3.69	6.97	6	10	16	60	72	132	1.83	2.71	2.30	18.29	19.51	18.94	0.94
2010	3.29	3.73	7.02	9	8	17	68	80	148	2.74	2.14	2.42	20.67	21.45	21.08	0.96
2011	3.30	3.77	7.07	8	3	11	75	83	158	2.42	0.80	1.56	22.73	22.02	22.35	1.03
2012	3.33	3.83	7.15	3	2	5	77	85	162	0.90	0.52	0.70	23.12	22.19	22.66	1.04
2013	3.33	3.86	7.19	2	3	5	79	87	166	0.60	0.78	0.70	23.72	22.54	23.09	1.05
2014	3.35	3.90	7.24	2	8	10	82	94	175	0.60	2.05	1.38	24.48	24.10	24.17	1.02
2015	3.37	3.94	7.31	6	0	6	86	93	179	1.78	0.00	0.82	25.52	23.60	24.49	1.08
2016	3.38	3.96	7.34	5	4	9	89	96	185	1.48	1.01	1.23	26.33	24.24	25.20	1.09

M: Male; F: Female; T: Total.

rate of Wilson's disease was 1.44 per million person-years. There was a decrease in the annual incidence rate from 1.65 to 1.23 per million person-years (Poisson *P* = 0.010) (Table 5). The average annual prevalence was 17.93 per million. The annual prevalence increased from 7.80 to 25.20 per million (Poisson *P* < 0.001).

Wilson's disease was more commonly diagnosed in the younger age groups, with the incidence rate peaking in the 15- to 19-year age group (Table 6). For patients aged less than 25 years, the average annual incidence rate ranged from 2.02 to 3.06 per million person-years. For those aged between 25 and 44 years, the average annual incidence rate ranged from 1.71 to 1.97 per million person-years. Patients were less likely to have disease onset at 45 years or older (age group 45-49: 0.86 per million person-years; age group ⩾ 50: 0.29 per million person-years).

Adverse events

Among the 176 patients with hepatic involvement, 38 had cirrhosis (21.6%). Nineteen patients (10.8%) had cirrhotic complications, with the two most common being the development of ascites (*n* = 10; 5.7%) and esophageal varices (*n* = 10; 5.7%). Around 20% of the cirrhotic cases were men (21 out of 104), while 15.9% were women (17 out of 107) (*P* = 0.416). The median age for the diagnosis of cirrhosis was 33.4 years [IQR 23.9 to 42.8 years; 32.4 years (IQR 23.7 to 45.5 years) and 34.1 years (IQR 24.4 to 41.4 years) in men and women, respectively; *P* = 0.772].

Three cases were newly diagnosed with HCC (1.7% of cases with hepatic involvement), all of which developed in men (age at diagnosis ranged from 45.8 to 56.0 years). The 5-year and 10-year cumulative incidences of HCC were 1.3% (95%CI: 0%-3.1%) and 2.3% (95%CI: 0%-4.8%), respectively. The time from diagnosis of Wilson's disease to HCC development ranged from 1.7 to 8 years. All three patients had underlying cirrhosis, and one of them had concomitant CHB infection. None of them received liver transplantation, and two died at the age of 47.2 and 56.8 years.

Liver transplantations were performed in 24 patients (11.3% of all cases; 13.6% of cases with hepatic involvement) -- 14 for cirrhosis and 10 for acute liver failure. Twelve out of 104 were men (11.1%)

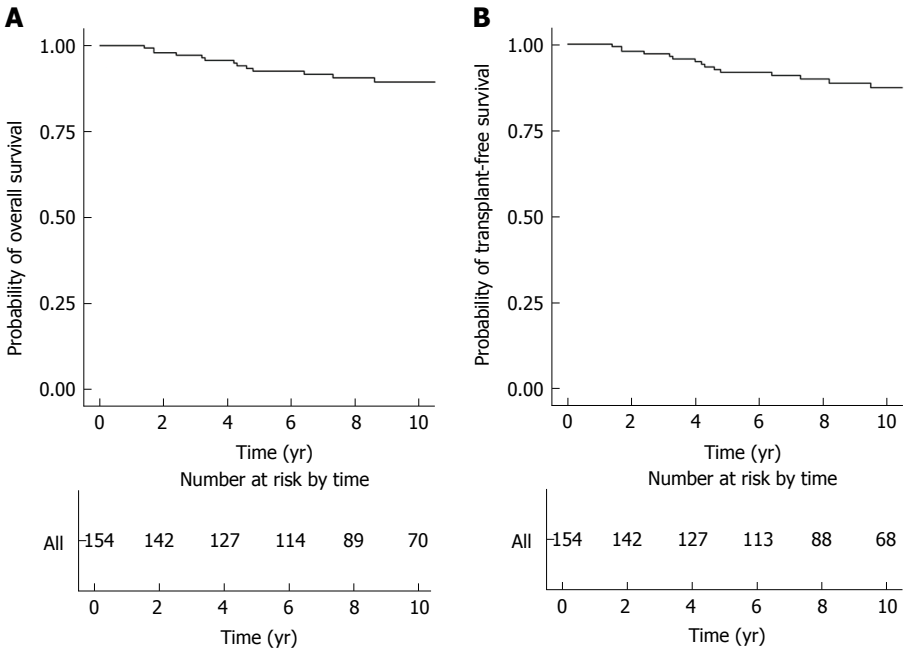

Figure 1 Kaplan-Meier survival plot. A: Overall survival; B: Transplant-free survival.

Table 6 Average age-specific incidence rate of Wilson's disease in Hong Kong between 2000 and 2016

	Case number			Incidence rate (per million person-years)		
Age group	M	F	T	M	F	T
0-4	6	7	13	2.81	3.38	2.61
5-9	3	7	10	1.35	2.73	2.02
10-14	4	9	13	1.30	2.94	2.09
15-19	10	12	22	2.70	3.43	3.06
20-24	10	10	20	2.64	2.52	2.58
25-29	9	6	15	2.33	1.21	1.71
30-34	11	8	19	2.72	1.45	1.97
35-39	9	10	19	1.92	1.70	1.80
40-44	10	9	19	1.92	1.54	1.74
45-49	4	5	9	0.81	0.92	0.86
⩾ 50	7	4	11	0.36	0.21	0.29

M: Male; F: Female; T: Total.

and 12 out of 107 were women (11.2%). Five liver transplantations were newly performed between 2000 and 2016. The 5-year and 10-year cumulative incidences of liver transplantation were 1.5% (95%CI: 0%-3.5%) and 4.2% (95%CI: 0%-8.4%), respectively. Patients underwent liver transplantation at a median age of 24.4 years (IQR 18.4 to 37.4 years). There were eight liver transplantation cases in patients aged less than 20 years, 11 cases in those aged between 20 and 39 years, and five cases in those aged between 40 and 59 years.

Twenty-six patients died between 2000 and 2016 [male cases 16 (15.0%), female cases 10 (9.6%); P = 0.182]. The median time from diagnosis to death for these 26 patients was 4.2 years (IQR 2.1 to 5.0 years). For those newly diagnosed patients receiving long-term treatment (n = 154), the 5-year and 10-year rates of overall survival were 92.6% (95%CI: 88.3%-97.1%) and 89.5% (95%CI: 84.1%-95.2%), respectively (Figure 1A). By univariate analysis, older age at diagnosis, cirrhosis, HCC , concomitant chronic viral hepatitis and metabolic factors were significant risk factors. By multivariate analysis, cirrhosis (HR 21.75; 95% CI 5.62 to 84.18) was the only independent risk factor, while concomitant chronic viral hepatitis was of borderline significance (HR 7.04; 95% CI 0.94 to 52.81)(Table 7).

Figure 2A illustrates the comparison of overall survival of patients according to their baseline cirrhosis. Patients without cirrhosis had a significantly higher rate of overall survival (log-rank P < 0.001), with the 5-year and 10-year rates of overall survival being 96.5% (95%CI: 93.2%-99.9%) and 95.3% (95%CI: 91.4%-99.5%), respectively. For patients with cirrhosis, the 5-year and 10-year rates of overall survival were 66.9% (95%CI: 48.3%-92.7%) and 51.0% (95%CI: 30.9%-84.1%), respectively. Figure 2B illustrates the comparison of overall survival of patients according to the presence of chronic viral hepatitis. Patients without chronic viral hepatitis had a significantly higher rate of overall survival (log-rank P = 0.021), with the 5-year and 10-year rates of overall survival being 93.1% (95%CI: 88.9%-97.6%) and 90.9% (95%CI: 85.8%-96.3%), respectively. For patients with chronic viral hepatitis, the 5-year and 10-year rates of overall survival were 80.0% (95%CI: 51.6%-100%) and 60.0% (95%CI: 29.3%-100%), respectively.

The 5-year and 10-year rates of transplant-free survival were 91.8% (95%CI: 87.3%-96.6%) and 87.4% (95%CI: 81.5%-93.8%), respectively (Figure 1B). By univariate analysis, older age at diagnosis, cirrhosis, HCC, concomitant chronic viral hepatitis and

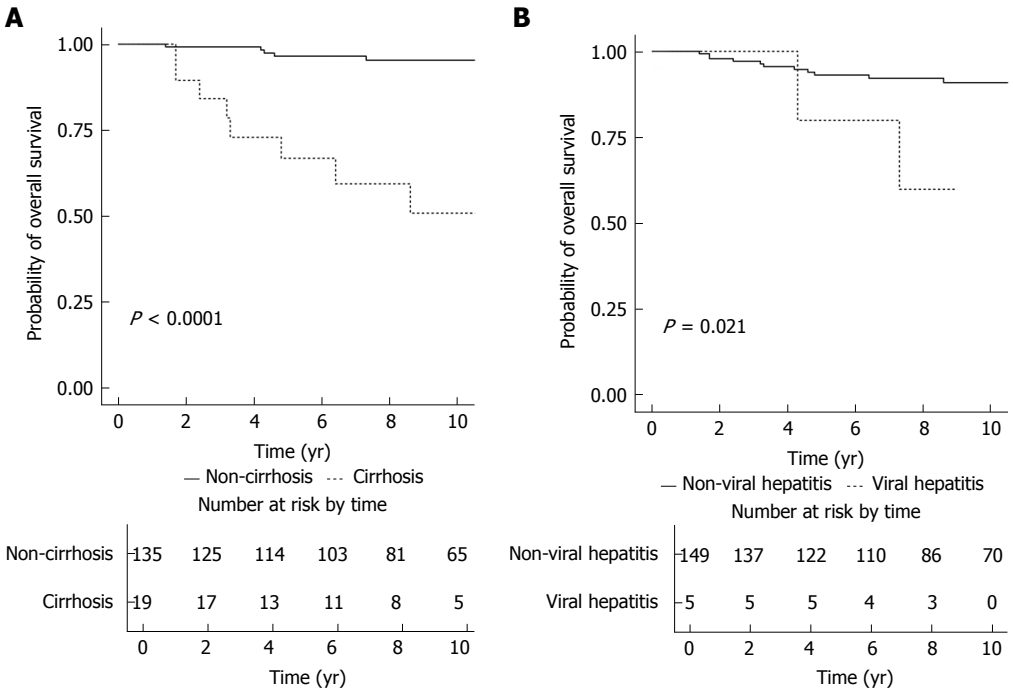

Figure 2 Kaplan-Meier survival plot for overall survival stratified according to baseline cirrhosis status(A) and presence of chronic viral hepatitis (B).

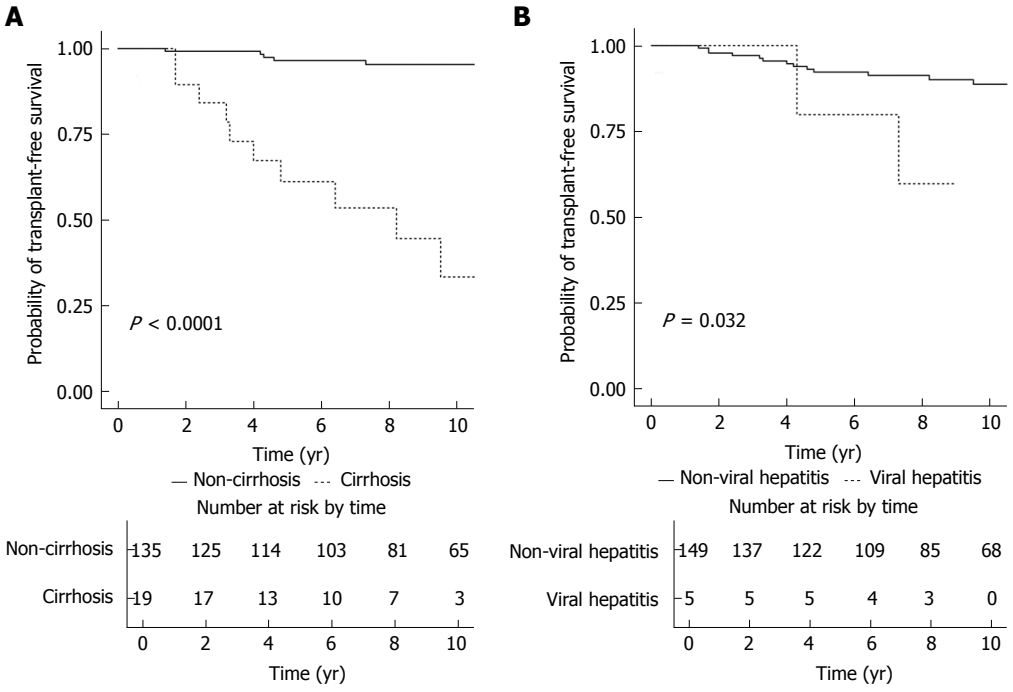

Figure 3 Kaplan-Meier survival plot for transplant-free survival stratified according to baseline cirrhosis status (A) and presence of chronic viral hepatitis (B).

metabolic factors were significant risk factors. By multivariate analysis, cirrhosis (HR = 32.16; 95%CI: 8.58-120.52) and concomitant chronic viral hepatitis (HR = 9.97; 95%CI: 1.36-73.05) were independent risk factors (Table 7).

Figure 3A illustrates the transplant-free survival of patients with and without baseline cirrhosis. Patients without cirrhosis had a significantly higher rate of transplant-free survival (log-rank $P < 0.001$), with the 5-year and 10-year rates of transplant-free survival being 96.5% (95%CI: 93.2%-99.9%) and 95.3% (95%CI: 91.4%-99.5%), respectively. For patients with cirrhosis, the 5-year and 10-year rates of transplant-free survival were 61.2% (95%CI: 42.3%-88.7%) and 33.5% (95%CI: 14.9%-75.2%), respectively. Figure 3B illustrates the transplant-free

Table 7 HRs and 95%CIs for the association between different covariates and overall survival, transplant-free survival

	Univariate analysis		Multivariate analysis	
	HR	95%CI	HR	95%CI
Overall survival				
Age[1]	1.04	1.02-1.07	1.03	0.99-1.08
Male sex	1.49	0.52-4.29		
Hepatic disease	0.92	0.21-4.13		
Cirrhosis	15.42	5.15-46.18	21.75	5.62-84.18
Hepatocellular carcinoma	12.42	2.71-56.93	2.23	0.38-13.32
Chronic viral hepatitis[2]	4.95	1.09-22.44	7.04	0.94-52.81
Neurological or psychiatric disease	1.11	0.35-3.55		
Metabolic factors[3]	4.37	1.37-13.96	2.68	0.59-12.17
Drug				
Penicillamine	Reference	-		
Trientine	2.30	0.61-8.63		
Zinc	0.30	0.04-2.39		
transplant-free survival				
Age[1]	1.04	1.01-1.07	1.03	0.98-1.07
Male sex	1.45	0.54-3.88		
Hepatic disease	1.09	0.25-4.80		
Cirrhosis	21.34	7.34-61.99	32.16	8.58-120.52
Hepatocellular carcinoma	11.25	2.48-50.92	1.93	0.34-11.07
Chronic viral hepatitis[2]	4.48	0.998-20.09	9.97	1.36-73.05
Neurological or psychiatric disease	1.27	0.44-3.66		
Metabolic factors[3]	3.59	1.16-11.16	2.51	0.60-10.54
Drug				
D-penicillamine	Reference	-		
Trientine	1.97	0.54-7.17		
Zinc	0.26	0.03-1.97		

[1]Age was treated as a continuous variable in the analysis; [2]Chronic viral hepatitis was defined as the presence of either chronic hepatitis B or C infection; [3]Metabolic factors were defined as the presence of one of the following: diabetes mellitus, hypertension, dyslipidemia, obesity or alcoholism. Significant risk factors were bolded.

survival of patients with and without chronic viral hepatitis. Patients without chronic viral hepatitis had a significantly higher rate of transplant-free survival (log-rank P = 0.032), with the 5-year and 10-year rates of transplant-free survival being 92.3% (95%CI: 87.9%-97.0%) and 88.8% (95%CI: 83.1%-94.9%), respectively. For patients with chronic viral hepatitis, the 5- and 10-year rates of transplant-free survival were 80.0% (95%CI: 51.6%-100%) and 60.0% (95%CI: 29.3%-100%), respectively.

Data validation

Of the 211 patients with Wilson's disease identified, 80 patients (37.9%) had follow-up in our hospital. After cross validating with the medical records, 77 were confirmed to have Wilson's disease (positive predictive value (PPV) of 96.3%).

Of the 77 patients, we could not ascertain in 10 patients whether they were index cases or diagnosed by family screening. For the remaining 67 cases, 59 (88.1%) were index cases and 8 (11.9%) were diagnosed by family screening. All cases were diagnosed of Wilson's disease in accordance to the AALSD or EASL guidelines. Sixty-five patients (84.4%) had liver manifestations, while 25 (32.5%) had neurological and/or psychiatric manifestations [18 (23.4%) and 11 (14.3%) had neurological and psychiatric manifestations, respectively].

DISCUSSION

Currently, population-based studies on the epidemiology as well as the natural history of Wilson's disease are lacking in both the western and Asian populations, and the majority of them were done before the 1990s. The present territory-based study was the first to describe both the epidemiology and natural history of Wilson's disease over a long period of time (a span of 17 years from 2000 to 2016) in the Chinese. The population-based nature of this study, in which the data were retrieved via electronic search of hospital medical registry, enables complete capture of all cases under the care of our public health system. This helps to minimize selection bias inherent in studies conducted in tertiary referral centers or selected hospitals. Cases were identified by ICD-9 coding, with local studies showing high coding accuracies[24,28]. We also confirmed a PPV of 96.3% for the coding accuracy of Wilson's disease in the CDARS.

The incidence rate and prevalence of Wilson's disease were estimated to be 17 to 29 per million person-years[8,9] and 30 per million, respectively[1,11] In comparison, the estimates from our study were lower, with an average annual incidence rate of 1.44

per million person-years, and an average annual prevalence of 17.93 per million. These estimates were in line with what were reported in Taiwan between 2000 and 2005 (an average annual incidence rate of 2.7 per million person-years and a prevalence ranging from 8.4 to 16.0 per million). The lower incidence rate may be partly due to under diagnosis. However, whether Wilson's disease is genuinely less common in Asia requires confirmation from more population-based studies.

From the present study, the annual incidence rate of Wilson's disease in Hong Kong had been decreasing, from 1.65 to 1.23 per million person-years between 2000 and 2016. This is likely attributed to the ageing population. As shown in Table 4, the incidence rate of Wilson's disease peaked in the 15- to 19-year age group (3.06 per million person-years), while it was extremely low among individuals aged 45 or more. Between 2000 and 2016, population aged less than 20 has decreased from 1.59 million to 1.17 million, while the population aged 50 or more has increased from 1.63 million to 2.92 million in Hong Kong[31]. On the other hand, the prevalence of Wilson's disease has been increasing, from 7.80 to 24.49 per million. This is likely related to the availability of effective treatment. It has been shown that treatment is associated with hepatic and neurological improvements in 82%-90% and 55%-69% of patients, respectively[4,6].

In the current study, the median age at diagnosis was 27.2 years, with the highest incidence rate observed in the 15- to 19-year age group. The proportions of cases in different age strata in our study [age < 20: 58 cases (30.8%); age 20-39: 73 cases (44.5%); age ⩾ 40: 39 cases (24.6%)] were consistent with that reported by the Taiwanese population-based study [age < 20: 115 cases (37.5%); age 20-39: 127 cases (41.4%); age ⩾ 40: 65 cases (21.2%)][14]. Men and women were equally affected as shown in our study and also previous studies[3,4,14,32]. The proportion of patients with neurological and/or psychiatric involvement was relatively low in our study (n = 49, 23.2%), although some studies also reported a similar rate of 24%-27%[3,33]. As for our center, 25 out of 77 patients (32.5%) had neurological and/or psychiatric involvement. This slightly higher proportion of neurological/psychiatric involvement is likely to be explained by the fact that our center is a tertiary referral hospital and is the only center which provides liver transplantation services in our locality.

However, a significant proportion of patients with hepatic involvement still had liver-related complications, with 21.6% having cirrhosis, 10.8% having cirrhotic complications, 1.7% developing HCC, and 13.6% undergoing liver transplantations. Around 12% of the total cases died during the observation period, with the 5-year and 10-year rates of overall survival being 92.6% and 89.5%, respectively.

Factors that could modify the transplant-free survival included the presence of cirrhosis (HR = 32.16) and concomitant chronic viral hepatitis (HR = 9.97). This highlights the importance of early diagnosis and treatment in reducing the copper load in the body to prevent development of cirrhosis. Future prospective studies with larger sample size are required to ascertain whether early treatment to suppress hepatitis B virus replication and to cure hepatitis C virus infection will be useful in improving the prognosis of patients with concomitant chronic viral hepatitis. The presence of metabolic factors was shown to be associated with lower transplant-free survival by univariate but not multivariate analysis. There are two possible reasons. First, as metabolic factors are associated with increasing age and cirrhosis[17,18,34], the potential risk of metabolic factors per se as shown by univariate analysis was attenuated after adjusting for the effect of age and cirrhosis in multivariate analysis. Second, the present study may be underpowered to detect this potential effect due to the relatively small sample size.

There are some limitations of the current study. First, a small proportion of healthcare services (around 10%) is not covered by the Hospital Authority[19], and hence some patients attending private hospitals will not be captured in the CDARS. As such, the incidence rate and prevalence of Wilson's disease might be underestimated. Nevertheless, the majority of these patients are likely to be captured in the CDARS eventually, because of preference for long-term follow-up in the public hospitals or having consultation for other illnesses in our locality. Second, information regarding biochemical response to various treatment (D-penicillamine, trientine and zinc) could not be retrieved. Third, whether Wilson's disease was diagnosed in accordance to the international guidelines (AALSD or EASL guidelines) could not be confirmed in other centers. However, this limitation was unlikely to have significant impact as it is the standard practice that all patients with suspected Wilson's disease in Hong Kong are referred to hepatologists. As an example, all 77 cases who followed up in our center were diagnosed according to the international guidelines. Fourth, we could not ascertain whether these patients were index cases or were detected by family screening. As for our center, only 8 cases (11.9%) were diagnosed by family screening. In addition, diagnosis of Wilson's disease in patients with neurological symptoms is usually delayed for a longer time from onset than in patients with hepatic symptoms[35]. The incidence rate of Wilson's disease in our cohort should therefore be interpreted in this context. Lastly, some of the medications used to treat the neurological and/or psychiatric symptoms (*e.g.,* anticonvulsants) could potentially lead to deranged liver function, which may be wrongly regarded as liver involvement due to Wilson's disease by simply referring to the ICD coding.

These limitations may be addressed by collaboration between all public and private hospitals in the

future. In this way, an even more precise estimate of the epidemiology of Wilson's disease could be derived, and other potential risk factors for reduced transplant-free survival (*e.g.,* metabolic factors) could be further investigated.

In conclusion, the epidemiology of Wilson's disease was described in a well defined Chinese population, with identification of risk factors for overall and transplant-free survival. There was a significant increase in the prevalence of Wilson's disease in Hong Kong. Early diagnosis and treatment as well as control of concomitant chronic viral hepatitis to improve prognosis could potentially reduce the disease complications.

ARTICLE HIGHLIGHTS

Research background

There are few studies on the epidemiology and natural history of Wilson's disease in the Chinese population. The authors conducted a territory-based study in Hong Kong (HK) with a population of 7.3 million to address this issue.

Research motivation

Epidemiology data are important for recognizing the temporal trend of a particular disease, understanding the natural history and risk factors, as well as for resource allocation.

Research objectives

To investigate the epidemiology and natural history of Wilson's disease in the Chinese population.

Research methods

Data were retrieved from the Clinical Data Analysis and Reporting System (CDARS) and Clinical Management System (CMS) of the Hong Kong Hospital Authority. The study observation period was from 2000 to 2016. Cases of Wilson's disease between 1999 and 2016 were identified from CDARS by the International Classification of Diseases (ICD)-9 code of 275.1. The incidence rate and prevalence of Wilson's disease between 2000 and 2016 were calculated. Evaluation of the count data and temporal trends was assessed by Poisson regression model. Cox proportional hazards model was used to identify variables that were associated with adverse outcomes. Kaplan-Meier method was used to analyze the adverse outcomes.

Research results

The authors identified 211 patients (male-to-female ratio 0.97:1; median age 27.2 years, IQR: 17.1-38.6 years; median follow-up 8.0 years, IQR: 5.0-14.0 years). The average annual incidence rate and prevalence were 1.44 per million person-years and 17.93 per million, respectively. Between 2000 and 2016, the annual incidence rate decreased from 1.65 to 1.23 per million person-years (Poisson $P = 0.010$), while the annual prevalence increased from 7.80 to 25.20 per million (Poisson $P < 0.001$). Among the 176 patients with hepatic involvement, 38 (21.6%) had cirrhosis, three (1.7%) developed hepatocellular carcinoma, 24 (13.6%) underwent liver transplantations, and 26 (14.8%) died. Seven patients had concomitant chronic viral hepatitis B or C. The 5- and 10-year overall survival rates were 92.6% and 89.5%, and for transplant-free survival rates 91.8% and 87.4%, respectively. Cirrhosis and possibly chronic viral hepatitis were associated with poorer overall survival.

Research conclusions

There was a considerable increase in the prevalence of Wilson's disease in the Chinese population. The long-term survival was good except in patients with cirrhosis or concomitant viral hepatitis.

Research perspectives

The epidemiology of Wilson's disease was described in a well defined Chinese population, and factors associated with overall survival and transplant-free survival were identified. Future collaboration with the hepatology units from all public and private hospitals is warranted to allow for an even more precise estimate of the epidemiology of Wilson's disease and to investigate other potential risk factors for reduced transplant-free survival (*e.g.,* metabolic factors).

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P- Reviewer: Codoñer-Franch P, Garcia-Fernandez MI
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7727-7734

DOI: 10.3748/wjg.v23.i43.7727

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

ORIGINAL ARTICLE

Retrospective Study

Efficacy of thalidomide therapy in pediatric Crohn's disease with evidence of tuberculosis

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Author contributions: Wang L, Hong Y and Wu J collected the data; Wang L analyzed the data and drafted the manuscript; Leung YK and Huang Y designed and supervised this study; all authors approved the final version to be published.

Supported by Medical Science and Technology Planning Project of Zhejiang Province, China, No. 2015KYB430 (to Hong Y).

Institutional review board statement: The study was approved by the ethics committee of Children's Hospital of Fudan University, Shanghai, China.

Informed consent statement: All the patients and/or their legal guardians provided written consent forms for the study.

Conflict-of-interest statement: All the authors listed here declare no conflicts of interest related to this study.

Data sharing statement: The dataset is available from the corresponding author at yhuang815@163.com.

Manuscript source: Unsolicited manuscript

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Received: July 26, 2017
Peer-review started: July 26, 2017
First decision: August 10, 2017
Revised: August 28, 2017
Accepted: September 13, 2017
Article in press: September 13, 2017
Published online: November 21, 2017

Abstract

AIM

To evaluate the efficacy of thalidomide for treating troublesome cases of pediatric Crohn's disease (CD) with tuberculosis infection.

METHODS

A retrospective study of clinical outcome among children treated with thalidomide was conducted. All patients had evidence of tuberculosis infection with a failure of anti-tuberculosis treatment for more than one year, and were subsequently diagnosed with CD. All the patients received thalidomide treatment with a starting dose of 1.2-2.5 mg/kg per day. Remission was defined as pediatric CD activity index less than or equal to 10.

RESULTS

Ten patients with CD were treated with thalidomide at an average age of 7.2 years and followed up for a median of 22.2 mo. Clinical remission rate was 60% after 9-12 mo of thalidomide treatment. One pati-

ent with no response had an interleukin-10 receptor alpha gene mutation. Erythrocyte sedimentation rate, C-reactive protein and platelet count showed a dramatic decrease; hemoglobin level and weight improved significantly after thalidomide treatment when compared with the baseline values.

CONCLUSION
Thalidomide is an effective and safe drug for remission of CD in pediatric patients who have been treated for tuberculosis.

Key words: Inflammatory bowel disease; Intestinal tuberculosis; Anti- tubercular treatment; Thalidomide; Children

Core tip: Therapies for Crohn's disease (CD) and intestinal tuberculosis are totally different, and anti-TNF alpha treatment may increase the risk of tuberculosis reactivation. That makes it still tough to treat patients with severe CD with concomitant tuberculosis, especially in high tuberculosis prevalence areas. In the current study, all patients had evidence of tuberculosis infection and diagnosed with CD. Thalidomide yielded a positive result for those special cases, and it could be an alternative drug after treatment of tuberculosis is completed.

Wang L, Hong Y, Wu J, Leung YK, Huang Y. Efficacy of thalidomide therapy in pediatric Crohn's disease with evidence of tuberculosis. *World J Gastroenterol* 2017; 23(43): 7727-7734 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7727.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7727

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder affecting an increasing number of patients each year around the world[1]. It is characterized by abdominal pain, diarrhea, bloody stool and other extra-intestinal manifestations, and impacts on growth in children and adolescents. Wang *et al*[2] reported a multicenter retrospective study in China which demonstrated that childhood-onset inflammatory bowel disease is an emerging disease with a 12-fold increase of incidence over the past decade. Intestinal tuberculosis (ITB) shares a close resemblance in clinical, endoscopic and histological manifestations with CD, making the differential diagnosis of these two diseases difficult[3]. At the same time, tuberculosis (TB) is and has always been a major public health problem worldwide, especially in the lower income countries[4]. With the changing epidemiology of TB and CD, it is not unusual to encounter the coexistence of these two diseases, especially in high TB endemic areas[5].

Managing CD with steroids, immunomodulatory therapy and biological agents in high TB endemic regions is challenging since those treatments are associated with an increased risk of tuberculosis reactivation for active or latent TB[6]. Considering a high incidence of TB in developing countries, empirical anti-tubercular treatment (ATT) is used to differentiate between ITB and CD[7]. However, the use of anti-TB medications may pose a risk of toxicity and cause unnecessary delay for management of CD patients.

In 2011, we reported in our pilot study our experience that three pediatric patients with CD concomitant with tuberculosis achieved clinical remission after six months of thalidomide treatment[8]. Similarly, a later case report presented an adult patient with CD and pulmonary TB who was steroid-dependent and unresponsive to infliximab, but the patient exhibited an excellent response to thalidomide treatment[9]. In the present study, we enrolled 10 pediatric-onset patients who had symptoms and evidence suggestive of CD and tuberculosis in attempt to evaluate the efficacy of thalidomide in clinical remission of these cases in a long-term follow-up.

MATERIALS AND METHODS

Patients

This is a tertiary medical center, retrospective study of pediatric CD patients (aged < 18 years) with tuberculosis treated with thalidomide at the Children's Hospital of Fudan University from July 2009 to April 2016. Tuberculosis diagnosis was established based on at least one of the following criteria: (1) histological manifestation of acid fast bacilli (AFB) in intestinal tissues; (2) positive TB culture; and (3) positive tuberculin skin testing. All of the patients received a full course of anti-TB medications prior to thalidomide administration. The diagnosis of CD was based on endoscopic and clinical symptoms, which were defined as no improvement of clinical and endoscopic symptoms after anti-TB treatment for at least one year.

Treatment

Thalidomide (Changzhou Pharmaceutical Factory, Changzhou, China) was administered orally at a starting dose of 1.2-2.5 mg/kg per day. The decision to modify the dosage was made by the director of gastroenterology department according to the response and disease activity. To minimize adverse events, thalidomide was taken every evening. This study was approved by the Ethics Committee of Children's Hospital, Fudan University. Written informed consent was obtained from either the parents or legal guardians of the patients after they were informed about possible adverse events. Contraception was controlled in the patients who were in reproduction age.

Outcome

A retrospective chart review of medical records was performed to collect baseline demographic and disease characteristics, results of clinical indices and adverse events during follow-up. Pediatric CD Activity Index (PCDAI)[10], as primary outcome, was used to evaluate the response to the treatment from the time of thalidomide initiation and at 9-12 mo thereafter. Each patient acted as his/her own historical control. Clinical remission was defined by PCDAI less than or equal to 10, significant response was a decrease in PCDAI at least 12.5 points from baseline[11]. The clinical indices of erythrocyte sedimentation rate (ESR; normal range 0-20 mm/h), C-reactive protein (CRP; normal range 0-8 mg/L), platelet count and hemoglobin were compared before and after treatment. Weight for age *Z* score by Chinese standardized growth curve was evaluated as a measurement of nutritional index.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL). Quantitative variables were presented as mean ± SD or median with range. Continuous variables were evaluated using paired Student's *t* test with normal distribution and Wilcoxon test with non-normal distribution. Statistical significance level was set at two-sided $P < 0.05$.

RESULTS

Baseline characteristics

Ten pediatric patients treated with thalidomide were enrolled in the study. There were 6 females and 4 males, with an average age of 7.2 years at thalidomide treatment (range: 2-13.5 years). The mean disease duration before thalidomide therapy was 24 mo (range: 16-42 mo). The average length of follow-up was 22.2 mo (range: 9-44 mo) after the initiation of thalidomide. Clinical characteristics are summarized in Table 1.

All patients have received ATT treatment for more than one year with the average age of 5.3 years (range: 0.2-12.0 years). Seven children were previously treated with isoniazide, rifampicin and pyrzinamide (HRZ). All patients had evidence of tuberculosis; positive AFB was observed in 8 cases and positive tuberculin skin testing in 2. One patient suffered from spleen TB, and cell culture of Mycobacterium was positive. The mean duration of ATT treatment was 18 mo (range: 12-36 mo).

In the present cases, 2 showed prominently colonic involvement, 7 had ileocolonic diseases and the remaining one had isolated ileal disease. Six patients presented with perianal disease, one suffered from joint involvement and one had oral ulcers.

Outcome

Disease activity: At baseline, 7 patients had moderate-to-severe disease activity (PCDAI > 30). There was a significant decrease in the PCDAI score from 37.3 ± 14.1 in the beginning to 16.0 ± 17.9 after 9-12 mo treatment ($P < 0.05$) (Figure 1). Clinical remission was achieved in 6 patients (60.0%) and there was a response in 3 cases (30.0%) at 9-12 mo after commencement of thalidomide treatment. For those 3 patients with follow-up duration longer than 36 mo, they were still in remission. Case 4 was unresponsive to infliximab prior to positive AFB, but was responsive to thalidomide. In case 2, the PCDAI increased from 52.5 to 60, indicating no response to thalidomide. Given early-onset symptoms and severe perianal abscess of the patient, we performed the whole exome sequencing and found a causative interleukin-10 receptor alpha (IL10 RA) mutation. One of the compound heterozygous variants was c.301C > T inherited from her father, and the other was c.537G > A from her mother. She has been treated with umbilical cord blood transplantation, which was described in our previous studies[12,13].

Given the lack of complete data, we only evaluated the response of some cases at different time points. Case 1 showed significant clinical response in the first one month (PCDAI decreased from 45 to 15), case 5 and case 7 attained clinical remission (PCDAI < 10) after 2 mo and one month of treatment, respectively. With regard to TB status, the positive AFB findings were turned to negative in case 3 and case 8 after 16 mo and 10 mo of treatment, respectively.

Overall, the laboratory assessment showed significant drop after thalidomide therapy in ESR (baseline: 26.1 mm/h ± 14.5 mm/h; follow-up: 11.5 mm/h ± 12.0 mm/h) (normal range: 0-20 mm/h) ($P = 0.037$) (Figure 2A), CRP (baseline: 64.0 mg/L ± 46.5 mg/L; follow-up: 25.1 mg/L ± 47.8 mg/L) (normal range: 0-8 mg/L) ($P = 0.004$) (Figure 2B) and platelet (baseline: 471.1 × 10^9/L ± 178.3 × 10^9/L; follow-up: 328.4 × 10^9/L ± 163.8 × 10^9/L) ($P = 0.002$) (Figure 2D), with a marked improvement in hemoglobin level (baseline: 111.0 g/L ± 13.4 g/L; follow-up: 119.5 g/L ± 19.7 g/L) (normal range: 110-160 g/L) ($P = 0.105$) (Figure 2C). Weight values at 9-12 mo after starting the treatment showed a dramatic increase when compared with the baselines (21.03 ± 13.4 *vs* 27.8 ± 19.3) ($P < 0.05$). The changes of weight for age *Z* score are shown in Figure 3.

Dose escalation: The majority of patients (70%) started on an initial dose of 2 mg/kg per day or more. During follow-up, four cases had thalidomide dose increased (to a maximum of 3 mg/kg per day). And the details of dose changes in each individual patient are listed in Table 2. Doses were successfully decreased for all cases after responding to thalidomide. Two cases discontinued thalidomide administration during follow-up due to clinical remission, and one patient with IL10 RA deficiency stopped the treatment

Table 1 Clinical characteristics of all cases treated with thalidomide

Patient No.	Sex	Age at thalidomide treatment	Disease duration before thalidomide	Age at ATT treatment	Disease distribution	Extra-intestinal symptoms	TB status before ATT	ATT medications and duration	Thalidomide starting dose	Thalidomide final dose (duration)	Follow-up time (mo)	Response
1	M	2 yr 8 mo	26 mo	1 yr 3 mo	Ileocolonic	Joint lesions	AFB (+), PPD (+)	HRZ, 6 m; HRZEP, 6 mo	2.5 mg/kg per day	- (34 m)	38 mo	Remission
2	F	2 yr	23 mo	2 mo	Colon	Perianal abscess	AFB (+)	HRZ, 3 mo; HR, 15 mo	2.5 mg/kg per day	- (7 m)	10 mo	No response
3	F	2 yr 4 mo	28 mo	7 mo	Colon	Perianal abscess	AFB (+)	HRZ, 9 mo; HREP, 27 mo	2 mg/kg per day	0.33 mg/kg per day	33 mo	Response
4	F	11 yr 7 mo	42 mo	8 yr 1 mo	Ileocolonic	Perianal skin tag	AFB (+)	HREZ, 12 mo	1.8 mg/kg per day	0.8 mg/kg per day	15 mo	Response
5	M	12 yr 6 mo	18 mo	11 yr 3 mo	Ileocolonic	Oral ulcers	TB culture (+), Spleen TB	HRS, 5 mo; HRE, 12 mo	2 mg/kg per day	0.5 mg/kg per day	36 mo	Remission
6	M	12 yr 5 mo	20 mo	11 yr 5 mo	Ileocolonic	Anal fistula	AFB (+)	HRZ, 12 mo	1.2 mg/kg per day	0.6 mg/kg per day	12 mo	Remission
7	M	8 yr 6 mo	38 mo	5 yr 4 mo	Ileal	Pleural and ascetic fluid	PPD (+)	HRE, 3 mo; HRZ, 12 mo	2 mg/kg per day	- (44 m)	44 mo	Remission
8	F	3 yr 8 mo	16 mo	2 yr 4 mo	Ileocolonic	-	AFB (+)	HRZ, 14 mo; HRE, 12 mo	2 mg/kg per day	1.6 mg/kg per day	16 mo	Response
9	F	2 yr 4 mo	16 mo	1 yr	Ileocolonic	Anal fissure	AFB (+)	HRZ, 14 mo	2.2 mg/kg per day	1.8 mg/kg per day	9 mo	Remission
10	F	13 yr 6 mo	17 mo	12 yr 1 mo	Ileocolonic	Perianal skin tag	AFB (+)	HREZ, 3 mo; HR, 15 mo	1.8 mg/kg per day	1.3 mg/kg per day	9 mo	Remission

ATT: Anti-tubercular treatment; H: Isoniazid; R: Rifampicin; Z: Pyrazinamide; E: Ethambutol; S: Streptomycin; P: Para-aminosalicylic acid.

due to no response (Table 1). The median cumulative dose for all patients until the end of follow-up time was 16.0 g. For case 5, the cumulative dose was over 28 g. He discontinued thalidomide after 22 mo of treatment, but the symptom of oral ulcers relapsed. However, the patient later opted to restart thalidomide and responded quickly. He was in remission with a minimum dose of 0.5 mg/kg per day. Among all cases, 4 completely discontinued anti-tuberculous drugs and then took thalidomide, the other 6 patients continued anti-tuberculous medications. In terms of the treatment for CD, 6 cases were treated with 5-ASA as well as thalidomide, one patient received nutritional treatment.

No significant relations were seen between the disease duration, disease location or age of diagnosis and remission or response to thalidomide treatment.

Adverse events

One patient (case 5) was complained of drowsiness and one (case 1) had dryness in eyes, the symptoms were relieved without reducing dose, or discontinuation. One patient (case 8) developed dryness in eyes and knee pain, and recovered after discontinuation of thalidomide. Subsequent thalidomide was readminstered without any adverse effects. None of these patients presented with symptoms and signs of sensory impairment during the follow-up period.

DISCUSSION

This study presents an experience with thalidomide for CD patients who have been treated for tuberculosis at a young age. And 60% of patients in our study achieved clinical remission after 9-12 mo of treatment and the measured parameters were significantly improved in most patients.

Previous studies have well described the efficacy of thalidomide in adult-onset CD patients[14-17]. Facchini *et al*[18] first reported five pediatric CD patients who were administered with thalidomide as refractory cases or the last medical resort before surgical intervention, 4 of them were in remission after 19-24 mo of treatment. In a long-term retrospective study, remission was achieved with thalidomide in 17 of 19 children and adolescent patients with CD and 80% of patients suspended

Table 2 Dose changes at different time points after thalidomide treatment

Patient No.	Dose (mg/kg per day) at different follow-up time (mo)
1	2.5 (baseline) - 2.5 (1 mo) - 1.2 (12 mo) - discontinue (34 mo)
2	2.5 (baseline) - discontinue (7 mo)
3	2 (baseline) - 1.1 (12 mo) - 0.7(18 mo) - 0.4 (21 mo) - 0.33 (33 mo)
4	1.8 (baseline) - 3 (5 mo) - 1 (12 mo) - 0.8 (15 mo)
5	2 (baseline) - 0.5 (2 mo) - 1.8(9 mo) - discontinue (22 mo) - 0.5 (36 mo)
6	1.2 (baseline) - 0.6 (12 mo)
7	2 (baseline) - 1.8 (1 mo) - 1.1 (10 mo) – stop (44 mo)
8	2 (baseline) – 2.4 (10 mo) - 1.6 (16 mo)
9	2.2 (baseline) -1.4 (5 mo) - 1.8 (9 mo)
10	1.8 (baseline) -1.3 (9 mo)

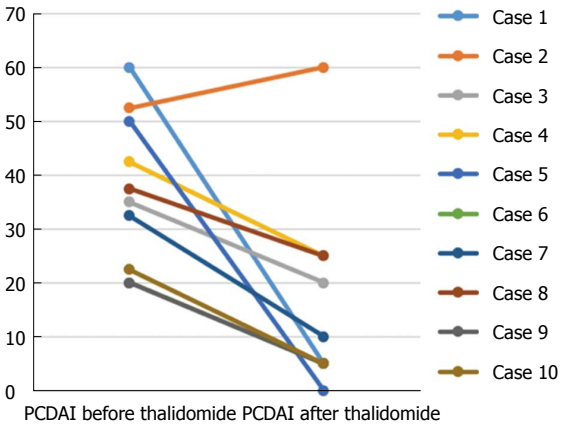

Figure 1 Changes in pediatric Crohn's disease activity index before and after 9-12 mo of thalidomide treatment in 10 patients. There was a decrease of the pediatric CD activity index (PCDAI) scores in 9 patients ($P < 0.05$), 6 of them achieved clinical remission (PCDAI < 10). PCDAI: Pediatric Crohn's disease activity index.

steroids successfully[19]. In a later retrospective series of 12 children with severe refractory CD who failed to respond to infliximab and adalimumab, a 83.3% clinical remission rate and a rate of 71.4% complete fistula closure after thalidomide treatment as a rescue therapy were achieved[20]. In our study, one case (case 4) also failed to respond to infliximab, but responded to thalidomide therapy. Recently, the first multicenter, double-blind randomized clinical trial provided more information concerning the efficacy and safety of thalidomide on active pediatric CD despite immunosuppressive treatment. In that study, 31 of 49 (63.3%) children achieved clinical remission and 65.3% achieved a 75% response rate after 1.5 to 2.5 mg/kg per day thalidomide treatment[21]. The 60% remission and 30% response rates in our study are comparable with those studies.

One of the most important features in the current study is that all patients had laboratory findings consistent with tuberculosis infection. Of note, we found evidence of AFB in 80% of patients, which indicated tuberculosis infection. Thus, it is reasonable that they were first treated with anti-TB medications, however, the treatment failed despite administration for more than one year. Given that CD and ITB have marked overlap in clinical, endoscopic and histologic features, CD diagnosis was then established due to the failure of ATT therapy, and the majority of our cases had perianal diseases which were more common in CD than ITB. While it is undeniable that the two conditions could coexist in countries with a high TB prevalence, one hypothesis suggests that *Mycobacterium avium* subspecies might be a cause of CD[22]. In 2011, a successful use of thalidomide under such circumstances in patients with CD and also tuberculosis infection was also reported[9]. In fact, thalidomide has been shown to be effective as an adjuvant treatment for intractable intracranial tuberculosis and central nervous system tuberculosis infection that did not respond to standard medical and surgical therapy[23-25]. It has been postulated that the mechanism of action of thalidomide is associated with inhibiting tumor necrosis factor-alpha secretion. It could also co-stimulate T lymphocytes and have a greater effect on CD8+ than CD4+ T cells since CD8+ T cells have a protective immunological effect in Mycobacterium tuberculosis infection[26]. Therefore, due to its positive role in tuberculosis infection, the application of thalidomide seems to be able to avoid the contraindication for the use of infliximab in patients with latent or active TB and reduce the damage from delaying treatment of CD.

As a sedative and antiemetic agent during pregnancy in the 1950s, thalidomide was withdrawn from the market due to potential teratogenicity. The most commonly encountered adverse effect of thalidomide treatment is the peripheral neuropathy which impedes its long-term use. Our experience showed that drowsiness was the most common adverse reaction to thalidomide. The relationship between peripheral neuropathy and cumulative dose has been investigated in a previous study which showed that 25% of patients complained of peripheral neuropathy and all received cumulative doses over 28 g[19]. In our cases, only one reached high cumulative dose during the follow-up period. The low-dose administration may be one of the

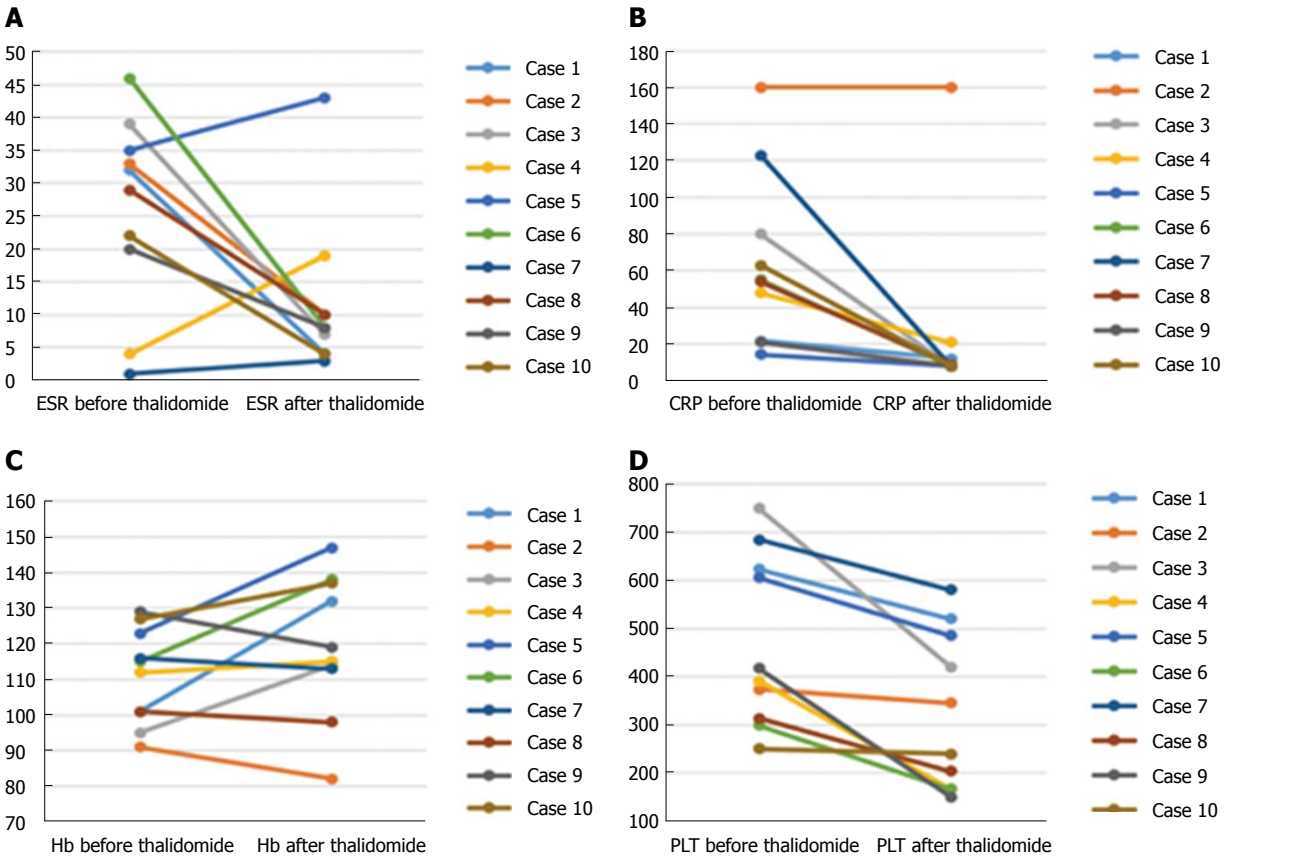

Figure 2 Changes of laboratory indices before and after 9-12 mo treatment in 10 Crohn's disease patients with tuberculosis. A: Erythrocyte sedimentation rate (ESR); B: C-reactive protein (CRP); C: Hemoglobin (Hb); D: Platelet (PLT). It showed significant reductions in ESR, CRP and platelet levels ($P < 0.05$); and an increasing trend in Hb levels.

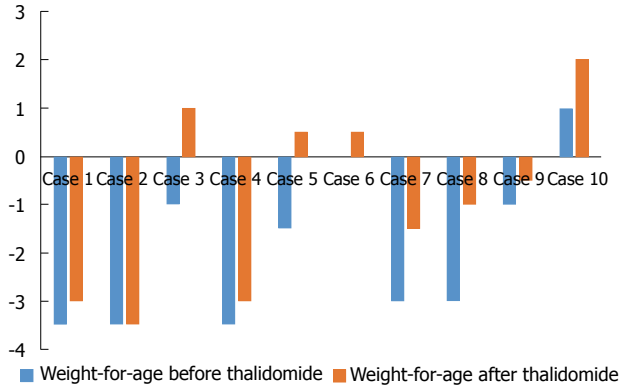

Figure 3 Changes in weight for age Z score before and after 9-12 mo treatment in 10 cases treated with thalidomide.

reasons for no obvious adverse events, or it could be due to the short-term follow-up in our study. However, it is noteworthy that one case with a cumulative dose over 28 g experienced disease relapse after thalidomide discontinuation and recovered soon after restarting thalidomide at a very low daily dose, being indicative of thalidomide-dependency. Clearly, this still needs further investigation with similar cases.

Our study had some limitations. There was no control group to compare the effect of other medications because the natural course of CD can achieve spontaneous remission and exacerbation. We selected the self-control study to reduce the bias. Although our study is the largest reported series treated with thalidomide in pediatric patients with CD who has been treated for tuberculosis, its retrospective design may create several limitations. A prospective study should be carried out in the future. We also did not perform *Mycobacterium* culture and electromyography because no patient presented with symptoms and signs of sensory impairment.

In conclusion, thalidomide is an effective and safe drug in inducing clinical remission and improving laboratory parameters for pediatric patients with CD who have been treated for tuberculosis infection. It can be used as an alternative drug after treatment of TB is completed. A controlled, long-term trial of thalidomide in patients with those conditions awaits further investigation.

COMMENTS

Background

Since Crohn's disease (CD) and tuberculosis (TB) could be present in the same patient, especially in high tuberculosis endemic areas. However, biological agents or immunosuppressive drugs, as effective medications for CD, may increase the risk of tuberculosis reactivation. Therefore, it is urgent to develop a

suitable therapy for those troublesome cases of CD with tuberculosis infection.

Research frontiers

Anti-TNF alpha treatment may increase the risk of tuberculosis reactivation. This makes it difficult to treat severe inflammatory bowel disease patients in high endemic area of tuberculosis. Thalidomide is reported to be effective in pediatric CD, and it is also safe for treatment of intracranial tuberculosis as an adjuvant therapy.

Innovations and breakthroughs

The authors describe a small series of 10 pediatric patients with evidence of tuberculosis. Following at least one year of anti-tubercular therapy, patients started on thalidomide due to persistence of gastrointestinal symptoms and endoscopic abnormalities consistent with CD. Clinical remission rate reached 60% after 9-12 mo of thalidomide treatment. Importantly, no exacerbation of TB was reported during a mean follow-up period of 22.2 mo. The results of the study may be relevant for clinicians dealing with CD in countries with high prevalence of tuberculosis.

Applications

This is a retrospective case series of 10 pediatric CD patients complicated with tuberculosis from one institution. In TB prevalent region where CD is also prevalent, the potential benefit using thalidomide provides a reasonable option.

Terminology

Anti-tubercular treatment refers to the use of response to the therapy to differentiate between tuberculosis and CD. PCDAI is the Pediatric CD Activity Index, a scale to assess the severity of the disease.

Peer-review

This study presented the retrospective experience with thalidomide to treat children with CD who had laboratory evidence of infection of Mycobacterium tuberculosis. The patient numbers included in this study are small. However, it provides useful information for the management of pediatric CD.

ACKNOWLEDGMENTS

We would like to express the gratitude to all the patients and their families for their participation and support of this study.

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P- Reviewer: Tommasini A, Zhang L, Lakatos PL **S- Editor**: Ma YJ
L- Editor: Ma JY **E- Editor**: Huang Y

World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7735-7745

DOI: 10.3748/wjg.v23.i43.7735

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

ORIGINAL ARTICLE

Retrospective Study

Combined endovascular brachytherapy, sorafenib, and transarterial chemobolization therapy for hepatocellular carcinoma patients with portal vein tumor thrombus

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Author contributions: Ma JQ, Luo JJ and Yan ZP were involved in study conception and design; Zhang ZH and Zhang W acquired the data; Liu QX, Wang JH, and Liu LX analyzed and interpreted the data; All authors were involved in the drafting and critical revision of the manuscript and approved the final version of the manuscript.

Supported by the Project of Advanced and Appropriate Technique Generalization of Shanghai Health and Family Planning Committee, No. 2013SY060; and the Scientific Program of Shanghai Municipal Heath Bureau, No. 20124188.

Institutional review board statement: The study protocol was reviewed and approved by the institutional ethics committee of the respective hospital involved.

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

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author, Dr. Jian-Jun Luo, at zsluojianjun@126.com.

Manuscript source: Unsolicited manuscript

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Received: August 17, 2017
Peer-review started: August 18, 2017
First decision: August 30, 2017
Revised: September 18, 2017
Accepted: October 17, 2017
Article in press: October 17, 2017
Published online: November 21, 2017

Abstract

AIM

To evaluate the safety and efficacy of combined endovascular brachytherapy (EVBT), transarterial chemoembolization (TACE), and sorafenib to treat hepatocellular carcinoma (HCC) patients with main portal vein tumor thrombus (MPVTT).

METHODS

This single-center retrospective study involved 68 patients with unresectable HCC or those who were unfit for liver transplantation and percutaneous frequency ablation according to the BCLC classification. All patients had Child-Pugh classification grade A or B, Eastern Cooperative Oncology Group (ECOG)

performance status of 0-2, and MPVTT. The patients received either EVBT with stent placement, TACE, and sorafenib (group A, *n* = 37), or TACE with sorafenib (group B, *n* = 31). The time to progression (TTP) and overall survival (OS) were evaluated by propensity score analysis.

RESULTS

In the entire cohort, the 6-, 12-, and 24-mo survival rates were 88.9%, 54.3%, and 14.1% in group A, and 45.8%, 0%, and 0% in group B, respectively ($P < 0.001$). The median TTP and OS were significantly longer in group A than group B (TTP: 9.0 mo *vs* 3.4 mo, $P < 0.001$; OS: 12.3 mo *vs* 5.2 mo, $P < 0.001$). In the propensity score-matched cohort, the median OS was longer in group A than in group B (10.3 mo *vs* 6.0 mo, $P < 0.001$). Similarly, the median TTP was longer in group A than in group B (9.0 mo *vs* 3.4 mo, $P < 0.001$). Multivariate Cox analysis revealed that the EVBT combined with stent placement, TACE, and sorafenib strategy was an independent predictor of favorable OS (HR = 0.18, $P < 0.001$).

CONCLUSION

EVBT combined with stent placement, TACE, and sorafenib might be a safe and effective palliative treatment option for MPVTT.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Endovascular brachytherapy; Main portal vein tumor thrombus; Sorafenib

Core tip: As portal vein tumor thrombus occurs in a high proportion of hepatocellular carcinoma patients and no standard treatment has been established, we aimed to evaluate the effect of endovascular brachytherapy (EVBT) combined with stent placement, transarterial chemoembolization (TACE), and sorafenib and compared this strategy with TACE plus sorafenib alone. The results of our study revealed that EVBT along with stent placement, TACE, and sorafenib is a safe and effective palliative treatment option for main portal vein tumor thrombus.

Zhang ZH, Liu QX, Zhang W, Ma JQ, Wang JH, Luo JJ, Liu LX, Yan ZP. Combined endovascular brachytherapy, sorafenib, and transarterial chemobolization therapy for hepatocellular carcinoma patients with portal vein tumor thrombus. *World J Gastroenterol* 2017; 23(43): 7735-7745 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7735.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7735

INTRODUCTION

Portal vein tumor thrombus (PVTT) occurs in a substantial proportion of patients with hepatocellular carcinoma (HCC), with 44% of the patients presenting at the time of death and about 10%-40% at the time of diagnosis[1]. In particular, HCC with tumor thrombus in the main portal trunk or the opposite side portal branch represents an end-stage condition with poor prognosis due to malignant hepatic tumor cells occluding the blood flow and deteriorating the portal hypertension[2], with a perioperative mortality rate of 0%-28% and a 5-year overall survival (OS) rate of 0%-26.4%[3]. As main PVTT (MPVTT) is contraindicated to surgical resection and transplantation due to a high tumor recurrence rate, no standard treatment has been established[4]. Three-dimensional conformal radiotherapy (3-DCRT) has shown survival benefits in HCC patients with PVTT[5]. However, blood flow to the obstructed main portal vein (MPV) cannot be restored immediately with radiotherapy alone. Further, PVTT is generally considered a contraindication for TACE due to the interruption of hepatic arterial flow which could result in a large segment of hepatic necrosis in patients whose blood supply is already compromised[6]. Furthermore, tumor thrombus in the MPV could not be effectively controlled by TACE combined with intra-portal stent, leading to a shorter stent patency rate along with increased risk of liver necrosis and treatment-related death[7]. Thus, the use of TACE is limited to only selected group of patients with good hepatic function and adequate collateral circulation around the occluded portal vein[8]. Given the limitation of TACE, transarterial radioembolization (brachytherapy) has emerged as a safer and more effective treatment for HCC with PVTT than TACE[9,10]. Endovascular brachytherapy (EVBT) by interstitial implantation of iodine-125 (^{125}I) seeds has been studied extensively[11-13]. Combined endovascular implantation of ^{125}I seed strand with stent placement and TACE provided long-term survival benefits and increased patency rates of the stent[14,15]. Although sorafenib has been recommended as the first-line treatment for advanced-stage disease (*i.e.*, Barcelona Clinic Liver Cancer (BCLC) stage C with PVTT), the survival outcomes obtained were also only modest[16]. Recent studies have reported survival benefits in patients with PVTT who underwent combination treatments of TACE with sorafenib, radiotherapy with sorafenib, and hepatic arterial infusion chemotherapy with sorafenib[17-19]. A recent study by Huang *et al*[20] reported survival benefit of chemoembolization plus ^{125}I seed implantation in unresectable hepatitis B-related HCC with PVTT. However, reports of such combined therapeutic strategies aiming at MPVTT are obscure. Endovascular implantation of ^{125}I seeds strand and portal vein stenting followed by TACE combined with sorafenib could improve the progression free survival (PFS) of HCC patients with MPVTT[21]. In the present retrospective study, we aimed to evaluate the safety and efficacy of EVBT combined with stent

placement, TACE, and sorafenib compared with TACE with sorafenib in the treatment of HCC patients with MPVTT.

MATERIALS AND METHODS

Study design

This was a single-center, retrospective study conducted on advanced HCC patients with MPVTT from January 2009 and December 2015. The study protocol was approved by the institutional ethics committee of the respective hospital involved. MPVTT was detected based on the presence of low-attenuation intraluminal mass expanding the main portal vein, and/or filling defects in the main portal vein, as determined by three-phase dynamic computed tomography (Figure 1).

Patient selection and grouping

Patients aged between 18-75 years with unresectable HCC or unfit for liver transplantation and percutaneous frequency ablation according to the BCLC classification were included. All patients had Child-Pugh classification grade A or B, Eastern Cooperative Oncology Group (ECOG) performance status of 0-2, and MPVTT confirmed by demonstration of tumor thrombus in MPV and HCC in three-phase dynamic CT images 7 d before treatment.

Patients who had undergone surgery, local-regional therapies (radiofrequency ablation, percutaneous ethanol injection, or -^{125}I seed implantation), liver transplantation, previous sorafenib therapy, systemic chemotherapy, intra-arterial chemoinfusion, or TACE; patients with serious medical comorbidities such as encephalopathy and uncorrectable bleeding diathesis; patients who currently had or had a history of malignant tumors in addition to HCC; and those with intrahepatic portal vein completely occluded by HCC, tumor thrombus extending into the superior mesenteric vein (SMV) or splenic vein (SV), advanced liver disease, or contraindication for chemoembolization (HCC burden > 70% of total liver volume or high flow intrahepatic arterial venous shunt) were excluded from the study.

Written informed consent was obtained from all eligible patients who were recommended to choose either combined EVBT-stenting-TACE-sorafenib (group A) or TACE-sorafenib (group B) treatment. TACE-sorafenib was recommended for patients who refused EVBT-stenting-TACE-sorafenib treatment.

Treatment procedures

Sorafenib treatment: Sorafenib (Nexavar; Bayer, Leverkusen, Germany) was taken for 3 d after the first TACE procedure at a recommended dose of 400 mg twice daily in all patients and with a 3-d interruption after subsequent TACE cycles (30 d).

Stent and iodine-125 seed: Nitinol self-expandable stent (Luminxx Ⅲ; Bard, Covington, Georgia; diameter: 12-14 mm; length: 60-100 mm) was used. Brachytherapy source was titanium encapsulated model 6711 ^{125}I seed (XinKe; Shanghai, China; active length: 3.25 mm), with radioactivity and half-life of each ^{125}I seed of 25.9 MBq and 59.4 d, respectively. The principal photon emissions of X-ray and gamma ray and incipient dose rate were 31.4 keV, 35.5 keV, and 7 cGy/h, respectively. The 240-d accumulated dose at 10 mm from the axis of the ^{125}I seed strand source ($Z = 0$, $r = 10$ mm) was calculated with radiation field distribution calculation software (version 0.1, Institute of Radiation Medicine, Fudan University, Shanghai, China) based on the American Association of Physicists in Medicine TG43U1 brachytherapy formalism.

Intra-MPV stent and ^{125}I seed strand implantation: In group A patients, the patent second-order branch of the intrahepatic portal vein in the unaffected side with hepatopetal flow was punctured with a 22-gauge Chiba needle (Cook, Inc., Bloomington, Indiana) under ultrasound guidance, followed by insertion of a 0.018-inch wire (Cook Inc.) into the portal vein. A 5-F calibrated pigtail catheter (Cook Inc.) was used to gauge the pressure in filling splenic mesenteric veins and portography was performed to measure the diameter and length of the obstructed MPV (stenosis). The number of ^{125}I seeds to be implanted was calculated by the following formula: N = Length of obstructed MPV (mm)/4.5 + 4.

These seeds were arranged linearly and sealed into a 4-F catheter continuously to construct a ^{125}I seed strand. After 50 U/kg heparin (XinYi, Shanghai, China) was administered intravenously, two 0.035-inch, 260-cm-long stiff wires (Terumo) were inserted into the superior mesenteric vein through the 7-F sheath. After the sheath had been removed, the outer cannula of the NEFF set and a self-expendable stent of appropriate size were introduced to the MPV over one of the stiff wires, respectively. The stent was deployed from the distal MPV into the proximal patent intrahepatic portal vein. The ^{125}I seed strand was delivered to the target position *via* outer cannula of the NEFF set and released between the stent and MPV (Figure 2A and B). Portography and pressure measurement were repeated.

TACE procedure

Segmental TACE was performed by experienced interventional radiologists immediately after stent and ^{125}I seed implantation. Regardless of the type of HCC (unilobar or bilobar), all feeding arteries of tumor identified by angiography of the celiac, hepatic, superior mesenteric, left gastric, and bilateral inferior phrenic arteries were chemoembolized using a 5-F RH catheter (Cook). The target artery was catheterized with a 2.7-F microcatheter (Renegade, Boston

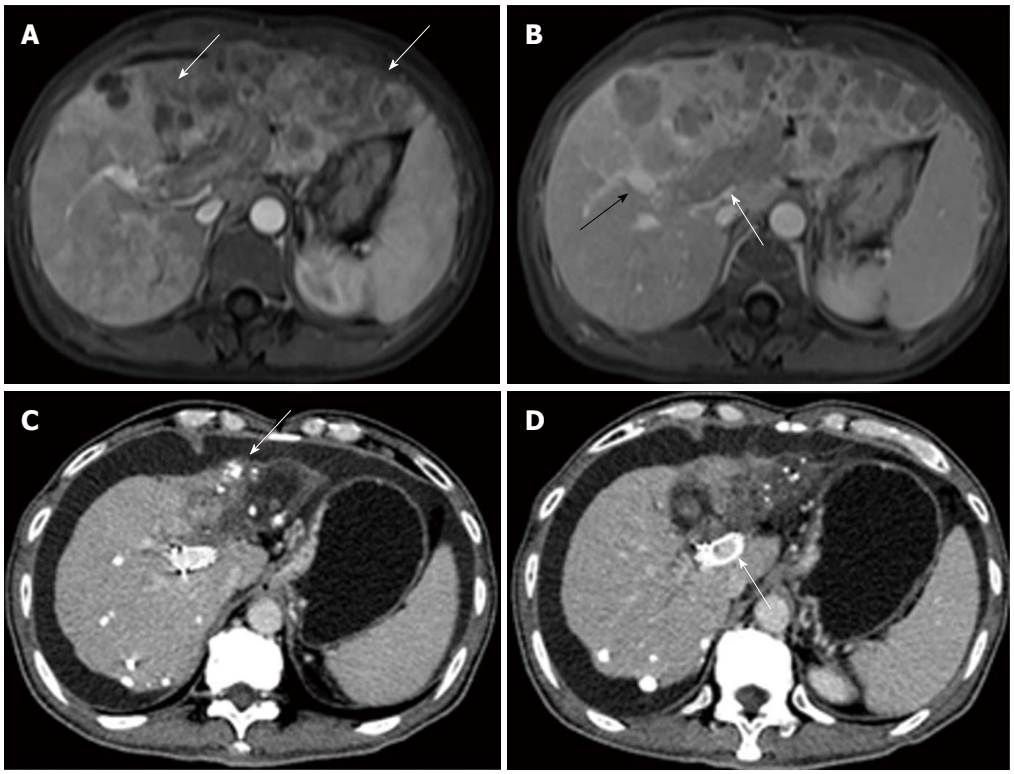

Figure 1 Images from a 50-year-old man who had hepatocellular carcinoma with main portal vein tumor thrombus. A: Contrast-enhanced abdominal magnetic resonance imaging before therapy. Diffuse hepatocellular carcinoma (white arrow) was detected in the left lobe. B: The second order of the right portal vein (black arrow) was patent. Tumor thrombus (white arrow) was observed in the left portal vein, the first order of the right portal vein, and the main portal vein. C: Contrast-enhanced abdominal CT image 24 mo after first therapy. Deposition of iodized oil (white arrow) within tumor was observed and the left lobe was atrophied. D: The stent was still patent (white arrow).

Scientific, Natick, MA). Under fluorescence imaging, a mixture of 10-50 mg/m^2 of epirubicin (Pharmorubicin, Pfizer, NY) and 5-20 mL of iodized oil (Lipiodol Ultrafluide, Laboratoire Guerbet, Aul-nay-sous-Bois, France) was infused at a rate of 0.5-1 mL/min through the microcatheter until stasis flow in the tumor vascularity was achieved. Finally, gelatin sponge (Jingling, Jiangsu, China) was used to embolize the feeding artery of the tumor.

Post-procedural evaluation

Single photon emission computed tomography (SPECT) combined with CT (SPECT/CT) scan was performed on day 1 of the therapy to evaluate the distribution of radiation by the ^{125}I seed strand implanted in group A patients (Figure 2C).

Follow-up and repeat TACE

The total number of hospitalization days was 5-7 d, which were prolonged if grade 3-4 adverse events occurred. All the patients were followed every 30 d until death or till March 1, 2016. Repeat TACE with the same protocol was performed upon detection of residual tumors or new lesions in all patients.

Efficacy and safety endpoints

Efficacy was evaluated *as per* the Modified Response Evaluation Criteria in Solid Tumor (mRECIST)[22]. The primary endpoints were OS and time to progression (TTP). OS was defined as the period from the day of the procedure to patients' death or to their last follow-up. TTP was defined as the period from the day of the procedure until the radiologic confirmation of tumor progression in liver parenchyma. For HCC in liver parenchyma, disease control rate (DCR) was defined as the percentage of patients with complete response (CR), partial response (PR), or stable disease (SD). Tolerance and AEs were measured as secondary endpoints. Sorafenib- and TACE-related AEs were monitored using the Common Terminology Criteria for Adverse Events (CTCAE) v.4.0[23].

As MPVTT would increase the incidence of tumor dissemination, elevate portal vein pressure, and impair the liver functional reserve, occurrence of events like intrahepatic HCC spread, variceal bleeding, and liver function decompensation was also compared between the two groups.

Statistical analysis

SPSS version 22.0 (SPSS, Chicago, Illinois) was used for all the analyses. Continuous variables are presented as mean ± SD and were compared by *t* test. Categorical variables are expressed as frequencies and were compared by χ^2 test. OS and

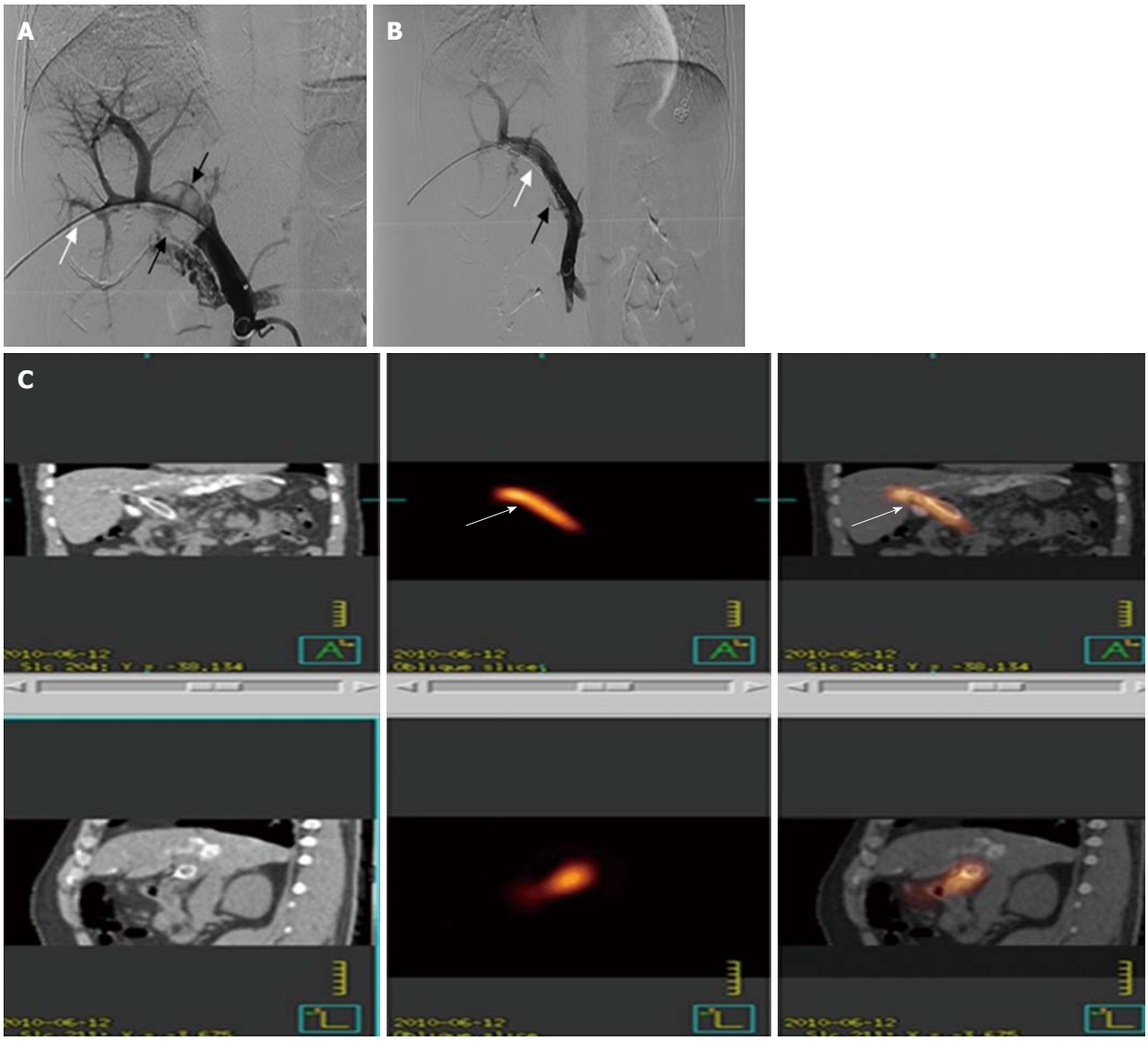

Figure 2 Images of iodine-125 seed strand and stent placement. A: After the patent second-order branch of the left portal vein was catheterized, a 5-F calibrated pigtail catheter (white arrow) was placed in the splenic vein. Tumor thrombus (black arrows) in the proximal MPV and sagittal segment of right portal vein was shown clearly on portography, but the right portal vein did not develop. B: A 14-mm x 80-mm self-expandable stent (black arrow) and ^{125}I seed strand (white arrow) with 20 seeds loaded were placed precisely in the obstructed MPV. C: Images of SPECT/CT scan 1 d after therapy. MPV: Main portal vein.

disease-free survival were analyzed using the Kaplan-Meier curves and log-rank test. A *P*-value of less than 0.05 was considered statistically significant. Variables with $P < 0.05$ were chosen for multivariate analysis using the Cox proportional hazards model. Regression analysis was used to determine independent predictors of survival. Sex, age, type of tumor, HCC maximum diameter, degree of MPVTT, Child-Pug class, ECOG performance status, etiology of liver disease, serum alfa-fetoprotein level, and extrahepatic metastasis were considered within the propensity model. Propensity score matching analysis was performed, with a matching ratio of 1:1 for two groups, using the nearest-neighbor matching method with a caliper distance of 0.2 without replacement.

RESULTS

Baseline characteristics of patients and tumors

A total of 83 unresectable HCC patients were identified. Of them, 15 patients were excluded due to reasons listed in Figure 3. Finally, 68 patients were included in this study (group A, $n = 37$; group B, $n = 31$). Baseline characteristics before score matching are shown in Table 1. After propensity score matching, we created 24 matched pairs of patients in Table 2.

Technical success

The ^{125}I seeds strand and stent placement procedure was completed in all patients in group A (technical success rate, 100%) and the TACE procedure was

Table 1 Baseline characteristics of overall patients *n* (%)

Characteristic	Group A (*n* = 37)	Group B (*n* = 31)
Sex		
Male	34 (91.9)	26 (83.9)
Female	3 (8.1)	5 (16.1)
Age, yr (%)		
⩾ 55 yr	18 (48.9)	14 (45.2)
< 55 yr	19 (51.4)	17 (54.8)
Type of tumor		
Nodular	30 (81.1)	29 (93.5)
Infiltrative	7 (18.9)	2 (6.5)
HCC maximum diameter		
⩾ 5 cm	26 (70.3)	19 (61.3)
< 5 cm	11 (29.7)	12 (38.7)
Degree of MPVTT		
Stenosis	31 (83.8)	24 (77.4)
Occlusive	6 (16.2)	7 (22.6)
Child-pugh class		
A	33 (89.2)	24 (77.4)
B	4 (10.8)	7 (22.6)
ECOG performance status		
0/1	33 (89.2)	26 (83.9)
2	4 (10.8)	5 (16.1)
Etiology of liver disease		
HBV	35 (94.6)	29 (93.5)
Other	2 (5.4)	2 (6.5)
Serum AFP level (in ng/mL)		
⩾ 400	20 (54.1)	17 (54.8)
< 400	17 (45.9)	14 (45.2)
Extrahepatic metastasis	3 (8.1)	3 (9.7)

AFP: Alfa-fetoprotein; ECOG: Eastern cooperative oncology group; HBV: Hepatitis B virus; MPVTT: Main portal vein tumor thrombus; HCC: Hepatocellular carcinoma.

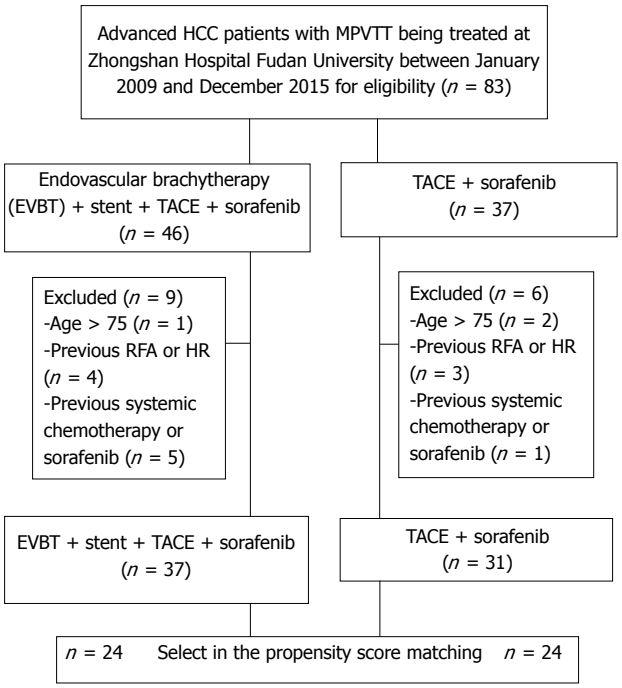

Figure 3 Patient selection and cohorting. MPVTT: Main portal vein tumor thrombus; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; EVBT: Endovascular brachytherapy.

Table 2 Baseline characteristics of propensity-matched patients *n* (%)

Characteristic	Group A (*n* = 24)	Group B (*n* = 24)
Sex		
Male	22 (91.7)	23 (95.8)
Female	2 (8.3)	1 (4.2)
Age		
⩾ 55 yr	14 (58.3)	9 (37.5)
< 55 yr	10 (41.7)	15 (62.5)
Type of tumor		
Nodular	21 (87.5)	22 (91.7)
Infiltrative	3 (12.5)	2 (8.3)
HCC maximum diameter		
⩾ 5 cm	16 (66.7)	17 (70.8)
< 5 cm	8 (33.3)	7 (29.2)
Degree of MPVTT		
Stenosis	21 (87.5)	19 (79.2)
Occlusive	3 (12.5)	5 (20.8)
Child-pugh class		
A	20 (83.3)	21 (87.5)
B	4 (16.7)	3 (12.5)
ECOG performance status		
0/1	20(83.3)	22(91.7)
2	4(16.7)	2(8.3)
Etiology of liver disease		
HBV	22 (91.7)	23 (87.5)
Other	2 (8.3)	1 (12.5)
Serum AFP level		
⩾ 400	12 (50.0)	10 (41.7)
< 400	12 (50.0)	14 (58.3)
Extrahepatic metastasis	1 (3.8)	3 (11.5)

AFP: Alfa-fetoprotein; ECOG: Eastern cooperative oncology group; HBV: Hepatitis B virus; MPVTT: Main portal vein tumor thrombus; HCC: Hepatocellular carcinoma.

completed in all patients in both groups.

OS and TTP in the entire cohort

The median follow-up durations were 12.7 mo and 5.9 mo for the prematched groups A and B, respectively. The 6-, 12-, and 24-mo survival rates were 88.9%, 54.3%, and 14.1% in group A, and 45.8%, 0%, and 0% in group B, respectively ($P < 0.001$). However, there were no procedure-related deaths. Kaplan-Meier curves for survival outcomes in the two groups showed significantly higher OS in group A compared to group B (12.3 mo *vs* 5.2 mo; $P < 0.001$; Figure 4A).

The median TTP was longer (9.0 mo) in group A, compared to group B (3.4 mo) ($P < 0.001$; Figure 4B).

OS and TTP in the matched cohort

In the propensity score-matched cohorts, the median OS was longer in group A than in group B (10.3 mo *vs* 6.0 mo; $P < 0.001$; Figure 4C). Similarly, the median TTP was significantly longer in group A than in group B (9.0 mo *vs* 3.4 mo; $P < 0.001$; Figure 4D).

Predictive factors for OS in the entire cohort

In multivariate Cox analysis, treatment regimen (HR

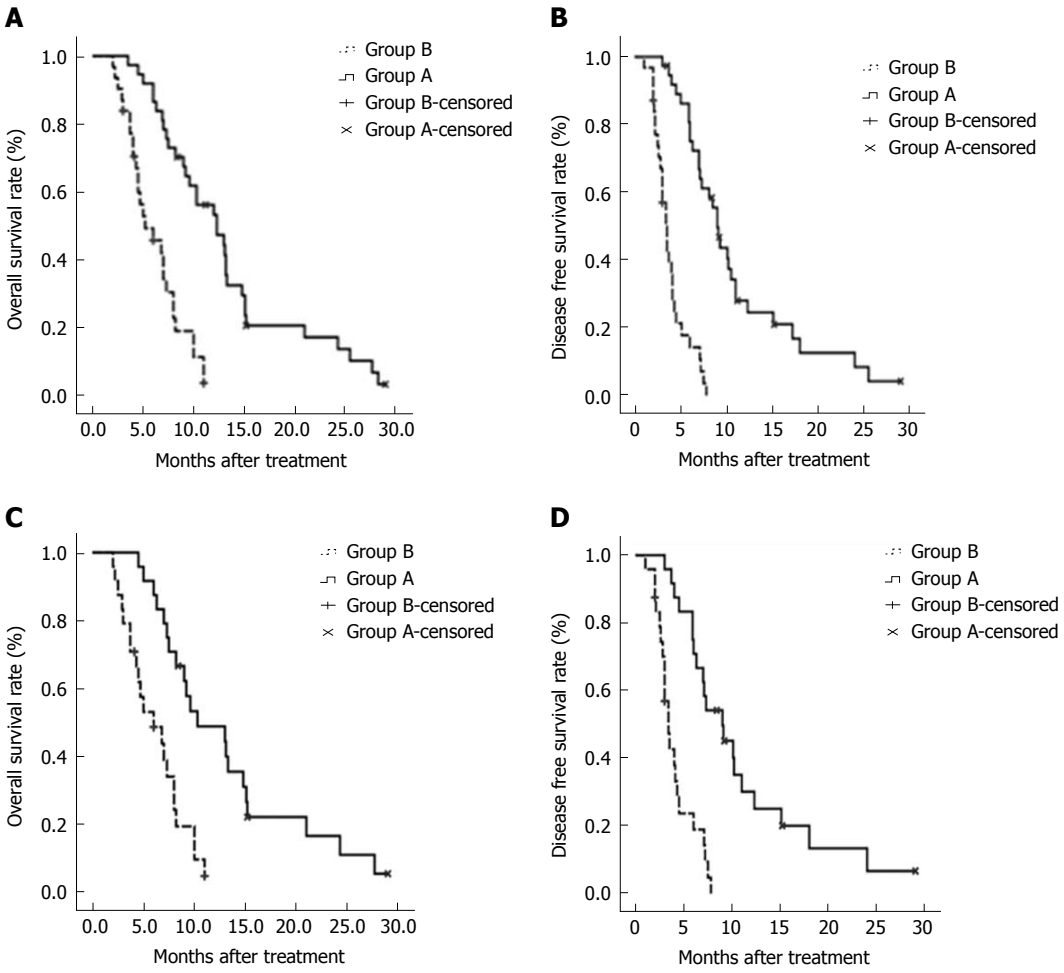

Figure 4 Overall survival of the overall cohort and matched cohort. A: Kaplan-Meier curves for the overall patient cohort. OS differed between the two groups (OS, 12.3 *vs* 5.2 mo; $P < 0.001$); B: Kaplan-Meier curves for disease free survival in the overall patient cohort. C: Kaplan-Meier curves for OS in propensity score-matched patiens. Median OS was longer in group A than in group B (10.3 *vs* 6.0 mo; $P < 0.001$); D: Kaplan-Meier curves for disease free survival in propensity score-matched patients. OS: Overall survival.

= 0.18, $P < 0.001$) was identified as an independent predictor of OS for group A *vs* group B (Table 3).

Iodine-125 seed dose and stent patency

After stent placement in group A patients who were implanted with 17.2 ± 4.9 (range, 10-31) ^{125}I seeds, the mean pressure of MPV dropped from 38.1 ± 5.8 cm H_2O (range, 23-46 cm H_2O) to 32.0 ± 5.6 cm H_2O (range, 16-37 cm H_2O) ($P = 0.002$). The estimated mean accumulated dose (R = 10 mm, z = 0, 240 d) was 62.9 ± 2.3 Gy (range, 57.4-65.3 Gy). Stent occlusion was observed in 9 (24.3%) patients and the median stent patency period was 22.1 ± 6.1 mo (95%CI: 9.5-34.7 mo).

Response of HCC and MPVTT

HCC response was assessed using the mRECIST criteria. During the course of the study, 4.4 ± 3.2 and 2.9 ± 1.2 TACE procedures were performed in groups A and B, respectively. The ORR and DCR in group A were significantly higher than the rates observed in group B (ORR: 45.9% *vs* 16.1%, $P = 0.009$; DCR: 67.6% *vs* 29.0%, $P = 0.002$).

During the course of the study, the occurrence rate of complications related to MPVTT, such as intrahepatic metastasis, variceal bleeding, and liver function decompensation, were observed in 15 (40.5%), 6 (16.2%), and 11 (29.7%) patients in group A and 22 (71.0%), 18 (58.1%), and 25 (80.6%) patients in group B, respectively ($P = 0.012$, $P < 0.001$, and $P < 0.001$, respectively).

Treatment-related toxicities

A total of 49 TACE-related AEs occurred in the two groups. The percentages of patients who experienced new ascites, liver dysfunction, and gastrointestinal hemorrhage were significantly higher in group B than in group A.

A total of 140 sorafenib related AEs occurred in 91.2% of patients. Seven patients required a sorafenib dose reduction to 400 mg once daily for grade 3 hand-foot skin reactions (4.2%) and grade 3 diarrhea (5.3%) and resumed a regular dose after the AEs subsided. One patient with grade 4 hypertension was subjected

Table 3 Log-rank test and cox regression analysis of factors potentially related to overall survival

	Group A *vs* Group B			
	Log-rank test		Cox regression	
Factors	*n*	*P*	HR (95%CI)	*P*
Treatment regimen	-	< 0.001	-	-
EVBT-stenting-TACE-sorafenib	37		0.18 (0.09-0.35)	< 0.001
TACE-sorafenib	31		1	-
Type of tumor		0.466	-	-
Nodular type	59		-	-
Diffuse type	9		-	-
HCC maximum diameter	-	0.320	-	-
⩾ 5 cm	45		-	-
< 5 cm	23		-	-
Child-pugh class		0.298	-	-
A	57		-	-
B	11		-	-
ECOG performance status	-	0.125	-	-
0 and 1	59		-	-
2	9		-	-
Extrahepatic metastasis	-	0.742	-	-
Yes	62		-	-
No	6		-	-
Serum AFP level (ng/mL)	-	0.586	-	-
⩾ 400	37		-	-
< 400	31		-	-

AFP: Alfa-fetoprotein; ECOG: Eastern cooperative oncology group; TACE: Transarterial chemoembolization.

to a drug interruption period of 20 d until the AEs subsided (Table 4).

DISCUSSION

MPVTT is the most important independent predictive factor for poor prognosis of patients with HCC[24]. Although TACE is effective and safe for intrahepatic primary HCC lesions including few cases of HCC-cholangiocellular carcinoma, its benefit in PVTT has less importance, especially in type Ⅲ PVTT or MPVTT. A combined treatment of TACE with novel drugs or other therapies might be a better alternative strategy for HCC with PVTT[22-25]. Huang *et al*[20] reported better survival outcomes with TACE plus ^{125}I-seed implantation than with TACE alone in patients with type Ⅰ and Ⅱ PVTT. Currently, sorafenib is the recommended standard treatment for advanced HCC with PVTT[26]. Novi *et al*[27] reported that 15 wk of sorafenib monotherapy played a key role in PVTT revascularization. TACE combined with sorafenib could be a feasible alternative treatment option in patients with HCC and PVTT in the first-order or lower order portal vein branches but not in MPVTT[12]. However, a propensity-score analysis reported a significantly shorter OS in the sorafenib group than in the radiotherapy group[28].

According to these reports, the benefits of OS and TTP were lower in patients with MPVTT than in patients with PVTT of first-order or lower order portal vein branches. The main reason is that the occlusion of MPV is associated with an increased risk of tumor spread, elevated portal venous pressure causing variceal hemorrhage, and decreased portal flow resulting in ascites, jaundice, hepatic encephalopathy, and liver failure[1]. Restoring the flow of obstructed MPV and effectively inhibiting tumor thrombus progression might confer further survival benefit to patients with advanced HCC and MPVTT[13,21].

The strategy of implantation of ^{125}I seed strand combined with stent placement and TACE has been reported to treat MPVTT[14,15]. This method of treatment has two advantages. On one hand, the blood flow to the portal vein is increased immediately and the portal vein pressure elevated by MPV obstruction is reduced effectively after stent deployment. One the other hand, the half-life of the gamma ray emitted by ^{125}I seeds is 59.4 d. Sustained radiation can inhibit tumor cell growth by inducing apoptosis. Therefore, it is rational to hypothesize that placing a stent to restore the blood flow of obstructed MPV and implantation of ^{125}I seeds might inhibit the progression of tumor thrombus. This increases the safety of subsequent TACE because of previous concerns that hepatic arterial flow interruption in TACE procedure would result in serious liver necrosis in patients whose hepatic blood supply has been already compromised[29].

Previously, combined brachytherapy with TACE and sorafenib showed greater OS compared to combined brachytherapy with TACE alone in HCC patients with MPVTT[30]. The success rate of TACE with stent placement and ^{125}I implantation was 88.5%, with a mOS and mTTP of 8.9 mo and 7.9 mo, respectively, both of which were higher than mOS (5.7 mo) and mTTP (5.3 mo) associated with TACE with portal vein stenting alone[6]. Further, in our study addition of sorafenib to the TACE plus portal vein stenting and

Table 4 Adverse events related to sorafenib administration and transarterial chemoembolization in the two groups *n* (%)

Adverse event	Group A (*n* = 37)	Group A (*n* = 31)	*P*
Sorafenib related AEs			
Hand-foot skin reaction			
Grade 1-2	26 (92.9)	27 (96.4)	0.143
Grade 3-4	2 (7.1)	1 (3.6)	0.593
Diarrhea			
Grade 1-2	23 (88.5)	18 (94.7)	0.994
Grade 3-4	3 (11.5)	1 (3.6)	0.620
Hypertension			
Grade 1-2	8 (21.6)	6 (19.4)	0.818
Grade 3-4	1 (3.2)	0	1.000
Alopecia			
Grade 1-2	2 (7.1)	4 (12.9)	0.400
Grade 3-4	0	0	
Fatigue			
Grade 1-2	9 (24.3)	4 (12.9)	0.354
Grade 3-4	0	0	
Voice change			
Grade 1-2	0	1 (3.6)	464
Grade 3-4	0	0	
Epistaxis			
Grade 1-2	2 (7.1)	2 (6.5)	1.000
Grade 3-4	0	0	
TACE related AEs			
New ascites	4 (10.8)	11 (35.5)	0.020
Liver dysfunction	2 (16.2)	15 (48.4)	< 0.000
Gastrointestinal hemorrhage	0	8 (25.8)	0.001
Hepatorenal syndrome	0	2 (6.5)	0.204
Liver abscess	0	0	-
Spontaneous bacterial peritonitis	0	0	-
Inguinal hematoma	0	0	-
Pulmonary/cerebral oil embolization	0	0	-

TACE: Transarterial chemoembolization.

^{125}I implantation increased the OS and TTP to 10.3 mo and 9.0 mo, respectively. This study reported encouraging efficacy for the combination of sorafenib, EVBT, stent placement, and TACE in advanced HCC patients with MPVTT. The OS and TTP were longer in the EVBT-stenting-TACE-sorafenib group than in the TACE-sorafenib group. Although our results show moderate sorafenib-related side effects, they were mostly manageable after TACE and were comparable among the groups. However, TACE-related toxicities were lower in the combination group compared to the sorafenib plus TACE group. The data also demonstrated that the combination therapy has significant benefit in term of ORR and DCR compared with sorafenib-TACE. Zhang *et al*[31] reported that sorafenib monotherapy is a better treatment strategy over sorafenib plus TACE therapy for MPVTT due to the adverse events related to TACE. However, the results of our study suggest that combining EVBT with the sorafenib-TACE combination offers added benefits to sorafenib and decreases the toxicities of TACE. After overall analysis of all the side effects observed in the sorafenib-TACE group, we believe that combined EVBT with sorafenib and TACE may be a better approach for managing this specific subgroup of patients with advanced HCC and MPVTT.

The major limitations of this study are the single-center retrospective design, which may affect the generalization of results, and small sample size. Further, cost-benefit analysis was not performed for the expensive procedures in this study, which may be a topic of interest to be covered in our future studies.

In conclusion, EVBT combined with sorafenib and TACE might be a safe and effective palliative treatment option for MPVTT.

ARTICLE HIGHLIGHTS

Research background

Despite the beneficial outcomes of individual therapies, studies pertaining to the clinical outcome of endovascular brachytherapy (EVBT) combined with stent placement, transarterial chemoembolization (TACE), and sorafenib to treat hepatocellular carcinoma (HCC) with main portal vein tumor thrombus (MPVTT) are scarce.

Research motivation

Recent studies have reported survival benefits in patients with PVTT who underwent combined treatments of TACE with sorafenib, radiotherapy with sorafenib, hepatic arterial infusion chemotherapy with sorafenib, and iodine-125 seed implantation with TACE. However, reports of such combined therapeutic strategies aiming at MPVTT are obscure. According to these previous studies, we aimed to find an effective therapy for HCC patients with MPVTT.

Research objectives
To evaluate the safety and efficacy of combined EVBT, stent placement, TACE, and sorafenib to treat HCC with MPVTT.

Research methods
We conducted this retrospective study involving 68 patients with unresectable HCC. The patients received either EVBT with stent placement, TACE, and sorafenib or TACE with sorafenib. The time to progression (TTP) and overall survival (OS) were evaluated by propensity score analysis.

Research results
In the EVBT with stent placement, TACE, and sorafenib group, the 6-, 12-, and 24-mo survival rates were 88.9%, 54.3%, and 14.1%, respectively, and in the TACE with sorafenib group, they were 45.8%, 0%, and 0%, respectively. The median TTP and OS were significantly longer in the EVBT with stent placement, TACE, and sorafenib group ($P < 0.001$). In the propensity score-matched cohort, the median OS was longer in the EVBT with stent placement, TACE, and sorafenib group ($P < 0.001$).

Research conclusions
EVBT combined with stent placement, TACE, and sorafenib might be a safe and effective palliative treatment option for MPVTT.

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P- Reviewer: El-Bendary MM, Mizuguchi T, Ohkohchi N, Sergi CM **S- Editor**: Wei LJ **L- Editor**: Wang TQ **E- Editor**: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7746-7755

DOI: 10.3748/wjg.v23.i43.7746

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

ORIGINAL ARTICLE

Retrospective Study

Procedure-related complications in gastric variceal obturation with tissue glue

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Author contributions: Guo YW and Miao HB contributed equally to this work; Guo YW designed the study; Guo YW, Miao HB, Wen ZF, Xuan JY and Zhou HX performed the research; Guo YW and Miao HB analyzed the data and wrote the paper.

Supported by Guangdong Science and Technology Program, No. 2016A020216012.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the Third Affiliated Hospital of Sun Yat-Sen University.

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

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

Manuscript source: Unsolicited manuscript

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Received: July 21, 2017
Peer-review started: July 24, 2017
First decision: August 30, 2017
Revised: September 12, 2017
Accepted: October 28, 2017
Article in press: October 28, 2017
Published online: November 21, 2017

Abstract

AIM

To focus on procedure-related complications, evaluate their incidence, analyze the reasons and discuss the solutions.

METHODS

Overall, 628 endoscopic gastric variceal obturation (EGVO) procedures (case-times) with NBC were performed in 519 patients in the Department of Endoscopy of the Third Affiliated Hospital of Sun Yat-Sen University from January 2011 to December 2016. The clinical data of patients and procedure-related complications of EGVO were retrospectively analyzed.

RESULTS

In the 628 EGVO procedures, sticking of the needle to the varix occurred in 9 cases (1.43%), including 1 case that used lipiodol-diluted NBC and 8 cases that used undiluted NBC (P = 0.000). The needle was successfully withdrawn in 8 cases. Large spurt bleeding occurred in one case, and hemostasis was achieved by two other injections of undiluted glue. The injection catheter became blocked in 17 cases (2.71%) just during the injection, and 4 cases were complicated with the needle sticking to the varix. Large glue adhesion to the endoscope resulted in difficulty

withdrawing the endoscope in 1 case. Bleeding from multiple sites was observed in the esophagus and gastric cardia after the endoscope was withdrawn. Hemostasis was achieved by 1% aethoxysklerol injection and intravenous somatostatin. The ligation device stuck to the varices in two cases during the subsequent endoscopic variceal ligation. In one case, the ligation device was successfully separated from the esophageal varix after all bands were released. In another case, a laceration of the vein and massive bleeding were observed. The bleeding ceased after 1% aethoxysklerol injection.

CONCLUSION

Although EGVO with tissue glue is usually safe and effective, a series of complications can occur during the procedure that may puzzle endoscopists. There is no standard operating procedure for addressing these complications. The cases described in the current study can provide some reference for others.

Key words: Endoscopic gastric variceal obturation; Tissue glue; N-butyl-2-cyanoacrylate; Complications

Core tip: Tissue glue has been widely used in endoscopic gastric variceal obturation (EGVO) but there is little discussion on procedure-related complications. In our study, the procedure-related complications in 628 EGVO procedures with tissue glue were retrospectively analyzed. These complications include sticking of the needle to the varix, glue adhesion to the endoscope, blockage of the catheter, and sticking of the ligation device to the esophageal varices in the subsequent endoscopic variceal ligation. Although these complications were rare, they may be fatal and always puzzle the endoscopists. Besides incidence, how to tackle and prevent these complications were discussed in the current study.

Guo YE, Miao HB, Wen ZF, Xuan JY, Zhou HX. Procedure-related complications in gastric variceal obturation with tissue glue. *World J Gastroenterol* 2017; 23(43): 7746-7755 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7746.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7746

INTRODUCTION

Esophageal varices (EV) and gastric varices (GV) are pathological portosystemic shunts that often occur in patients with portal hypertension (PH). Compared with esophageal variceal hemorrhage, gastric variceal bleeding is more severe and has a higher mortality rate. Endoscopic gastric variceal obturation (EGVO) with the intravariceal injection of tissue glue has become the first choice for the treatment of gastric variceal hemorrhage and is one of the most important therapies in the treatment of GV in many countries, including China[1-11].

Although EGVO is usually safe and effective, a series of complications can occur, some of which are fatal. Post-endoscopic treatment complications such as abdominal pain, pyrexia, organ embolization and local ulceration have been widely reported and discussed, but the incidence of procedure-related complications was calculated in only some studies and was not thoroughly analyzed[12-16]. Most of the procedure-related complications were only described as case reports. These complications include sticking of the needle to the varix, glue adhesion to the endoscope resulting in difficulty withdrawing the endoscope, blockage of the catheter during the injection, and more seriously, sticking of the ligation device to the esophageal varices in the subsequent endoscopic variceal ligation (EVL). Although these complications were rare, they may be fatal and always puzzle operators, especially those in training. In the current study, we focus on procedure-related complications, evaluate their incidence, analyze the reasons and discuss the solutions.

MATERIALS AND METHODS

Patients

Overall, 628 EGVO procedures were performed in 519 patients in the Department of Endoscopy of the Third Affiliated Hospital of Sun Yat-Sen University from January 2011 to December 2016. The 519 patients underwent at least one EGVO. A total of 82 patients underwent repeated EGVO 1-3 times in the subsequent sequential endoscopic therapy for esophageal and gastric varices. The clinical data of patients and procedure-related complications of EGVO were retrospectively analyzed. The study was reviewed and approved by the Institutional Review Board of the Third Affiliated Hospital of Sun Yat-Sen University.

Sequence endoscopic treatment for esophageal and gastric varices

In our Department of endoscopy, patients with esophageal and gastric varices, most of whom have a history of variceal hemorrhage, undergo a sequence of endoscopic treatments unless there were endoscopic contraindications or more suitable for transjugular intrahepatic porto-system stent shunt (TIPSS) and surgery, including EGVO approximately 1-2 times and EVL 3-5 times, sometimes followed by endoscopic variceal sclerotherapy 1-2 times on smaller esophageal varices that were not suitable for EVL. The interval between the two procedures was approximately 4 wk until the varices were eradicated or considerably alleviated. We performed the treatment mainly according to Baveno Consensus[17,18] and United Kingdom guidelines on the management of variceal haemorrhage in cirrhotic patients[19].

Technique for Injection

The endoscopes used for glue injection were all

Table 1 Clinical features of patients with gastric varices

Clinical features	*n* (%)
Total number of patients	519
Male	392 (75.5)
Female	127 (24.5)
Mean age (yr)	47.9 ± 10.8
Etiology of gastric varices	
HBV cirrhosis	386 (74.3)
HCV cirrhosis	21 (4.0)
Alcoholism cirrhosis	18 (3.5)
Combined cirrhosis	19 (3.7)
Cryptogenic cirrhosis	29 (5.6)
Others (PBC, Budd-Chiari syndrome, PVT, *etc.*)	46 (8.9)
Classification of gastric varices	
GOV1	53 (10.2)
GOV2	153 (29.5)
GOV1 + GOV2	304 (58.6)
IGV1	7 (1.3)
IGV2	2 (0.4)
Child-Pugh classification	
A	195 (37.6)
B	231 (44.5)
C	93 (17.9)

GOV: Gastroesophageal varices; IGV: Isolated gastric varices; PBC: Primary biliary cirrhosis; PVT: Portal vein thrombosis. The patients in this category had portal vein thrombosis but had no primary liver diseases.

Olympus (Japan), including GIF-H260 and GIF-H290. Injection catheters (Olympus Japan) with 21 or 23G needles and a 6 mm long needle tip were used. The tissue glue was N-butyl-2-cyanoacrylate (NBC). There were two brands of NBC used in our department: Histoacryl (German) and Compant (SMR China). All injections were performed by the same medical team.

Complete endoscopic reports were obtained in 473 out of 628 EVGO procedures and included the detailed injection methods written by endoscopists. The detailed injection methods were not written by endoscopists in the remaining 155 EVGO procedures. In the 473 EVGO procedures, 263 procedures were performed using the classical "sandwich" injection with lipiodol-diluted NBC[20], 159 procedures were performed with undiluted NBC, and the remaining 51 procedures were performed using other modified "sandwich" injections. The endoscope tip and channel were protected by simethicone.

In the classical "sandwich" injection, the injection order was lipiodol, lipiodol-diluted NBC, and then lipiodol. The injection catheter was pre-filled with 1.0-1.2 mL of lipiodol. NBC was diluted with lipiodol at a ratio of 0.5:0.8 to 0.5:0.5. The target varix was located and punctured with the needle, and the lipiodol-diluted NBC was pushed into the varix followed by another 1.0-1.2 mL of lipiodol flush. The needle was quickly withdrawn into the catheter and removed from the varix.

After 2013, all brands of lipiodol in China required a complicated sensitivity test before use, and nearly all of them were forbidden to be used for intravascular injection. Therefore, the lipiodol- lipiodol-diluted NBC-lipiodol method is inconvenient, especially for emergent patients. Several modified "sandwich" methods were attempted. Undiluted NBC must be used as a working solution. The solution used for the pre-fill catheter included 50% glucose, sterile saline, distilled water, and 1% aethoxysklerol. Flush water used after the working solution injection included 50% glucose, distilled water and sterile saline.

Table 2 Procedure-related complications in gastric variceal obturation

Complications	*n* (%)
Total number of EGVO procedures	628
Sticking of the needle to the varix	9 (1.43)
Blockage of the injection catheter	17 (2.71)
Glue adhesion to the endoscope resulted in difficulty withdrawing the endoscope	1 (0.159)
Ligation device sticking to the varices	2 (0.318)

Statistical analysis

Statistical data were expressed as mean ± SD or as a percentage. A χ^2 test was used to compare the constituent ratio of non-continuous variables between the two groups. A statistical significance threshold of $P = 0.05$ was adopted.

RESULTS

Clinical features of the patients with gastric varices

In the 519 patients, HBV cirrhosis was the most cause of gastric varices and the most common type of gastric varices was gastroesophageal varices 1 (GOV1) + GOV2. Most of the patients had a liver function of Child-Pugh A or Child-Pugh B (Table 1).

A summary of procedure-related complications

In the 628 EGVO procedures, the most common procedure-related complications was blockage of the injection catheter just when the glue was being injected, the very rare but intractable complications were glue adhesion to the endoscope resulted in difficulty withdrawing the endoscope and ligation device sticking to the varices (Table 2).

Sticking of the needle to the varix

In the 628 EGVO procedures, sticking of the needle to the varix occurred in 9 cases (1.43%). Among the 9 cases, 1 case out of 263 procedures (0.843%) involved the use of NBC diluted with lipiodol, and 8 out of 159 procedures (5.03%) involved the use of undiluted NBC ($\chi^2 = 23.202$, $P = 0.000$). Among the 9 cases, 2 cases had a liver function of Child-Pugh A, 4 cases had a liver function of Child-Pugh B and 3 cases had a liver function of Child-Pugh C. There was no statistical difference of the complication among the patients with liver function of Child-Pugh A, B and C ($\chi^2 = 0.927$, $P = 0.629$). Once the sticking of the needle to the varix was noted, the tip of the endoscope was rapidly drawn near the varix

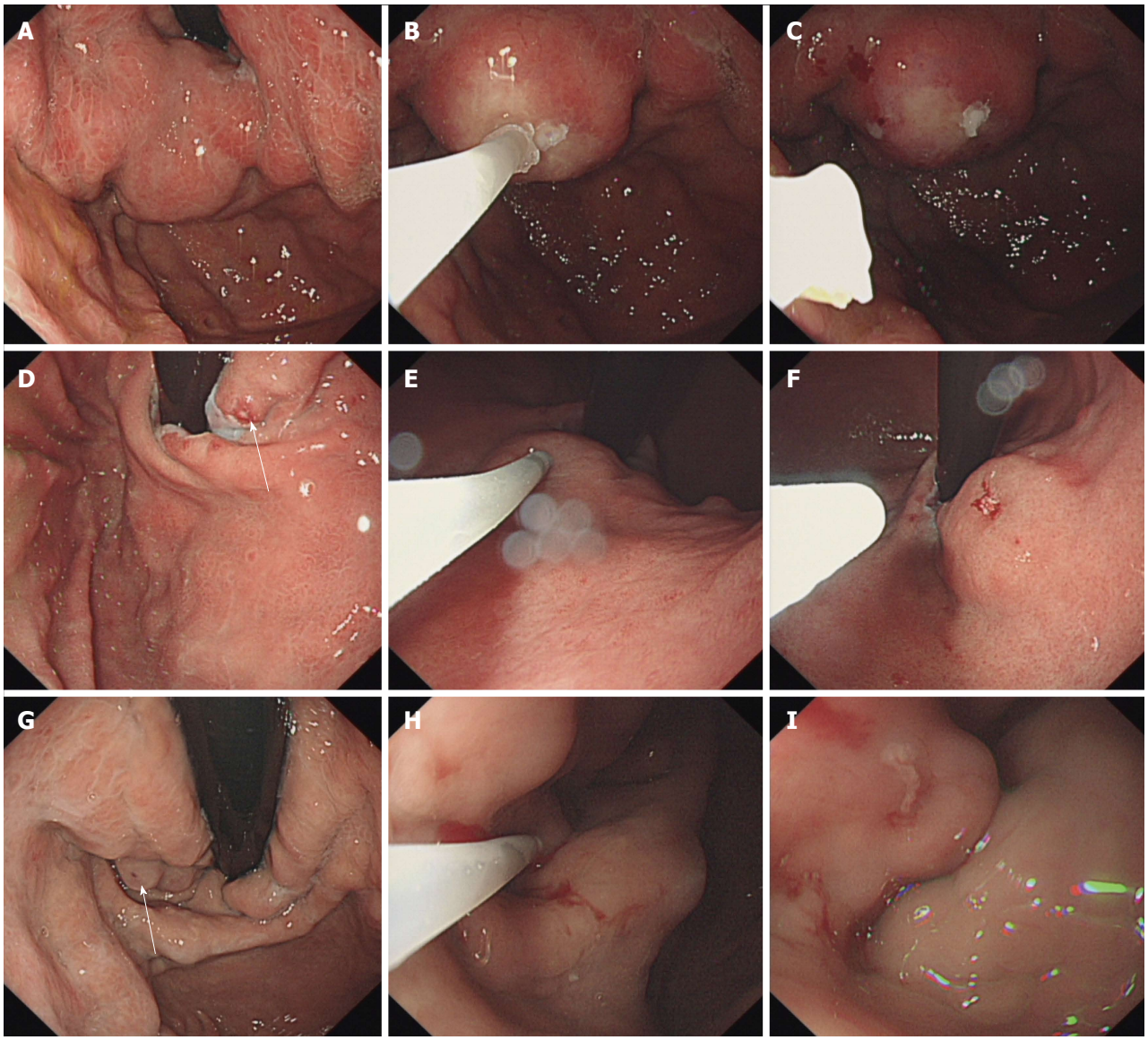

Figure 1 Sticking of the needle to the varix in three patients. A-C: Large GOV2 varices were obturated with tissue glue. The needle stuck to the varix and was successfully withdrawn without bleeding. The varices exhibited full solidification; D-F: A GOV1 varix with a post-bleeding break was obturated with tissue adhesive. The needle stuck to the varix and was successfully withdrawn, leaving little errhysis; G-I: A GOV1 varix with post-bleeding erosion was obturated with tissue glue and was successfully withdrawn without bleeding. The varix was soft, and another injection was performed. GOV: Gastroesophageal varices.

without hesitation and then directly withdrawn along the direction of the needle that was inserted inserting the varix as soon as possible. In 7 of 9 cases of needle sticking, the needle was successfully withdrawn with no bleeding or errhysis but could stop on its own (Figure 1). Persistent dropwise bleeding was observed in one patient and another NBC injection was needed to stop the bleeding. In one case, blockage of the catheter during injection and sticking of the needle to the varix occurred at the same time. A large spurt bleeding occurred, and two other injections of undiluted NBC with a total volume of 4 mL were performed immediately and hemostasis was achieved (Figure 2). The patient had an increase in heart rate but not a drop in blood pressure. Somatostatin was administered to prevent rebleeding. The patient finished the sequence therapy of esophageal and gastric varices in the following 6 mo.

Blockage of the injection catheter

If several gastric varices needed to be obliterated in one procedure, blockage of the injection catheter was common during the whole procedure and several injection catheters were needed. However, it was relatively rare that the catheter became blocked just when the glue was being injected. In the 628 EGVO procedures, the catheter became blocked in 17 cases (2.71%) during the injection. Four cases also included the needle sticking to the varix. Rapid withdrawal and change of the injection catheter was the only option for a simple injection catheter obstruction. Bleeding from the injection position, such as errhysis, dropwise bleeding and even spurt could be observed in many cases. Unlike the needle sticking to the varix, the pin pole was small, and the bleeding caused by simply withdrawing the needle was not usually massive.

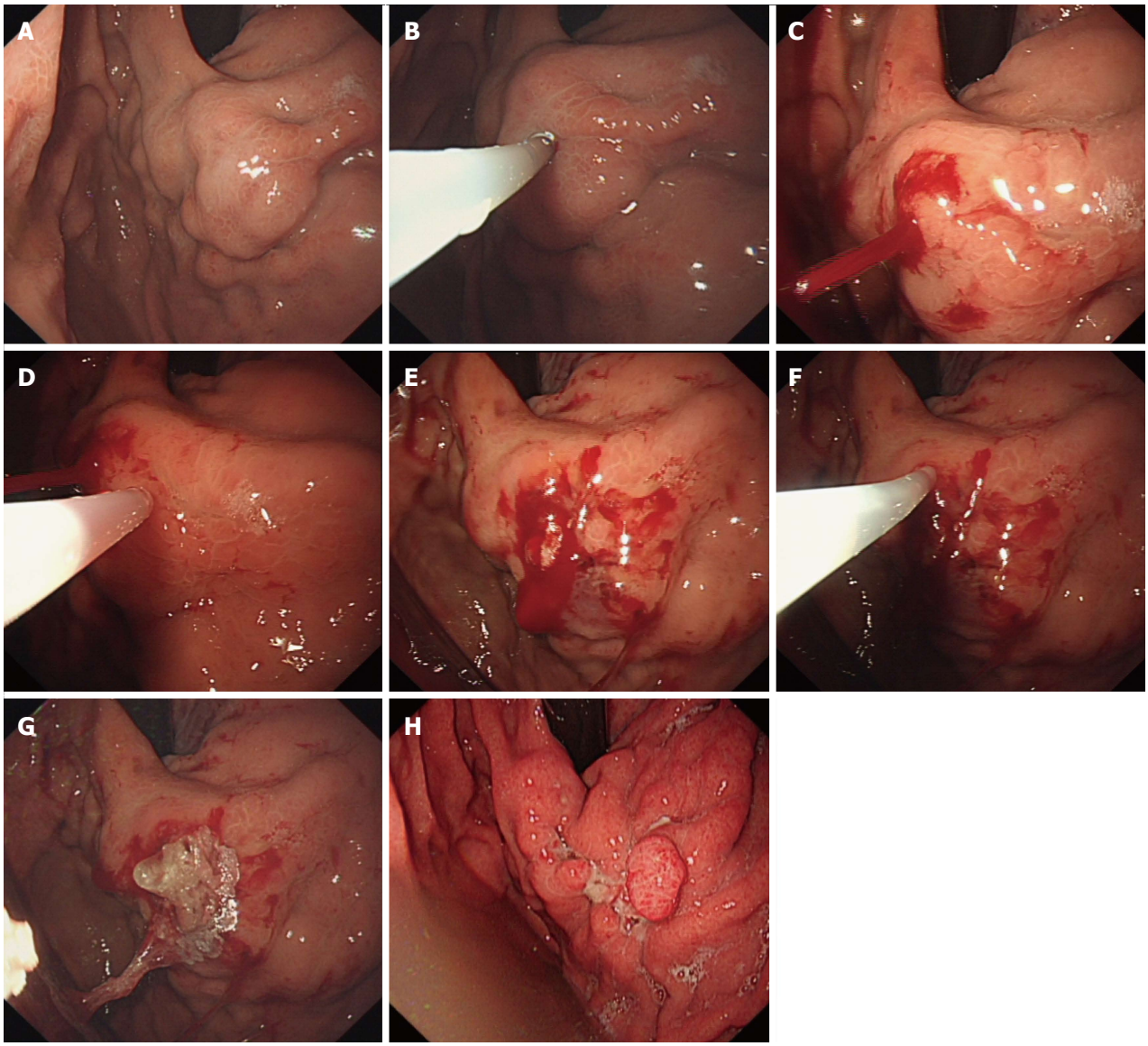

Figure 2 Sticking of the needle to the varix in one patient. A: A mass of GOV2 varices in one patient with HBV cirrhosis; B: Blockage of the catheter and sticking of the needle occurred at the same time when the tissue glue was being injected; C: Laceration of the varix and large spurt bleeding occurred; D and E: An injection with 3 mL of undiluted tissue glue was performed, and dropwise bleeding was observed; F and G: Another injection with 1 mL of undiluted tissue glue was performed, and hemostasis was achieved; H: A repeat endoscopy 6 mo later shows that the GOV2 varices were dramatically alleviated. Extrusion of the glue and some hyperplasia of the local gastric mucosa were observed. GOV: Gastroesophageal varices.

Another rapid injection of NBC could solve the problem. Maintenance of clear vision and skillful and efficient assistance were important.

Glue adhesion to the endoscope

Despite the protection of the endoscopic tip by simethicone, the glue would sometimes adhere to the endoscope. Most endoscopists did not record this event in their endoscopic reports, because the endoscope could be withdrawn smoothly so long as the adhesion was noted in time and the glue adherence was minimal. Acetone was used for the removal of the adhesion glue. In one case, a large amount of glue had adhered to the surface of endoscope. The endoscopist did not note or address this issue the first time. When two injections of NBC were completed on the same varix, a large mass of solidified glue was discovered but had become too hard. Biopsy forceps and foreign body forceps were used to try to remove the glue from endoscope, but both failed. The patient had a liver function of Child C and the surgery was difficult. Finally, the endoscope had to be withdrawn very slowly with the adhesive glue. The glue was 1.5 cm in width and 3.0 cm in length. Another endoscope was immediately inserted. A large area of mucous was damaged in the esophagus and gastric cardia and bleeding was observed in multiple places. An injection of 1% aethoxysklerol was used to stop the bleeding at several severe points (Figure 3). The patient achieved hemostasis when treated with somatostatin and blood transfusion and underwent EVL 5 wk later.

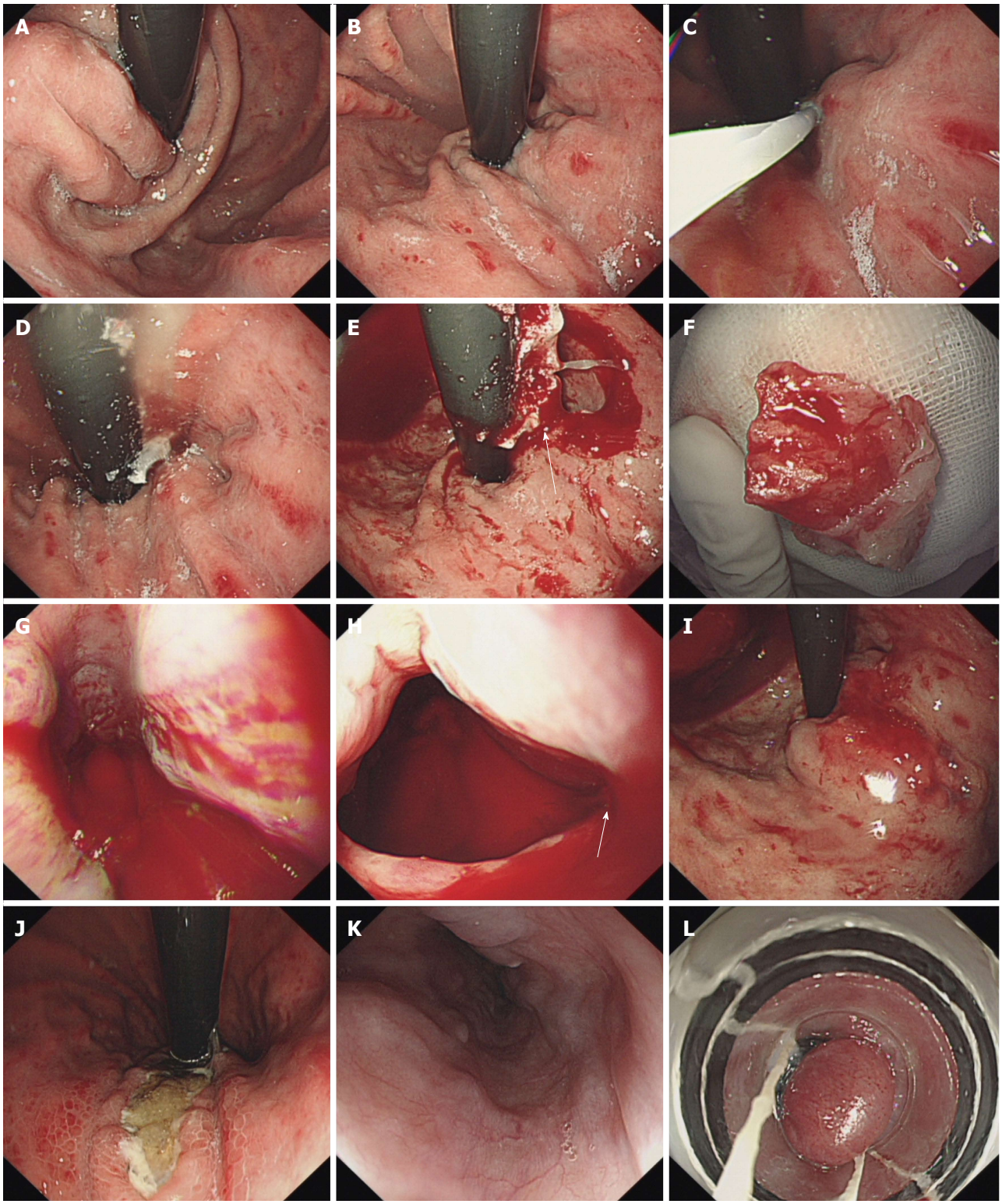

Figure 3 Glue adhesion to the endoscope in one patient. A and B: Three GOV1 varices were observed; C: Obturation of one varix with tissue glue; D: The glue leaked out but the reversed endoscope blocked the vision; E: A large mass of solidified glue adhered to the endoscope and damaged the cardia; F: The glue was 1.5 cm in width and 3.0 cm in length; G and H: A large area of mucous was damaged in the esophagus and gastric cardia. Bleeding was observed, especially a gush of blood at the cardia; I: The bleeding at the cardia ceased after a 1% aethoxysklerol injection; J-L: The patient underwent follow-up 5 wk later. Extrusion of the glue from the gastric varices was observed, and EVL was performed to eradicate the esophageal varices. GOV: Gastroesophageal varices; EVL: Endoscopic variceal ligation.

Ligation device sticking to the varices

The sticking of the ligation device to the varices was extremely rare in the treatment of esophageal and gastric varices *via* endoscopy. It occurred in two cases, when the patients underwent EVL after EGVO during the same procedure or 4 wk later. Some of the glue injected into the gastric varices would extend to the esophageal varices along the blood flow. In one case,

the patient underwent EVL immediately after EGVO. When the esophageal varices were sucked by ligation device, the glue leaked from the varices and stuck to the ligation device. The ligation device was successfully separated from the esophageal varix after the release of all bands and no persistent bleeding occurred. In another case[21], the patient underwent EVL 3 times after EGVO. The first and second EVL both went well. The patient underwent the third EVL at 4 mo after EGVO. When the varix was sucked by ligation device, the variceal bulb containing the glue was embedded in the cavity of the transparent cap and could not be released. The laceration of the vein and massive bleeding were observed. The bleeding ceased after 10 mL of 1% aethoxysklerol were injected with the aid of the transparent cap.

DISCUSSION

Unlike other reports, the current study mainly focused on the analysis of the complications that occur during the EGVO procedure that puzzle operators, especially beginners. The tissue glue, also called tissue adhesive, that is primarily used in gastric variceal obliteration is N-butyl-2-cyanoacrylate (NBC). NBC will solidify within 10-15 s once it contacts the hydroxyl ions present in plasma. When it is used in variceal obturation, a smooth and skillful operation is important to prevent procedure-related complications. The instrument, different methods of injection, and different working solutions also affect the outcome of treatment.

It had been reported that the use of undiluted glue might contribute to the impaction of the needle tip in the varices and appeared to relate to blockage of the injection catheter. The present study also revealed that the sticking of the needle to the varices occurred more easily with an undiluted glue injection[22-24]. The use of lipiodol-diluted NBC can delay solidification and enable radiographic visualization but might increase the chances of distal embolization. In contrast, undiluted NBC solidifies more quickly, but distal embolization seems less[20,25-27]. When the diluted glue was used, most of the endoscopists in our department preferred to keep the needle in the target varix for 5-10 s after the injection; thus, the full solidification could avoid a spurt or dropwise bleeding from the injection site. When performing an injection with undiluted glue, it is wise to withdraw the catheter rapidly once the injection is completed, which could avoid some cases of needle sticking. The speed of injection by the assistant is also important; an injection that is too slow might result in blockage of the injection catheter or the needle sticking to the varices, and an injection that is too fast might contribute to the complication of distal embolism[28]. The use of an injector with a 21G needle rather than a 23G needle appears not to easily cause blockage of the injector. In the current study, in some cases that EGVO was performed with undiluted glue, the injector with a 21G needle was used, the endoscopist withdrew the catheter soon after injection, and the speed of the injection was not slow; however, sticking of needle to the varices and blockage of the injection catheter still occurred. We considered other risk factors. In some of our procedures and in other reported studies, sterile saline was used to flush the catheter to measure or prefill the dead space. In our observation, solidification would occur after NBC was mixed with saline *in vitro*, so flushing the catheter with distilled water is a better choice. In China, some endoscopists prefer to inject a certain volume of 1% aethoxysklerol before the injection of the glue to decrease the volume of glue and glue-related complications in the treatment of large gastric varices. However, we observed that the glue would solidify very soon after mixing with 1% aethoxysklerol *in vitro*, suggesting it might be a risk factor for blockage of the catheter. The 50% glucose and 5% glucose solutions would not solidify after mixing with glue in vitro and are both safe for flushing the catheter.

What to do once the needle sticks to the varices? Some experts suggest that the needle can be kept in the varices for several minutes until the glue is hard enough. Then, the endoscopic tip of the varices can be closed, the varices can be pressed slightly, and the needle can be withdrawn. However, no one can guarantee that the needle would stick more tightly to the varices while waiting, and when the endoscopic tip presses the varices, the view is not clear, and the endoscopic tip may also stick to the varices. In the current study, once the sticking of the needle to the varix was noted, the tip of the endoscope was rapidly drawn near the varix, but did not touch, and then was directly withdrawn along the direction of needle that was inserted into the varix as soon as possible. The solidification of the glue was incomplete at that time, and the needle could be withdrawn relatively easily. In 7 of 9 cases of needle sticking, the needle was successfully withdrawn. One case needed another injection of glue, but the bleeding was not massive. One patient with Child C liver function and large gastric varices was unfortunate. Sticking of the needle and blockage of the catheter occurred at the same time, and there was massive bleeding from the injection site. Although hemostasis was finally achieved, it could have been fatal[22-24]. In some experts' experience, if the needle is too difficult to withdraw, the needle should remain in the varices, and TIPPS or surgery should be performed. In case of uncontrolled bleeding, TIPPS or surgery is also needed[10]. However, there is no a standard method for how to address the sticking needle; each method could lead to massive bleeding or a fatal outcome.

The protection of the endoscopic tip by simethicone, lipiodol or another oil-based contrast agents and the performance of a careful operation can noticeably decrease the incidence of glue adhesion to the endoscope[10,25]. Most of the time, timely observation and rapid withdraw of the endoscope

always works because a small amount of glue has adhered to the endoscope and the adhesion is loose at the beginning. If a large amount of glue has adhered to the endoscope and the adhesion was not noted in time, it is very difficult to manage because there is an increased chance of multiple sites of laceration of varices when the endoscope is withdrawn. An injection of 1% aethoxysklerol or other sclerosants can be used to control acute bleeding, especially when the accurate bleeding position is difficult to locate and clear vision cannot be obtained. Sclerosants can be injected either intravariceally or paravariceally. These types of injection can be managed more easily and quickly compared with EVL and EGVO and do not require a second oral intubation[29]. Thus, precious time is gained for patients, and further treatments can be considered. Sometimes, TIPPS and surgery are needed.

EVL is usually performed after EGVO during the same procedure or after a period of time[18,19]. The glue that is injected into the gastric varices usually extends to the esophageal varices along the blood flow. Ligation device sticking to the esophageal varices is extremely rare. No report about this complication was found. First, it is wise to avoid the ligation of a varix with a tissue glue plug. When the varices are sucked by the ligation device with a negative pressure and ligated with a band, some ruptures at the site of ligation, and bleeding from the rupture can occur. This kind of bleeding is usually small and can spontaneously stop. However, if the varices contain some glue that did not fully solidify, the glue would be pressed out and stick to the ligation. Usually, we can compress a varix with the tip of the ligation device or suck the varix gently with a lower suction pressure. If the varix is hard and difficult to suction, the varix may contain glue, and ligation on this site should be abandoned. If the ligation device sticks to a varix, some actions can be tried, such as releasing all bands and cutting the string from the handle, flushing the ligation site with air and water, and even more surgery. If laceration occurs, it would be fatal. We have little experience addressing this situation, and it merits more discussion. Since endoscopic ultrasound-guided NBC injection has been successfully applied in clinical practice[30-32], it may be used in the identification of varices that contain glue.

In conclusion, to avoid procedure-related complications, comprehensive preparation, careful and skillful operation, and smooth assistance are very important. There is currently no standard operating procedure for addressing these complications. We hope that the cases described in the current study can provide some reference for others.

ARTICLE HIGHLIGHTS

Research background

Tissue glue has been widely used in endoscopic gastric variceal obturation (EGVO). Although EGVO is usually safe and effective, a series of complications can occur. Post-endoscopic treatment complications have been widely reported and discussed, but there is little in-depth discussion on procedure-related complications. The complications associated with tissue glue that occur during gastric variceal obturation were retrospectively evaluated in the current study.

Research motivation

Post-endoscopic treatment complications of EGVO such as abdominal pain, pyrexia, organ embolization and local ulceration have been widely reported and discussed, but the incidence of procedure-related complications was calculated in only some studies and was not thoroughly analyzed. Most of the procedure-related complications were only described as case reports. In the current study, the authors focused on procedure-related complications, evaluated their incidence, analyzed the reasons and discuss the solutions. The authors hope that the cases described in the current study can provide some reference for others.

Research objectives

The main objectives of the current study was to evaluate four procedure-related complications of EGVO, including sticking of the needle to the varix, glue adhesion to the endoscope resulting in difficulty withdrawing the endoscope, blockage of the catheter during the injection, and more seriously, sticking of the ligation device to the esophageal varices in the subsequent endoscopic variceal ligation. Investigation of the incidence, reasons and solutions of these complications is expected to help the endoscopists especially those in training to avoid some troubles.

Research methods

Six hundred and twenty-eight EGVO procedures (case-times) with tissue glue were performed in 519 patients in the Department of Endoscopy of the Third Affiliated Hospital of Sun Yat-Sen University from January 2011 to December 2016. The tissue glue used in EGVO was N-butyl-2-cyanoacrylate (NBC). The endoscopic reports and medical records of all patients were collected. The clinical data of patients and procedure-related complications of EGVO were retrospectively analyzed.

Research results

In the 519 patients, HBV cirrhosis was the most cause of gastric varices (75.5%) and the most common type of gastric varices was GOV1 + GOV2 (58.6%). Most of the patients had a liver function of Child-Pugh A (37.6%) or Child-Pugh B (44.5%). Detailed injection methods were written by endoscopists in 473 out of 628 EVGO procedures, including 263 procedures that were performed using the classical "sandwich" injection with lipiodol-diluted NBC, 159 procedures that were performed with undiluted NBC, and the remaining 51 procedures that were performed using other modified "sandwich" injections. In the 628 EGVO procedures, sticking of the needle to the varix occurred in 9 cases (1.43%), including 1 case that used lipiodol-diluted NBC and 8 cases that used undiluted NBC ($P = 0.000$). There was no statistical difference of the complication among the patients with liver function of Child-Pugh A, B and C ($P = 0.629$). The needle was successfully withdrawn in 8 cases. Large spurt bleeding occurred in one case, and hemostasis was achieved by two other injections of undiluted glue. The injection catheter became blocked in 17 cases (2.71%) just during the injection, and 4 cases were complicated with the needle sticking to the varix. Large glue adhesion to the endoscope resulted in difficulty withdrawing the endoscope in 1 case. Bleeding from multiple sites was observed in the esophagus and gastric cardia after the endoscope was withdrawn. Hemostasis was achieved by 1% aethoxysklerol injection and intravenous somatostatin. The ligation device stuck to the varices in two cases during the subsequent endoscopic variceal ligation (EVL). In one case, the ligation device was successfully separated from the esophageal varix after all bands were released. In another case, a laceration of the vein and massive bleeding were observed. The bleeding ceased after 1% aethoxysklerol injection.

Research conclusions

The findings of this study verified that procedure-related complications were rare but sometimes were extremely dangerous. There is currently no standard operating procedure for addressing these complications. To avoid procedure-related complications, comprehensive preparation, careful and skillful operation,

and smooth assistance are very important.

Research perspectives

Although EGVO with tissue glue is usually safe and effective, a series of complications can occur during the procedure. Some factors might influence the occurrence of the complications and their outcomes, including Child-Pugh Class, type of gastric varices (GOV1, GOV2, IGV1 and IGV2), platelets, INR, volume of tissue glue using during EGVO, and diameter of the injection needle. These factors were not fully investigated and discussed in the current retrospective study. A well designed prospective study with a large sample is expected to solve that problem.

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P- Reviewer: Cui J, Keyashian K, Vujasinovic M **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Lu YJ

World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7756-7764

DOI: 10.3748/wjg.v23.i43.7756

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

ORIGINAL ARTICLE

Retrospective Study

Gastric xanthelasma and metabolic disorders: A large retrospective study among Chinese population

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Author contributions: Chen Y and He XJ contributed equally to this work; Chen Y and Zhou MJ designed the research; Chen Y and He XJ performed the research; Chen Y and He XJ analyzed data; Chen Y and Li YM wrote the paper.

Supported by Science Foundation of Health Bureau of Zhejiang Province, No. 2017183691 (to Chen Y).

Institutional review board statement: This manuscript was approved Department of Gastroenterology, the First Affiliated Hospital, College of Medicine, Zhejiang University for its conduction.

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

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

Data sharing statement: All data of this study was shown and available in manuscript.

Manuscript source: Unsolicited manuscript

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Received: July 3, 2017
Peer-review started: July 3, 2017
First decision: August 10, 2017
Revised: August 25, 2017
Accepted: September 13, 2017
Article in press: September 13, 2017
Published online: November 21, 2017

Abstract

AIM

To gain knowledge of xanthelasma, a large population-based study was conducted.

METHODS

Patients who underwent upper gastrointestinal endoscopy at the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China during Jan 2009 to Nov 2016 were included. General characteristics as well as clinical data were collected, including blood routine, serum biochemical analysis, endoscopic findinds, histological evaluation and comorbiditie. Statistical analyses was performed using SPSS 20.0 software for Windows (IBM Inc., Chicago, IL, United States) using Student's *t*-test, Mann-Whitney *U* test, χ^2 test, univariable and multivariable logistic analysis. 2-tailed *P* value less than 0.05 was considered to be statistically significant.

RESULTS

A total of 176006 endoscopies were retrieved and we included 1370 xanthelasma participants (703 men, 667 women) in this study. Prevalence of xanthelasma was 0.78% with average age of 56.6 ± 11.2 years. Chief complaint of xanthelasma consisted abdominal pain

(24.2%), up-abdominal discomfort (14.1%), abdominal distention (10.1%), dyspepsia (9.1%), *et al*. Most xanthelasma occurred as single lesion in gastric antrum. Xanthelasma patients witnessed higher *Helicobacter pylori* (*H. pylori*) infection rate, more of other gastric lesions including atrophy, intestinal metaplasia and dysplasia ($P < 0.01$). In xanthelasma patients, serum carcinoembryonic antigen, triglyceride, fasting glucose, neutrophil, neutrophil-to-lymphocyte ratio were significantly higher, and high density lipoprotein-cholesterol, lymphocyte was lower ($P < 0.05$). Xanthelasma accompanied with more fatty liver disease and hepatic cyst, but fewer gallbladder polyp ($P < 0.05$). In logistic regression, it revealed that fasting plasma glucose (OR = 3.347, 1.170-9.575, $P < 0.05$), neutrophil (OR = 1.617, 1.003-2.605, $P < 0.05$), and carcinoembryonic antigen (OR = 2.011, 1.236-3.271, $P < 0.01$) were all independent risk factors in xanthelasma.

CONCLUSION

Current study described a large xanthelasma cohort in Chinese population, revealed its relationship with *H. pylori* infection, carcinogenesis, metabolic dysfunction and inflammation as well.

Key words: Gastric xanthelasma; *Helicobacter pylori* infection; Gastric dysplasia; Metabolic disorder

Core tip: Xanthelasma was a relatively rare endoscopic finding, characterized by accumulation of lipid in histiocytic foam cells in mucosa. Current study described a large xanthelasma cohort in Chinese population and revealed its relationship with *Helicobacter pylori* infection, atrophy, intestinal metaplasia, dysplasia, and metabolic disorder, indicating role of xanthelasma in both carcinogenesis and metabolic dysfunction.

Chen Y, He XJ, Zhou MJ, Li YM. Gastric xanthelasma and metabolic disorders: A large retrospective study among Chinese population. *World J Gastroenterol* 2017; 23(43): 7756-7764 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7756.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7756

INTRODUCTION

Gastric xanthelasma, characterized by lipid accumulation in histiocytic foam cells, was a relatively scarce endoscopic finding, with prevalence varied from 0.8% to 7% in different study population[1-3]. As is frequently observed in the gastric mucosa of patients with early gastric cancer, xanthelasma was assumed to be a predictive biomarker or pre-tumorigenesis change of gastric adenocarcinoma[1,4]. But there is no large descriptional study among Chinese population.

Mechanism of xanthelasma was unknown. Early small sample studies have showed relationship between xanthelasma and *Helicobacter pylori* (*H. pylori*) infection[5,6]. Later on, a Korean cohort further supported this finding, indicating a role of *H. pylori* as well as inflammation in xanthelasma[2]. As these countries were high *H. pylori* prevalent places, till now, whether *H. pylori* infection was result of or just casual finding in xanthelasma was not clear, neither was the association between chronic inflammation and xanthelasma.

As xanthelasma was defined as lipid deposit in stomach, its relevance to metobolic disorders was of great interest. Etiologically, these conditions may be associated with a primary dyslipoproteinemic state, such as diabetes, nephrosis, obesity or cholestasis. In two Korean studies, dyslipidemia, representative of lower mean high density lipoprotein (HDL)-cholesterol and higher mean triglyceride levels was found in gastric xanthelasma subjects in comparison with the controls, accompanied with higher body mass index (BMI)[2,7]. But, there were no reports regarding xanthelasma and metabolic factors in Chinese population yet.

So, herein, we conducted a large retrospective study in China. General aspects of xanthelasma were described, including clinical aspect, endoscopic and histological findings. Furthermore, we focused on relationship between xanthelasma and metabolic disorders, as well as inflammation property.

MATERIALS AND METHODS

Study population

This study was performed among adults who underwent upper gastrointestinal endoscopy at the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China during Jan., 2009 to Nov., 2016. Endoscopic xanthelasma group was defined as patients with xanthelasma as one of their endoscopic findings. Biopsy-proven xanthelasma were those who did biopsy at suspicious xanthelasma lesion and histological staining showed typical foam cells which supported diagnosis of xanthelasma.

The study protocol was approved by the Hospital Ethics Committee and performed in accordance with Declaration of Helsinki. All persons gave their informed consent prior to their inclusion. Patients with history of gastric cancer, gastrectomy, proton pump inhibitor use within the last 2 wk, antibiotics use within the last 4 wk, poor general condition not suitable for prolonged procedure time, and surveillance or inability to give informed consent were excluded.

Clinical evaluations

Clinical evaluations were performed according to procedures as previously described[8]. In brief, demographic data, health habits and outpatient records (chief complaint, medical history, home medications, et al) were collected by trained physicians. Standing height and body weight without shoes and with light clothes

were measured using standard procedures. BMI was calculated as body weight (kg) divided by square of height (m). Waist circumference was measured at the level of the narrowest point between iliac crest and rib cage using a non-stretchable tape. Systolic and diastolic blood pressures were measured using an automated sphygmomanometer, with participants in sitting position. Overnight fasting blood samples were obtained. Blood routine, as well as biochemical factors, including liver enzymes, serum lipids, glucose, and uric acid, was measured as previously described[9,10]. Carbohydrate antigen 199 (CA199) level and carcinoembryonic antigen (CEA) was measured by ECLIA on a Modular Analytics E module (Roche Diagnostics Co., Tokyo, Japan).

Endoscopy procedure

Procedure of upper gastrointestinal endoscopy was described in previous study[11]. Two endoscopists worked together on each patient during the whole procedure including biopsy. For controversial or non-classifiable cases, consensus was reached by discussion with one or more senior endoscopists. Gastric antrum was routine biopsies site. Additional biopsy were collected where lesion was found. The biopsy samples were fixed in formalin and sent to pathology laboratory in two separate vials, labeled according to their demographic data and anatomic sites.

Endoscopic xanthelasma diagnosis was based on observation of typical lesions appearing as a yellow-white nodule or plaque. Anatomic location and size of all xanthelasma lesion were recorded during procedure. Size was estimated by comparing it with open biopsy forceps[12]. Non-gastric gastrointestinal xanthelasma refers to xanthelasma within GI tract, but not located in stomach; extra-gastrointestinal xanthelasma refers to xanthelasma other than GI tract, such as skin xanthelasma or eye xanthelasma.

Histologic evaluation

Histology of each specimen was determined by two experienced pathologists independently, who were blinded to endoscopic findings. Procedures and definitions of different lesion were described in previous reports[11,12].

H. pylori status was evaluated by modified Giemsa staining. Dysplasia refers to phenotypically neoplastic epithelium confined to glandular structures inside the basement membrane[13]. Atrophy of gastric mucosa indicates that the gastric glands proper in the gastric mucosa become sparse.

Foamy cells were typical histological findings and diagnostic prior criteria of xanthelasma[2]. The histologic appearance of lipid islands was described as lamina propria occupied by large ovoid to polygonal histiocytes with an abundant, finely vacuolated (foamy) cytoplasm staining lightly with eosin. Nuclei of foam cell were regular, round to ovoid, and occupied a small portion of cell area. Intestinal metaplasia was characterized by presence of histologically typical goblet cells. Grade of intestinal metaplasia was classified according to goblet cell density (low for 1-10, medium for 10-50 and high as > 50 goblet cells per low power field). If any inconsistence of two pathologists, the biopsy specimens were assessed by a third experienced pathologist, and the final diagnosis was based on diagnosis of the majority.

Statistical analysis

Statistical analyses was performed using SPSS 20.0 software for Windows (IBM Inc., Chicago, IL, United States). Continuous variables were shown as mean and standard deviation and compared using Student's *t*-test or Mann-Whitney *U* test. Categorical variables were analyzed using χ^2 test. Univariable and multivariable logistic analysis were adopted in constructing a predicting model of xanthelasma. 2-tailed *P* value less than 0.05 was considered as statistically significant.

RESULTS

General aspect of gastric xanthelasma among study population

From 2009 to 2016, a total of 176006 endoscopies was retrieved in the First Affiliated Hospital. Among them, 1370 xanthelasma were identified, with prevalence being 0.78%. Proportion of female and male was almost equivalent (667 *vs* 703). Xanthelasma was seen in a relative elderly group, with average age of 56.6 ± 11.2 years (57.2 ± 11.5 for male and 55.9 ± 10.9 for female).

Among all patients with xanthelasma, 24.2% complained of abdominal pain, 9.1% visited hospital for dyspepsia, 10.1% came with abdominal distention, and 14.1% for up-abdominal discomfort. Other symptoms like constipation, regurgitation, and diarrhea were seen in minority.

Xanthelasma appeared in most cases as single lesion, 6.1% were found as double or multiple. Average size of xanthelasma was 0.40 ± 0.24 cm. Most common site of xanthelasma was antrum (as high as 70.7%), corpus being the second (15.2%), and angulus xanthelasma accounted for 10.1% patients. None was seen in the fundus. Among all antrum xanthelasma, 35.7% were found in greater curvature, 26.3% in less curvature, 11.1% in the frontier wall and the rest in the posterior. No non-gastric gastrointestinal xanthelasma or extra-gastrointestinal xanthelasma was identified in current study.

Gastric xanthelasma, atrophy, H. pylori infection, intestinal metaplasia and dysplasia

In order to investigate relationship between xanthelasma and other gastric lesion (such as atrophy, *H. pylori* infection, intestinal metaplasia and dysplasia), 207 patients without biopsy were excluded in current

Table 1 χ^2 analysis of *Helicobacter pylori*, atrophy, infection, intestinal metaplasia and dysplasia rate in xanthelasma and non-xanthelasma group *n* (%)

Variables	Xanthelasma	Non-xanthelasma	χ^2	*P*
n (male/female)	1163 (602/561)	1163 (602/561)	-	1
Age (yr)	57 ± 11	56 ± 13	-	0.619
Helicobacter pylori infection	358 (30.8)	341 (29.4)	0.269	0.603
Atrophy	155 (13.3)	58 (4.9)	13.1	< 0.01
Intestinal metaplasia	576 (49.5)	166 (14.3)	44.6	< 0.001
Low grade	331 (28.5)	119 (10.3)	15.6	< 0.001
Medium grade	199 (17.1)	43 (3.7)	27.1	< 0.001
High grade	46 (4.0)	4 (0.3)	13.7	< 0.001
dysplasia	24 (2.1)	36 (3.1)	14.3	< 0.01

Table 2 Metabolic factors in xanthelasma and non-xanthelasma groups

Variables	Non-xanthelasma	Xanthelasma	*P*
n (male/female)	99 (41/58)	99 (41/58)	1.000
Age (yr)	55 ± 13	56 ± 11	0.537
Body mass index (kg/m^2)	22.1 ± 2.3	23.2 ± 2.3	0.288
Systolic blood pressure	119 ± 16	117 ± 13	0.818
Diastolic blood pressure	73 ± 10	73 ± 13	0.785
Alanine aminotransferase	20 ± 13	20 ± 9	0.953
Aspartate aminotransferase (U/L)	21 ± 6	21 ± 4	0.669
Alkaline phosphatase (U/L)	67 ± 18	72 ± 21	0.202
γ-Glutamyltransferase (U/L)	23 ± 18	27 ± 32	0.327
Total bilirubin (μmol/L)	12.2 ± 3.8	13.4 ± 8.5	0.246
Triglyceride (mmol/L)	1.07 ± 0.55	1.33 ± 0.63	< 0.05
Albumin (g/L)	47.5 ± 3.2	46.2 ± 3.8	0.063
Total cholesterol (mmol/L)	4.51 ± 0.77	4.59 ± 0.78	0.614
HDL-cholesterol (mmol/L)	1.45 ± 0.35	1.26 ± 0.33	< 0.01
LDL cholesterol (mmol/L)	2.42 ± 0.61	2.56 ± 0.52	0.259
Fasting plasma glucose (mmol/L)	4.71 ± 0.50	5.08 ± 0.58	< 0.001
Hb1Ac (%)	5.49 ± 1.19	5.5 ± 0.17	0.984
Serum uric acid (μmol/L)	303 ± 79	328 ± 91	0.119
CEA (ng/mL)	1.8 ± 1.2	2.8 ± 1.6	< 0.001
CA199 (U/mL)	8.3 ± 9.5	9.4 ± 12	0.780

HDL-cholesterol: High density lipoprotein cholesterol; LDL-cholesterol: Low density lipoprotein cholesterol; CA199: Carbohydrate antigen 199; CEA: Carcinoembryonic antigen.

analysis (Table 1). Among all 1163 xanthelasma, *H. pylori* infection rate was 30.8%. In 13.3% xanthelasma patients, atrophy was identified. Prevalence of dysplasia was 2.1%. To look deep into transition from gastritis to dysplasia, intestinal metaplasia was investigated. Pathological evaluation of intestinal metaplasia was conducted and sorted into three degrees, and it showed that high, medium and low grade intestinal metaplasia was found in 28.5%, 17.1% and 4.0% xanthelasma population, respectively.

To dig deep into relationship of these findings with xanthelasma, 1163 patients without endoscopic xanthelasma were also included among people who underwent endoscopy in our hospital (Table 1). Comparing with control group, patients with xanthelasma showed a slightly higher rate of *H. pylori* infection but not significant. Atrophy was more prevalent in xanthelasma (13.3% *vs* 4.9% in control group, $P < 0.01$). Similarly, all three grades of intestinal metaplasia was seen more common in xanthelasma. Prevalence of dysplasia was not seven-fold higher in xanthelasma than non-xanthelasma group (2.1% *vs* 0.3% in control group, $P < 0.001$).

Association between gastric xanthelasma and metabolic disorders

Among 1370 endoscopic diagnosed xanthelasma, 99 were biopsy-proven, which were included in further analysis, with 41 being male and 58 female (Table 2, Figure 1). 99 healthy controls were collected from annual health examination who underwent endoscopy with negative finding of xanthelasma. Mean age in xanthelasma was 56 ± 11 years, and 55 ± 13 in non-xanthelasma. Body mass index was similar in these two group (23.2 ± 2.3 in xanthelasma *vs* 22.1 ± 2.3 in non-xanthelasma), as well as blood pressure (systolic 117 ± 13 and diastolic 73 ± 13 in xanthelasma *vs* systolic 119 ± 16 and 73 ± 10 in non-xanthelasma).

Serum lipid profile was found distinct between two groups (Figure 1). Xanthelasma patients showed significant higher TG (1.33 ± 0.63 *vs* 1.07 ± 0.55 in non-xanthelasma, $P < 0.05$) and lower HDL-cholesterol (1.45 ± 0.35 *vs* 1.26 ± 0.33 in non-xanthelasma, $P < 0.01$), but no difference in LDL-cholesterol or total cholesterol. As for glucose metabolism, serum fasting glucose was increased in xanthelasma (5.08 ± 0.58 *vs* 4.71 ± 0.49 in non-xanthelasma, $P < 0.001$), but Hb1Ac was similar.

Tumor marker CEA was higher in xanthelasma group (2.8 ± 1.6 *vs* 1.8 ± 1.2 in non-xanthelasma, $P < 0.001$), but CA199 was similar in two groups (9.4 ± 12 *vs* 8.7 ± 9.5 in non-xanthelasma).

No significant disparity was seen in liver enzyme, including ALT, AST, ALP and GGT. Other factors, such as total bilirubin, albumin and serum uric acid were not different in two groups.

Metabolic comorbidities in xanthelasma

Data of metabolic comorbidities were collected in xanthelasma and non-xanthelasma group (Table 3). In xanthelasma, fatty liver was more prevalent than non-

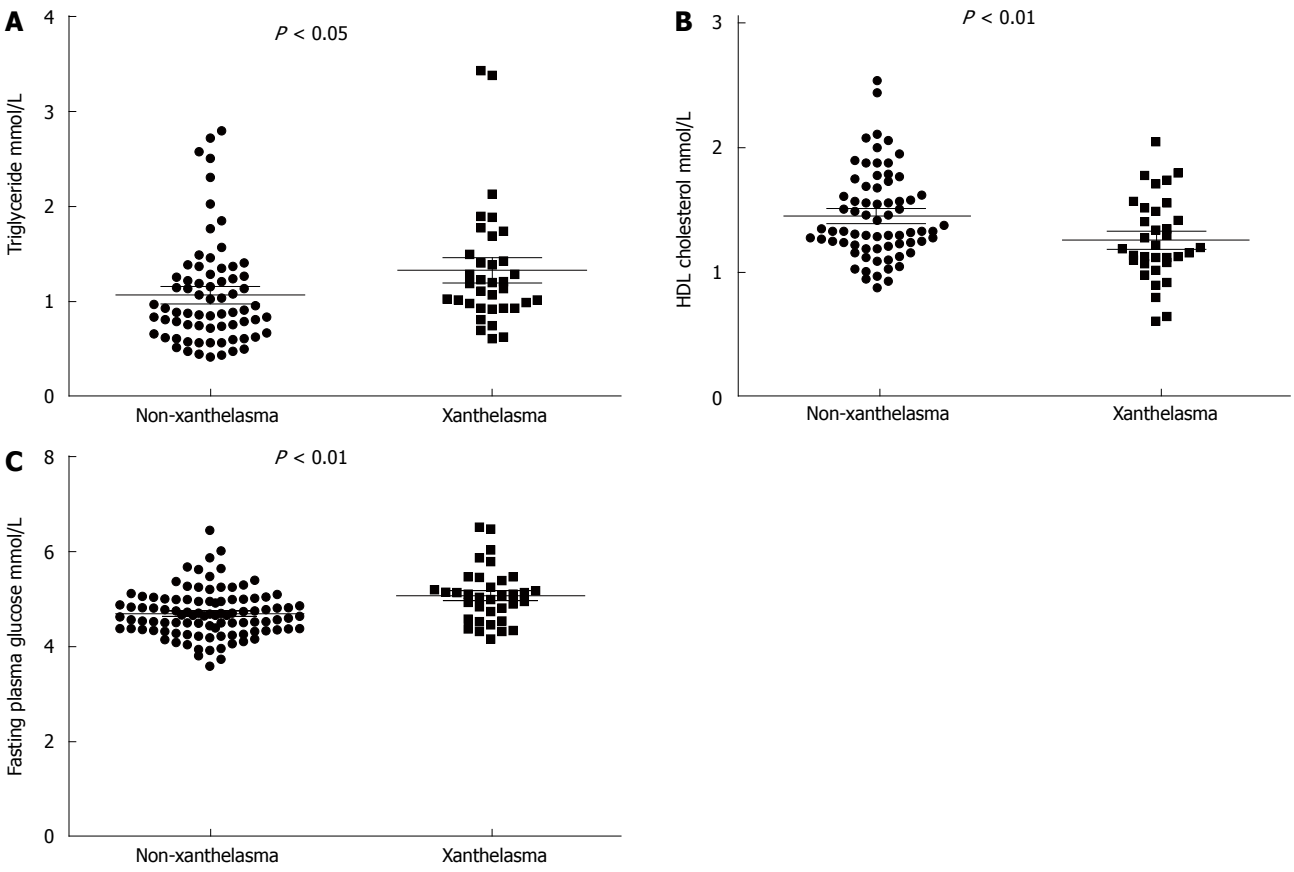

Figure 1 Metabolic changes in xanthelasma. A: TG; B: HDL-cholesterol; C: Fasting plasma glucose. HDL-cholesterol: High density lipoprotein cholesterol.

Table 3 Metabolic comorbidities in xanthelasma and non-xanthelasma group *n* (%)

Metabolic comorbidity	Non-xanthelasma	Xanthelasma	*P*
Biliary and liver diseases			
Chronic liver disease	2 (2.0)	4 (8.9)	0.911
Fatty liver	15 (15.2)	13 (28.9)	< 0.05
Hemangioma	1 (1.9)	1 (2.1)	0.568
Cyst	10 (10.1)	8 (17.0)	< 0.05
Post-cholecystectomy	2 (2.0)	4 (8.5)	0.911
Gallstone	4 (4.0)	3 (6.8)	0.132
Gallbladder polyp	9 (9.1)	3 (6.8)	< 0.01
Cardio-cerebral-vascular disease			
Coronary artery disease	1 (1.0)	2 (4.9)	0.877
Atrial fibrillation	0 (0)	1 (2.4)	0.522
Carotid atherosclerosis	5 (5.1)	6 (14.6)	0.223
Stroke	0 (0)	1 (2.4)	0.522
Thyroid disease	4 (4.0)	4 (9.8)	0.187
Diabetes mellitus	1 (1.0)	2 (4.9)	0.877

xanthelasma (28.9% *vs* 15.2% in non-xanthelasma). Liver cyst was found more frequent in xanthelasma (10.1% *vs* 17% in non-xanthelasma) and fewer gallbladder polyp was discovered in xanthelasma (9.1% *vs* 6.8% in xanthelasma). Prevalence of other diseases, including chronic liver disease, hemangioma, gallstone coronary artery disease, atrial fibrillation, carotid atherosclerosis, stroke, thyroid disease and diabetes mellitus were similar in two groups.

Difference in serum inflammatory markers in gastric xanthelasma

Inflammation markers were analyzed in our study as well (Table 4, Figure 2). In xanthelasma group, neutrophil was higher (3.4 ± 1.3 *vs* 4.0 ± 1.3 in non-xanthelasma, $P < 0.05$), lymphocyte was lower (2.1 ± 0.5 *vs* 1.8 ± 0.8 in non-xanthelasma, $P < 0.05$), newly developed inflammation related index NLR (Neutrophil-to-lymphocyte ratio) was significantly increased (1.74 ± 0.74 *vs* 2.62 ± 1.92 in non-xanthelasma, $P < 0.001$), but C-reactive protein (CRP) was similar (2.4 ± 4.6 *vs* 2.4 ± 2.8 in non-xanthelasma, $P = 0.124$).

Independent risk factors in gastric xanthelasma

In order to investigate diagnostic and predictive role of metabolic and inflammatory factors in xanthelasma, univariable logistic analysis was adopted. It turned out that fasting plasma glucose (OR = 3.740, 95%CI: 1.721-8.129, $P < 0.01$), neutrophil (OR = 1.329, 95%CI: 1.004-1.758, $P < 0.05$), NLR (OR = 2.097, 95%CI: 1.234-3.564, $P < 0.01$), and CEA (OR = 1.882, 95%CI: 1.195-2.964, $P < 0.01$) were significantly associated with gastric xanthelasma (Table 5).

To gain more specific knowledge of whether these four factors are independent, multivariable logistic regression was used. As NLR was a calculated index from neutrophil, we herein included fasting plasma glucose, neutrophil, and CEA in further analysis to

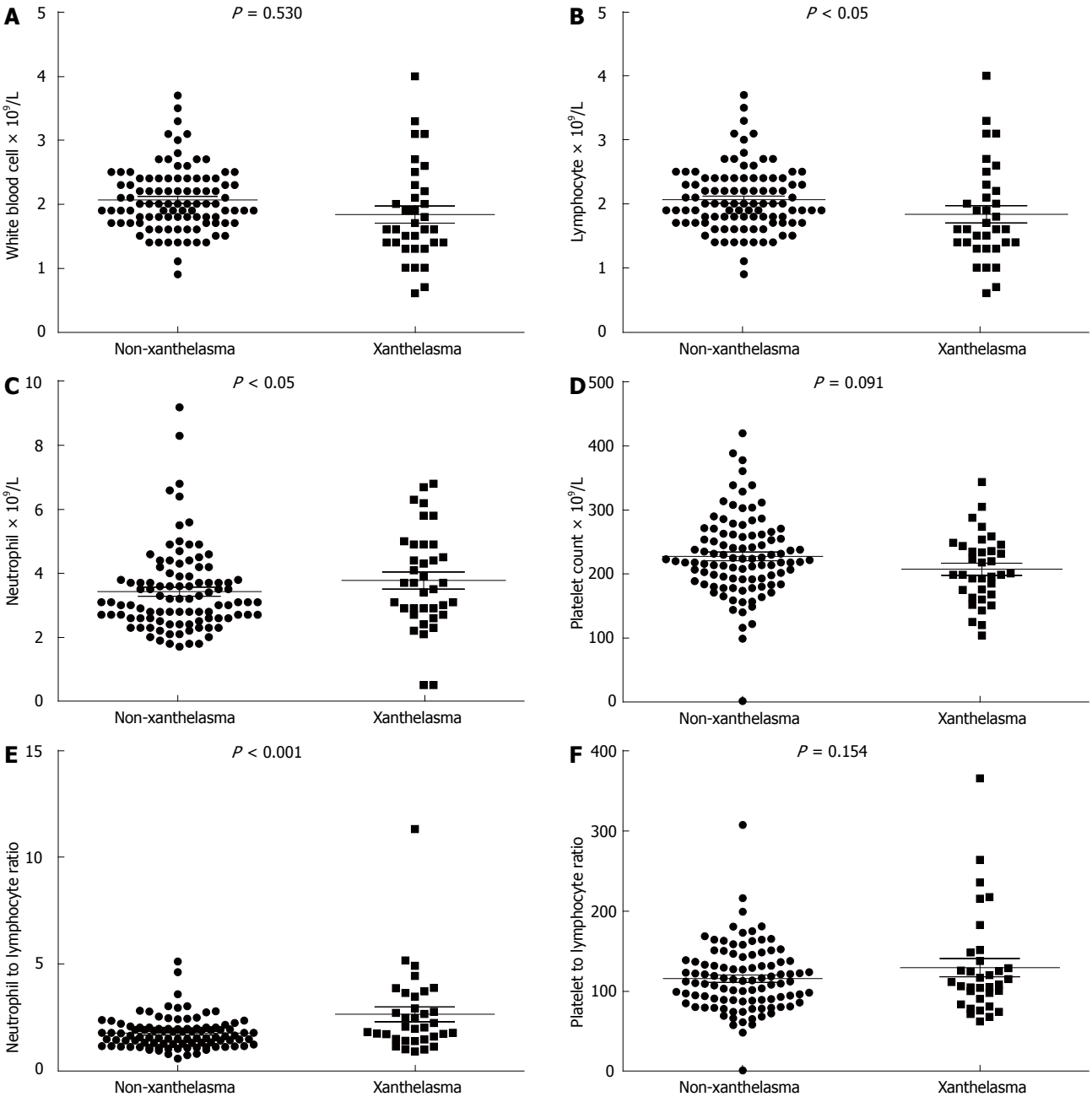

Figure 2 Blood routine changes in xanthelasma. A: WBC; B: Lymphocyte; C: Neutrophil; D: Platelet count; E: NLR; F: PLR; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio.

avoid potential confounding. It manifested that fasting plasma glucose (OR = 3.347, 95%CI: 1.170-9.575, $P < 0.05$), neutrophil (OR = 1.617, 95%CI: 1.003-2.605, $P < 0.05$), and CEA (OR = 2.011, 95%CI: 1.236-3.271, $P < 0.01$) were all independent risk factors in xanthelasma (Table 6).

DISCUSSION

Current study was the first populational based cohort of xanthelasma in China. This retrospective study collected a large xanthelasma group in Chinese population. Prevalence of endoscopic xanthelasma was 0.78% in our cohort with average age of 56.6 ± 11.2. Reasons of hospital visiting included abdominal pain (24.2%), up-abdominal discomfort (14.1%), abdominal distention (10.1%), and dyspepsia (9.1%), *et al*. Xanthelasma was most frequently seen in antrum as a single lesion. Chi-square analysis revealed positive relationship between prevalence of xanthelasma and *H. pylori* infection rate, more atrophy, intestinal metaplasia, dysplasia respectively. Metabolic and inflammatory factors were found associated with xanthelasma, as with higher CEA, TG, fasting glucose, neutrophil, NLR index, fatty liver comorbidity and lower HDL-cholesterol, lymphocyte. Among all these factors, fasting plasma glucose, neutrophil, and CEA were proved to be independent factors in xanthelasma.

Table 4 Serum inflammatory markers in xanthelasma and non-xanthelasma groups

Variables	Non-xanthelasma	Xanthelasma	*P*
White blood cell (× 10^9/L)	6.0 ± 1.5	5.8 ± 1.6	0.530
Neutrophil (× 10^9/L)	3.4 ± 1.3	4.0 ± 1.3	< 0.05
Lymphocyte (× 10^9/L)	2.1 ± 0.5	1.8 ± 0.8	< 0.05
Hemoglobin (g/L)	141 ± 15	141 ± 17	0.950
Platelet count (× 10^9/L)	228 ± 63	208 ± 53	0.091
NLR	1.74 ± 0.74	2.62 ± 1.92	< 0.001
PLR	116 ± 41	129 ± 65	0.154
CRP (mg/L)	2.4 ± 4.6	2.4 ± 2.8	0.124

NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; CRP: C-reactive protein.

Table 5 Univariable logistic regression of xanthelasma-related factors

Variables	OR	95%CI	*P*
n (male/female)	1.006	0.571-1.773	0.983
Age (yr)	1.007	0.984-1.131	0.535
Body mass index (kg/m^2)	1.188	0.866-1.629	0.286
Systolic blood pressure	0.972	0.904-1.045	0.437
Diastolic blood pressure	1.045	0.938-1.163	0.425
Alanine aminotransferase	1.051	0.975-1.131	0.192
Aspartate aminotransferase (U/L)	0.911	0.801-1.037	0.159
Alkaline phosphatase (U/L)	1.011	0.986-1.036	0.393
γ-Glutamyltransferase (U/L)	0.992	0.961-1.024	0.623
Total bilirubin (μmol/L)	1.071	0.971-1.180	0.171
Triglyceride (mmol/L)	1.145	0.361-3.632	0.818
Albumin (g/L)	0.902	0.805-1.009	0.072
Total cholesterol (mmol/L)	0.178	0.010-3.133	0.238
HDL-cholesterol (mmol/L)	9.130	0.448-185.8	0.150
LDL-cholesterol (mmol/L)	4.764	0.183-123.9	0.348
Fasting plasma glucose (mmol/L)	3.740	1.721-8.129	< 0.01
Hb1Ac (%)	1.011	0.350-2.924	0.983
Serum uric acid (μmol/L)	1.006	0.999-1.012	0.088
White blood cell (× 10^9/L)	0.919	0.708-1.194	0.527
Neutrophil (× 10^9/L)	1.329	1.004-1.758	< 0.05
Lymphocyte (× 10^9/L)	0.471	0.222-1.000	0.050
Hemoglobin (g/L)	1.012	0.955-1.072	0.695
Platelet count (× 10^9/L)	0.971	0.927-1.016	0.200
NLR	2.097	1.234-3.564	< 0.01
PLR	1.071	0.995-1.152	0.069
CRP (mg/L)	0.998	0.838-1.188	0.980
CEA (ng/mL)	1.882	1.195-2.964	< 0.01
CA199 (U/mL)	1.007	0.961-1.054	0.777

HDL-cholesterol: High density lipoprotein cholesterol; LDL-cholesterol: Low density lipoprotein cholesterol; CA199: Carbohydrate antigen 199; CEA: Carcinoembryonic antigen; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; CRP: C-reactive protein.

Current analysis revealed co-existence of atrophy, intestinal metaplasia and dysplasia in xanthelasma, indicating a potential pro-tumorigenesis role in gastric pathology. In previous prospective study, age/sex/atrophy-matched control analysis demonstrated that the presence of gastric xanthelasma was significantly associated with the presence of gastric cancer, indicating that xanthelasma could serve as a warning sign of gastric cancer[4]. Furthermore, in a Japanese cohort, with follow-up being more than three years, gastric xanthelasma was shown to be a useful marker for gastric cancer development[1]. Other than endoscopic diagnosis of gastric cancer, serum CEA level in xanthelasma group was significantly elevated. The exact mechanism behind this remained unknown. But it was speculated that reactive oxygen species (ROS) might be the mediator. First, increased release of ROS may be involved in accumulation of oxidized LDL cholesterol and development of gastric xanthelasma[14]. On this basis, ROS can cause DNA damage and associated oncogenic changes, thus lead to tumorigenesis[15,16]. However, molecular and cell biology experiments were needed to support the idea.

Table 6 Multivariable logistic regression of xanthelasma-related factors

Variables	OR	95%CI	*P*
Fasting plasma glucose (mmol/L)	3.347	1.170-9.575	< 0.05
Neutrophil (× 10^9/L)	1.617	1.003-2.605	< 0.05
CEA (ng/mL)	2.011	1.236-3.271	< 0.01

CEA: Carcinoembryonic antigen.

This study identified a slightly higher *H. pylori* infection rate in gastric xanthelasma. Recent years witnessed rapid increase rate in infection, especially in China[17]. As high as 57.6% people in population carried *H. pylori*[18]. *H. pylori* is a well-known cause of various gastrointestinal issues, such as peptic ulcer disease, gastroesophageal reflux disease and chronic active gastritis as well[19]. Chronic gastritis is thought to be involved in gastric glandular atrophy and intestinal metaplasia, which is considered as a precursor of gastric tumorigenesis[20]. In consistent with our findings, Hori and Tsutsumi first reported that *H. pylori* infection was seen on the surface of foveolar cells in 48% of biopsy samples of xanthelasma in 1996[5]. To further support that, Isomoto, in 1999, also reported a close relationship among *H. pylori* infection, xanthelasma, and atrophic gastritis[6]. Similarly, a large cohort in Korea also identified this association and proposed that xanthelasma may be provoked by *H. Pylori* infection[2].

In order to better understand role of chronic inflammation and xanthelasma, this study also investigated relationship between serum inflammatory biomarkers and xanthelasma, which revealed an increasing trend of neutrophil and a decrease in lymphocyte with negative relationship of CRP, an acute phase protein. Moreover, NLR index, defined as neutrophil counts divided by lymphocyte counts, was introduced. In most cases, lymphopenia well reflects impaired cell-mediated immunity, while neutrophilia represents a response to systematic inflammation, so high NLR represents systemic and local inflammatory state[21]. Emerging evidences have shown an important role of NLR in different cancer types and metabolic disease such as cardiovascular disease as well[22,23]. These results further

supported an inflamed environment in xanthelasma.

Xanthelasma was characterized by deposit of lipid in gastrointestinal tract. In this study, we found a positive association between xanthelasma and serum TG level accompanied by decreased HDL cholesterol. As a major component in metabolic syndrome, fatty liver was seen more frequent in xanthelasma patients. Glucose metabolism was also impaired in xanthelasma, supported by significantly higher fasting plasma glucose. However, Hb1Ac did not differ between groups. This indicated a scenario of slight fasting glucose intolerance, rather than well-established diabetes. Moreover, the amount of epicardial adipose tissue found in subjects with xanthelasma was higher and, the presence of xanthelasma was independently associated with supra-median epicardial fat thickness[24]. Whether these were random findings or potential causal relationship in pathogenesis still need further studies.

However, this study has several limitations. Firstly, with the nature of retrospective case-control study, no causal relationship can be identified between either xanthelasma and other gastric pathology or xanthelasma with metabolic disorders. Further prospective studies are needed in this concern and in other groups to improve generalizability. Secondly, as was a retrospective study, no standard procedure of xanthelasma treatment was set. Difference might existed among endoscopists. Rate of xanthelasma biopsy would be underestimated.

Current study described a large xanthelasma cohort in Chinese population, and revealed its positive relationship with *H. pylori* infection, atrophy, intestinal metaplasia, dysplasia, and metabolic disorder as well, indicating an important role of xanthelasma in both carcinogenesis and metabolic dysfunction. Logistic regression revealed role of fasting plasma glucose, neutrophil, and CEA as independent risk factors in xanthelasma.

COMMENTS

Background

Xanthelasma was a relatively rare endoscopic finding, characterized by accumulation of lipid in histiocytic foam cells in mucosa. To gain knowledge of xanthelasma, a large population-based study was conducted.

Research frontiers

As xanthelasma was defined as lipid deposit in stomach, its relevance to metobolic disorders was of great interest. Dyslipidemia, representative of lower mean high density lipoprotein-cholesterol and higher mean triglyceride levels was found in two Korean studies, accompanied with higher body mass index in gastric xanthelasma subjects in comparison with the controls. But, there were no reports in this concern in Chinese population yet.

Innovations and breakthroughs

Current study described a large xanthelasma cohort in Chinese population, revealed its positive relationship with *H. pylori* infection, atrophy, intestinal metaplasia, dysplasia, and metabolic disorder as well, indicating an important role of xanthelasma in both carcinogenesis and metabolic dysfunction.

Applications

With better understanding of xanthelasma pathogenesis and its relationship with metabolic, immunogenic and carcinogenic factors, clinical value of xanthelasma as predicting factor will be envisaged.

Peer-review

The authors studied the relationship between gastric xanthelasma and metabolic disorders in Chinese patients. They collected patient information from endoscopy archives and analysis the relationship of xanthelasma with several biochemical, metabolic and tumor markers.

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P- Reviewer: Ding SZ, Lankarani KB, Lee CL **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Lu YJ

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7765-7775

DOI: 10.3748/wjg.v23.i43.7765

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

ORIGINAL ARTICLE

Clinical Trials Study

Application of superb microvascular imaging in focal liver lesions

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Author contributions: He MN, Lv K, and Jiang YX designed the research; He MN and Lv K performed the research; Jiang TA contributed new reagents or analytic tools and analyzed the data; He MN wrote the paper.

Institutional review board statement: The study was reviewed and approved by the ethical committee of Peking Union Medical College Hospital.

Informed consent statement: Informed consent was provided by each patient before examination.

Conflict-of-interest statement: This article has no conflict of interests to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: August 13, 2017
Peer-review started: August 15, 2017
First decision: August 30, 2017
Revised: September 14, 2017
Accepted: September 19, 2017
Article in press: September 19, 2017
Published online: November 21, 2017

Abstract

AIM

To explore the ability of superb microvascular imaging (SMI) in differential diagnosis of focal liver lesions (FLLs) and to compare SMI morphology findings to those of color Doppler ultrasound and enhanced imaging.

METHODS

Twenty-four patients with 31 FLLs were included in our study, with diagnoses of hemangioma (HE) (n = 17), hepatocellular carcinoma (HCC) (n = 5), metastatic lesions (n = 5), primary hepatic lymphoma (n = 1), focal nodular hyperplasia (FNH) (n = 2), and adenoma (n = 1). Nine lesions were pathologically diagnosed, and 22 lesions were radiologically confirmed, all of which were evaluated by at least two types of enhanced imaging techniques. All patients had undergone SMI. Patients were divided into subgroups based on pathological and radiological diagnoses to analyze SMI manifestations. We also compared the SMI manifestations of the most common malignant FLLs (HCCs and metastatic lesions) with those of the most common benign FLLs (HEs).

RESULTS

HEs were classified into three SMI subgroups: diffuse dot-like type (n = 6), strip rim type (n = 8), and

nodular rim type (n = 3). The sizes of the three types of HEs were significantly different (P = 0.00, < 0.05). HCCs were classified into two subgroups: diffuse honeycomb type (n = 2) and non-specific type (n = 3). Four of the metastatic lesions were the strip rim type, and the other metastatic lesion was the thick rim type, which is the same as that of lymphoma. FNH was described as a spoke-wheel type, and adenoma as a diffuse honeycomb type. The SMI types of HCCs and metastatic lesions were significantly different from those of HEs (P = 0.048, < 0.05).

CONCLUSION

SMI technology enables microvascular evaluation of FLLs without using any contrast agent. For HEs, lesion size may affect SMI performance. SMI is able to provide useful information for differential diagnosis of HCCs and metastatic lesions from HEs.

Key words: Primary hepatic lymphoma; Hemangioma; Color doppler ultrasound; Focal liver lesions; Superb microvascular imaging

Core tip: We utilized a novel ultrasound technique, superb microvascular imaging (SMI), to assess the microvascular morphology of focal liver lesions to provide additional diagnostic information. The focal liver lesions consisted of hemangiomas, hepatocellular carcinomas, metastatic lesions, primary hepatic lymphoma, focal nodular hyperplasia, and adenoma. We also compared SMI manifestations to color Doppler ultrasound and enhanced imaging features.

He MN, Lv K, Jiang YX, Jiang TA. Application of superb microvascular imaging in focal liver lesions. *World J Gastroenterol* 2017; 23(43): 7765-7775 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7765.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7765

INTRODUCTION

The detection rate of focal liver lesions (FLLs) is clearly increasing because of the widespread application of imaging techniques, especially ultrasound examinations. Kaltenbach *et al*[1] investigated 45319 hospitalized patients and found that the prevalence of benign FLLs was 15.1%. Hepatocellular carcinoma (HCC) is the second most common cause of mortality from cancer[2,3]. The current diagnostic challenge not only involves effectively distinguishing between malignant and benign FLLs[4,5] but also precisely identifying the characteristics of all types of FLLs since they require different clinical treatments and have different outcomes. For example, treatments for hemangioma (HE) and adenoma and those for HCC and lymphoma differ significantly.

To address this challenge, it is essential to establish a diagnostic method that is inexpensive, easy to operate, and has high diagnostic accuracy. Contrast-enhanced ultrasound (CEUS) has been gradually recognized as a comparable imaging technique to contrast-enhanced computed tomography (CECT) and contrast-enhanced magnetic resonance imaging (CEMRI) in the diagnosis of FLLs[6,7]. The common advantage of the above three techniques is visualization of the microvascular structure, which is one of the most important elements of the tumor microenvironment[8], plays an important role in the development and progression of lesions, and is essential for their differential diagnosis. However, these techniques also have drawbacks. Some patients may be ineligible for these examinations because of contraindications to contrast agents, such as the risk of triggering or worsening renal failure with the iodinated contrast agents used for computed tomography (CT) and gadolinium diethylene-trianmine pentaacetic acid (Gd-DTPA) used for magnetic resonance imaging (MRI)[9], as well as the risk of hypersensitivity reactions caused by the agents used in CEUS, CECT, and CEMRI[10]. Additionally, these techniques are expensive and time-consuming, limiting their widespread use.

Superb microvascular imaging (SMI) is a novel Doppler technique developed by Toshiba Medical System (Tokyo, Japan)[11], which was designed to simulate CEUS by using advanced clutter elimination to obtain only vascular flow signals without using any contrast agents[12]. Similar to color Doppler and power Doppler imaging, SMI can provide a real-time examination of vascularity in FLLs, but it has the additional advantages of detecting slower blood flow and revealing micro-vessels. Studies on SMI technology in superficial tissues, such as thyroid and breast tumors, have been reported, with some useful information obtained for differential diagnoses[11,13]. Machado *et al*[13] evaluated the capability of SMI to show microvascular flow in normal thyroid tissue and in thyroid nodules compared with that of CDFI and found that SMI was able to better depict vessel branching. Zhan *et al*[14] compared the abilities of CDFI and SMI to reveal penetrating vessels (PVs) in breast cancer and discovered that more PVs were evident by SMI than by CDFI. By contrast, very few studies have been conducted on SMI for FLLs[15]. The purpose of this study was to investigate the SMI features of FLLs and to analyze the ability of SMI to provide additional information for the differential diagnosis of FLLs.

MATERIALS AND METHODS

Patients and FLLs

This study was performed from November 2016 to March 2017 at Peking Union Medical College Hospital. The study was approved by the ethical committee of the hospital, and informed consent was provided by

Table 1 Characteristics of focal liver lesions and corresponding patients

No.	Clinical diagnosis	Pathological diagnosis	SMI type (Ⅰ-Ⅶ)	Size (cm)	Age (yr)	Sex	Other
1	HE	-	Ⅰ	1.7	79	M	
2	HE	-	Ⅰ	1.1	58	F	
3	HE	-	Ⅰ	2.6	54	M	
4	HE	-	Ⅰ	2.5	52	F	
5	HE	-	Ⅰ	1.8	24	F	Same person
6	HE	-	Ⅰ	2.7	24	F	
7	HE	-	Ⅱ	2.3	63	M	
8	HE	-	Ⅱ	3.9	61	M	Same person
9	HE	-	Ⅱ	4.8	61	M	
10	HE	-	Ⅱ	3.7	61	F	
11	HE	-	Ⅱ	2.8	48	M	Same person
12	HE	-	Ⅱ	3.1	48	M	
13	HE	-	Ⅱ	3.1	41	M	
14	HE	-	Ⅱ	5.7	33	F	
15	HE	-	Ⅲ	6.3	63	F	
16	-	HE	Ⅲ	8.4	51	F	
17	-	HE	Ⅲ	8.5	47	F	
18	B-M	-	Ⅱ	1.4	39	F	Same person
19	B-M	-	Ⅱ	2.3	39	F	
20	B-M	-	Ⅱ	2.6	39	F	
21	B-M	-	Ⅱ	3.3	39	F	
22	P-M	-	Ⅵ	3.9	64	M	
23	-	HCC	Ⅳ	7.4	68	M	
24	-	HCC	Ⅳ	6.1	57	M	
25	-	HCC	Ⅴ	2.5	60	M	
26	-	HCC	Ⅴ	2.9	48	M	Same person
27	-	HCC	Ⅴ	4.7	48	M	
28	-	HA	Ⅳ	5.3	41	M	
29	-	LYM	Ⅵ	5.8	71	M	
30	FNH	-	Ⅶ	4.1	62	F	
31	FNH	-	Ⅶ	4.3	39	F	

Type Ⅰ: Diffuse dot-like type; Type Ⅱ: Strip rim type; Type Ⅲ: Nodular rim type; Type Ⅳ: Diffuse honeycomb type; Type Ⅴ: Non-specific type; Type Ⅵ: Thick rim type; Type Ⅶ: Spoke-wheel type; FLL: Focal liver lesion; HE: Hemangioma; B-M: Metastatic lesion from breast; P-M: Metastatic lesion from pancreas; M: Metastatic lesion; HCC: Hepatocellular carcinoma; FNH: Focal nodular hyperplasia; HA: Hepatic adenoma; PHL: Primary hepatic lymphoma.

each patient before examination. Twenty-four patients (mean age, 53.5 ± 12.9 years, range 24-79 years; 13 men and 11 women) with 31 FLLs were included in our study. All FLLs had been detected by at least two modalities of CEUS/CT/MRI. Of all FLLs, nine were pathologically diagnosed, including two HEs in two patients, five HCCs in four patients, one hepatic adenoma, and one primary hepatic lymphoma, and 22 were radiologically confirmed, including 15 HEs in 12 patients (a single lesion in nine patients and two lesions in three patients), which were diagnosed by CECT and/or CEMRI and showed typical manifestations of nodular or strip type, with peripheral to centripetal enhancement and persistent enhancement in the portal venous phase and the delayed phase. Five metastatic masses (one patient had a single lesion and another patient had four lesions) were diagnosed by the primary tumor history and CECT/CEUS, which showed malignant characteristics with arterial enhancement that disappeared quickly in the portal venous phase. Two focal nodular hyperplasia (FNH) lesions were diagnosed by CECT based on persistent enhancement from the arterial phase to the portal venous/delayed phase, clearly defined outlines, and a central scar. Table 1 summarizes the clinical, SMI, and pathologic features of the 31 FLLs.

SMI examination and imaging analysis

Patients were placed in the supine or left lateral position after 6 h of fasting. All US examinations, including B-mode US, CDFI, and SMI, were performed with a curved transducer (6C1Aplio 500; Toshiba Medical Systems Corporation, Tochigi, Japan). B-mode US was performed first to thoroughly scan the liver for FLLs. Once detected, the general features of FLLs were observed and their sizes (maximal diameter) were measured. Subsequently, conventional CDFI and SMI were performed to observe the vascular structures of the FLLs. For the CDFI examination, the scale was set as low as possible until the appropriate level was reached without any pseudo color flow, such as color flow spillover (the lowest scale was 4 cm/s), and the flow gain was adjusted until noise emerged. For the SMI examination, the parameter settings were as follows: color velocity scale of no more than 2.0 cm/s, frame rate > 30 fps, color frequency 5-7 MHz, and the gain setting adjusted to show optimal imaging. All US examinations were performed by a single operator, and

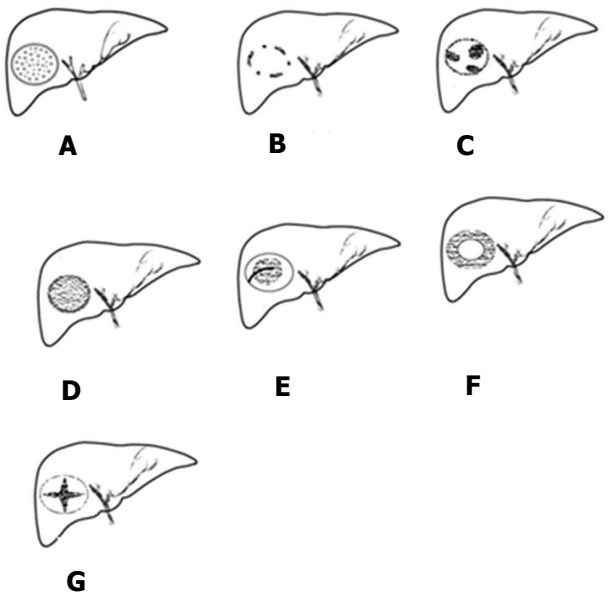

Figure 1 A simplified diagram of the seven superb microvascular imaging types. A: Type Ⅰ, diffuse dot-like type; B: Type Ⅱ, strip rim type; C: Type Ⅲ, nodular rim type; D: Type Ⅳ, diffuse honeycomb type; E: Type Ⅴ, non-specific type; F: Type Ⅵ, thick rim type; G: Type Ⅶ, spoke-wheel type.

Table 2 Size, average age, and sex distribution of the three types of hemangiomas

Type	Size	Yr	Sex
Ⅰ	2.07 ± 0.63	48.5 ± 21.3	2\4
Ⅱ	3.68 ± 1.12	52.0 ± 11.2	6\2
Ⅲ	7.73 ± 1.24	53.7 ± 8.3	0\3
P value	0	0.89	0.06

Type Ⅰ: Diffuse dot-like type; Type Ⅱ: Strip rim type; Type Ⅲ: Nodular rim type.

the imaging data were analyzed by two experienced radiologists. Both the operator and readers had > 10 years of experience in liver ultrasound. The readers classified the SMI characteristics of the FLLs into seven types (Figure 1): Ⅰ, diffuse dot-like type; Ⅱ, strip rim type; Ⅲ, nodular rim type; Ⅳ, diffuse honeycomb type; Ⅴ, non-specific type; Ⅵ, thick rim type with lymphoma; and Ⅶ, spoke-wheel type[15]. If a disagreement occurred, the decision was determined by consensus after consultation with a third experienced doctor. We divided the 31 FLLs into a ⩽ 3.0 cm group and a > 3.0 cm group[16], and then compared vascular visibility between CDFI and SMI. We also compared the SMI types of the most common malignant FLLs (HCCs and metastatic lesions) and those of the most common benign FLLs (HEs).

Statistical analysis

Differences in SMI types between the most common malignant FLLs (HCCs and metastatic lesions) and the most common benign FLLs (HEs) were evaluated by Fisher's exact test. The χ^2 test was used to compare the sex distribution of HEs between different SMI types. Differences in size and age were evaluated by a one-way ANOVA test. $P < 0.05$ was considered statistically significant. SPSS 16.0 was used for all data analyses.

RESULTS

All 31 FLLs underwent successful US examinations, including B-mode US, CDFI, and SMI, and satisfactory images were obtained. Of the 17 HEs, the SMI features could be divided into three types: diffuse dot-like type (Type Ⅰ; *n* = 6; Figure 2), strip rim type (Type Ⅱ; *n* = 8; Figure 3), and nodular rim type (Type Ⅲ; *n* = 3; Figure 4). The sizes of the three types of HEs were significantly different (*P* = 0.00, < 0.05), but the average age and sex distributions of the patients showed no significant differences (Table 2).

The SMI features of the 14 remaining FLLs were as follows: two of the HCCs were described as the diffuse honeycomb type (Type Ⅳ, Figure 5) and three were defined as the non-specific type (Type Ⅴ, Figure 6). Four of the metastatic lesions were from breast cancer in one patient and were classified as the strip rim type (Type Ⅱ), and one was from pancreatic cancer and was classified as the thick rim type (Type Ⅵ, Figure 7), with the lesion reflecting primary hepatic lymphoma. Two FNH lesions were described as the spoke-wheel type (Type Ⅶ, Figure 8), and the adenoma was described as the diffuse honeycomb type (Type Ⅴ). The distributions of SMI types between the most common malignant FLLs (HCCs and metastatic lesions) and the most common benign FLLs (HEs) were significantly different (*P* = 0.048, < 0.05) (Table 3), and these morphological findings of SMI types in different FLLs were consistent with the findings from contrast-enhanced ultrasound/CT/MRI. The characteristics of all FLLs and the corresponding patients are summarized in Table 1.

Among the 31 FLLs, 13 were small FLLs with a maximum diameter of less than 3.0 cm and 17 were larger than 3.0 cm. SMI could detect the vascular structures of all 31 lesions, while CDFI failed to detect the vascular structures of nine (69.2%) lesions in the < 3.0 cm group and two (11.8%) lesions in the > 3.0 cm group.

DISCUSSION

Angiogenesis, an important part of the tumor microenvironment[17], plays a key role in the development of FLLs. The morphology of blood vessels in lesions is also important for differential diagnoses. CDFI had been widely used to depict tumor vessels, but it is limited in identifying low-speed flow signals because of the wall filter, which suppresses clutter and motion artifacts and results in the loss of low-speed flow signals. SMI, a novel technique, can overcome this limitation and can effectively distinguish low-speed flow signals from

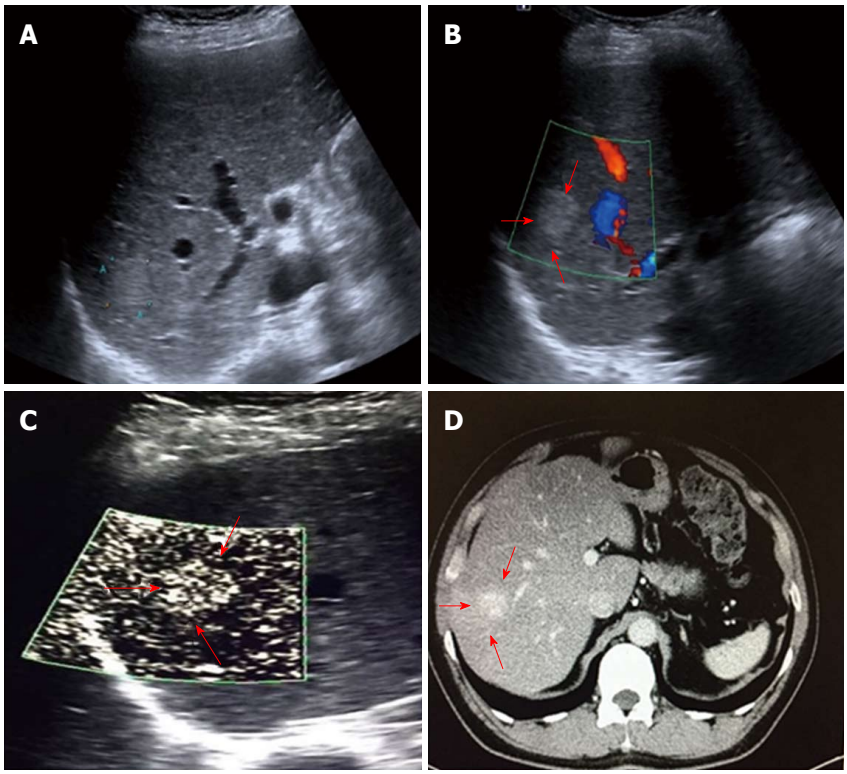

Figure 2 Diffuse dot-like type (type Ⅰ) in a 52-year-old male diagnosed with hemangioma. A: A high-echo lesion with a clear margin was evident in the right liver lobe; B: CDFI showed no blood flow signals for this lesion; C: SMI showed a diffuse dot-like microvascular structure; D: Contrast-enhanced CT showed diffuse enhancement of the lesion in the arterial phase. SMI: Superb microvascular imaging.

Table 3 Distributions of the superb microvascular imaging types in focal liver lesions *n* (%)

Group	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ	Ⅶ
HE (17)	6 (35.2)	8 (47.1)	3 (17.6)	0 (0)	0 (0)	0 (0)	0 (0)
M (5)	0 (0)	4 (80)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)
HCC (5)	0 (0)	0 (0)	0 (0)	2 (40)	3 (60)	0 (0)	0(0)
FNH (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)
HA (1)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
PHL (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)

The distributions of the superb microvascular imaging types between the most common malignant focal liver lesions (hepatocellular carcinoma and metastatic lesions) and the most common benign focal liver lesions (hemangiomas) were significantly different (P = 0.048, < 0.05). Type Ⅰ: Diffuse dot-like type; Type Ⅱ: Strip rim type; Type Ⅲ: Nodular rim type; Type Ⅳ: Diffuse honeycomb type; Type Ⅴ: Non-specific type; Type Ⅵ: Thick rim type; Type Ⅶ: Spoke-wheel type; HE: Hemangioma; M: Metastatic lesion; HCC: Hepatocellular carcinoma; FNH: Focal nodular hyperplasia; HA: Hepatic adenoma; PHL: Primary hepatic lymphoma.

artifacts without the use of any contrast agent. In the current study, we compared the abilities of CDFI and SMI to detect the vascular structures of all 31 FLLs, and the results showed that SMI could detect flow information in all lesions, but CDFI failed to detect the vascular structures of nine (69.2%) lesions in the < 3.0 cm group and two (11.8%) lesions in the > 3.0 cm group, suggesting that SMI has obvious advantages in detecting the blood vessels of FLLs. Therefore, SMI overcomes the limitation of CDFI, especially for the description of micro-vessels in small lesions.

Studies on the application of SMI for FLLs are limited, and only two investigations have been reported: one was reported by Wu *et al*[18], in which SMI clearly demonstrated the typical spoke-wheel vascular type of FNH in the liver without the use of any contrast agent, and the other was reported by Lee *et al*[15], who used SMI for 29 FLLs, including HE, HCC, and FNH, and concluded that the SMI types were significantly different between FLLs. In the present study, we analyzed the SMI features of 31 FLLs, including HEs, HCC, metastatic lesions, FNH, hepatic adenoma, and primary hepatic lymphoma. We used seven SMI types to depict the vessel distributions and morphologies of the 31 FLLs. We found that the SMI type distribution between the most common malignant FLLs (HCCs and metastatic lesions) and the most common benign FLLs (HEs) differed significantly, which could provide meaningful differential diagnostic information. Meanwhile, we also found that various

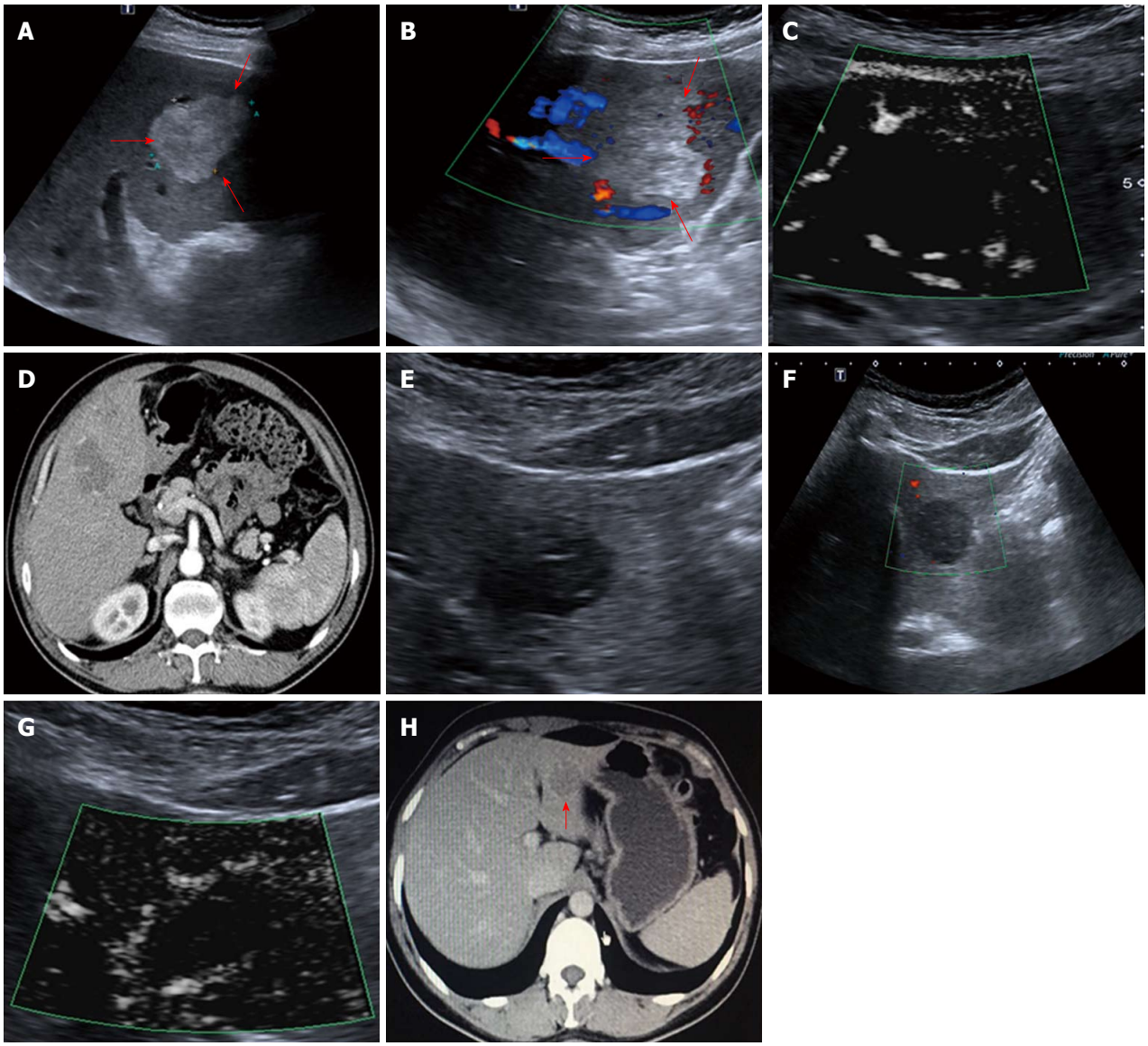

Figure 3 Strip rim type (type Ⅱ). A-D: A 61-year-old male diagnosed with hemangioma. A: A high-echo lesion with a clear margin was evident in the left liver lobe; B: CDFI showed an interrupted strip blood flow signal around the edge of this lesion; C: SMI showed a relatively continuous strip rim-distributed microvascular structure; D: Contrast-enhanced CT showed strip rim enhancement of the lesion in the arterial phase. E-H: A 63-year-old male diagnosed with hemangioma. E: A low-echo lesion with a clear margin was evident in the left liver lobe; F: CDFI showed no blood flow signal for this lesion; G: SMI showed a continuous strip rim microvascular structure; H: Contrast-enhanced CT showed strip rim enhancement of the lesion in the arterial phase. SMI: Superb microvascular imaging.

FLLs shared the same SMI type. For example, the metastatic lesions from breast cancer and HEs shared the strip rim type (Type Ⅱ), and the other metastatic lesion from pancreatic cancer and primary hepatic lymphoma both showed the thick rim type (Type Ⅵ). Hepatic adenoma and some HCCs had the same diffuse honeycomb type (Type Ⅳ). Therefore, when an FLL is suspected to be a certain SMI type, other clinical information, such as a medical history, is still needed to determine an accurate diagnosis.

For HEs, the average maximum diameters of type Ⅰ, type Ⅱ, and type Ⅲ lesions were 2.07 ± 0.63 cm, 3.68 ± 1.12 cm, and 7.73 ± 1.24 cm, respectively, and the lesion sizes of the three different SMI types were significantly different. The histology of the HEs can explain the SMI manifestations. First, the original etiology of HEs was not clear, but congenital vessel malformation caused by hyperplastic endothelial cells may be the cause[19]. Consequently, the microscopic features of HEs appeared like a blood pool constituted by cavernous vascular spaces, which were lined by a single layer of flat endothelial cells of different sizes. The vascular cavities varied in size and corresponded to the size of HE lesions. Some HEs may contain a thrombus that will gradually turn into a fibrous scar or nodules, especially in large lesions[20]. Therefore, for larger HEs, the internal blood flow may be slower or even completely replaced by the thrombus, so an enhanced examination can reveal peripheral nodules or ring-enhanced patterns. SMI also reveals these features, especially in type Ⅱ and type Ⅲ. For small HEs, both enhanced techniques and SMI could show

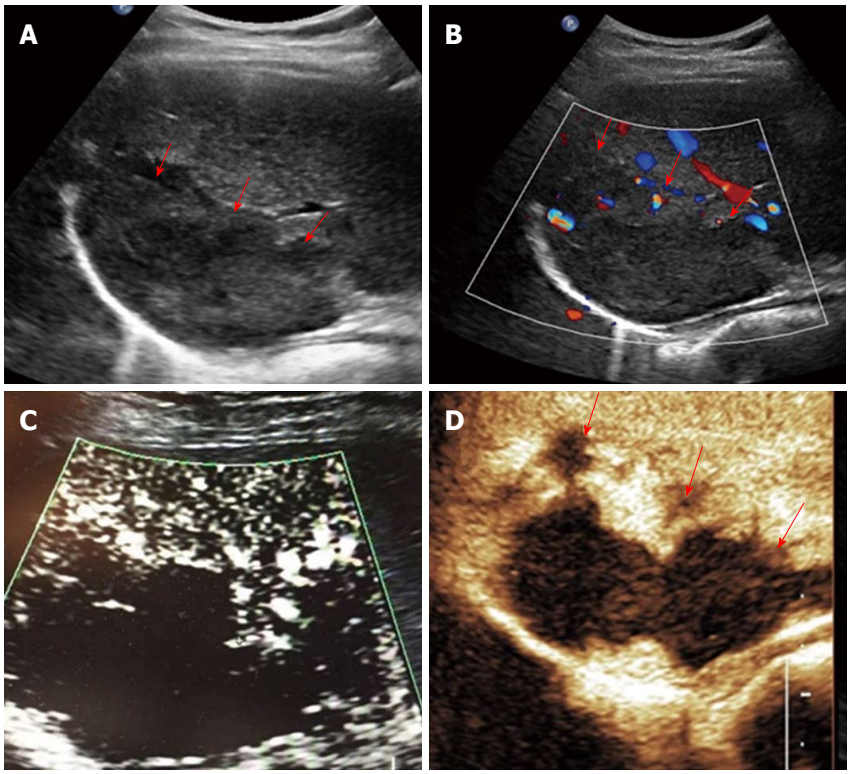

Figure 4 Nodular rim type (type Ⅲ) in a 51-year-old female diagnosed with hemangioma. A: A mixed-echo lesion with a relatively clear margin was evident in the right liver lobe; B: CDFI showed a sporadic short strip blood flow signal around the edge of this lesion; C: SMI showed a nodular rim-distributed microvascular structure; D: Contrast-enhanced ultrasound showed nodular rim enhancement of the lesion in the arterial phase. SMI: Superb microvascular imaging.

slow internal vascular signals, as in type Ⅰ. According to the previous statements, type Ⅰ and type Ⅲ had distinctive features enabling the discrimination of HEs from other FLLs.

Regarding the other types of FLLs, the previous SMI study on FLLs by Lee *et al*[15] showed that the SMI manifestations of HCCs revealed non-specific vascular types, while in the present study, we used two SMI types to summarize HCCs, including type Ⅳ, the diffuse honeycomb type, and type Ⅴ, the non-specific type. The general pathology of the former type showed that the inter-tumor blood vessels were distributed in a grid pattern resembling honeycombs. The SMI feature of the latter type was a strip-like trunk with tiny branches, but we do not have enough evidence from other enhanced techniques or pathology to support it. Therefore, we classified it as the non-specific type. In this research, including two cases of FNH with both CDFI and SMI showing the typical spoke-wheel type without a basic echo of the liver parenchyma, SMI seemed to show the vascular structure more clearly, which is consistent with the previous studies[15,18].

For metastatic lesions, it is always assumed that their imaging results are particularly confusing because metastatic lesions can simulate various other types of FLLs, as in the present study. The SMI types of metastatic lesions were similar to those of HEs and lymphoma. Therefore, in the diagnosis of metastatic lesions, a clinical history including the primary tumor is critical in addition to imaging studies.

Research on the ultrasound features of hepatic adenomas is relatively rare because of the low morbidity compared to other FLLs. Dong *et al*[21] conducted a retrospective study to analyze differences in ultrasound and CEUS features between hepatic adenoma and HCC, and the results showed that most cases of hepatic adenomas manifested as homogenous, rapid, and complete enhancement in the arterial phase, which is similar to HCC. In this study, our case of hepatic adenoma showed the same SMI type (type Ⅳ, diffuse honeycomb type) as some HCCs. This patient was diagnosed with a benign lesion or a relatively mild malignant mass before surgery because of the very clear margin and the slow wash-out pattern on CECT. SMI for this case did not show unique characteristic performance and was limited to the diagnosis of hepatic adenoma, requiring supplementation with an enhanced imaging examination.

Primary hepatic lymphoma (PHL) is also a rare disease, accounting for only 0.016% of all cases of non-Hodgkin's lymphoma (NHL)[22]. The treatment for PHL includes surgery, chemotherapy, and radiotherapy, which is significantly different from that for HCC or other malignant FLLs. Therefore, an accurate diagnosis before surgery is essential. Research by Lu *et al*[23] showed that one CECT manifestation of some PHLs was rim-like enhancement, which was similar to the features revealed by CEUS and SMI. Therefore, SMI

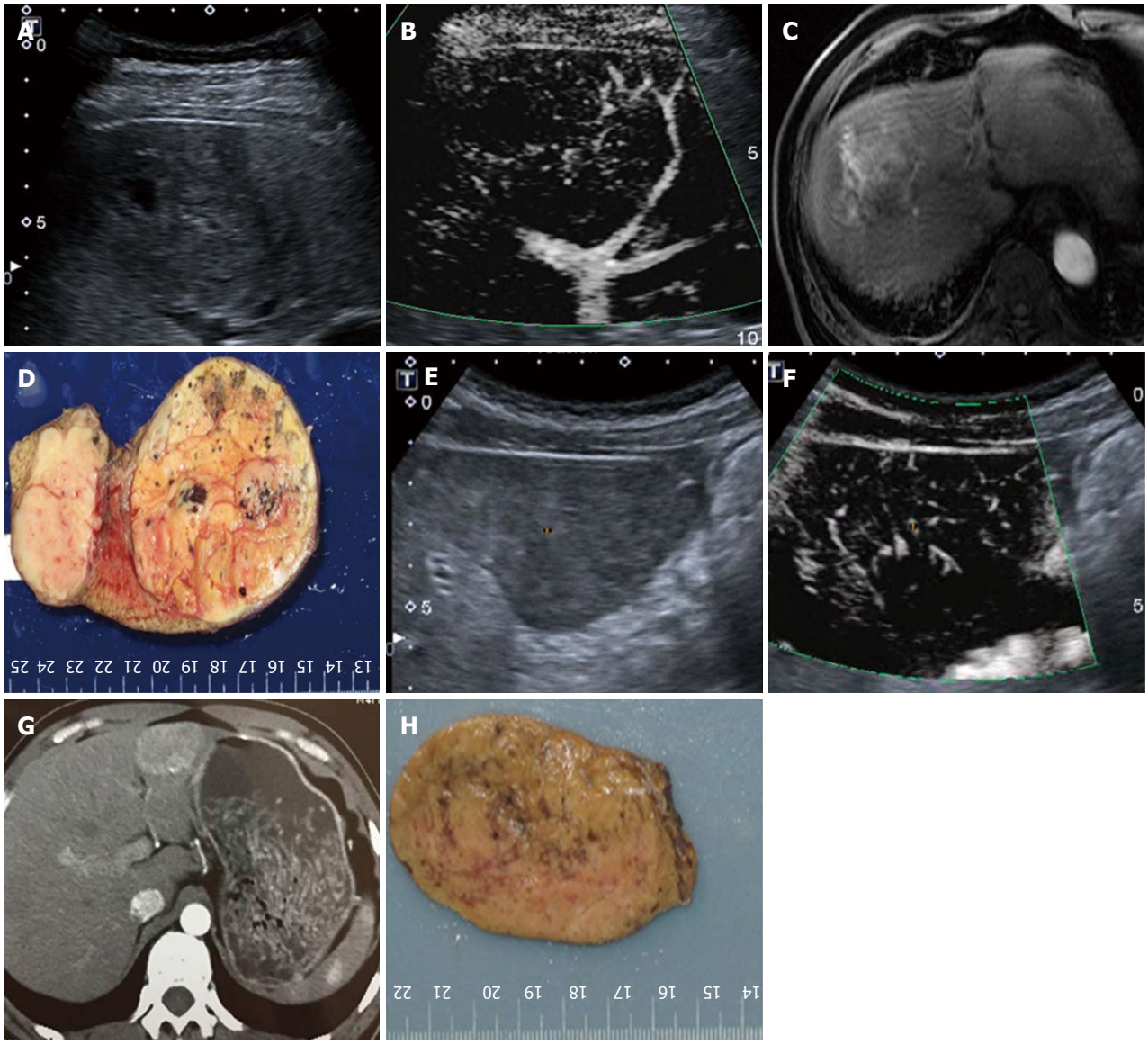

Figure 5 Diffuse honeycomb type (type Ⅳ). A-D: A 68-year-old male diagnosed with hepatic cellular carcinoma. A: A mixed-echo lesion with a relatively unclear margin was evident in the right liver lobe; B: SMI showed a diffuse honeycomb-distributed microvascular structure; C: Contrast-enhanced MRI showed diffuse enhancement of the lesion in the arterial phase; D: The pathology result showed that the inter-tumor blood vessels were distributed in a grid pattern resembling honeycombs. E-H: A 41-year-old male diagnosed with hepatic adenoma. E: A hypo-echo lesion with a clear margin was evident in the left liver lobe; F: SMI showed a diffuse honeycomb-distributed microvascular structure; G: Contrast-enhanced CT showed diffuse enhancement of the lesion in the arterial phase; H: The pathology result showed that the inter-tumor blood vessels were distributed in a grid pattern resembling honeycombs. SMI: Superb microvascular imaging.

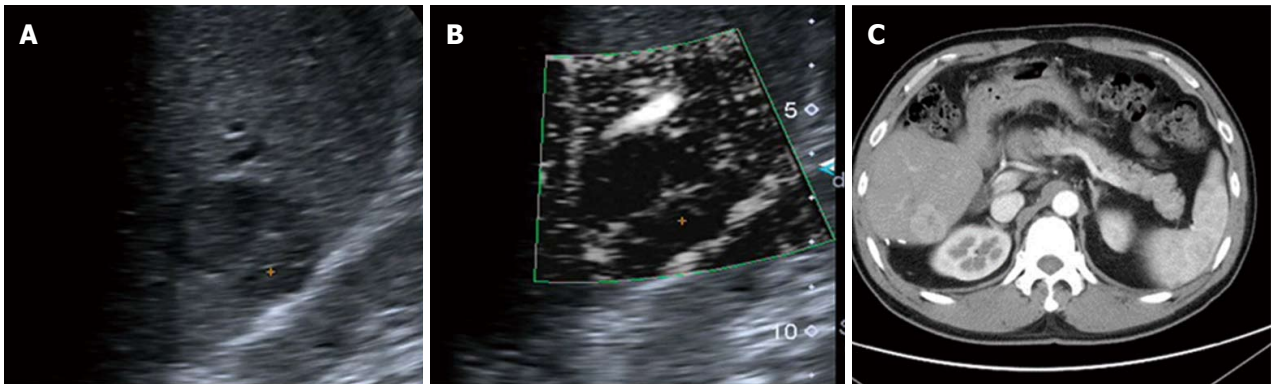

Figure 6 Non-specific type (type Ⅴ) in a 48-year old male diagnosed with hepatic cellular carcinoma. A: A low-echo lesion with a relatively clear margin was evident in the right liver lobe; B: SMI showed a microvascular distribution of a strip trunk with tiny branches ; C: Contrast-enhanced CT showed diffuse enhancement of the lesion in the arterial phase. SMI: Superb microvascular imaging.

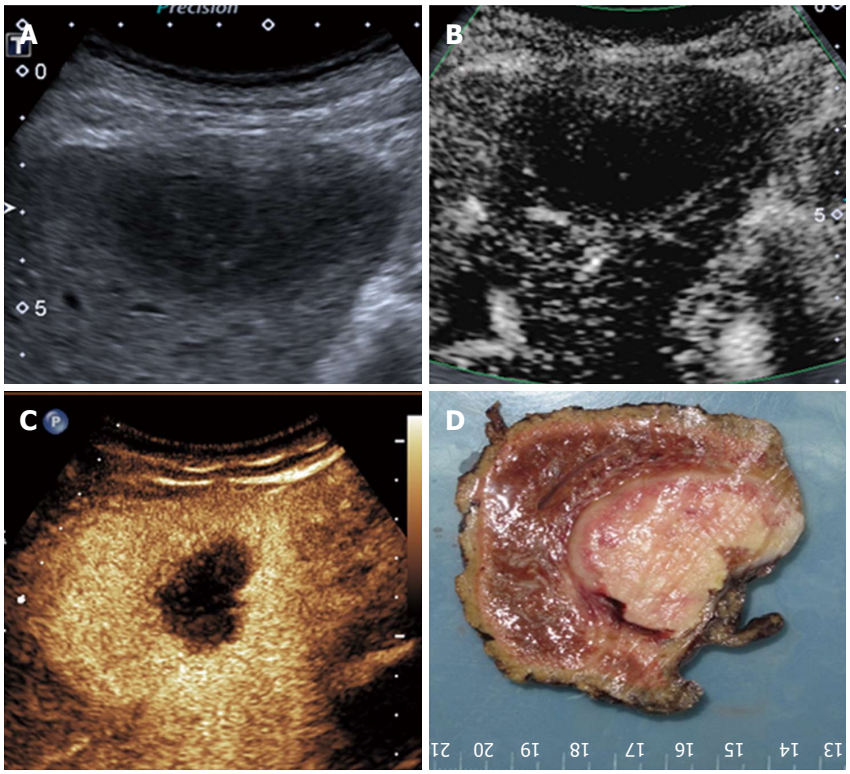

Figure 7 Thick rim type (type Ⅵ) in a 71-year-old male diagnosed with primary hepatic lymphoma. A: A low-echo lesion with a relatively clear margin was evident in the left liver lobe; B: SMI showed a thick rim-distributed microvascular structure; C: Contrast-enhanced ultrasound showed thick rim enhancement of the lesion in the arterial phase; D: The gross pathology result showed a thick rim distribution of the vasculature. SMI: Superb microvascular imaging.

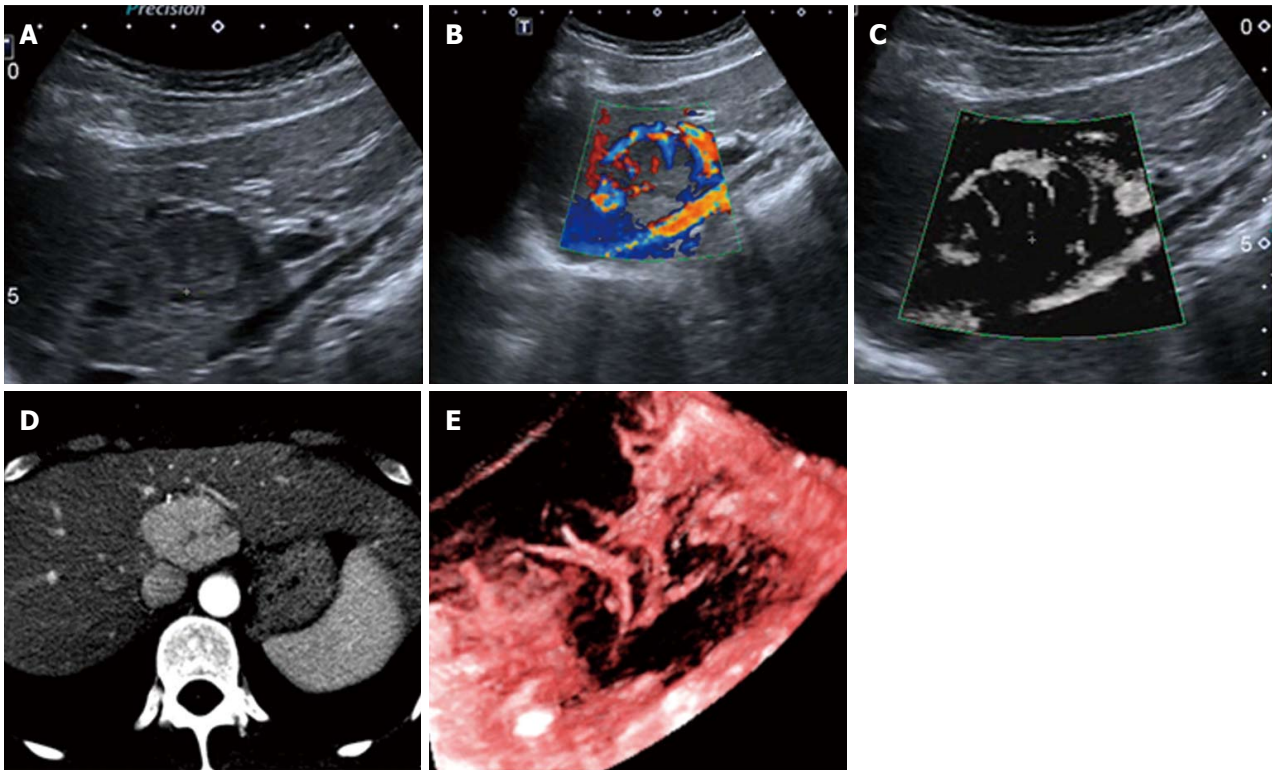

Figure 8 Spoke-wheel type (type Ⅶ) in a 39-year-old female diagnosed with focal nodular hyperplasia. A: A low-echo lesion with a relatively clear margin was evident in the caudate liver lobe; B: CDFI showed a spoke-wheel blood flow signal of this lesion; C: SMI showed a spoke-wheel-distributed microvascular structure; D: Contrast-enhanced CT showed diffuse enhancement with a central scar of the lesion in the arterial phase; E: 3-D vascular remodeling of this lesion was successfully achieved and showed spoke-wheel blood flow. SMI: Superb microvascular imaging.

may provide some helpful information for the diagnosis of some PHLs.

Our study has some limitations. First, for hepatic adenoma and primary hepatic lymphoma, the incidence rates are very low and sample errors are inevitable. Second, only nine lesions were pathologically diagnosed in this study. Therefore, a very low possibility of missed diagnosis remains for the other 22 lesions that were radiologically confirmed because as the results showed, different types of FLLs may have similar imaging manifestations despite the use of at least two kinds of enhanced imaging techniques.

In conclusion, SMI technology allows evaluation of the microvascular structures of FLLs without using any contrast agent. For HEs, the lesion size may affect SMI performance. SMI can overcome the limitation of CDFI, especially for micro-vessel descriptions in small lesions. The SMI characteristics between the most common malignant FLLs (HCCs and metastatic lesions) and the most common benign FLLs (HEs) are significantly different.

ARTICLE HIGHLIGHTS

Research background

The frequency of focal liver lesion (FLL) detection is increasing because of the development and prevalence of imaging technology, especially ultrasound examinations. The subsequent challenge not only involves efficiently distinguishing between malignant and benign FLLs but also precisely identifying the characteristics of all types of FLLs as different clinical treatments and outcomes may be inherent to each type.

Addressing this challenge involves choosing a diagnostic method that requires minimal time and effort, but can achieve high diagnostic accuracy. Some patients may be ineligible for the currently used imaging techniques, such as CEUS/CT/MRI, because of the risk of triggering or worsening renal failure due to contrast agents, such as iodine used for CT and Gd-DTPA (gadolinium diethylene-trianmine pentaacetic acid) used for MRI. In addition, the agents used in CEUS, CECT, and CEMRI are foreign bodies, and each one could cause hypersensitivity reactions. Additionally, the three techniques are expensive and time-consuming, limiting their widespread application.

Research motivation

Superb microvascular imaging (SMI) is a novel Doppler technique that simulates enhanced ultrasound by using advanced clutter elimination to obtain only vascular flow signals without using any contrast agent. The purpose of our study was to investigate the SMI features of focal liver lesions and to analyze their ability to provide additional information for differential diagnoses.

Research objectives

To explore the ability of SMI to differentially diagnose focal liver lesions and compare SMI morphologies to those of color Doppler ultrasound and enhanced imaging.

Research methods

Twenty-four patients with 31 focal liver lesions (FLLs) were included in our study, with diagnoses of hemangioma (HE) (n = 17), hepatocellular carcinoma (HCC) (n = 5), metastatic lesions (n = 5), primary hepatic lymphoma (n = 1), focal nodular hyperplasia (FNH) (n = 2), and adenoma (n = 1). Nine lesions were pathologically diagnosed, and 22 lesions were radiologically confirmed, all of which were evaluated by at least two types of enhanced imaging techniques. All patients had undergone SMI. Patients were divided into subgroups based on pathological and radiological diagnoses to analyze SMI manifestations. We also compared the SMI manifestations of the most common malignant FLLs of HCCs and metastatic lesions with those of the most common benign FLLs of HEs.

Research results

HEs were classified into three SMI subgroups: diffuse dot-like type (n = 6); strip rim type (n = 8); and nodular rim type (n = 3). The sizes of the three types of HEs were significantly different (P = 0.00, < 0.05). HCCs were classified into two subgroups: diffuse honeycomb type (n = 2) and non-specific type (n = 3). Four of the metastatic lesions were the strip rim type of HE, and the other metastatic lesion was the thick rim type, which is the same as that of lymphoma. FNH was described as a spoke-wheel type, and adenoma as a diffuse honeycomb type. The SMI types of HCCs and metastatic lesions were significantly different from that of HEs (P = 0.048, < 0.05).

Research conclusions

SMI technology enables microvascular evaluation of focal liver lesions without using any contrast agent. For HEs, lesion size may affect SMI performance. SMI is able to provide useful information for differentially diagnose HCCs and metastatic lesions from HEs.

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P- Reviewer: Dietrich CF, Eleftheriadis NP, Tarantino G
S- Editor: Wei LJ **L- Editor**: Wang TQ **E- Editor**: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 November 21; 23(43): 7776-7784

DOI: 10.3748/wjg.v23.i43.7776

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

ORIGINAL ARTICLE

Observational Study

Chronic liver disease is universal in children with biliary atresia living with native liver

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Author contributions: Lee WS and Ong SY conceived the concept of the present research; Ong SY, Foo HW, Kong CX, Seah RB and Ng RT collected the clinical data; Wong SY provided statistical analysis; Lee WS and Ong SY wrote the first draft; Lee WS provided critical analysis to the draft; Lee WS revised the final draft; All authors approved the final draft.

Supported by University Malaya High Impact Research (UM.C/625/HIR/MOHE/CHAN/13/1) from Ministry of Higher Education, Malaysia.

Institutional review board statement: The study was reviewed and approved by the University Malaya Medical Centre, Kuala Lumpur Medical Ethical Committee (MEC 902.15).

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

Conflict-of-interest statement: None of the authors has any potential conflict of interest to declare.

Manuscript source: Unsolicited manuscript

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Received: June 9, 2017
Peer-review started: June 10, 2017
First decision: July 13, 2017
Revised: August 3, 2017
Accepted: August 15, 2017
Article in press: August 15, 2017
Published online: November 21, 2017

Abstract

AIM

To examine the medical status of children with biliary atresia (BA) surviving with native livers.

METHODS

In this cross-sectional review, data collected included complications of chronic liver disease (CLD) (cholangitis in the preceding 12 mo, portal hypertension, variceal bleeding, fractures, hepatopulmonary syndrome, portopulmonary hypertension) and laboratory indices (white cell and platelet counts, total bilirubin, albumin, international normalized ratio, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase). Ideal medical outcome was defined as absence of clinical evidence of CLD or abnormal laboratory indices.

RESULTS

Fifty-two children [females = 32, 62%; median age 7.4 years, *n* = 35 (67%) older than 5 years] with BA (median age at surgery 60 d, range of 30 to 148 d) survived with native liver. Common complications of CLD noted were portal hypertension (40%, *n* = 21; 2 younger than 5 years), cholangitis (36%) and bleeding varices (25%, *n* = 13; 1 younger than 5 years). Fifteen (29%) had no clinical complications of CLD and three

(6%) had normal laboratory indices. Ideal medical outcome was only seen in 1 patient (2%).

CONCLUSION

Clinical or laboratory evidence of CLD are present in 98% of children with BA living with native livers after hepatoportoenterostomy. Portal hypertension and variceal bleeding may be seen in children younger than 5 years of age, underscoring the importance of medical surveillance for complications of BA starting at a young age.

Key words: Biliary atresia; Medical status; Chronic liver disease

Core tip: Previous study showed that more than 90% of children with biliary atresia (BA) surviving with native livers have clinical and laboratory evidence of chronic liver disease (CLD). In the present cohort, we found that 71% of patients with BA living with native livers had no clinical complications of CLD and 90% had normal liver synthetic function, only 2% had ideal medical outcome. Common medical complications encountered were cholangitis, portal hypertension and bleeding oesophageal varices. Portal hypertension and bleeding oesophageal varices were seen in 12% and 6% of children younger than 5 years of age. Medical surveillance in children with BA after Kasai surgery for medical complications should start even before 5 years of age.

Lee WS, Ong SY, Foo HW, Wong SY, Kong CX, Seah RB, Ng RT. Chronic liver disease is universal in children with biliary atresia living with native liver. *World J Gastroenterol* 2017; 23(43): 7776-7784 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7776.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7776

INTRODUCTION

Biliary atresia (BA) is a progressive fibro-obliterative cholangiopathy presenting only in the first 3 mo of life[1,2]. Without surgery, BA is a fatal disease with children rarely surviving beyond 3 years of age[3]. Worldwide, the reported prevalence of BA ranges from 1 in 5000 to 18000 new-borns[4]. Since the introduction of Kasai hepatoportoenterostomy (HPE), long term survival of children with BA has been possible[1,2]. However, the surgery can only provide temporary relief to the biliary obstruction and has been considered as a bridge to eventual liver transplantation (LT)[3,5,6].

In individuals surviving with native livers after HPE, long term follow up is necessary to ascertain the health status and to detect complications of biliary cirrhosis[7-9]. Malnutrition[10,11], cholangitis[12,13], and fractures are common medical complications[14,15]. Ng *et al*[7] reported that cholangitis and bone fractures are the two major complications in long-term survivors of children with BA living with native livers. More importantly, the majority of these children have clinical or biochemical evidence of chronic liver disease (CLD)[7]. Most of the studies addressing the medical complications of children with BA surviving with native livers were in a setting where LT is readily available[7-9]. Children who needed LT were transplanted when indicated. Those children who survived with native livers were probably still in "optimal health"[7].

We have previously reported that the short-to-medium term outcome of Malaysian children with BA were favourable[16]. However, like in other developing countries, LT is not widely available in Malaysia[16]. Many children with BA who had unsuccessful surgery died within the first few years of life due to a lack of timely LT[4,16]. Nevertheless, in those who survived with their native livers after initial successful surgery, the quality of life was comparable to healthy children[17]. However, the prevalence and types of medical complications were not addressed[16,17].

The aim of the present study was to describe the medical status of children with BA living with their native livers in a setting where LT is not common. The present study is unique as most of the current studies on the long-term outcome and medical status of BA were conducted in countries where LT is readily available.

MATERIALS AND METHODS

This was a cross-sectional study conducted among children with BA attending the Paediatric Gastroenterology Unit of University Malaya Medical Centre (UMMC), Malaysia. Patients with BA diagnosed between January 1993 and December 2015 were identified. The medical record, latest clinic review and laboratory data were reviewed. The present study was approved by the institutional ethic review committee (MEC reference: 902.15).

Inclusion criteria

Only children who had surgery performed at UMMC were included. The diagnosis of BA was confirmed *via* an operative cholangiogram and a histology compatible with BA. The following children were excluded: (1) patients who had surgery performed at other centres but were subsequently followed up at UMMC; (2) patients younger than 6 mo of age at the last clinic review; (3) patients with inadequate data; and (4) patients who had LT.

Post-operative care

After HPE, prophylactic oral antibiotics were given for 3 mo. All episodes of cholangitis were treated with 10-14

d of intravenous antibiotics. Children with persistent abnormal liver indices were given ursodeoxycholic acid. All children received fat-soluble vitamins, including vitamin D supplementation.

Data collection

Demographic, clinical and laboratory data were collected from chart review. Data collected included age, sex, ethnicity, date of birth, age at surgery, latest age at review, physical examination, growth status, size of liver and spleen, and signs of chronic liver disease. Laboratory data obtained were part of routine laboratory assessment performed on patients, and included the following: indices of hypersplenism [white cell (WBC) and platelet counts], synthetic [international normalized ratio (INR), albumin] and excretory (total bilirubin) functions of liver, and indices of hepatic inflammation [aspartate aminotransaminase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γGT)].

Age at surgery

Age at Kasai surgery was considered as 'early' if the surgery was performed ⩽ 60 d of age, and 'late' if > 60 d of life.

Growth parameters

For children aged ⩽ 5 years old, the World Health Organization (WHO) age- and sex-specific growth charts were used[18]. For children aged > 5 years, the Centers for Disease Control and Prevention (CDC) age- and sex-specific growth charts were used[19]. Failure to thrive (FTT) was characterized as the weight-for-age z-score < -2.0, while short stature was defined as height-for-age z-score of < -2.0.

Medical complications

Medical complications of CLD included portal hypertension (PHT), bleeding oesophageal varices, cholangitis in the preceding 12 mo, hepatopulmonary syndrome (HPS), portopulmonary hypertension (POPT), and fracture.

Criteria for the diagnosis of PHT were the presence of at least one of the following: (1) complication of PHT (such as ascites); (2) splenomegaly (⩾ 2 cm palpable below the costal margin) and thrombocytopenia (platelet count ⩽ 150 × 10^9/L); or (3) endoscopic evidence of esophageal varies, esophageal variceal bleeding, or hypertensive gastropathy[7].

As the yield of a positive blood culture in cholangitis is low, a positive blood culture was not required. Its diagnosis was based on the presence of fever of > 38 ℃ without other obvious source of infection, abdominal pain and new onset of acholic stools, and an elevation of conjugated bilirubin and/or (γGT) from the previous baseline[7].

The presence of HPS required evidence of reduced partial pressure of oxygen < 80 mmHg under room air and evidence of intrapulmonary shunting by contrast echocardiography with agitated saline[20]. POPH was defined as the presence of echocardiographic evidence of raised pulmonary arterial pressure (⩾ 25 mmHg)[20, 21].

CLD

Laboratory evidence of hypersplenism included the presence of WBC < 4.0 × 10^9/L and/or platelet count < 150 × 10^9/L in the absence of other identifiable causes, such as virus infection. Indices of CLD included: (1) impaired excretory function [total serum bilirubin ⩾ 17 μmol/L]; (2) impaired synthetic function [albumin level < 35 g/L or INR ⩾ 1.3]; and (3) presence of hepatitis (ALT ⩾ 40 IU/L, or AST ⩾ 40 IU/L, or γGT ⩾ 55 IU/L).

Definition of an ideal medical outcome

The definition of an ideal medical status in survivors of BA with native livers was modified from that of Ng *et al*[7] and included: (1) absence of clinical complications of CLD; (2) absence of cholangitis in the preceding 12 mo; and (3) normal liver laboratory indices.

Statistical analysis

Data were entered by using SPSS 21.0 (SPSS Inc., Chicago, IL, United States) for Windows XP (Microsoft, Seattle, WA, United States). Data are quoted as medians and range. χ^2 tests were used for categorical data, while one-sample *t*-test was used for comparison of numerical data. Independent samples *t*-test was used when two different groups of continuous variables were compared.

RESULTS

During the study period between 1993 and 2015, 140 children were diagnosed with BA at UMMC, Kuala Lumpur (Figure 1). Of these, 112 (80%) had Kasai surgery. Twenty of these 112 patients developed liver cirrhosis and had LT. Seven of these 20 patients died after LT. Forty of the remaining 92 patients who had Kasai surgery died of liver cirrhosis without LT. Six of the 28 children who did not have Kasai surgery had LT as primary treatment, while the remaining 22 patients died without LT. Fifty two patients (37% of the original cohort) survive with their native livers and were included in the present analysis. The overall survival rate (native livers and LT) was 51%.

Patient characteristics and associated congenital anomalies

The median age of the 52 patients [females = 32, (62%); Table 1] at review was 7.4 years (range of 10 mo to 22 years). Two-thirds (*n* = 35, 67%) were aged 5 years or older. The median age at Kasai surgery was 60 d (range of 30 d to 148 d). Twenty nine (59%) of the patients had HPE performed before 60 d of age. Three (6%) patients had associated congenital

Table 1 Clinical and laboratory characteristics of the 52 patients with biliary atresia surviving with native livers after Kasai surgery

Characteristic		*n* (%)
Sex	Male	20 (38.4)
	Female	32 (61.6)
Ethnicity	Chinese	32 (61.5)
	Malays	15 (28.8)
	Indians	5 (9.6)
Age at Kasai surgery in d	mean ± SD	65.5 ± 26.3
	Median	60
	Range	30-148
Kasai surgery ⩽ 60 d	Yes	30 (57.7)
	No	22 (42.3)
Age at latest follow-up in yr	mean ± SD	8.3 ± 6.1
	Median	7.4
Medical conditions present	No	46 (88.5)
	Yes	6 (11.5)
	Congenital anomalies	3 (5.7)
Presence of failure to thrive		
All, *n* = 52	Yes	14 (26.9)
	No	38 (73.1)
< 5 yr old, *n* = 17	Yes	3 (17.6)
	No	14 (82.4)
⩾ 5 yr old, *n* = 35	Yes	11 (31.4)
	No	24 (68.6)
Short stature		
All, *n* = 51	Yes	10 (19.6)
	No	41 (80.4)
< 5 yr old, *n* = 16	Yes	1 (5.9)
	No	16 (94.1)
⩾ 5 yr old, *n* = 32	Yes	9 (26.5)
	No	25 (73.5)
Laboratory indices at review	White cell count, $\times 10^9$/L	6.2 (4.8)
Median (IQR)	Platelet count, $\times 10^9$/L	131 (169)
	Total bilirubin, μmol/L	18 (39)
	Serum albumin, g/L	41 (8)
	International normalised ratio	1.1 (0.1)
	Alanine transferase, IU/L	54 (64)
	Aspartate transferase, IU/L	70 (80)
	Gamma glutamyl-transpeptidase, IU/L	109 (174)

Congenital anomalies present: One each for aortic anomalies, atrial septal defect and craniosynostosis. IQR: Interquartile range.

anomalies: one each for aortic stenosis, atrial septal defect and craniosynostosis. Another 3 patients developed unrelated medical conditions: asthma, Sjogren's syndrome and polycystic ovarian syndrome. None of the patients in the present cohort had BA splenic malformation (BASM) syndrome.

Growth parameters

Presence of FTT and short stature are shown in Table 1, while the distribution of weight and height-adjusted z-score are shown in Figure 1. Overall, approximately one-quarter (*n* = 14; 27%) had a weight-for-age *z*-score < - 2.0, while 10 children (20%) had a height-for-age *z*-score < -2.0. Data on parental height were not available for comparison. FTT was present in 17.6% of children younger than 5 years of age, and in 31.4% of children older than 5 years (*P* = 0.29). There was no difference between the proportion of children with short stature in those younger (6.3%) and older than 5 years of age (27%; < 5 years old *vs* ⩾ 5 years old; *P* = 0.089).

Medical complications

PHT (*n* = 21, 40%), the most common complication encountered, was seen in 4 out of every 10 patients, but bleeding oesophageal varices only occurred in one-quarter of the patients (*n* = 13, 25%). Another indicator of PHT, ascites was uncommon (*n* = 3, 6%). Cholangitis was common and was diagnosed in approximately one-third (*n* = 19, 36%) of the patients in the preceding 12 mo. No patients with bone fracture were noted in the present cohort. No patients were found to have HPS. However, 2 patients (aged 19 and 20 years, respectively) were diagnosed to have POPH. One was asymptomatic and the hepatopulmonary hypertension was diagnosed during pre-LT assessment, while the second patient presented with chest symptoms. Both patients are receiving appropriate therapy while being assessed for LT (Table 2).

Laboratory indices

The distribution of various laboratory indices is shown in Figure 2. Leukopenia and thrombocytopenia were

Table 2 Medical status of the 52 patients with biliary atresia surviving with native livers after Kasai surgery *n* (%)

Medical variable	Data available, *n*	All, *n* = 52	< 5 yr old, *n* = 17	≥ 5 yr old, *n* = 35	*P* value, < 5 yr old *vs* ≥ 5 yr old
Failure to thrive, weight-for-age z-score < 2 SD	52	14 (26.9)	3 (17.6)	11 (31.4)	0.29
Short stature, height-for-age z-score < 2 SD	51	10 (19.6)	1 (5.9)	9 (26.5)	0.089
Medical complications					
Portal hypertension	52	21 (40.4)	2 (11.8)	19 (54.3)	0.034
Variceal bleeding	52	13 (25.0)	1 (5.8)	12 (34.3)	0.026
Ascites	52	3 (5.8)	2 (11.8)	1 (2.9)	0.20
Cholangitis	52	19 (36.3)	8 (47.1)	11 (31.4)	0.27
Portopulmonary hypertension	52	2 (3.8)	0 (0)	2 (5.7)	0.31
Hepatopulmonary syndrome	52	0 (0)	0 (0)	0 (0)	-
Bone fracture	52	0 (0)	0 (0)	0 (0)	-
Laboratory indices					
White cell count, < 4×10^9/L	49[A]	13 (25.0)	1 (5.9)	13 (40.6)	0.017
Platelet count, < 150×10^9/L	50[A]	28 (53.8)	8 (47.1)	22 (66.7)	0.28
Total bilirubin, ≥ 17 μmol/L	52	26 (50.0)	6 (35.3)	21 (60.0)	0.09
Albumin, < 35 g/L	52	6 (11.5)	1 (5.8)	5 (14.3)	0.37
International normalized ratio, ≥ 1.3	52	12 (23.1)	5 (29.4)	7 (20.0)	0.70
Alanine transferase, ≥ 40 IU/L	52	35 (67.3)	13 (76.5)	24 (68.6)	0.56
Aspartate transferase, ≥ 40 IU/L	52	38 (73.1)	15 (88.2)	25 (71.4)	0.18
Gamma glutamyl-transpeptidase, ≥ 55 IU/L	52	36 (69.2)	11 (64.7)	27 (77.1)	0.34
Absence of any medical complications	52	15 (28.8)	6 (35.3)	9 (25.7)	0.98
Absence of abnormal laboratory indices	49	3 (6.1)	1 (5.9)	2 (6.3)	0.47
Ideal medical outcome	49	1 (2.0)	0 (0)	1 (2.9)	

A: Data for white cell count for 3 patients and platelet count for 2 patients were not available for review. Ideal medical outcome was defined as an absence of any medical complications and presence of normal laboratory indices.

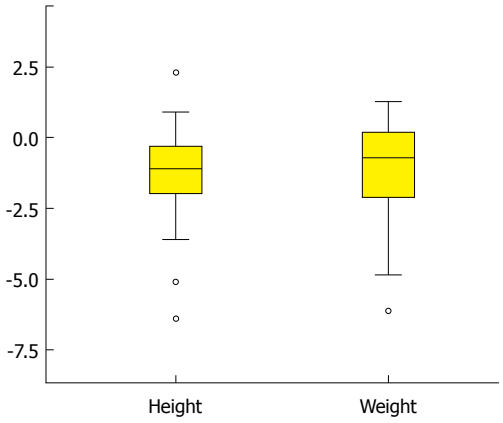

Figure 1 Boxplot showing distribution of weight-for-age and height-for-age z-score in 52 children with biliary atresia surviving with native livers. From top to bottom, the five horizontal lines represent the largest data point, which is not more than 1.5 times from the box, the third quartile, the median, the first quartile and the smallest data point which is not more than 1.5 times the interquartile range from the box, respectively.

noted in 25% and 54% of the patients, respectively. Half (*n* = 26, 50%) of the patients had raised total bilirubin level. Elevated liver enzymes were also common (ALT in 67%, AST in 73% and γGT in 69%; Figure 2). A great majority of patients (*n* = 46, 90%) had normal serum albumin level, while more than half (*n* = 27 of 39, 59.2%) had a normal INR value.

Influence of age on the presence of chronic liver disease

Generally, as compared to patients younger than 5 years of age, patients older than 5 years of age were more likely to develop complications of liver cirrhosis, *i.e.*, portal hypertension (*P* = 0.034), bleeding oesophageal varices (*P* = 0.026) and leukopenia (*P* = 0.017; Table 2).

Ideal medical outcome

Fifteen patients (29%) had no complications associated with CLD noted (Table 2). These included 6 of the 17 (36%) patients younger than 5 years old and 9 of the 35 (26%) older than 5 years of age. Three patients had normal laboratory indices. Of these, only 1 patient (2% of the entire cohort) had an ideal outcome, *i.e.* absence of any clinical complications and normal laboratory indices. The remaining 2 patients who had normal laboratory indices had an episode of cholangitis in the preceding 12 mo. Ninety eight percent of the patients in the present study had either presence of medical complications or abnormal laboratory indices.

DISCUSSION

The present study confirmed that the vast majority of patients with BA surviving with native livers after HPE had either clinical or laboratory evidence of CLD[7]. Although 71% of the patients had no clinical complications of CLD and 90% had normal liver synthetic function, only 1 of the 49 patients (2%) in the present study fulfilled the criteria for an 'ideal' outcome after HPE. This is consistent with the finding from Childhood Liver Disease Research and Education Network (CHiLDREN), where 98% of children with BA either had clinical complications of CLD or biochemical

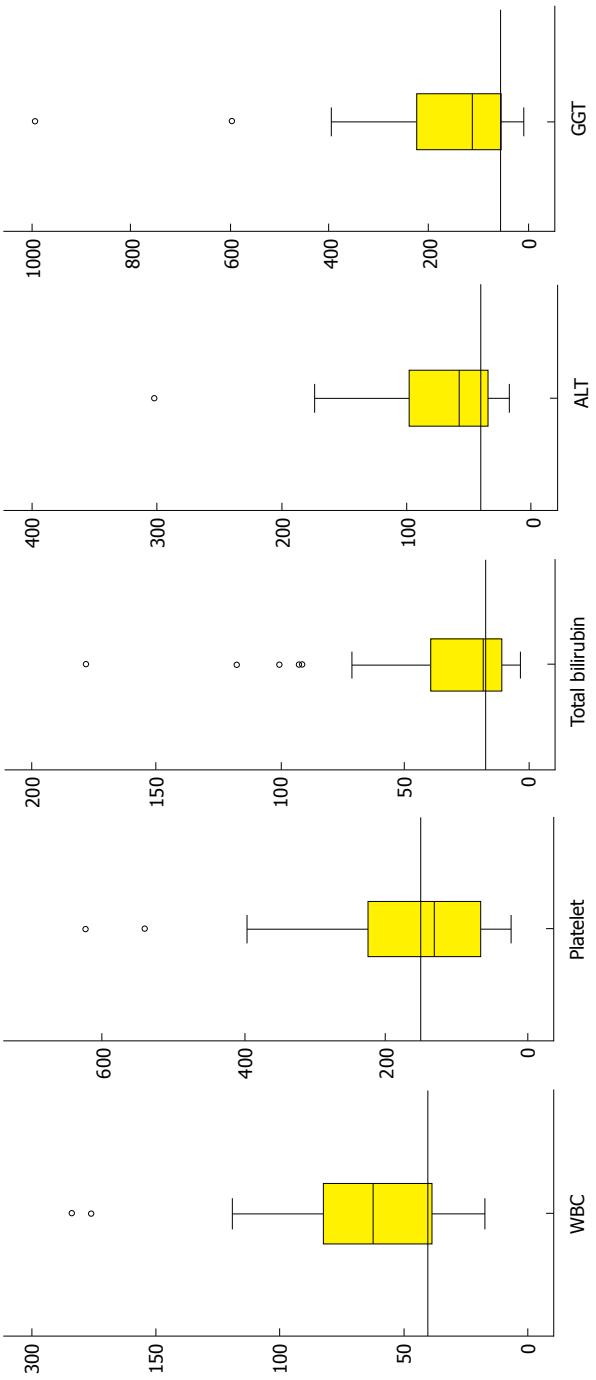

Figure 2 Boxplot showing distribution of white cell count, platelet count, total serum bilirubin and liver enzymes in 52 children with biliary atresia surviving with native livers. From top to bottom, the five horizontal lines represent the largest data point, which is not more than 1.5 times from the box, the third quartile, the median, the first quartile and the smallest data point which is not more than 1.5 times the interquartile range from the box, respectively.

abnormalities[7]. Hadzić *et al*[22], using slightly different criteria, also noted that only 11% of children with BA living with native livers had absence of surgical complications and normal laboratory indices.

In the present study, although clinical complications of CLD were absent in 30% of the children living with native livers after HPE, most had raised liver enzymes indicating persistent hepatitis. Normal liver biochemistries were only seen in 3 children in the present cohort, 2 of whom had cholangitis in the preceding 12 mo. This underscores the importance of continuing surveillance in children with BA after HPE as well as regular laboratory assessment, even in the absence of clinical complications of CLD.

There are major similarities and differences in the findings between the present study and the CHiLDREN study[7]. In both studies, PHT and cholangitis were the two most common complications noted. Bleeding oesophageal varices and cholangitis were noted in 19% and 17%, respectively, in the CHiLDREN cohort and 13% and 19%, respectively, in the present cohort[7].

However, fracture of the long bone, which was common in the CHiLDREN cohort (27%), was not seen in the present study. Hepatic osteodystrophy is a well-known complication in children with cholestatic CLD[23]. Radiological change of long bone has been reported to be as high as 76% in children with BA[23]. The reported incidence of bone fractures in children with BA before LT was between 8% and 35%[24,25]. The reason for the discrepancies in the incidence of fractures observed in the CHiLDREN as well as other studies and present cohort is unknown. We previously reported that 28% of children with CLD had vitamin D non-sufficiency despite being on vitamin D supplementation in a tropical climate setting[26]. Thus, it is unlikely that adequate exposure to sunlight in the tropical climate among the patients in the present study is a satisfactory reason.

Malnutrition in patients with BA after HPE, a well-known complication[10], is associated with LT or death by 24 mo of age[10]. It is also an independent risk factor for pre- and post-LT mortality, and even graft failure[4]. Careful monitoring of the nutritional intake, early nutritional supplementation, regular monitoring of growth parameters including triceps skinfold thickness and mid-upper arm circumference, are important[27]. When adequate nutrition has been provided and malnutrition persisted, LT should be considered[28].

In the present study, the prevalence of weight and height *z*-score of < -2.0 were 28% and 20%, respectively. This is much higher as compared to the findings in the CHiLDREN cohort, where only 0.5% and 3.2% of the patients had a weight and height z-score of < -2.0[7]. These discrepancies can be partly explained by a much higher prevalence of both underweight (13.0%, 95%CI: 11.7, 14.5) and stunting (13.4%, 95%CI: 15.1, 21.5) in Malaysian children under 18 years of age[29]. Nevertheless, underweight and stunting in children with BA post-HPE in the present cohort was common. Intensive efforts in improving the nutritional status of these children is important. In the present study, we did not include growth parameters in the analysis of ideal medical status. Besides the high prevalence of underweight and stunting in Malaysian children, the growth status of the parents of these children were not available.

Thus, we were unable to exclude familial short stature.

PHT is a common occurrence in BA, commonly presenting as splenomegaly and thrombocytopenia[30]. In the present study, 40% of the patients had PHT while 25% had bleeding varices. This is consistent with another study on PHT from the CHiLDREN cohort, where 49% and 15% of the 211 patients had PHT and bleeding varices, respectively[30]. Ascites, another complication of PHT, was uncommon, being observed in only 6% of the patients. More importantly, 12% and 6% of the patients in the present study who were younger than 5 years old had PHT and bleeding oesophageal varices. This underscores the importance of monitoring of PHT and its complications even in younger patient with BA[30].

In the present study, 36% of the patients experienced at least one episode of cholangitis. This included 31% of the patients older than 5 years of age. A total of 8 children aged older than 10 years experienced cholangitis. Thus, the risk of cholangitis after HPE continues throughout early childhood to the adolescent period and even into early adulthood[7].

POPH and HPS are reportedly uncommon complications of BA[21]. In the present study, POPH was diagnosed in 2 young adults (3.8%).

As compared to the CHiLDREN cohort, the strength of the current study was that it was conducted in a single unit, where consistent post-surgical clinical care was practiced throughout the study period. The variability in clinical care as seen in the CHiLDREN cohort was not seen in our study[7]. Other practice, such as routine administration of fat-soluble vitamins in BA after HPE, could also explain the low incidence of bone fracture seen in the present study.

However, there are several weaknesses in the present study. Firstly, it was a single-centre study and the number of patients studied was relatively small. Thus, children younger than 5 years of age were included in this review. Nevertheless, even in young children, PHT and bleeding oesophageal varices were seen in 12% and 6% of patients, respectively.

Health-related quality of life survey was not included in the present study but was studied separately[17]. The health-related quality of life in children with BA was similar to that of healthy children, and was not adversely affected by the presence of complications of CLD, such as PHT, cholangitis or impaired synthetic function of the liver[17].

The most important factor adversely affecting the overall outcome of children with BA in Malaysia was a lack of timely LT in those with unsuccessful surgery[16]. The median age for HPE in the present study was 60 d, which was comparable to figures from other centres[31], ranging from 54 days in England and Wales[32] to 68 d in Switzerland[33].

The survival rate of patients with BA living with native livers in the present study was 37%. This was comparable to the survival rates in the review by Verkade *et al*[31], where the survival rates with native livers ranged from as low as 20% in Germany[34] to 60% in Japan[6]. The overall survival rate of the present study at 51%, however, is much lower that similar figures of 73% in the Netherlands[35] to 92% in Switzerland[33].

Thus, the lack of timely LT in those with unsuccessful surgery is the biggest unmet medical need in children with BA in Malaysia. Factors contributing to a lack of LT included a low deceased organ donation rate and the relative high cost of LT surgery[36]. It is unlikely that the lack of deceased organ donation in Malaysia will be overcome in the near future.

We have previously shown that early referral for surgery significantly affected the outcome of surgery[16]. Children with BA who were operated before 60 d of age had a 65% chance of surviving with native liver at two years of age as compared to those who had surgery at or after 60 d of age. The median age of survival for those with unsuccessful surgery was 14 mo (range of 3 mo to 25 mo)[16]. Thus, in a country such as Malaysia where LT remains limited, early referral for successful surgery remains the most important opportunity to avoid early LT.

Thus, clinicians in Malaysia caring for children need to familiarize themselves with signs of an unsuccessful surgery to facilitate early referral for assessment of LT. We have shown that children with unsuccessful surgery were more likely to have a significantly bigger liver and spleen size, a more deranged coagulation profile, a significantly lower serum albumin level, and a significantly higher serum γGT level, as compared to children who had successful surgery. Thus, monitoring of children with BA after surgery should include regular measurement of liver and spleen size, assessment of liver synthetic function, such as coagulation and albumin level, and determination of liver enzymes.

As stated earlier, the opportunity to improve the overall survival rate of BA in Malaysia includes strategies to enhance early referral and surgery[31]. The provision of stool colour cards to parents of new-borns for identification of acholic stools has been practiced in Taiwan and Switzerland[37,38]. In Taiwan, 5 years after starting the stool colour card screening, the rate of HPE performed at < 60 d increased from 49% to 66% while the 5-year survival rate with native liver improved from 27% to 64%[37]. This strategy should be adopted in countries where LT is limited, such as Malaysia.

In conclusion, about 98% of children with BA living with native livers after HPE had clinical or laboratory evidence of CLD, underscoring the importance of continuing medical surveillance. PHT and variceal bleeding may be seen in children younger than 5 years of age. We recommend that surveillance for the complications of CLD in children with BA living with native livers should be started before 5 years of age. The major unmet medical need for children with BA living with their native livers is a lack of timely LT in those with unsuccessful surgery. Opportunities to

improve the overall survival rate include universal newborn screening in all infants to improve early referral rate and the surgical outcome of HPE.

COMMENTS

Background

In individuals with biliary atresia (BA) surviving with native livers after hepatoportoenterostomy, biliary cirrhosis is an inevitable long-term sequelae. Malnutrition, cholangitis and fractures are common medical complications encountered in these children, arising as a result of biliary cirrhosis. Previous study has shown that cholangitis and bone fractures are the two major complications in long-term survivors of children with BA living with native livers.

Research frontiers

It is important to determine the prevalence of medical complications in children with BA surviving with their native livers, particularly in a setting where liver transplantation (LT) is limited.

Innovations and breakthroughs

The present study shows that clinical or laboratory evidence of chronic liver disease are present in 98% of children with BA living with native livers after surgery. Portal hypertension and variceal bleeding may be seen in children younger than 5 years of age, underscoring the importance of medical surveillance for complications of BA starting at a young age.

Applications

The authors recommend that in all children with BA who survive with their native livers, systemic surveillance to ascertain medical complications be started at an age younger than 5 years. Ascending cholangitis, portal hypertension and variceal bleeding are important medical complications encountered.

Peer-review

This manuscript describes the current situation of treatment of BA in Malaysia. In Malaysia, LT is not common. Therefore, most patients with BA must live with their native livers after Kasai's hepatic portoenterostomy. This manuscript reemphasized the devastating nature of BA and essentialness of LT for patients with BA.

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P- Reviewer: Hashimoto D, Neri V **S- Editor**: Ma YJ
L- Editor: Filipodia **E- Editor**: Huang Y

World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7785-7790

DOI: 10.3748/wjg.v23.i43.7785

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

ORIGINAL ARTICLE

Prospective Study

How severe is moderately severe acute pancreatitis? Clinical validation of revised 2012 Atlanta Classification

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Institutional review board statement: The study was reviewed and approved by the Kaunas Regional Biomedical Research Ethics Committee.

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

Conflict-of-interest statement: All authors have nothing to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: September 2, 2017
Peer-review started: September 4, 2017
First decision: September 20, 2017
Revised: October 1, 2017
Accepted: November 1, 2017
Article in press: November 1, 2017
Published online: November 21, 2017

Abstract

AIM

To explore the outcomes and the appropriate treatment for patients with moderately severe acute pancreatitis (AP).

METHODS

Statistical analysis was performed on data from the prospectively collected database of 103 AP patients admitted to the Department of Surgery, Hospital of Lithuanian University of Health Sciences in 2008-2013. All patients were confirmed to have the diagnosis of AP during the first 24 h following admission. The severity of pancreatitis was assessed by MODS and APACHE Ⅱ scale. Clinical course was re-evaluated after 24, 48 and 72 h. All patients were categorized into 3 groups based on Atlanta 2012 classification: Mild, moderately severe, and severe.

Outcomes and management in moderately severe group were also compared to mild and severe cases according to Atlanta 1992 and 2012 classification.

RESULTS

Fifty-three-point four percent of patients had edematous while 46.6 % were diagnosed with necrotic AP. The most common cause of AP was alcohol (42.7%) followed by alimentary (26.2%), biliary (26.2%) and idiopathic (4.9%). Under Atlanta 1992 classification 56 (54.4%) cases were classified as "mild" and 47 (45.6%) as "severe". Using the revised classification (Atlanta 2012), the patient stratification was different: 49 (47.6%) mild, 27 (26.2%) moderately severe and 27 (26.2%) severe AP cases. The two severe groups (Atlanta 1992 and Revised Atlanta 2012) did not show statistically significant differences in clinical parameters, including ICU stay, need for interventional treatment, infected pancreatic necrosis or mortality rates. The moderately severe group of 27 patients (according to Atlanta 2012) had significantly better outcomes when compared to those 47 patients classified as severe form of AP (according to Atlanta 1992) with lower incidence of necrosis and sepsis, lower APACHE Ⅱ (P = 0.002) and MODS (P = 0.001) scores, shorter ICU stay, decreased need for interventional and surgical treatment.

CONCLUSION

Study shows that Atlanta 2012 criteria are more accurate, reduce unnecessary treatments for patients with mild and moderate severe pancreatitis, potentially resulting in health costs savings.

Key words: Acute pancreatitis; Atlanta 1992; Atlanta 2012; Severity stratification; Treatment; Outcomes

Core tip: The revised (2012) Atlanta classification proved to be superior to the former classic (1992) Atlanta classification. The results of this study support the use of Atlanta 2012 classification in clinical setting and suggest that "moderately" severe AP cases could be treated as "mild" AP once temporary organ failure is controlled, and should result in significant health costs savings without compromising the patient's outcomes.

Ignatavicius P, Gulla A, Cernauskis K, Barauskas G, Dambrauskas Z. How severe is moderately severe acute pancreatitis? Clinical validation of revised 2012 Atlanta Classification. *World J Gastroenterol* 2017; 23(43): 7785-7790 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7785.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7785

INTRODUCTION

Acute pancreatitis (AP) encompasses a wide spectrum of disease severity from a brief, self-limited presentation to a fulminant progression to multi-organ failure and death[1,2]. In response to the need for a comprehensive classification system in the treatment of AP, the 1992 Atlanta classification was established. The 1992 Atlanta Classifications identified two categories of AP, "mild" and "severe" and recommended the clinical treatment for each type[3,4]. However, a subgroup of AP patients who fell in-between the two 1992 severity categories were often observed to have relatively good outcomes and respond positively to less aggressive treatment protocols than those with severe disease, thus calling for the revision of the existing classification system. In 2012 the Atlanta classification was revised by adding a third category defined as "moderately severe".

To the best of our knowledge, no studies have evaluated the outcomes and clinical course of "moderately severe" pancreatitis to test their true value in clinical setting. No studies have focused on complications, mortality and outcomes of patients with moderately severe AP. It raises the question if the recommended aggressive treatment and expensive interventions are necessary in moderately severe category patients.

The main aim of this study was to stratify the same cohort of patients into the mild and severe categories using Atlanta 1992 classification and into mild, moderate and severe categories according to the Atlanta 2012 revised version to highlight the severity of moderate AP and to assess the outcomes of these patients. We also aimed to ascertain whether this new category aids predicting the outcomes and complications while optimizing the use of medical resources and interventional procedures.

MATERIALS AND METHODS

Patients

Since 2008 data of patients with acute pancreatitis, admitted to the Department of Surgery, Hospital of Lithuanian University of Health Sciences were prospectively collected and entered into a specially designed database (The Regional Ethics Committee and IRB approval No. BE-2-47 and P1-113/2005, all patients provided a written informed consent). Statistical analysis was performed on data from the prospectively collected database of 103 AP patients. All patients were confirmed to have diagnosis of AP during the first 24 h since admission according to Atlanta 2012 classification (acute abdominal pain, localized in epigastrium, commonly radiating to the back, 3-fold elevated serum levels of lipase/amylase content, typical findings on abdominal computed tomography (CT) scan with intravenous enhancement).

In addition, the severity of pancreatitis was assessed by MODS and APACHE Ⅱ scale. Clinical course was reevaluated after 24, 48 and 72 h. A contrast enhanced CT scan performed on Days 5-7 after the onset of the disease to confirm the presence and extent of pancreatic/peripancreatic necrosis. Clinical data relating to the severity of the disease, development of organ dysfunction

Table 1 Patients' characteristics *n* (%)

Variable	All patients (*n* = 103)
Male	54 (52.4)
Necrotic	48 (46.6)
Edematous	55 (53.4)
Etiology	
Alcohol	44 (42.7)
Alimentary	27 (26.2)
Biliary	27 (26.2)
Idiopathic	5 (4.9)
Atlanta 1992	
Mild	56 (54.4)
Severe	47 (45.6)
Atlanta 2012	
Mild	49 (47.6)
Moderately severe	27 (26.2)
Severe	27 (26.2)
Interventions	
US guided drainage	6 (5.8)
Fasciotomy	1 (1)
Necrosectomy	5 (4.9)
APACHE Ⅱ (mean ± SD)	7.1 ± 5.32
MODS (mean ± SD)	2.6 ± 2.91
Sepsis	6 (5.8)
Mortality	13 (12.6)

APACHE: Acute Physiology and Chronic Health Evaluation; US: Ultrasound; MODS: Multiple organ dysfunction syndrome.

and/or septic complications were prospectively collected in standardized fashion. All patients were re-categorized into 3 groups based on severity: Mild (no organ failure, no local or systemic complications), moderately severe (organ failure that resolves within 48 h (transient organ failure) and/or local or systemic complications without persistent organ failure), severe [persistent organ (single/multiple) failure (> 48 h)] (Atlanta 2012) and mild (minimal organ dysfunction and an uneventful recovery, absence of the described features of severe acute pancreatitis) and severe (organ failure and/or local complications, such as necrosis, abscess, or pseudocyst) acute pancreatitis groups (Atlanta 1992).

Severe AP groups according to Atlanta 1992 and Atlanta 2012 were compared with each other. Moderately severe (Atlanta 2012) cases were compared to mild and severe cases according to Atlanta 1992 classification. Outcomes and management were re-assessed in all groups.

Statistical analysis

Data are expressed as mean ± SD of the number of replicate. Differences between two groups are evaluated with *t*-test. Differences among three or more groups are evaluated using the nonparametric one-way ANOVA test. Differences are considered significant when $P < 0.05$. SPSS 20.0 (SPSS Inc., Chicago, IL, United States) was employed to analyze the data.

RESULTS

There were a total of 103 patients with acute pancreatitis included in the study. Alcohol abuse was the most common cause of the disease 42.7%, while biliary etiology was obvious in less than 30% of patients. According to the Atlanta 1992 classification mild AP was diagnosed in 56 (54.4%) and severe AP in 47 (45.6%) cases. The group of moderately severe acute pancreatitis (Atlanta 2012) was mainly derived from the severe AP group (Atlanta 1992), while only 7 patients moved from the mild AP group (Atlanta 1992). Overall mortality reached 12.6 % (Table 1).

Table 2 Comparison of severe acute pancreatitis group outcomes

	Atlanta 1992	Atlanta 2012	*P* value
ICU admission (*n*)	5	6	0.32
US drainage (*n*)	5	4	0.71
Infected necrosis (*n*)	12	11	0.19
Deaths (*n*)	13	13	0.75

US: Ultrasound; ICU: Intensive care unit.

While comparing the disease course and outcomes of severe AP according to 1992 and 2012 classifications, there were no statistically significant differences in clinical outcomes, including intensive care unit (ICU) stay, need for ultrasound (US) guided drainage, occurrence of infected necrosis or mortality rates (Table 2).

According to the severity of disease, organ failure, complication rates and treatment outcomes, majority of moderately severe acute pancreatitis cases according to Atlanta 2012 classification matched to being "severe" according to Atlanta 1992 classification, as only 7 patients according to Atlanta 1992 classification would have been classified as "mild" and the rest 20 patients as "severe" category, if the former criteria were followed. Both mild and severe acute pancreatitis patients according to Atlanta 2012 classification matched the groups identically to Atlanta 1992 classification (Figure 1).

When comparing mild AP to severe AP according to Atlanta 1992 classification, there were more patients who had SIRS and MODS (confirmed by APACHE Ⅱ and MODS scores). Incidence of pancreatic and extrapancreatic necrosis, infected necrosis, number of surgical interventions was also significantly higher. In severe AP group, there were 13 (27.7%) deaths, while there was none in the mild AP group (Table 3).

Comparison of moderately severe and severe AP groups according to Atlanta 2012 classification is presented in Table 4. In moderately severe AP group rate of surgical interventions [FNA 2 (7.4%), US-guided drainage 1 (3.7%), surgical treatment 0 (0%), mortality rate, deaths 0 (0%)] and disease severity (APACHE Ⅱ 7.7 ± 3.07, MODS scores 2.9 ± 1.78) was significantly lower when comparing to severe AP accordingly: [FNA 10 (37%), US-guided drainage 4 (14.8%), surgical treatment 6 (22.2%), mortality rate, deaths 13(48.1%)] and disease severity (APACHE Ⅱ 13.2 ± 5.43, MODS scores 5.2 ± 3.90).

DISCUSSION

Since the Atlanta conference established a classification system in the early 1990s, it has been criticized for

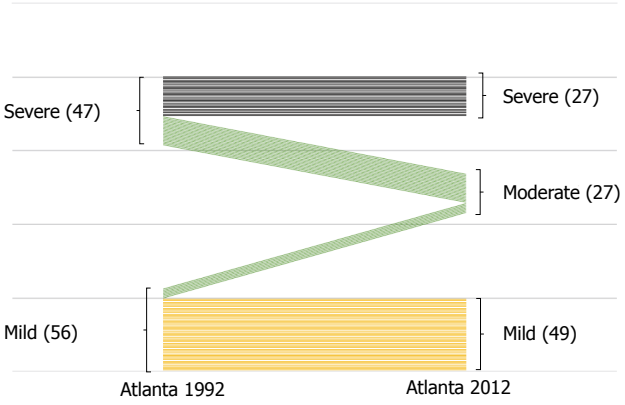

Figure 1 Distribution of patient allocation between the Atlanta 1992 and Atlanta 2012 subgroups. Majority (20) of moderately severe (APACHE Ⅱ score range: 6-9) AP cases according to Atlanta 2012 classification matched to being severe according to Atlanta 1992 classification, as only 7 patients would have been classified as "mild". Both mild (APACHE Ⅱ score range: 0-6) and severe (APACHE Ⅱ score range: 9-27) AP patients according to Atlanta 2012 classification matched the groups identically to Atlanta 1992 classification. AP: Acute pancreatitis.

being overly simplistic in categorizing acute pancreatitis into only "mild" and "severe" disease[5-8]. As a result, a heterogeneous group of patients were categorized as having severe AP, making it difficult to appropriately stratify patients therapeutically and compare research outcomes in this disease[4,9-11].

Our study supports the literature finding that Atlanta 1992 classification (mild and severe AP) is not sufficient because patients may experience transitory organ failure and/or have local pancreas and peripancreatic complications and were categorized as being "severe" AP[4-13]. The updated Atlanta 2012 classification addresses this missing group and "moderately severe" category is introduced[9,10]. While performing analysis of our clinical database, patients who had less than 48 hours transitory one organ system failure and previously categorized as "severe" pancreatitis ("moderately severe" according to Atlanta 2012), usually had self-limited disease, little risk of local and systemic complications, and the course of AP was like "mild" AP[2,3,14-18]. As a result, these patients require shorter ICU stay if any at all, less frequently develop infected pancreatic necrosis and/or sepsis, furthermore, require little or no US guided or surgical interventions. There were no deaths reported in moderately severe AP group. Failure to categorize precisely patient's according to disease severity and initiation of aggressive treatment results in increased costs.

Our study compared the accuracy of two AP severity classifications for predicting important outcomes using a prospective clinical database. In addition, we evaluated different course (mild, moderately severe, severe) of the disease, treatment outcomes and compared among the groups while applying the most recent classification. Both classifications (Atlanta 1992, Atlanta 2012) were accurate for predicting "severe" group of the patients. They both were essentially equivalent

Table 3 Comparison of mild and severe acute pancreatitis (Atlanta 1992) *n* (%)

	Mild	Severe	*P* value
Male	29 (51.8)	25 (53.2)	1.000
Necrosis			
Sterile	55 (98.2)	41 (87.2)	0.343
Infected	1 (1.8)	6 (12.8)	0.054
Sepsis	0 (0)	6 (12.8)	0.011
Interventions			
Fine needle aspiration	1 (1.8)	12 (25.5)	0.002
US guided drainage	1 (1.8)	5 (10.6)	0.101
Necrosectomy	0 (0)	6 (12.7)	0.011
APACHE Ⅱ, mean ± SD	3.51 ± 1.94	11.48 ± 4.79	< 0.001
MODS, mean ± SD	1.17 ± 1.28	4.29 ± 3.38	< 0.001
Deaths	0 (0)	13 (27.7)	< 0.001

APACHE: Acute Physiology and Chronic Health Evaluation; US: Ultrasound; MODS: Multiple organ dysfunction syndrome.

Table 4 Comparison of moderately severe and severe acute pancreatitis (Atlanta 2012) *n* (%)

	Moderately severe	Severe	*P* value
Male	15 (55.6)	16 (59.3)	1.000
Necrosis			
Sterile	26 (96.3)	22 (81.5)	0.696
Infected	1 (3.7)	5 (18.5)	0.201
Sepsis	0 (0)	6 (22.2)	0.028
Interventions			
Fine needle aspiration	2 (7.4)	10 (37.0)	0.053
US guided drainage	1 (3.7)	4 (14.8)	0.356
Necrosectomy	0 (0)	6 (22.2)	0.028
APACHE Ⅱ, mean ± SD	7.7 ± 3.07	13.2 ± 5.43	0.002
MODS, mean ± SD	2.9 ± 1.78	5.2 ± 3.90	< 0.001
ICU admission	5 (18.5)	24 (88.9)	0.004
Deaths	0 (0)	13 (27.7)	0.001

APACHE: Acute Physiology and Chronic Health Evaluation; US: Ultrasound; MODS: Multiple organ dysfunction syndrome; ICU: Intensive Care Unit.

in predicting mortality, need for ICU stay and surgical interventional procedure for "severe" AP group. Our study also demonstrates that all patients with persistent organ failure do not have the same risk of mortality and should be further stratified.

Like previously reported by Kadiyala *et al*[11] that those with multisystem persistent organ failure experienced a significantly higher mortality than those with single-system persistent organ failure (7.4% *vs* 56.3%, respectively, $P = 0.001$). Furthermore, the study by Kadiyala *et al*[11]. reports that multisystem persistent organ failure was a stronger predictor of mortality than single-system persistent organ failure, sterile necrosis, or infected necrosis. The same study suggested that patient classified as having severe AP based on persistent organ failure should be further stratified by the presence or absence of multisystem persistent organ failure. Therefore, our study results suggest that patients classified as "severe" based on organ failure should be further evaluated for the

presence or absence of multiple persistent organ failure.

The biggest advantage of the Revised Atlanta 2012 classification is that patients with transitory organ failure previously classified as severe AP are now allocated to the moderately severe AP group. Our findings suggest that "moderately severe" AP has a clinical course similar to "mild AP" and often is self-limited or if the treatment is initiated they have less complications and they rarely need intervention (drainage, surgical, *etc*.). Furthermore, if organ insufficiency regresses within the first 48 hours, antibiotic usage may be limited or not even started, no need for enteric or parenteral feeding or use of catheterization (central vein, urinary) while aiming to limit complications related to interventions and treatment costs.

The present study has several important strengths. The primary strength of this study is that the data were collected prospectively. This minimized missing data and selection bias. Also, our study explicatively analyzed patient outcomes and economic impact while comparing two versions (1992 and 2012) of Atlanta classification. All patients had a CT scan performed at regular intervals according to the protocol

According to our data, moderately severe AP (Atlanta 2012) group has similar disease course to mild AP (Atlanta 1992 and 2012). As a result, the disease often resolves without any adverse events and temporary organ failure is overcome by the timely treatment, patients tend to have less complications and interventional treatment is rarely needed.

In conclusion, the revised (2012) Atlanta classification proved to be superior to the former classic (1992) Atlanta classification. The results of the study support the use of Atlanta 2012 classification and suggest that "moderately" severe AP cases should be treated as "mild" AP once temporary organ failure is controlled. Use of the classification system in this way will result in significant costs savings with improved outcomes of the patients.

ARTICLE HIGHLIGHTS

Research background

The 1992 Atlanta Classifications identified two categories of acute pancreatitis (AP), "mild" and "severe". However, a subgroup of AP patients who fell in-between the two 1992 severity categories were often observed to have relatively good outcomes and respond positively to less aggressive treatment protocols than those with severe disease. In 2012 the Atlanta classification of AP was revised by adding a third category defined as "moderately severe".

Research motivation

To the best of authors' knowledge, there are no studies have evaluated the outcomes and clinical course of "moderately severe" pancreatitis to test their true value in clinical setting. There are no studies have focused on complications, mortality and outcomes of patients with moderately severe AP. The question if the recommended aggressive treatment and expensive interventions are necessary in moderately severe category patients is raises.

Research objectives

The main objectives of this study were to explore the outcomes and the appropriate treatment for patients with moderately severe AP. These objectives were achieved and to the best of our knowledge, no studies have evaluated the outcomes and clinical course of "moderately severe" pancreatitis to test their true value in clinical setting.

Research methods

The study is based on the data from specially designed database. Since 2008 data of patients with AP, admitted to the Department of Surgery, Hospital of Lithuanian University of Health Sciences were prospectively collected and entered into this database. Statistical analysis was performed on data of 103 AP patients. After stratifying patients into different categories, severe AP groups according to Atlanta 1992 and Atlanta 2012 were compared with each other. Moderately severe (Atlanta 2012) cases were compared to mild and severe cases according to Atlanta 1992 classification and the outcomes and management were re-assessed in all groups.

Research results

Both classifications (Atlanta 1992, Atlanta 2012) are accurate for predicting "severe" group of the patients. They both are essentially equivalent in predicting mortality, need for ICU stay and surgical interventional procedure for "severe" AP group. The study also demonstrates that all patients with persistent organ failure do not have the same risk of mortality and should be further stratified. Findings suggest that "moderately severe" AP has a clinical course similar to "mild AP" and often is self-limited or if the treatment is initiated they have less complications and they rarely need intervention (drainage, surgical, *etc*.).

Research conclusions

The revised (2012) Atlanta classification proved to be superior to the former classic (1992) Atlanta classification. Use of the classification system in this way will result in significant costs savings with improved outcomes of the patients.

Research perspectives

Similar validation studies could be performed with larger patient cohorts in multicenter setting. The focus of such studies in the future should be on "severe" group of AP patients as the patients of this group require the most intensive treatment and the mortality rate is high.

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P- Reviewer: Kikuyama M, Xu CF **S- Editor**: Gong ZM
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World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7791-7806

DOI: 10.3748/wjg.v23.i43.7791

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

META-ANALYSIS

Laparoscopic *VS* open hepatectomy for hepatolithiasis: An updated systematic review and meta-analysis

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Supported by National Natural Science Foundation of China, No. 81372243, No. 81570593 and No. 81370575; Key Scientific and Technological Projects of Guangdong Province, No. 2014B020228003 and No. 2014B030301041; Natural Science Foundation of Guangdong Province, No. 2015A030312013; Science and Technology Planning Project of Guangzhou, No. 201400000001-3, No. 201508020262 and No. 2014J4100128; Science and Technology Planning Project of Guangdong Province, No. 2017A020215178.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this report.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: June 17, 2017
Peer-review started: July 10, 2017
First decision: July 28, 2017
Revised: July 31, 2017
Accepted: August 15, 2017
Article in press: August 15, 2017
Published online: November 21, 2017

Abstract

AIM

To perform a meta-analysis on laparoscopic hepatectomy *VS* conventional liver resection for treating hepatolithiasis.

METHODS

We conducted a systematic literature search on PubMed, Embase, Web of Science and Cochrane Library, and undertook a meta-analysis to compare

the efficacy and safety of laparoscopic hepatectomy *VS* conventional open liver resection for local hepatolithiasis in the left or right lobe. Intraoperative and postoperative outcomes (time, estimated blood loss, blood transfusion rate, postoperative intestinal function recovery time, length of hospital stay, postoperative complication rate, initial residual stone, final residual stone and stone recurrence) were analyzed systematically.

RESULTS

A comprehensive literature search retrieved 16 publications with a total of 1329 cases. Meta-analysis of these studies showed that the laparoscopic approach for hepatolithiasis was associated with significantly less intraoperative estimated blood loss [weighted mean difference (WMD): 61.56, 95% confidence interval (CI): 14.91-108.20, P = 0.01], lower blood transfusion rate [odds ratio (OR): 0.41, 95%CI: 0.22-0.79, P = 0.008], shorter intestinal function recovery time (WMD: 0.98, 95%CI: 0.47-1.48, P = 0.01), lower total postoperative complication rate (OR: 0.52, 95%CI: 0.39-0.70, P < 0.0001) and shorter stay in hospital (WMD: 3.32, 95%CI: 2.32-4.32, P < 0.00001). In addition, our results showed no significant differences between the two groups in operative time (WMD: 21.49, 95%CI: 0.27-43.24, P = 0.05), residual stones (OR: 0.79, 95%CI: 0.50-1.25, P = 0.31) and stone recurrence (OR: 0.34, 95%CI: 0.11-1.08, P = 0.07). Furthermore, with subgroups analysis, our results proved that the laparoscopic approach for hepatolithiasis in the left lateral lobe and left side could achieve satisfactory therapeutic effects.

CONCLUSION

The laparoscopic approach is safe and effective, with less intraoperative estimated blood loss, fewer postoperative complications, reduced length of hospital stay and shorter intestinal function recovery time than with conventional approaches.

Key words: Hepatolithiasis; Laparoscopic hepatectomy; Conventional liver resection; Systematic review; Meta-analysis

Core tip: Application of the laparoscopic approach in symptomatic hepatolithiasis has gradually attracted more attention. However, its advantages over the open approach are still unclear. We analyzed 16 articles, comprising 1329 patients, to compare the two techniques for treating hepatolithiasis. We concluded that the laparoscopic approach is safe, effective and feasible for liver resection, with less intraoperative estimated blood loss, fewer postoperative complications, reduced length of hospital stay and shorter intestinal function recovery time than with conventional approaches.

Li H, Zhang J, Cai JY, Li SH, Zhang JB, Wang XM, Chen GH, Yang Y, Wang GS. Laparoscopic *VS* open hepatectomy for hepatolithiasis: An updated systematic review and meta-analysis. *World J Gastroenterol* 2017; 23(43): 7791-7806 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7791.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7791

INTRODUCTION

Hepatolithiasis is a gallstone disorder that involves the intrahepatic biliary duct (IHD), which may occur alone or accompanying extrahepatic gallstones. IHD stones may occur in any segments of the liver, and are particularly prevalent in the left lateral segment[1]. A recent report has shown that only 0.6%-1.3% of patients have intrahepatic stones in western countries, being more prevalent in eastern countries, especially Southeast Asia[2]. Hepatolithiasis over a long period of time may cause secondary cholangitis-originated cirrhosis and even cholangiocarcinoma[1,3], which can seriously affect the health and quality of life of patients.

There are many approaches to treat this disease, including percutaneous transhepatic cholangioscopic lithotripsy, IHD exploration and hepatectomy[4-7]. Among these treatment methods, hepatectomy is considered the most radical option for hepatolithiasis. In the past, open hepatectomy was preferred, with bile duct exploration and stone removal[8,9]. In recent years, with the development of laparoscopic technology and refinement of laparoscopic instruments, laparoscopic hepatectomy is now identified as a safe and flexible technique for hepatolithiasis.

However, few meta-analyses have evaluated the efficacy and safety of the laparoscopic approaches and open surgery that are routinely used in hepatolithiasis. It is unclear whether laparoscopic hepatectomy can be performed as effectively and safely as conventional hepatectomy or is superior to it in treating hepatolithiasis in the left or right hepatic lobes. Here, we performed a meta-analysis to assess the safety and efficacy of laparoscopic hepatectomy for treating intrahepatic bile duct stones. Furthermore, we evaluated left lateral sectionectomy and left hemihepatectomy by performing subgroups analysis.

MATERIALS AND METHODS

Search strategy and criteria

This meta-analysis was performed to compare laparoscopic hepatectomy and conventional open hepatectomy for hepatolithiasis. In January 2017, PubMed, Embase, Web of Science and Cochrane Library were searched for studies comparing laparoscopic hepatectomy with open liver resection for hepatolithiasis. There were no restrictions on

publication date, type or language. Search terms were confined to Title/Abstract: "hepatolithiasis" OR "intrahepatic stone" AND "laparoscopic" OR "laparoendoscopic". The reference lists of all selected articles were manually searched to determine if they should be included. Two reviewers browsed the titles and abstracts independently. Articles were included if they: (1) compared the outcomes of laparoscopic and open approaches for hepatolithiasis; and (2) reported at least some of the outcomes that we were interested in. Articles were excluded if they were submitted by the same authors or they reported duplicate data, to avoid duplication of patient populations. Editorials, case reports, conference abstracts and animal studies were excluded.

Data management

Data from the included studies were summarized by two of the authors independently. They were blinded to journals of publication, authors and study institutions of all available articles. Any disagreements between the reviewers were settled by the senior author. Perioperative outcomes were compared, including operative time, estimated blood loss (EBL), intraoperative transfusion, length of hospital stay (LOS), time to oral intake and postoperative complications. Outcomes regarding residual rate of intrahepatic stones containing initial residual, final residual and stone recurrence were also analyzed.

Quality assessment and statistical analysis

The level of evidence of these articles was estimated using the UK Cochrane Centre of Evidence (2009)[10]. The methodological quality of randomized controlled trials (RCTs) was assessed by the Cochrane Risk of Bias Tool[11]. The modified Newcastle-Ottawa scale was used to assess the quality of retrospective studies, which consists of three factors: patient selection, comparability of the study groups, and assessment of outcome[12-14]. The maximum total score on this scale was 9, and studies with scores ≥ 7 were defined as high quality[12].

All data were pooled with the Cochrane Collaboration's Review Manager 5.3 (Cochrane Collaboration, Oxford, United Kingdom). Mean differences and 95% confidence intervals (CIs) were calculated to pool functional outcomes. Statistical heterogeneity among studies was assessed using the χ^2 test with significance set at $P < 0.1$, and heterogeneity was quantified using the I^2 statistic. A fixed-effects model was used routinely only if there was obvious heterogeneity among the included literature[15].

Subgroups and publication bias

Intrahepatic duct stones were located in different liver segments. Patients were subgrouped by type of operation, including left lateral sectionectomy (LLS), left hemihepatectomy (LH) and right hepatectomy (RH). Subgroup analysis was performed to compare outcomes resulting from different excision extension. Funnel plots were used to signify the publication bias. If outcomes were associated with significant heterogeneity, a random-effects model was used to minimize bias.

RESULTS

Characteristics of selected articles

The literature search identified 515 articles, 115 from PubMed, 187 from Embase and 213 from Web of Science; no studies were available in Cochrane Library (Figure 1). Of the 515 identified articles, 203 were duplications, 194 did not focus on hepatolithiasis, 40 were not comparative studies, 35 were case reports, 4 were conference abstracts and 2 were editorials. The full text of the remaining 36 articles was carefully reviewed. Twenty more were excluded, including 2 case reports, 5 that were not comparative studies and 13 that had no data of interest. Finally, 16 articles were included in our meta-analysis[16-31]. The characteristics of the selected articles are shown in Table 1.

Of the 1329 patients included in the 16 articles, 624 were treated with the laparoscopic approach and 705 with the open approach (Table 2). All 16 studies were retrospective except for 1 RCT (level of evidence: 2b)[17]. Among the remaining 15 studies, 3 compared contemporary series of patients (level of evidence: 3a)[20,25,30], 11 were retrospective case-control studies (level of evidence: 3b)[16,19,21-24,26-29,31], and 1 was a retrospective study using historical series as controls (level of evidence: 4)[18] (Table 3).

Duration of operation in the 15 studies[16-21,23-31] was similar between the two groups [weighted mean difference (WMD): 21.49, 95%CI: -0.27 to 43.24, $P = 0.05$] (Figure 2A). EBL was analyzed among 1221 patients from 13 studies[16-20,23-26,28-31], and less EBL was found in the laparoscopic group (WMD: -61.56, 95% CI: -108.2 to -14.91, $P = 0.01$) (Figure 2B). Intraoperative transfusion was analyzed in 9 articles[19,21,24-26,28-31], showing lower transfusion rate in the laparoscopic group [odds ratio (OR): 0.41, 95%CI: 0.22-0.79, $P = 0.008$) (Figure 2C). All 16 articles[16-31] were analyzed for postoperative complications, indicating that the rate was significantly lower in the laparoscopic group (OR: 0.52, 95%CI: 0.39-0.70, $P < 0.001$) (Figure 2D). Seven articles[16,22-25,27,31] reported time to oral intake, with a significantly shorter time for recovery of bowel movement in the laparoscopic group (WMD: -0.98, 95%CI: -1.48 to -0.47, $P < 0.001$) (Figure 3A). Fifteen studies[16,17,19-31], including 1294 patients, evaluated LOS, which was significantly shorter in the laparoscopic group (WMD: -3.32, 95% CI: -4.32 to -2.32, $P < 0.001$) (Figure 3B). No significant difference was found in initial and final

Table 1 Characteristics of included studies

Ref.	Level of evidence	Design	Patient No.		Location of stone	F/U, mo	Matching	Quality score
			LH	OH		LH/OH		
Cai *et al*[16], 2007	3b	Re	29	22	L+R	16.1/16.1	1,2,3,4,5	7
Ding *et al*[17], 2015	2b	RCT	49	49	L	Perioperative	1,2,3,5	RCT
Li, H *et al*[18], 2008	4	Re	14	20	L+R	Perioperative	1,2	5
Jin *et al*[19], 2015	3b	Re	96	105	L	18-90	1,2,3,6,7	5
Kim *et al*[20], 2015	3a	Re	17	17	R	35/35	1,3,4,5	7
Lee *et al*[21], 2014	3b	Re	7	9	L	12.1/11.1	1,2,4,6	6
Li, J *et al*[22], 2014	3b	Re	35	40	L+R	41/41	1,2,3	6
Li, Y *et al*[23], 2015	3b	Re	23	22	L+R	15-51	1,2,3,6	5
Namgoong *et al*[24], 2014	3b	Re	37	112	L	NA	1,2,3,4	7
Peng *et al*[25], 2016	3a	Re	36	39	L	18.9/20	1,2,3,4	7
Shin *et al*[26], 2015	3b	Re	40	54	L	46.8/75.7	1,2,6	5
Song *et al*[27], 2010	3b	Re	7	10	L	Perioperative	1,2,3,4,5	7
Tian *et al*[28], 2013	3b	Re	116	78	L+R	29/29	1,2,4	6
Tu *et al*[29], 2010	3b	Re	28	33	L	17/17	1,2,3,4	7
Ye *et al*[30], 2015	3a	Re	46	51	L	33/33	1,2,3,4	7
Zhou *et al*[31], 2013	3b	Re	44	44	L+R	24/24	1,2,3,4,5,6,7	7

1: Age; 2: Sex; 3: Liver function; 4: Previous upper surgery history; 5: Surgeon experience; 6: Body mass index; 7: American Society of Anesthesiologists score. F/U: Follow-up, mean or median or range, month; L: Left intrahepatic; LH: Laparoscopic hepatectomy; NA: Not available; OH: Open hepatectomy; R: Right intrahepatic; RCT: Randomized controlled trail; Re: Retrospective.

Table 2 Results of meta-analysis in laparoscopic hepatectomy *vs* open hepatectomy

Outcomes of interest	Study, *n*	LH, *n*	OH, *n*	WMD/OR (95%CI)	*P*	Study heterogeneity			*P*
						χ^2	df	I^2, %	
Operative time, min	15	589	665	21.49 (-0.27, 43.24)	0.05	227.54	14	94	< 0.001
Estimated blood loss, mL	13	575	646	-61.56 (-108.2, -14.91)	0.01	124.6	12	90	< 0.001
Intraoperative transfusion	9	450	525	0.41 (0.22, 0.79)	0.008	13.36	8	40	0.10
Length of hospital stay, d	15	609	685	-3.32 (-4.32, -2.32)	< 0.001	75.37	14	81	< 0.001
Postoperative complications	16	624	705	0.52 (0.39, 0.70)	< 0.001	10.10	15	0	0.81
Time to oral intake, d	7	210	289	-0.98 (-1.48, -0.47)	< 0.001	188.28	6	97	< 0.001
Initial residual stone	12	517	604	0.79 (0.50, 1.25)	0.31	3.96	11	0	0.97
Final residual stone	5	136	146	0.34 (0.11, 1.08)	0,07	2.92	4	0	0.57
Stone recurrence	12	530	604	0.63 (0.34, 1.16)	0.14	4.08	11	0	0.97

df: Degrees of freedom; LH: Laparoscopic hepatectomy; OH: Open hepatectomy; WMD/OR: Weight mean difference/odds ratio.

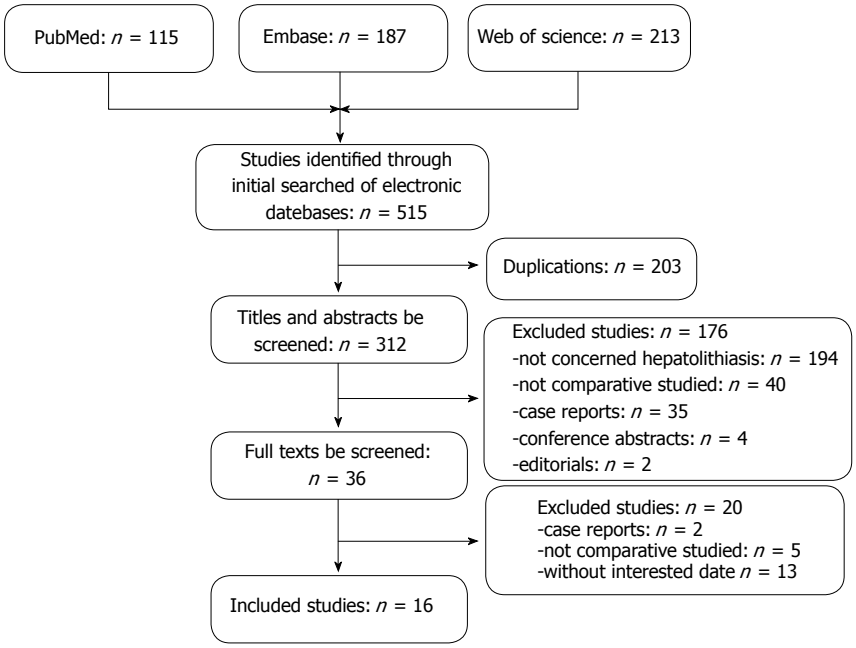


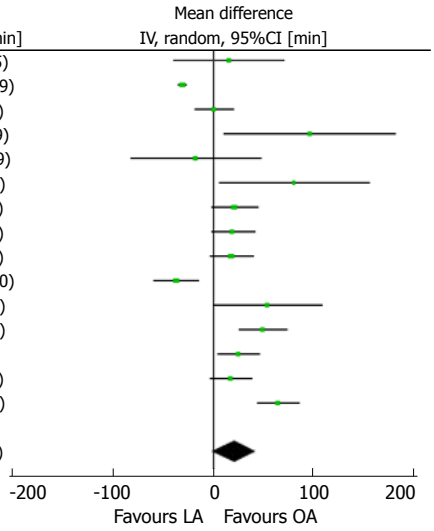
Figure 1 Flow chart showing study retrieval and selection process.

A

Study or subgroup	LA Mean [min]	LA SD [min]	LA Total	OA Mean [min]	OA SD [min]	OA Total	Weight	Mean difference IV, random, 95%CI [min]
Cai 2007	236	135	29	220	61	22	5.4%	16.00 (-39.65, 71.35)
Ding 2015	67.1	8.36	49	97.1	9.82	49	8.2%	-30.00 (-33.61, -26.39)
Jin 2015	207	81	96	206	53	105	7.7%	1.00 (-18.11, 20.11)
Kim 2015	432	158	17	335	85	17	3.6%	97.00 (11.71, 182.29)
Lee 2014	212	66	7	229	66	9	4.7%	-17.00 (-82.19, 48.19)
Li 2008	259	134	14	178	58	20	4.2%	81.00 (6.35, 155.65)
Li 2015	171.3	42.46	23	149.55	35.89	22	7.5%	21.75 (-1.19, 44.69)
Namgoong 2014	257	50.4	37	237	75.5	112	7.6%	20.00 (-1.43, 41.43)
Peng 2016	206.3	52.1	36	187.6	40.6	39	7.6%	18.70 (-2.56, 39.96)
Shin 2015	174.2	56.6	40	210.4	51.6	54	7.6%	-36.20 (-58.50, -13.90)
Song 2009	316.43	64.47	7	262	42.11	10	5.4%	54.43 (0.00, 108.86)
Tian 2013	323.3	103	116	272.8	66.8	78	7.5%	50.50 (26.60, 74.40)
Tu 2010	158	43	28	132	39	33	7.6%	26.00 (5.25, 46.75)
Ye 2015	254	52	46	236	50.75	51	7.7%	18.00 (-2.49, 38.49)
Zhou 2013	277.5	55	44	212.5	43.75	44	7.6%	65.00 (44.23, 85.77)
Total (95%CI)			589			665	100.0%	21.49 (-0.27, 43.24)

Heterogeneity: Tau^2 = 1499.21; Chi^2 = 227.54, df = 14 (P < 0.00001); I^2 = 94%

Test for overall effect: Z = 1.94 (P = 0.05)

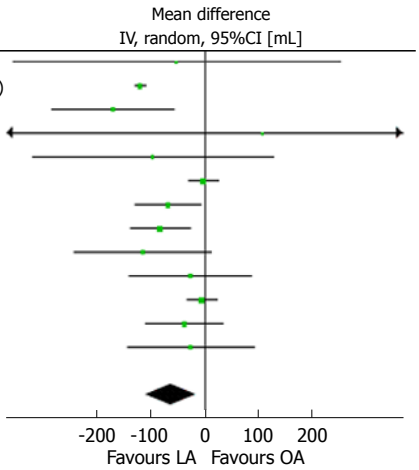


B

Study or subgroup	LA Mean [mL]	LA SD [mL]	LA Total	OA Mean [mL]	OA SD [mL]	OA Total	Weight	Mean difference IV, random, 95%CI [mL]
Cai 2007	603	525	29	655	569	22	2.0%	-52.00 (-357.03, 253.03)
Ding 2015	380	24.7	49	500	22.3	49	12.4%	-120.00 (-129.32, -110.68)
Jin 2015	383	281	96	554	517	105	7.1%	-171.00 (-284.75, -57.25)
Kim 2015	988	929	17	879	942	17	0.5%	109.00 (-519.92, 737.92)
Li 2008	454.5	314.2	14	550.9	348.1	20	3.2%	-96.40 (-320.82, 128.02)
Li 2015	214.57	42.58	23	216.36	50.74	22	11.9%	-1.79 (-29.22, 25.64)
Namgoong 2014	280	96.9	37	347	285.5	112	10.2%	-67.00 (-128.40, -5.60)
Peng 2016	215.8	75.8	36	298.7	158.9	39	10.6%	-82.90 (-138.58, -27.22)
Shin 2015	263.3	166.3	40	378.4	432.9	54	6.5%	-115.10 (-241.54, 11.34)
Tian 2013	479.2	402.1	116	505.8	396.9	78	7.1%	-26.60 (-141.11, 87.91)
Tu 2010	180	56	28	184	50	33	11.9%	-4.00 (-30.86, 22.86)
Ye 2015	332	166.75	46	369	193.75	51	9.6%	-37.00 (-108.76, 34.76)
Zhou 2013	367.5	262.5	44	392.5	300	44	6.9%	-25.00 (-142.79, 92.79)
Total (95%CI)			575			646	100.0%	-61.56 (-108.20, -14.91)

Heterogeneity: Tau^2 = 4557.70; Chi^2 = 124.60, df = 12 (P < 0.00001); I^2 = 90%

Test for overall effect: Z = 2.59 (P = 0.010)

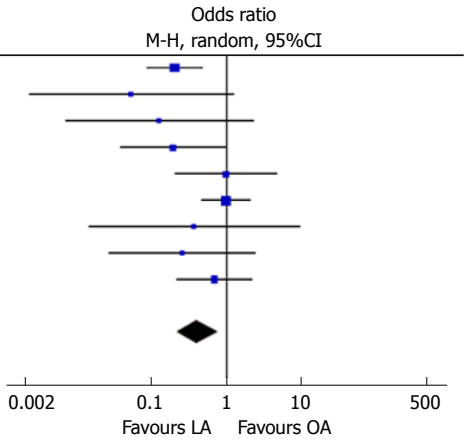


C

Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, random, 95%CI
Jin 2015	8	96	32	105	21.0%	0.21 (0.09, 0.48)
Lee 2014	0	7	5	9	3.8%	0.05 (0.00, 1.24)
Namgoong 2014	0	37	10	112	4.4%	0.13 (0.01, 2.28)
Peng 2016	2	36	9	39	10.8%	0.20 (0.04, 0.98)
Shin 2015	3	40	4	54	11.3%	1.01 (0.21, 4.80)
Tian 2013	21	116	14	78	22.6%	1.01 (0.48, 2.13)
Tu 2010	0	28	1	33	3.6%	0.38 (0.01, 9.70)
Ye 2015	1	46	4	51	6.7%	0.26 (0.03, 2.43)
Zhou 2013	6	44	8	44	15.9%	0.71 (0.22, 2.25)
Total (95%CI)		450		525	100.0%	0.41 (0.22, 0.79)
Total events	41		87			

Heterogeneity: Tau^2 = 0.34; Chi^2 = 13.36, df = 8 (P = 0.10); I^2 = 40%

Test for overall effect: Z = 2.67 (P = 0.008)



D

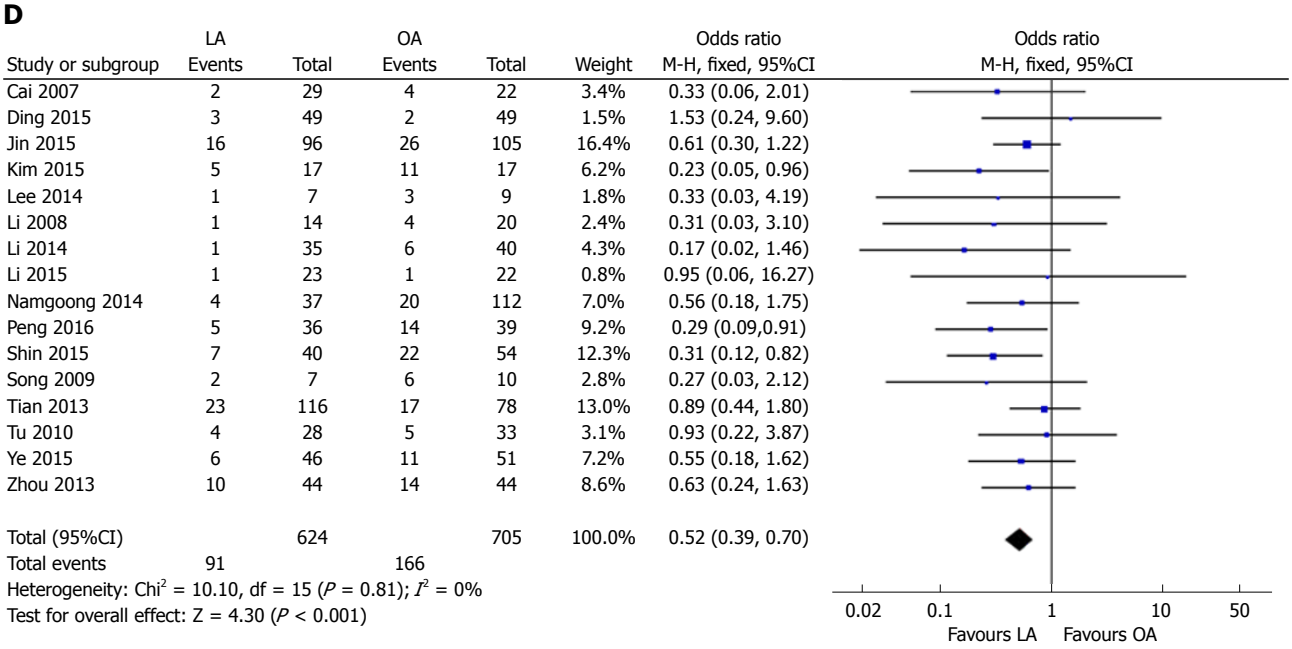


Figure 2 Forest plots comparing operative outcomes between laparoscopic and open liver resection for hepatolithiasis. A: Operative time; B: Intraoperative blood loss; C: Intraoperative transfusion; D: Postoperative complications.

residual rate (P = 0.31 and 0.07, respectively) (Figure 3C and D). Twelve studies[16,19-22,24-26,28-31] reported stone recurrence rate, with no significant difference between the two groups (OR: 0.63, 95%CI: 0.34-1.16, P = 0.14) (Figure 3E).

Subgroup analysis

Operative time, EBL, LOS, intraoperative transfusion, postoperative complications, initial residual stone and stone recurrence were included in subgroup analysis. In the subgroup assessment of operative time, 8 studies[17,19,20,23-26,30] with 793 patients were included. Pooled data of 5 studies[17,19,23,25,26] showed no significant difference in operating time in patients who underwent LLS by laparoscopic and open approach (WMD: -3.04, 95%CI: -28.19 to 22.11, P = 0.81) (Figure 4A). Pooled analysis of 4 studies[24-26,30] evaluating patients who underwent left hemihepatectomy showed no significant difference between the two groups (WMD: 6.72, 95% CI: -14.64 to 28.09, P = 0.54). In contrast, patients who underwent right hepatectomy tended to have a shorter operating time in the laparoscopic group (WMD: 97.00, 95%CI: 11.71-182.29, P = 0.03)[20].

Five studies[17,19,23,25,26] compared estimated blood loss for LLS, and showed significantly less blood loss for laparoscopic hepatectomy compared to open liver resection (WMD: -76.30, 95%CI: -144.45 to -8.15, P = 0.03) (Figure 4B). Four studies[24-26,30] comparing EBL for left hemihepatectomy found significantly less blood loss in the laparoscopic group (WMD: -72.86, 95%CI: -116.03 to -28.69, P = 0.001). One study[20] analyzed EBL for right hepatectomy, and indicated no significant difference between the two groups (WMD: 109.0, 95%CI: -519.92 to 737.92, P = 0.73).

Intraoperative transfusion was analyzed in 3 studies[19,25,26] of left lateral sectionectomy, and showed a lower transfusion rate for the laparoscopic approach (OR: 0.25, 95%CI: 0.12-0.52, P < 0.001) (Figure 4C). Similarly, 4 studies[24-26,30] comparing left hemihepatectomy indicated a lower transfusion rate for the laparoscopic approach (OR: 0.28, 95%CI: 0.08-0.90, P = 0.03).

Postoperative complication rate was analyzed in 8 studies[17,19,20,23-26,30], of which 5 involved LLS, 4 left hemihepatectomy[24-26,30] and 1 right liver resection[20] (Figure 4D). It revealed that the laparoscopic approach resulted in fewer postoperative complications than LLS and RH (P = 0.02 and 0.04, respectively). However, it suggested no significant difference between the two groups for left hemihepatectomy (OR: 0.55, 95%CI: 0.29-1.06, P = 0.07).

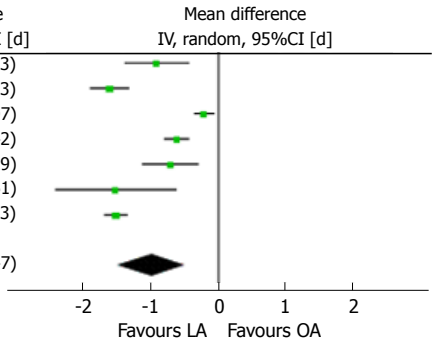
Eight studies[17,19,20,23-26,30] were included in the subgroup analysis of LOS. Five[17,19,23,25,26] evaluated LLS, showing shorter LOS in the laparoscopic group (WMD: -2.03, 95%CI: -2.44 to -1.62, P < 0.001) (Figure 4E). Four studies[24-26, 30] revealed that patients in the laparoscopic left hemihepatectomy group spent less time in hospital (WMD: -3.47, 95%CI: -4.33 to -2.61, P < 0.001). One article[20] suggested no significant difference between the two operative approaches for right hepatectomy (WMD: 4.0, 95%CI: -8.40 to 16.40, P = 0.53).

As for initial residual stone and stone recurrence, subgroup analysis suggested no significant difference

A

Study or subgroup	LA Mean [d]	LA SD [d]	LA Total	OA Mean [d]	OA SD [d]	OA Total	Weight	Mean difference IV, random, 95%CI [d]
Cai 2007	1.1	0.6	29	2	1	22	13.8%	-0.90 (-1.37, -0.43)
Li 2014	2.4	0.5	35	4	0.7	40	15.0%	-1.60 (-1.87, -1.33)
Li 2015	0.8	0.18	23	1	0.26	22	15.5%	-0.20 (-0.33, -0.07)
Namgoong 2014	2.2	0.48	37	2.8	0.46	112	15.4%	-0.60 (-0.78, -0.42)
Peng 2016	2.3	0.8	36	3	1	39	14.2%	-0.70 (-1.11, -0.29)
Song 2009	3.29	0.49	7	4.8	1.32	10	10.5%	-1.51 (-2.41, -0.61)
Zhou 2013	2.5	0.5	43	4	0.25	44	15.4%	-1.50 (-1.67, -1.33)
Total (95%CI)			210			289	100.0%	-0.98 (-1.48, -0.47)

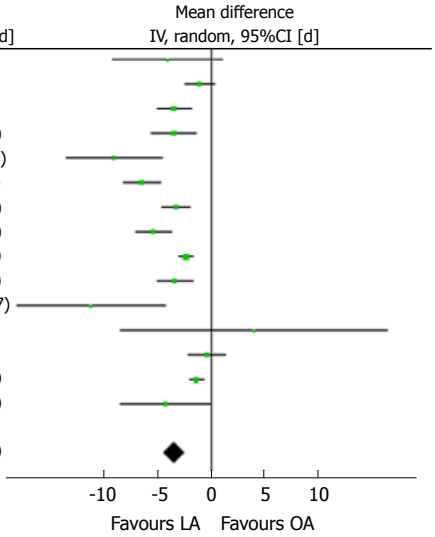
Heterogeneity: Tau^2 = 0.42; Chi^2 = 188.28, df = 6 (P < 0.00001); I^2 = 97%
Test for overall effect: Z = 3.79 (P = 0.0001)



B

Study or subgroup	LA Mean [d]	LA SD [d]	LA Total	OA Mean [d]	OA SD [d]	OA Total	Weight	Mean difference IV, random, 95%CI [d]
Zhou 2013	9.5	10.75	43	13.5	13.25	44	2.8%	-4.00 (-9.06, 1.06)
Ye 2015	11	3.25	46	12	3.75	51	8.9%	-1.00 (-2.39, 0.39)
Tu 2010	6.8	2.8	28	10.2	3.4	33	8.5%	-3.40 (-4.96,-1.84)
Tian 2013	13.1	5.6	116	16.5	8.3	78	7.3%	-3.40 (-5.51, -1.29)
Song 2009	10.14	2.41	7	19.1	6.52	10	3.5%	-8.96 (-13.38, -4.54)
Shin 2015	7.9	2.6	40	14.3	5.5	54	8.2%	-6.40 (-8.07, -4.73)
Peng 2016	7.7	2.2	36	10.9	3.3	39	9.2%	-3.20 (-4.46, -1.94)
Namgoong 2014	8.8	4.1	37	14.1	4.98	112	8.4%	-5.30 (-6.91, -3.69)
Li 2015	10.7	0.82	23	13	1.2	22	10.3%	-2.30 (-2.90, -1.70)
Li 2014	12.3	2.6	35	15.6	4.3	40	8.4%	-3.30 (-4.89, -1.71)
Lee 2014	10.9	4.7	7	22	9	9	1.8%	-11.10 (-17.93, -4.27)
Kim 2015	16	22	17	12	14	17	0.6%	4.00 (-8.40, 16.40)
Jin 2015	10.8	5.3	96	11.1	7.1	105	8.1%	-0.30 (-2.02, 1.42)
Ding 2015	4.5	2	49	5.8	1.5	49	10.2%	-1.30 (-2.00, -0.60)
Cai 2007	8.8	4.4	29	13	9.2	22	3.7%	-4.20 (-8.36, -0.04)
Total (95%CI)			609			685	100.0%	-3.32 (-4.32, -2.32)

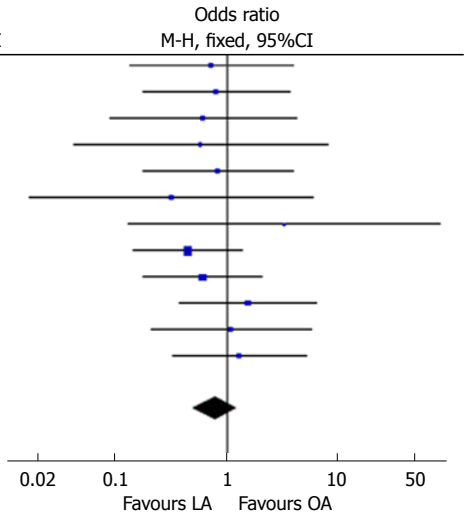
Heterogeneity: Tau^2 = 2.41; Chi^2 = 75.37, df = 14 (P < 0.00001); I^2 = 81%
Test for overall effect: Z = 6.52 (P < 0.00001)



C

Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
Cai 2007	3	29	3	22	7.2%	0.73 (0.13, 4.02)
Jin 2015	3	96	4	105	8.7%	0.81 (0.18, 3.74)
Kim 2015	2	17	3	17	6.2%	0.62 (0.09, 4.29)
Lee 2014	1	7	2	9	3.5%	0.58 (0.04, 8.15)
Li 2014	3	35	4	40	8.1%	0.84 (0.18, 4.06)
Namgoong 2014	0	37	4	112	5.3%	0.32 (0.02, 6.11)
Peng 2016	1	36	0	39	1.1%	3.34 (0.13, 84.60)
Shin 2015	5	40	13	54	22.9%	0.45 (0.15, 1.39)
Tian 2013	5	103	6	78	15.3%	0.61 (0.18, 2.08)
Tu 2010	5	28	4	33	7.1%	1.58 (0.38, 6.55)
Ye 2015	3	46	3	51	6.3%	1.12 (0.21, 5.83)
Zhou 2013	5	43	4	44	8.2%	1.32 (0.33, 5.27)
Total (95%CI)		517		604	100.0%	0.79 (0.50, 1.25)
Total events	36		50			

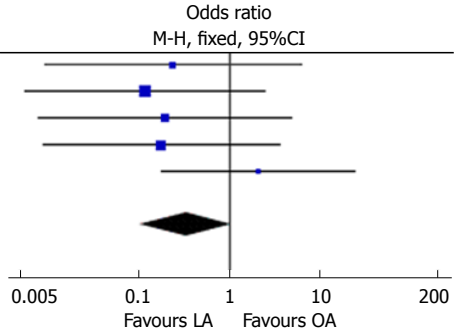
Heterogeneity: Chi^2 = 3.96, df = 11 (P = 0.97); I^2 = 0%
Test for overall effect: Z = 1.01 (P = 0.31)



D

Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
Cai 2007	0	29	1	22	15.1%	0.24 (0.01 6.26)
Kim 2015	0	17	3	17	30.8%	0.12 (0.01, 2.48)
Lee 2014	0	7	2	9	18.9%	0.20 (0.01, 4.91)
Shin 2015	0	40	3	54	26.7%	0.18 (0.01, 3.62)
Zhou 2013	2	43	1	44	8.5%	2.10 (0.18, 24.02)
Total (95%CI)		136		146	100.0%	0.34 (0.11, 1.08)
Total events	2		10			

Heterogeneity: Chi^2 = 2.92, df = 4 (P = 0.57); I^2 = 0%
Test for overall effect: Z = 1.83 (P = 0.07)



E

Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
Cai 2007	0	29	1	22	6.3%	0.24 (0.01, 6.26)
Jin 2015	1	96	0	105	1.8%	3.31 (0.13, 82.33)
Kim 2015	0	17	4	17	16.5%	0.09 (0.00, 1.73)
Lee 2014	0	7	1	9	4.7%	0.38 (0.01, 10.74)
Li 2014	2	35	2	40	6.6%	1.15 (0.15, 8.63)
Namgoong 2014	0	37	2	112	4.7%	0.59 (0.03, 12.55)
Peng 2016	2	36	3	39	10.3%	0.71 (0.11, 4.49)
Shin 2015	1	40	3	54	9.4%	0.44 (0.04, 4.35)
Tian 2013	3	116	2	78	8.8%	1.01 (0.16, 6.18)
Tu 2010	1	28	1	33	3.3%	1.19 (0.07, 19.86)
Ye 2015	2	46	3	51	10.3%	0.73 (0.12 4.56)
Zhou 2013	3	43	5	44	17.3%	0.58 (0.13, 2.62)
Total (95%CI)		530		604	100.0%	0.63 (0.34, 1.16)
Total events	15		27			

Heterogeneity: Chi^2 = 4.08, df = 11 (P = 0.97); I^2 = 0%
Test for overall effect: Z = 1.49 (P = 0.14)

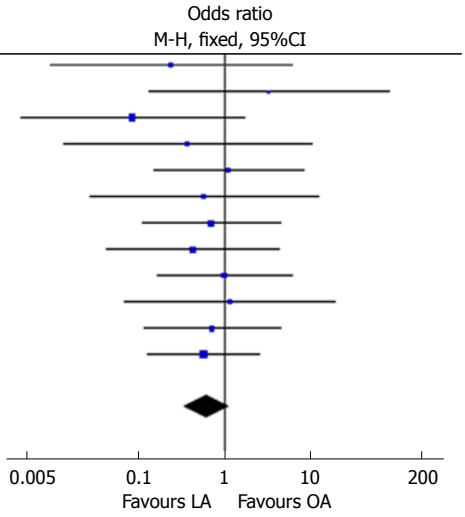


Figure 3 Forest plots comparing postoperative outcomes between laparoscopic and open liver resection for hepatolithiasis. A: Time to oral intake; B: Length of postoperative hospital stay.

Table 3 Quality of cohort studies evaluated by modified Newcastle-Ottawa scale

Ref.	Selection				Comparability		Outcomes		Quality score
	Definition of cases	Representativeness	Selection of controls	Definition of controls	Comparable for 1, 2, 3, 4	Comparable for 5, 6, 7	Assessment of outcomes	Integrity of follow-up	
Cai *et al*[16], 2007	Yes	No	No	Yes	Yes	5	Yes	Yes	7
Li, H *et al*[18], 2008	Yes	No	No	Yes	1, 2	5	Yes	Yes	5
Jin *et al*[19], 2015	Yes	No	No	Yes	1, 2, 3	No	Yes	Yes	5
Kim *et al*[20], 2015	Yes	Yes	No	Yes	1, 3, 4	6, 7	Yes	Yes	7
Lee *et al*[21], 2014	Yes	No	No	Yes	1, 2, 4	5	Yes	Yes	6
Li, J *et al*[22], 2014	Yes	No	No	Yes	1, 2, 3	6	Yes	Yes	6
Li, Y *et al*[23], 2015	Yes	No	No	Yes	1, 2, 3	No	Yes	Yes	5
Namgoong *et al*[24], 2014	Yes	No	No	Yes	Yes	6	Yes	Yes	7
Peng *et al*[25], 2016	Yes	Yes	No	Yes	Yes	No	Yes	Yes	7
Shin *et al*[26], 2015	Yes	No	No	Yes	1, 2	No	Yes	Yes	5
Song *et al*[27], 2010	Yes	No	No	Yes	Yes	6	Yes	Yes	7
Tian *et al*[28], 2013	Yes	No	No	Yes	1, 2, 4	5	Yes	Yes	6
Tu *et al*[29], 2010	Yes	Yes	No	Yes	Yes	No	Yes	Yes	7
Ye *et al*[30], 2015	Yes	Yes	No	Yes	Yes	No	Yes	Yes	7

1: Age; 2: Sex; 3: Liver function; 4: Previous upper surgery history; 5: Surgeon experience; 6: Body mass index; 7: American Society of Anesthesiologists score.

between the two approaches. *P* value for initial residual rate in the different subgroups was 0.09, 0.99 and 0.63, respectively (Figure 5). *P* value for postoperative stone recurrence rate in the different subgroups was 0.99, 0.53 and 0.11, respectively.

Sensitivity analysis and publication bias

The RCT and 8 retrospective studies that scored seven stars or more on the modified Newcastle-Ottawa scale were included in sensitivity analysis (Table 4). No significant changes were found in any of the outcomes. The degree of between-study heterogeneity decreased for operative time, EBL, intraoperative transfusion, LOS and time to oral intake. The degree of between-study heterogeneity remained significant for operating time, EBL, LOS and time to oral intake.

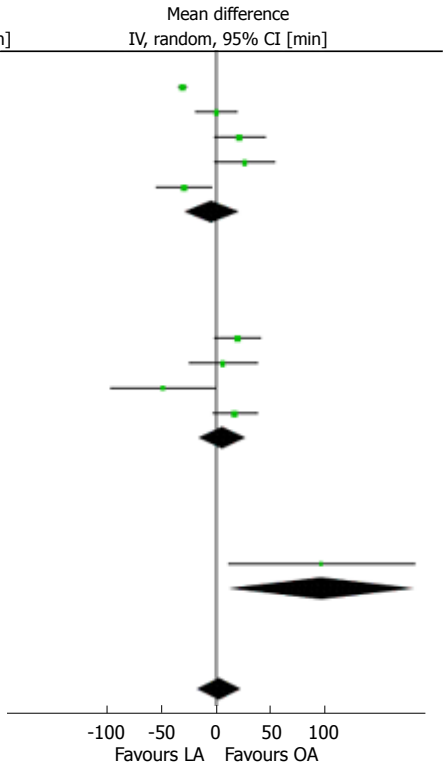
The funnel plot of postoperative complications showed that all articles included in this meta-analysis lay inside the 95% CIs and were symmetrically distributed around the center line, indicating a lack of obvious publication bias (Figure 6).

DISCUSSION

Treatment for symptomatic hepatolithiasis is still an intractable clinical problem. With appropriate therapy, a variety of complications would be avoided, including cholangitis, biliary stricture, recurrent stones, cirrhosis and even cholangiocarcinoma[32]. Traditionally, open hepatectomy was identified as the best method for this disease[33,34]. However, as laparoscopic approaches have been increasingly used in abdominal surgery

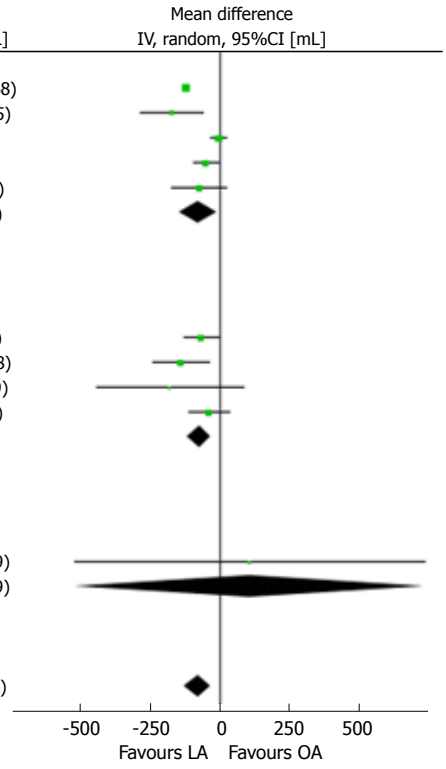
A

Study or subgroup	LA Mean [min]	LA SD [min]	LA Total	OA Mean [min]	OA SD [min]	OA Total	Weight	Mean difference IV, random, 95% CI [min]
2.1.1 LLS								
Ding 2015	67.1	8.36	49	97.1	9.82	49	12.7%	-30.00 (-33.61, -26.39)
Jin 2015	207	81	96	206	53	105	11.5%	1.00 (-18.11, 20.11)
Li 2015	171.3	42.46	23	149.55	35.89	22	11.0%	21.75 (-1.19, 44.69)
Peng 2016	199	56	21	172.2	32.6	23	10.4%	26.80 (-0.61, 54.21)
Shin 2015	175.1	57.2	33	203.8	43	30	10.7%	-28.70 (-53.55, -3.85)
Subtotal (95%CI)			222			229	56.2%	-3.04 (-28.19, 22.11)
Heterogeneity: Tau^2 = 710.69; Chi^2 = 43.07, df = 4 (P < 0.00001); I^2 = 91%								
Test for overall effect: Z = 0.24 (P = 0.81)								
2.1.2 LH								
Namgoong 2014	257	50.4	37	237	75.5	112	11.2%	20.00 (-1.43, 41.43)
Peng 2016	216.5	45.9	15	209.9	41.4	16	9.9%	6.60 (-24.24, 37.44)
Shin 2015	170.1	58.1	7	218.5	50.7	24	7.5%	-48.40 (-95.98, -0.82)
Ye 2015	254	52	46	236	50.75	51	11.3%	18.00 (-2.49, 38.49)
Subtotal (95%CI)			105			203	39.8%	6.72 (-14.64, 28.09)
Heterogeneity: Tau^2 = 263.83; Chi^2 = 7.17, df = 3 (P = 0.07); I^2 = 58%								
Test for overall effect: Z = 0.62 (P = 0.54)								
2.1.3 RH								
Kim 2015	432	158	17	335	85	17	3.9%	97.00 (11.71, 182.29)
Subtotal (95%CI)			17			17	3.9%	97.00 (11.71, 182.29)
Heterogeneity: Not applicable								
Test for overall effect: Z = 2.23 (P = 0.03)								
Total (95%CI)			344			449	100.0%	3.49 (-16.86, 23.84)
Heterogeneity: Tau^2 = 844.88; Chi^2 = 90.65, df = 9 (P < 0.00001); I^2 = 90%								
Test for overall effect: Z = 0.34 (P = 0.74)								
Test for subaroup difference: Chi^2 = 4.87, df = 2 (P = 0.09); I^2 = 59.0%								



B

Study or subgroup	LA Mean [mL]	LA SD [mL]	LA Total	OA Mean [mL]	OA SD [mL]	OA Total	Weight	Mean difference IV, random, 95%CI [mL]
2.3.1 LLS								
Ding 2015	380	24.7	49	500	22.3	49	15.7%	-120.00 (-129.32, -110.68)
Jin 2015	383	281	96	554	517	105	8.5%	-171.00 (-284.75, -57.25)
Li 2015	214.57	42.58	23	216.36	50.74	22	15.0%	-1.79 (-29.22, 25.64)
Peng 2016	186.7	54.2	21	235.2	94.7	23	13.9%	-48.50 (-93.61, -3.39)
Shin 2016	268.5	170.8	33	340	221.6	30	9.6%	-71.50 (-169.91, 26.91)
Subtotal (95%CI)			222			229	62.7%	-76.30 (-144.45, -8.15)
Heterogeneity: Tau^2 = 4983.84; Chi^2 = 72.08, df = 4 (P < 0.00001); I^2 = 94%								
Test for overall effect: Z = 2.19 (P = 0.03)								
2.3.2 LH								
Namgoong 2014	280	96.9	37	347	285.5	112	12.6%	-67.00 (-128.40, -5.60)
Peng 2016	256.7	84.5	15	394.7	185.2	18	9.5%	-138.00 (-238.32, -37.68)
Shin 2015	244.3	159.4	7	421.7	591	24	2.8%	-177.40 (-441.69, 86.89)
Ye 2015	332	166.75	46	369	193.75	51	11.8%	-37.00 (-108.76, 34.76)
Subtotal (95%CI)			105			203	36.7%	-72.86 (-117.03, -28.69)
Heterogeneity: Tau^2 = 151.10; Chi^2 = 3.21, df = 3 (P = 0.36); I^2 = 7%								
Test for overall effect: Z = 3.23 (P = 0.001)								
2.3.3 RH								
Kim 2015	988	929	17	879	942	17	0.6%	109.00 (-519.92, 737.29)
Subtotal (95%CI)			17			17	0.6%	109.00 (-519.92, 737.29)
Heterogeneity: Not applicable								
Test for overall effect: Z = 0.34 (P = 0.73)								
Total (95%CI)			344			449	100.0%	-77.53 (-126.55, -28.51)
Heterogeneity: Tau^2 = 3973.52; Chi^2 = 78.15, df = 9 (P < 0.00001); I^2 = 88%								
Test for overall effect: Z = 3.10 (P = 0.002)								
Test for subaroup difference: Chi^2 = 0.33, df = 2 (P = 0.85); I^2 = 0%								



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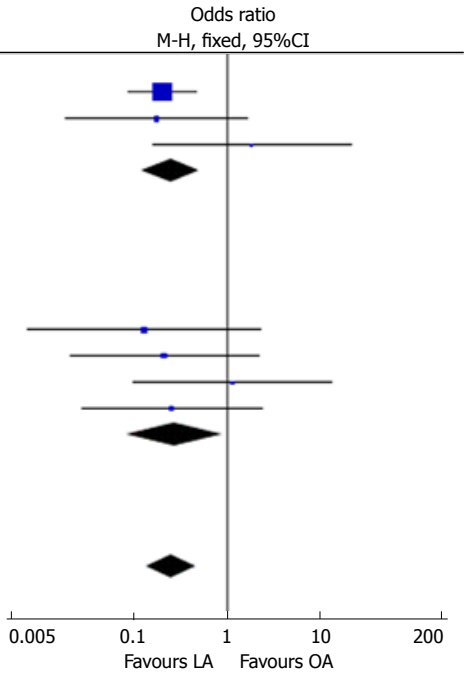
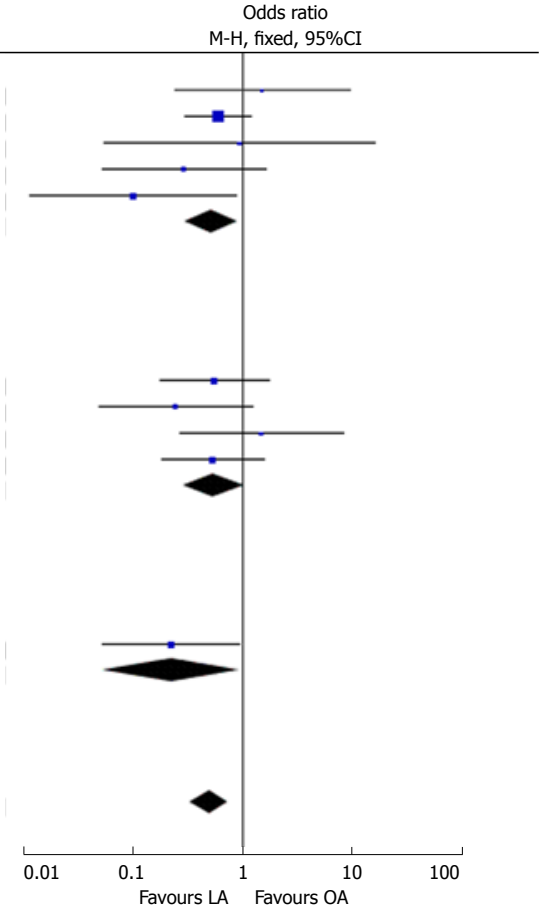


Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
2.5.1 LLS						
Jin 2015	8	96	32	105	59.3%	0.21 (0.09, 0.48)
Peng 2016	1	21	5	23	9.6%	0.18 (0.02, 1.69)
Shin 2015	2	33	1	30	2.1%	1.87 (0.16, 21.75)
Subtotal (95%CI)		150		158	71.0%	0.25 (0.12, 0.52)
Total events	11		38			
Heterogeneity: Chi^2 = 2.86, df = 2 (P = 0.24); I^2 = 30%						
Test for overall effect: Z = 3.77 (P = 0.0002)						
2.5.2 LH						
Namgoong 2014	0	37	10	112	11.0%	0.13 (0.01, 2.28)
Peng 2016	1	15	4	16	7.6%	0.21 (0.02, 2.19)
Shin 2015	1	7	3	24	2.5%	1.17 (0.10, 13.36)
Ye 2015	1	46	4	51	7.9%	0.26 (0.03, 2.43)
Subtotal (95%CI)		105		203	29.0%	0.28 (0.08, 0.90)
Total events	3		21			
Heterogeneity: Chi^2 = 1.66, df = 3 (P = 0.65); I^2 = 0%						
Test for overall effect: Z = 2.13 (P = 0.03)						
Total (95%CI)		225		361	100.0%	0.26 (0.14, 0.48)
Total events	14		59			
Heterogeneity: Chi^2 = 4.58, df = 6 (P = 0.60); I^2 = 0%						
Test for overall effect: Z = 4.30 (P < 0.0001)						
Test for subaroup difference: Chi^2 = 0.02, df = 1 (P = 0.90); I^2 = 0%						

D



Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
2.4.1 LLS						
Ding 2015	3	49	2	49	2.7%	1.53 (0.24, 9.60)
Jin 2015	16	96	26	105	29.7%	0.61 (0.30, 1.22)
Li 2015	1	23	1	22	1.4%	0.95 (0.06, 16.27)
Peng 2016	2	21	6	23	7.4%	0.30 (0.05, 1.68)
Shin 2015	1	33	7	30	10.2%	0.10 (0.01, 0.89)
Subtotal (95%CI)		222		229	51.4%	0.52 (0.30, 0.90)
Total events	23		42			
Heterogeneity: Chi^2 = 4.26, df = 4 (P = 0.37); I^2 = 6%						
Test for overall effect: Z = 2.31 (P = 0.02)						
2.4.2 LH						
Namgoong 2014	4	37	20	112	12.7%	0.56 (0.18, 1.75)
Peng 2016	3	15	8	16	8.9%	0.25 (0.05, 1.24)
Shin 2015	3	7	8	24	3.0%	1.50 (0.27, 8.38)
Ye 2015	6	46	11	51	13.0%	0.55 (0.18, 1.62)
Subtotal (95%CI)		105		203	37.5%	0.55 (0.29, 1.06)
Total events	16		47			
Heterogeneity: Chi^2 = 2.24, df = 3 (P = 0.52); I^2 = 0%						
Test for overall effect: Z = 1.78 (P = 0.07)						
2.4.3 RH						
Kim 2015	5	17	11	17	11.1%	0.23 (0.05, 0.96)
Subtotal (95%CI)		17		17	11.1%	0.23 (0.05, 0.96)
Total events	5		11			
Heterogeneity: Not applicable						
Test for overall effect: Z = 2.01 (P = 0.04)						
Total (95%CI)		344		449	100.0%	0.50 (0.33, 0.75)
Total events	44		100			
Heterogeneity: Chi^2 = 7.83 df = 9 (P = 0.55); I^2 = 0%						
Test for overall effect: Z = 0.36 (P = 0.0008)						
Test for subaroup difference: Chi^2 = 1.27, df = 2 (P = 0.53); I^2 = 0%						

E

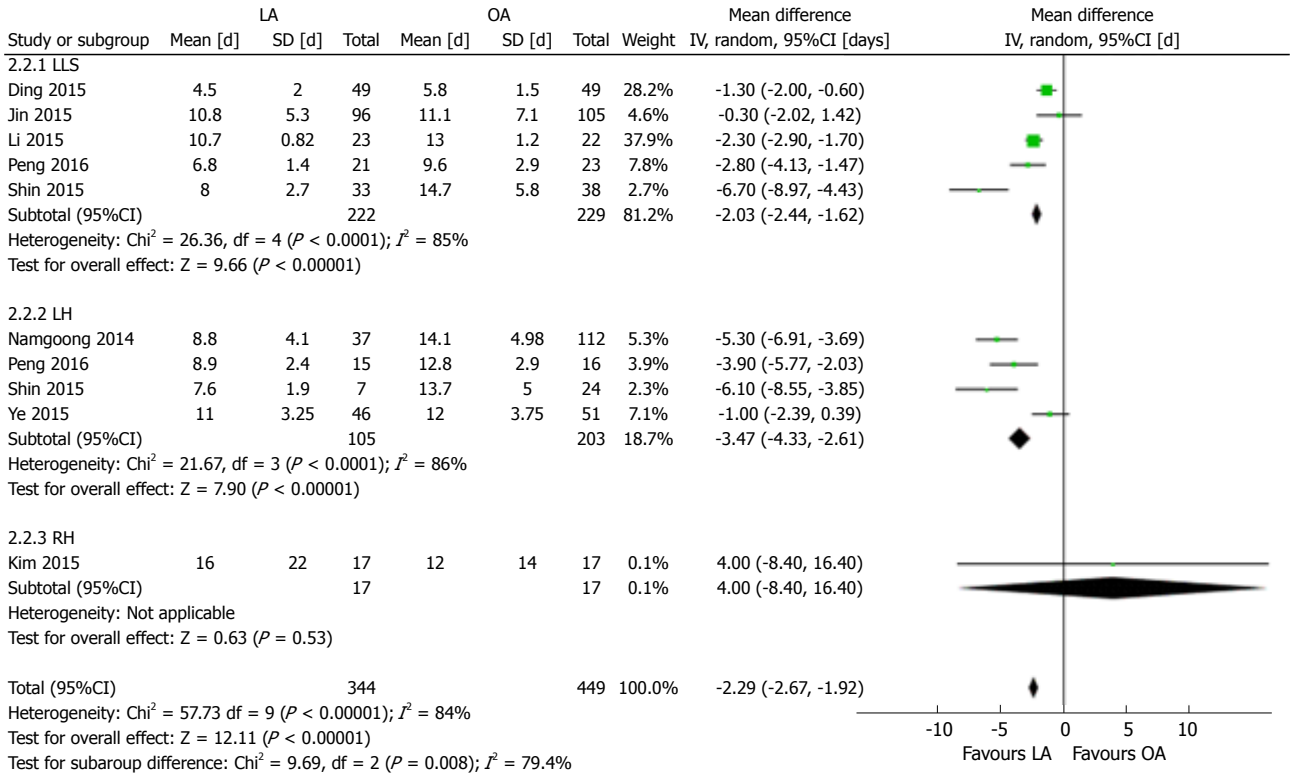


Figure 4 Forest plots and subgroup meta-analysis of operative time (A), blood loss during operation (B), intraoperative transfusion rate (C), postoperative complication rate (D) and length of hospital stay (E). IV: Inverse variance method; LA: Laparoscopic approach; LH: Left hemihepatectomy; LLS: Left lateral sectionectomy; OA: Open approach; RH: Right hemihepatectomy; SD: Standard deviation.

Table 4 Sensitivity analysis in laparoscopic hepatectomy *vs* open hepatectomy

Outcomes of interest	Study, *n*	LH, *n*	OH, *n*	WMD/OR (95%CI)	*P*	Study heterogeneity			*P*
						χ^2	df	I^2, %	
Operative time, min	11	416	464	26.58 (-1.78, 54.94)	0.07	201.48	10	95	< 0.001
Estimated blood loss, mL	9	402	445	-56.21 (-108.00, -4.43)	0.03	73.92	9	88	< 0.001
Intraoperative transfusion	7	314	366	0.47 (0.23, 0.97)	0.04	8.03	6	25	0.24
Length of hospital stay, d	12	450	504	-3.47 (-4.67, -2.27)	< 0.001	47.8	11	77	< 0.001
Postoperative complications	12	451	504	0.55 (0.38, 0.78)	< 0.001	8.33	11	0	0.68
Time to oral intake, d	6	187	267	-1.12 (-1.56, -0.68)	< 0.001	70.78	5	93	< 0.001
Initial residual stone	10	381	445	0.90 (0.53, 1.53)	0.71	2.74	9	0	0.97
Final residual stone	5	136	146	0.34 (0.11, 1.08)	0,07	2.92	4	0	0.57
Stone recurrence	10	394	445	0.59 (0.31, 1.15)	0.12	3.01	9	0	0.96

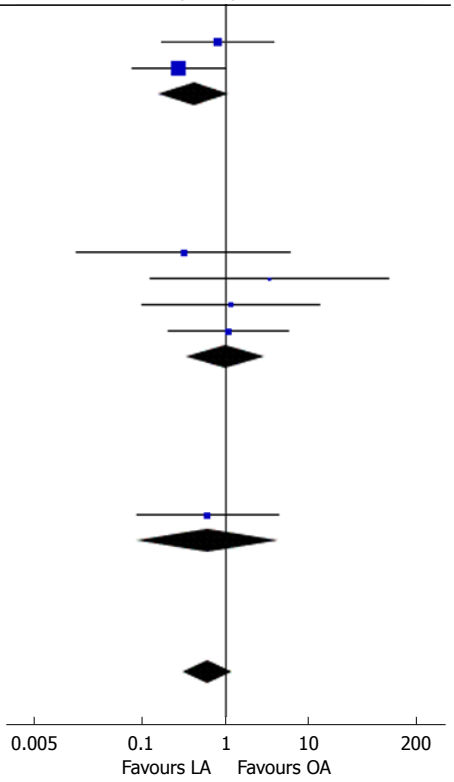
df: Degrees of freedom; LH: Laparoscopic hepatectomy; OH: Open hepatectomy; WMD/OR: Weight mean difference/odds ratio.

over the past two decades, laparoscopic hepatectomy for hepatolithiasis has been considered as standard practice for appropriate cases. Yet, laparoscopic hepatectomy for hepatolithiasis still has not been widely accepted, mainly due to the lack of convincing evidence by adequate comparison of surgical outcomes and long-term quality of life. Nevertheless, numerous studies[16,35] have reported the efficacy, safety and flexibility of laparoscopic hepatectomy for hepatolithiasis. In the current study, we aimed to conduct an extensive worldwide review and meta-analysis to evaluate whether laparoscopic liver surgery can replace open traditional approaches for symptomatic hepatolithiasis.

An earlier meta-analysis performed by Peng *et al*[36] in 2016 focused on left-sided hepatectomy for hepatolithiasis. It included studies of patients with hepatolithiasis in the left lobe and left lateral lobe that underwent laparoscopic or open hepatectomy. It included 8 studies, 1 RCT and 7 non-randomized trials. The conclusion was that the laparoscopic approach was a safe procedure for patients with hepatolithiasis. However, there were several limitations to that study. First, only 8 studies were included, comparing surgical outcomes between the two methods. Second, the

A

Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
2.6.1 LLS						
Jin 2015	3	96	4	105	16.8%	0.81 (0.18, 3.74)
Shin 2015	4	33	10	30	41.8%	0.28 (0.08, 1.00)
Subtotal (95%CI)		129		135	58.5%	0.43 (0.16, 1.13)
Total events	7		14			
Heterogeneity: Chi^2 = 1.13, df = 1 (P = 0.29); I^2 = 11%						
Test for overall effect: Z = 1.70 (P = 0.09)						
2.6.2 LH						
Namgoong 2014	0	37	4	112	10.1%	0.32 (0.02, 6.11)
Peng 2016	1	15	0	16	2.0%	3.41 (0.13, 90.49)
Shin 2015	1	7	3	24	5.3%	1.17 (0.10, 13.36)
Ye 2015	3	46	3	51	12.1%	1.12 (0.21, 5.83)
Subtotal (95%CI)		105		203	29.5%	1.01 (0.34, 2.98)
Total events	5		10			
Heterogeneity: Chi^2 = 1.14, df = 3 (P = 0.77); I^2 = 0%						
Test for overall effect: Z = 0.01 (P = 0.99)						
2.6.3 RH						
Kim 2015	2	17	3	17	12.0%	0.62 (0.09, 4.29)
Subtotal (95%CI)		17		17	12.0%	0.62 (0.09, 4.29)
Total events	2		3			
Heterogeneity: Not applicable						
Test for overall effect: Z = 0.48 (P = 0.63)						
Total (95%CI)		251		355	100.0%	0.62 (0.32, 1.22)
Total events	14		27			
Heterogeneity: Chi^2 = 3.61, df = 6 (P = 0.73); I^2 = 0%						
Test for overall effect: Z = 1.37 (P = 0.17)						
Test for subaroup difference: Chi^2 = 1.31, df = 2 (P = 0.52); I^2 = 0%						



B

Study or subgroup	LA Events	LA Total	OA Events	OA Total	Weight	Odds ratio M-H, fixed, 95%CI
2.7.1 LLS						
Jin 2015	1	96	0	105	3.3%	3.31 (0.13, 82.33)
Peng 2016	1	21	1	23	6.4%	1.10 (0.06, 18.77)
Shin 2015	1	33	2	30	14.3%	0.44 (0.04, 5.09)
Subtotal (95%CI)		150		158	24.0%	1.01 (0.23, 4.52)
Total events	3		3			
Heterogeneity: Chi^2 = 0.98, df = 2 (P = 0.61); I^2 = 0%						
Test for overall effect: Z = 0.01 (P = 0.99)						
2.7.2 LH						
Namgoong 2014	0	37	2	112	8.7%	0.59 (0.03, 12.55)
Peng 2016	1	15	2	16	12.7%	0.50 (0.04, 6.17)
Shin 2015	0	7	1	24	4.8%	1.04 (0.04, 28.44)
Ye 2015	2	46	3	51	19.1%	0.73 (0.12, 4.56)
Subtotal (95%CI)		105		203	45.3%	0.67 (0.19, 2.31)
Total events	3		8			
Heterogeneity: Chi^2 = 0.14, df = 3 (P = 0.99); I^2 = 0%						
Test for overall effect: Z = 0.63 (P = 0.53)						
2.7.3 RH						
Kim 2015	0	17	4	17	30.7%	0.09 (0.00, 1.73)
Subtotal (95%CI)		17		17	30.7%	0.09 (0.00, 1.73)
Total events	0		4			
Heterogeneity: Not applicable						
Test for overall effect: Z = 1.60 (P = 0.11)						
Total (95%CI)		272		378	100.0%	0.57 (0.24, 1.36)
Total events	6		15			
Heterogeneity: Chi^2 = 3.13, df = 7 (P = 0.87); I^2 = 0%						
Test for overall effect: Z = 1.26 (P = 0.21)						
Test for subaroup difference: Chi^2 = 2.08, df = 2 (P = 0.35); I^2 = 3.9%						

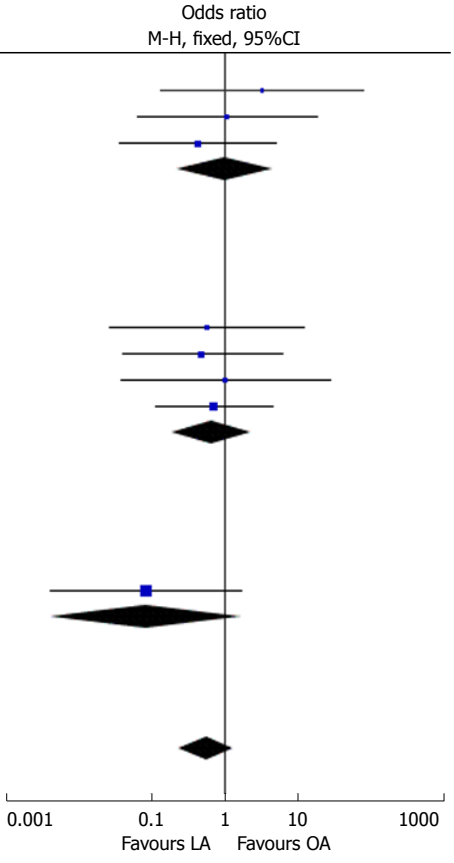


Figure 5 Forest plots and subgroup meta-analysis of initial residual stone rate (A) and stone recurrence rate (B). IV: Inverse variance method; LA: Laparoscopic approach; LH: Left hemihepatectomy; LLS: Left lateral sectionectomy; OA: Open approach; RH: Right hemihepatectomy; SD: Standard deviation.

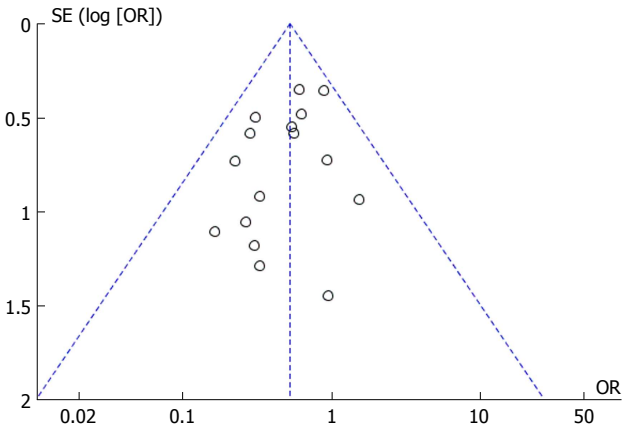


Figure 6 Funnel plot presenting meta-analysis of postoperative complication rate.

authors concluded the advantages of laparoscopic surgery for patients without any subgroup analysis. Furthermore, although hepatolithiasis is prevalent in the left-sided liver, stones may occur in any segment of the liver. The study only included studies that compared the safety and efficacy of laparoscopic and open approaches in left hemihepatectomy and left lateral segmentectomy for hepatolithiasis.

Our meta-analysis of an RCT and 15 retrospective studies, including 1329 patients, compared the efficacy and flexibility of two methods for hepatolithiasis. We showed that the laparoscopic approach was better than the open approach for both right and left sides of the liver, with significantly lower intraoperative blood loss and blood transfusion rate, shorter intestinal function recovery time, shorter LOS, and lower postoperative complication rate. However, no significant differences in operation time, residual stone and stone recurrence were found.

With respect to surgical outcomes, patient safety should be determined first in the application of any procedure. From the pooled data of postoperative complication rate, EBL and intraoperative blood transfusion, our results indicated that patients who underwent laparoscopic liver resection had better perioperative outcomes than those treated with the open approach.

In term of intraoperative outcomes, our study demonstrated that, compared with the open approach, laparoscopic hepatectomy for patients with hepatolithiasis had advantages of lower blood loss and less transfusion. Laparoscopic parenchymal dissection and the high intra-abdominal pressure during laparoscopic hepatectomy attained by pneumoperitoneum result in lower intraoperative blood loss[37]. Moreover, laparoscopy provided a magnified view of the liver, which contributed to bleeding control. Therefore, fewer patients undergoing laparoscopic surgery were in need of intraoperative transfusion. However, operating time did not differ significantly between the two approaches. This suggests that laparoscopic techniques are still a challenge for hepatic surgeons. The surgeons' experience had an impact on hepatic lobe dissection under laparoscopy, which contributed significantly to operating time[38,39]. The laparoscopic approach required frequent installation and removal of laparoscopic devices, resulted in additional operative time. In addition, the dissimilarity of the operating procedures in different institutions would have affected the result.

As for postoperative outcomes, the pooled outcomes of 16 studies with 1329 patients revealed that few patients experienced postoperative complications, including wound-related, vascular and biliary complications. Furthermore, fewer postoperative complications appeared in patients who underwent laparoscopic hepatectomy. In laparoscopic liver resection, the vessels and hepatic bile duct could be identified more precisely with the amplification effect of laparoscopy, and the probability of bile duct injury was reduced. Preoperative magnetic resonance cholangiopancreatography would help to reduce postoperative bile leak. With respect to postoperative recovery, the pooled outcomes of 7 studies suggested that the laparoscopic approach was associated with shorter time to oral intake and intestinal function recovery. Minimal incision, better intraoperative outcomes, and faster intestinal function recovery were confirmed to be in favor of shorter hospital stay in patients undergoing laparoscopic hepatectomy. Future well-designed studies should be performed to confirm these potential benefits.

Long-term outcomes after any procedure should also be taken into account. Our meta-analysis and subgroup analysis both showed that, compared with open surgery, there were no significant differences in residual stone rate and hepatolithiasis recurrence rate between the laparoscopic and open approaches. On the one hand, it means that there is no correlation between selection of the surgical procedure and stone residual/recurrent rate. Indeed, the generation and development of hepatolithiasis may have mainly been caused by anatomical variation and dietary habits in different regions[40,41]. Hepatolithiasis is likely to recur even if no residual stones exist after radical hepatectomy. On the other hand, it is known that the severity of abdominal adhesion after laparoscopic liver resection is significantly less than after open surgery[42]. Even though the patients have recurrence of intrahepatic bile duct stones, it would be easier for them to receive effective and safe treatment.

We conducted subgroup analysis, including left lateral hepatectomy, left hemihepatectomy and right hepatectomy for hepatolithiasis, to avoid the influence of heterogeneity. Similar outcomes were found for postoperative complication rate, blood loss and

intraoperative blood transfusion, whereas EBL in the subgroup of right hepatectomy did not differ between the two surgical approaches. Sensitivity analysis was also performed to assess the impact of study quality on the estimates. A meta-analysis of RCTs would be ideal. However, with ethical concerns and patient expectations, this kind of study is difficult to conduct. This situation highlights the importance of the present meta-analysis. Although only 1 RCT was included in the present study, most of the other studies were of high quality, and the results could be considered credible and evidential.

The present study confirmed that the laparoscopic approach was better than the open approach for hepatolithiasis. However, our meta-analysis had several limitations that should be taken into account. First, although there was no evidence of publication bias, all the included studies were retrospective studies, except for one RCT. This increased the risk of bias for inadequate random sequencing and blinding. Second, the different levels of surgical expertise would have affected the final outcomes, and multicenter studies with large patient samples are required. Third, the included studies were in English, Chinese and Korean, which could have caused language selection bias. Finally, only studies performed in eastern countries were included in our meta-analysis, which could have resulted in regional selection bias. Further studies are needed to overcome the above-mentioned limitations and confirm our findings.

Nevertheless, the results of this meta-analysis are encouraging, as laparoscopic surgical techniques are frequently applied in abdominal surgery. Moreover, sufficient data on a large patient cohort that underwent liver resection for treatment of hepatolithiasis have been accumulated, allowing evaluation by meta-analysis. Multiple strategies were used to identify applicable studies, with strict criteria used for study inclusion and evaluation. Subgroup analysis was performed to minimize heterogeneity. Future studies comparing laparoscopic and open approaches for treatment of intrahepatic bile duct stones should include larger numbers of patients, with a longer follow-up period.

In summary, this meta-analysis demonstrated that laparoscopic surgery was technically feasible and safe, and superior to open surgery for treatment of hepatolithiasis. Subgroups analysis showed consistent results, except for EBL during right hepatectomy. The laparoscopic approach provides a favorable option for patients seeking curative treatment for hepatolithiasis.

COMMENTS

Background

Hepatectomy has become an established treatment modality for patients with hepatolithiasis. With laparoscopic approach widely used in hepatic surgery in recent years, laparoscopic hepatectomy for treating symptomatic hepatolithiasis attracted more and more attention. Despite this, no consensus is available in the literature about which of these two approaches is more beneficial to the patient.

Research frontiers

Laparoscopy is applied in hepatic surgery more and more frequently. It has been reported about no less safety and efficacy of laparoscopic hepatectomy for liver cancer compared to the conventional approach. The worldwide research is directed towards a type of technique to guaranteeing the safety of patients with benign liver disease.

Innovations and breakthroughs

In this study, the authors investigate the perioperative outcomes of laparoscopic hepatectomy and conventional liver resection by pooling data from the literature. This is the first report of a meta-analysis comparing these two kinds of surgical approaches with large sample size comprehensively.

Applications

This work allows understanding the role of two surgical techniques for treating hepatolithiasis.

Peer-review

This systematic review and meta-analysis adds useful information for practice and research, and probably for policy.

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P- Reviewer: Lee HC, Pani SP, Specchia ML **S- Editor**: Qi Y
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World Journal of Gastroenterology

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World J Gastroenterol 2017 November 21; 23(43): 7807-7812

DOI: 10.3748/wjg.v23.i43.7807

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

CASE REPORT

Fatal gastrointestinal histoplasmosis 15 years after orthotopic liver transplantation

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Author contributions: All authors contributed to writing this case report and all authors apart from Agrawal N were involved in the care of this patient.

Informed consent statement: Consent for publication granted by next of kin.

Conflict-of-interest statement: No conflict of interests are declared by any authors.

Manuscript source: Unsolicited manuscript

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Received: July 19, 2017
Peer-review started: July 20, 2017
First decision: August 10, 2017
Revised: September 28, 2017
Accepted: October 17, 2017
Article in press: October 17, 2017
Published online: November 21, 2017

Abstract

We report a case of ileo-colonic Histoplasmosis without apparent respiratory involvement in a patient who had previously undergone an orthotopic liver transplant (OLT) for primary biliary cholangitis 15 years earlier. The recipient lived in the United Kingdom, a non-endemic region for Histoplasmosis. However, she had previously lived in rural southern Africa prior to her OLT. The patient presented with iron deficiency anaemia, diarrhoea, abdominal pain and progressive weight loss. She reported no previous foreign travel, however, it later became known that following her OLT she had been on holiday to rural southern Africa. On investigation, a mild granulomatous colitis primarily affecting the right colon was identified, that initially improved with mesalazine. Her symptoms worsened after 18 mo with progressive ulceration of her distal small bowel and right colon. Mycobacterial, Yersinia, cytomegalovirus and human immunodeficiency virus infections were excluded and the patient was treated with prednisolone for a working diagnosis of Crohn's disease. Despite some early symptom improvement following steroids, there was subsequent deterioration with the patient developing gram-negative sepsis and multi-organ failure, leading to her death. Post-mortem examination

revealed that her ileo-colonic inflammation was caused by Histoplasmosis.

Key words: Histoplasmosis; Orthotopic liver transplant; Primary biliary cholangitis; Primary biliary cholangitis

Core tip: Histoplasmosis is an endemic fungal infection in many parts of the world; the majority of hosts remain asymptomatic. Clinical manifestations are most commonly pulmonary. We present an unusual case of Histoplasmosis occurring in a patient who was living in a non-endemic region, developed the disease after 15 years of immunosuppression following an orthotopic liver transplant, she presented with no pulmonary symptoms but rather luminal GI and systemic symptoms. This highlights the importance of considering Histoplasmosis within the differential of immunosuppressed patients with a past relevant travel history who present with diarrhea, weight loss, abdominal pain and granulomatous colitis.

Agrawal N, Jones DEJ, Dyson JK, Hoare T, Melmore SA, Needham S, Thompson NP. Fatal gastrointestinal histoplasmosis 15 years after orthotopic liver transplantation. *World J Gastroenterol* 2017; 23(43): 7807-7812 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i43/7807.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i43.7807

INTRODUCTION

Granulomatous inflammation of the terminal ileum and right colon is most commonly caused by Crohn's disease in the United Kingdom, however chronic infections can cause similar appearances. Infectious causes include Mycobacteria tuberculosis and avium intracellulare complex, Yersinia, cytomegalovirus (CMV) and human immunodeficiency virus (HIV). Histoplasmosis is not endemic in the United Kingdom however this fungus can remain latent for many years following initial asymptomatic exposure and then may become active with a declining host immune response.

CASE REPORT

A 74-year-old Caucasian woman underwent orthotopic liver transplantation in 1997 for primary biliary cholangitis. She was referred initially to gastroenterology services in 2012 (15 years post-transplant), with new onset iron deficiency anaemia, loose watery stools with urgency occurring up to 3-4 times/wk, cramping lower abdominal pain and weight loss.

In 2009, she had been investigated for suspected interstitial pneumonitis with a trans-bronchial biopsy. No clear cause was established but she was commenced on steroids with subsequent normal chest imaging. Her other notable past medical history included hypercholesterolaemia, insulin-dependent diabetes mellitus and paroxysmal atrial tachycardia.

Her medications were mycophenolate mofetil (MMF) 1 g twice daily; tacrolimus (Prograf) 1 mg twice daily, prednisolone 5 mg daily, aspirin, diltiazem, irbesartan, perindopril, ursodeoxycholic acid (UDCA), simvastatin, insulin and modafinil. There was no relevant family history. The patient reported no recent foreign travel however she had lived in Africa for almost 20 years during her 2nd-4th decades, spending periods of time in rural housing. Nonetheless, this was many years prior to liver transplantation and she had no known exposure to tuberculosis (TB). Blood results showed: Hb 11.0 gm/dL, MCV 77.2 fL, WCC 8.2 × 10^9/L, CRP < 5 mg/L, albumin 44 g/L, ferritin 23 μg/L, bilirubin 3 μmol/L, ALP 129 μ/L and ALT 21 μ/L. Coeliac serology was negative.

Gastroscopy showed gastritis with scattered antral erosions with a negative Helicobacter urease test. The patient's gastritis and anaemia were managed with lansoprazole and oral iron replacement, respectively, and aspirin was stopped. Routine repeat gastroscopy 6 wk later showed improvement. A colonoscopy was performed that showed an indistinct mucosal vascular pattern in the ascending colon, with biopsies revealing a moderately active, right-sided, chronic granulomatous colitis. Given the lack of any past history of inflammatory bowel disease (IBD) and the patient's longstanding immunosuppression, infection was considered. The patient displayed no clinical or laboratory evidence of disseminated infection. She reported no fevers, night sweats, cough or haemoptysis. Inflammatory markers were within normal limits and her chest X-ray (CXR) was normal. Both a Quantiferon gold test and Yersinia serology were negative. A faecal calprotectin was mildly raised at 136 μg/g and small bowel imaging with both magnetic resonance (MR) enterography and a barium follow-through were performed. These showed no significant abnormality, with no evidence of inflammatory bowel disease affecting the small bowel.

As no infective cause had been found, a presumptive diagnosis of mild Crohn's colitis was made and the patient was started on a trial of mesalazine in November 2012 with almost complete remission of her symptoms when she was seen in clinic 4 mo later. At that time lansoprazole was replaced with ranitidine in case the proton pump inhibitor had contributed to her symptoms. In June 2013 the patient stopped her mesalazine as she felt so well.

Unfortunately her symptoms recurred in December 2013, and at clinic review in May 2014 she had lost 10 kg of weight, was having loose bowel motions 3-4 times/d with associated urgency, nocturnal symptoms and occasional faecal incontinence. She also complained of indigestion and early satiety although no dysphagia.

Gastroscopy in July 2014 showed mild oesophagitis and erythematous gastritis, with biopsy findings of mild focal duodenitis but no granulomatous changes, viral

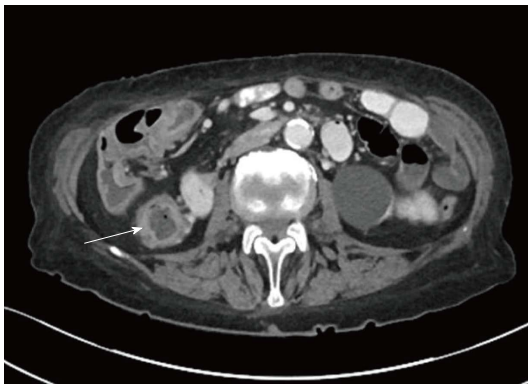

Figure 1 Axial image from computed tomography Chest/Abdomen/Pelvis performed with intravenous (IV) and oral contrast showing non-specific colonic wall thickening in the ascending colon (arrowed) and hepatic flexure characterised by discontinuous mucosal hyperenhancement and submucosal oedema reflecting ulceration and inflammation.

inclusions, parasites, metaplasia or changes suggestive of coeliac disease. Helicobacter urease test was now positive. Repeat colonoscopy showed more significant patchy inflammation and focal ulceration in the right colon. Colonic biopsies showed scattered giant cells and granulomas and terminal ileal biopsies revealed small bowel mucosa distorted with reactive lymphoid tissue and increased eosinophils, lymphocytes and plasma cells in the lamina propria. The pattern was most suggestive of IBD or unusual infections, rather than MMF-related changes, but other drug-related damage could not be excluded. Immunohistochemistry for CMV was negative and acid fast bacilli stain was negative. Mesalazine was restarted, however this time there was no significant symptomatic improvement-prompting a gastroenterology referral.

She was seen by the gastroenterology service in October 2014 at which time her stool frequency was 20 times/d, with ongoing urgency, incontinence and cramping abdominal pain. There was no evidence of blood in the stool or steatorrhoea. She continued to suffer from early satiety, anorexia and had now lost almost 20 kg in weight in the preceding year. A computed tomography (CT) scan of the chest, abdomen and pelvis showed no malignant lesion to explain her symptoms. However it did confirm the appearance of enterocolitis affecting the distal 15 cm of terminal ileum with associated stricturing, as well as the ascending colon up to the hepatic flexure (Figure 1). No abnormality was seen in the lungs. As atypical infections appeared to have been excluded a presumptive diagnosis of Crohn's disease was made and her steroid dose was increased from a maintenance dose of prednisolone 5 mg that she had been on since 2010, to 30 mg daily (with a tapering course) which resulted in some improvement of symptoms. Her severe diarrhoea continued and culminated in a hospital admission in November 2014 due to severe dehydration and acute kidney injury. Although this improved with intravenous fluids, a second course of increased steroids was not beneficial. Virology screen confirmed negative HIV status. Bloods tests revealed: Hb 112 g/L, MCV 77.2 fL, WCC 10.8 × 10^9/L, neutrophils 9.09 × 10^9/L, CRP 15 mg/L, ESR 9 mm/h, albumin 32 g/L, bilirubin 11 μmol/L, ALP 120 μ/L, ALT 11 μ/L and GGT 107 μ/L. A repeat Quantiferon test was again negative.

Given a presumptive diagnosis of progressive Crohn's disease by December 2014, both surgery and infliximab were considered. However, she rapidly deteriorated with evidence of gastrointestinal bleeding. The patient developed Enterococcus septicaemia likely as a secondary complication of her immunosuppressed state and gut inflammation, with subsequent multi-organ failure, and she sadly died in January 2015.

A limited post-mortem examination of the abdomen showed active inflammation and ulceration of the terminal ileum and most of the large intestine. Histological examination revealed abundant intracellular, small oval encapsulated narrow-based budding yeast cells some of which showed a peri-organism halo. The features were those of *Histoplasma capsulatum*, confirmed with positive Grocott and Giemsa histochemical staining (Figure 2). Her final diagnosis was therefore of gram-negative septicaemia as a complication of gastrointestinal Histoplasmosis, immunosuppression and diabetes. Review of her 2014 ileocolonic biopsies at this time with the addition of Grocott stain highlighted scant forms in keeping with Histoplasmosis.

We discovered on later discussion with her family that although the patient had not lived abroad for any prolonged period following her liver transplant, she had visited Southern Africa during this period and stayed in very basic accommodation. Thus, exposure to Histoplasmosis could have either been at this point, in her already immunosuppressed state, or earlier when she had lived in Africa with a prolonged period of dormancy until transplantation and increasing immunosuppressive treatments.

On reflection, there were opportunities for considering Histoplasmosis within the differential diagnosis. At the time of the second colonoscopy, the pattern of ulcerating ileo-colitis raised the possibility of an unusual infection especially in a long-term immunosuppressed individual with diabetes as well as Crohn's disease. Mycobacterial, Yersinia, HIV and CMV infections were excluded, though Histoplasmosis was not considered and as such Grocott stain was not performed at this time.

DISCUSSION

Histoplasmosis is a fungal infection that is typically acquired by inhalation of microscopic spores of the dimorphic fungus *Histoplasma capsulatum,* an organism found in parts of central and eastern North America, Central and South America, Africa, Asia and Australia[1-5]. Its spores thrive in and thus are predominantly found in nitrogen or phosphate- enriched soils that are associated with large amounts of bat and bird guano, including in

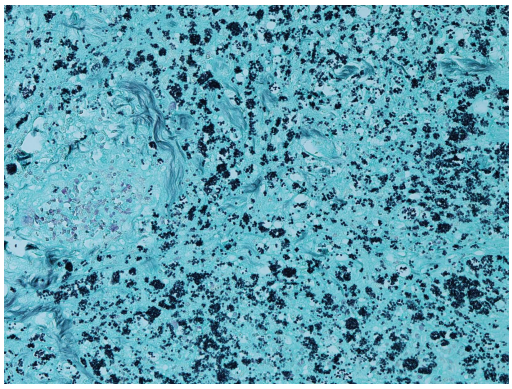

Figure 2 Extensive infiltration of bowel mucosa by the small histoplasmosis, Grocott stain.

caves colonized by bats[6,7] .

Host exposure occurs after inhalation of airborne microconidia (spores) following disturbance of contaminated material in the soil in daily activities, and is extremely common in endemic areas[7,8]. The inhaled *H. capsulatum* transforms from a mold to a yeast state in the lungs, and is phagocytosed by alveolar macrophages, in which it can multiply. These macrophages then disseminate the organism throughout the rest of the body *via* the reticuloandothelial system; generally before a cell mediated immune response can develop[7,9] .

The majority (> 99%) of people exposed to *H. capsulatum* will still be asymptomatic or as the lungs are the entry portals for the organism, show only a mild self-limiting respiratory illness[7]. These people have generally undergone haematogenous dissemination of the organism, however the subsequent development of T cell mediated immunity adequately controls and overcomes the primary infection-preventing it progressing or manifesting[7,8,10,11] .

In the < 1% of patients who do develop clinically appreciable infection, disease severity of Histoplasmosis, is dependent on the number of conidia inhaled and adequacy of the host's T-cell mediated immune response[7] . As such, the healthy host exposed to a relatively small inoculum of *H. capsulatum* would remain asymptomatic, while progressive primary disseminated disease would tend to occur in immunosuppressed patients (particularly with cellular immunity compromise), and especially if exposed to large inoculums of the organism.

Risk factors for developing disseminated Histoplasmosis disease at primary infection include: extremes of age, immunosuppressive conditions such as acquired immune deficiency syndrome (AIDS), haematological malignancies, solid organ transplants (SOTs), stem cell transplants and congenital T-cell deficiency syndromes, as well as immunosuppressive agents-particularly tumour necrosis factor antagonists[7,12] .

Nonetheless, despite SOT being a risk factor for disseminated histoplasmosis, the absolute incidence of the disease in this patient group still remains low. One prospective surveillance study estimated incidence at 1.6/1000 patients, with the highest incidence being in liver transplant patients, though the most cases overall in kidney transplant recipients, as the latter were so much more common[13]. Subsequent large American studies estimate the overall incidence of Histoplasmosis in patients with SOTs at < 0.5%, despite America having a higher endemic rate of the disease than in Europe[14].

The highest risk of developing Histoplasmosis in an exposed patient was in the first year post transplant, although the risk persists up to 20 years later[14]. Use of mycophenolate for immunosuppression and presence of fungaemia were the 2 specific risk factors for severe histoplasmosis in SOT patients identified by multivariate risk factor analysis[14]. Graft rejection appeared not to be a risk factor for Histoplasmosis in these patients[15].

Moreover it should be noted that even in healthy hosts, though the cell mediated immune response against *H. capsulatum* may potentially control the primary infection and prevent symptom manifestation, it does not eradicate the organism. As such *H.capsulatum* can remain silently viable in scattered foci throughout the body for years following initial infection[7]. Leaving these individuals prone to future reactivation of disease with any subsequent decline in cellular immunity; even if this occurs years after they have left the endemic region where they acquired the primary infection[7].

Alternatively, secondary infection can occur if an individual with past exposure to *H.capsulatum* returns to an endemic region and is exposed to a second large inoculum in a newly immunosuppressed state[7]. As our patient visited endemic regions for *H.capsulatum* both before and after her liver transplant, it is impossible to elucidate the mechanism by which she developed infection - new primary infection, secondary infection or reactivation.

In terms of clinical presentation of Histoplasmosis in the < 1% of patients who develop disease, there is a huge spectrum, with almost all the organ systems having potential for involvement following dissemination of the organism. There is a broad subdivision into either pulmonary Histoplasmosis (for predominantly respiratory disease) or disseminated Histoplasmosis (for clinical, laboratory or imaging evidence of extra pulmonary disease)[7].

The presentation of pulmonary Histoplasmosis itself is wide-ranging-from rapidly progressive forms which can present like an acute respiratory distress syndrome (ARDS) picture, more chronic cavitatory Histoplasmosis in older patients with underlying past pulmonary disease, and progressive infection of mediastinal lymph nodes causing granulomatous mediastinitis or mediastinal fibrosis[7]. Our patient did present in 2009 with an interstitial fibrosis noted on her CT scans, and was started on steroids for this as no obvious cause was found for it. This seemed to significantly improve both her symptoms and CT scan, her later chest radiology appearances did not suggest any pulmonary disease, so

it is presumed this was not a respiratory manifestation of Histoplasmosis.

Disseminated Histoplasmosis manifests even more diversely. It can present with single organ involvement as the sole feature of dissemination, or significant systemic features +/- multi-organ involvement or at the most aggressive end of the spectrum- a systemic inflammatory response syndrome mimicking severe sepsis with complications including hypotension, acute kidney injury and disseminated intravascular coagulation[7].

Systemic symptoms in disseminated histoplasmosis include anorexia, weight loss, fatigue and fevers; examination findings can include lymphadenopathy, hepatosplenomegaly, and in some, mucocutaneous lesions such as ulcerations. Blood tests might reveal raised inflammatory markers including CRP, ESR and ferritin[7].

Gastrointestinal histoplasmosis is believed to be a subset of disseminated disease[16,17] and though organisms are often found within the GI tract (one autopsy series identified the organism in 70% cases of disseminated histoplasmosis[18]), significant symptomatic disease affecting this system is strikingly less common with another study reporting the incidence of clinically diagnosed GI disease in only 3%-12% of patients with disseminated histoplasmosis[19]. This discrepancy between autopsy proven presence of the organism and symptomatic disease is present in various other organ systems too, likely because haematagenous dissemination ensures seeding of the organism throughout the body, though organism presence doesn't necessarily correlate with clinical disease.

Observed specific GI symptoms aside from the systemic ones already mentioned include intermittent abdominal pain and diarrhea (both watery and bloody), which in severe cases led to malabsorption[7]. The most serious complications were bowel perforation and haemorrhage[9]. The most commonly involved sites were the colon, then the small bowel. Radiological findings include bowel wall thickening, mass like lesions and signs of small bowel obstruction[9], while endoscopically mucosal ulcerations were most commonly seen (both unifocal and multiple), as well as polypoid lesions, obstructive masses and strictures[7,20].

In the case described here, a diagnosis of Histoplamosis was only made at post-mortem examination. However, if the diagnosis had been considered earlier, a urinary Histoplasma antigen test, which is one of the most sensitive diagnostic methods, could have been used[14]. Antigen testing is also suggested as a useful way of monitoring treatment response; antigen concentrations decrease with therapy and increase in disease relapse[8]. Primary detection on ileocolonic biopsy material may also have been possible if a fungal infection had been suspected and Grocott's methanamine silver stain had been performed at this time.

Histoplasmosis is an eminently treatable disease, with even severe disseminated forms responding well to IV amphotericin B, stepped down to oral azoles for generally at least a 12 mo course[8,14]. If left untreated, this condition often proves fatal.

Overall we have described a case of primarily luminal GI Histoplasmosis, with absent clinical/radiological evidence of pulmonary involvement, occurring 15 years following liver transplantation, in a non-endemic region.

Although Histoplasmosis is a relatively rare disease (even in immunosuppressed patients most prone to developing it); it is eminently treatable if appropriately considered and tested for. Severe disseminated Histoplasmosis if left untreated can often prove fatal. For immunosuppressed patients especially, regardless of where they reside, it is important to consider Histoplasmosis within the differential for ileo-colonic inflammation if a thorough travel history reveals stay in an endemic region, with the possibility of exposure to *H. capsulatum*, even if this was years prior to immunocompromise. The presence of additional pulmonary and/or systemic features may further suggest this disease, although the absence of these features is not enough to disregard the disease.

COMMENTS

Case characteristics

The patient presented 15 years post liver transplant for primary biliary cholangitis, with an iron deficiency anaemia, diarrhea, abdominal pain and progressive weight loss while living in the United Kingdom. Travel history revealed she had previously lived in rural Africa prior to transplantation and later that she had on holiday there again post transplantation, living in quite basic accommodation.

Clinical diagnosis

The patient had primarily luminal GI symptoms without any pulmonary symptoms of note since she presented in 2012. She went on to develop multi-organ failure ultimately; the diagnosis of Histoplasmosis was made at post-mortem examination.

Differential diagnosis

Includes Crohn's disease, as well as other atypical infective causes of colitis including human immunodeficiency virus, Yersinia, Tuberculosis and cytomegalovirus (all of which were excluded). Histoplasmosis could have also been considered within the differential diagnosis at this point, given the deterioration in spite of treatment as probable Crohn's disease, immunosuppression and the travel history to an endemic region for Histoplasmosis. Her initial travel history was reported as not having been abroad for many years.

Laboratory diagnosis

Although in the case, the diagnosis was only made at post mortem, a urinary Histoplasma antigen test is considered one of the most sensitive diagnostic methods for Histoplasmosis.

Imaging diagnosis

Computed Tomography imaging revealed colonic wall thickening, mucosal hyper-enhancement and submucosal oedema reflecting ulceration and inflammation of the ascending colon and hepatic flexure. Endoscopy revealed ileo-colonic ulceration with granulomatous inflammation.

Pathological diagnosis

Grocott's methanamine silver staining and Giemsa histochemical staining identifies Histoplasma capsulatum in biopsy material from affected sites (in this

case post mortem GI tract).

Treatment

Generally should be given to all those presenting with disseminated histoplamosis. Initially treatment consists of intravenous amphotericin B, which can subsequently be stepped down to usually at least a 12-mo treatment course of an oral azole, *e.g.,* itraconazole.

Related reports

Histoplasmosis is a usually an asymptomatic fungal infection in > 99% of exposed people, with the small percentage who do manifest symptoms having primarily pulmonary symptoms. It is largely immunosuppressed patients (with AIDS or other immune deficiencies) who develop disseminated Histoplasmosis, with primary GI luminal disease being rarely reported in the literature, especially post liver transplantation.

Term explanation

Histoplasmosis is an endemic fungal infection acquired through inhalation of the spores of *Histoplasma capsulatum* after disruption of soil containing bat and bird guano in endemic regions.

Experiences and lessons

We note the importance of considering Histoplasmosis within the differential diagnosis for immunosuppressed patients presenting with a granulomatous entero-colitis even in non-endemic areas. Taking a full and detailed history of travel to an endemic region at any point in the past is especially important.

Peer-review

The article of Nikita Agrawal and colaborators shows fatal gastrointestinal histoplasmosis 15 years after orthotopic liver transplantation. This is a good piece of work, data are consistent, paper is well written and conclusions based on presented data.

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P- Reviewer: Lu K, Hilmi I, Paramesh ASS, Zhang JJ **S- Editor**: Qi Y
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ISSN 1007-9327