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OPINION REVIEW

Annexin A2 promotion of hepatocellular carcinoma tumorigenesis *via* the immune microenvironment

Li-Wei Qiu, Yi-Fei Liu, Xiao-Qing Cao, Yan Wang, Xiao-Hong Cui, Xian Ye, Shuo-Wen Huang, Hong-Jun Xie, Hai-Jian Zhang

ORCID number: Li-Wei Qiu (0000-0001-5768-8684); Yi-Fei Liu (0000-0002-4571-2226); Xiao-Qing Cao (0000-0002-3827-1127); Yan Wang (0000-0002-8587-1811); Xiao-Hong Cui (0000-0002-7435-4317); Xian Ye (0000-0001-5747-7158); Shuo-Wen Huang (0000-0001-6409-5983); Hong-Jun Xie (0000-0001-6409-5983); Hai-Jian Zhang (0000-0003-2516-4215).

Author contributions: Qiu LW, Liu YF, and Cao XQ designed the research study, conducted the experiments, analyzed the data, and wrote the paper; Wang Y, Cui XH, Xian Ye, Huang SW, and Xie HJ collected the data and reviewed the manuscript; Zhang HJ is the guarantor; Qiu LW, Liu YF, and Cao XQ contributed equally to this work.

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Li-Wei Qiu, Hai-Jian Zhang, Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Yi-Fei Liu, Department of Pathology, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Xiao-Qing Cao, Department of Thoracic Surgery, Beijing Chest Hospital, Capital Medical University (Beijing Tuberculosis and Thoracic Tumor Research Institute), Beijing 101149, China

Yan Wang, Emergency Department, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Xiao-Hong Cui, Department of General Surgery, Shanghai Electric Power Hospital, Shanghai 200050, China

Xian Ye, Shuo-Wen Huang, Hong-Jun Xie, Medical School of Nantong University, Nantong 226001, Jiangsu Province, China

Corresponding author: Hai-Jian Zhang, MD, PhD, Associate Professor, Doctor, Postdoc, Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, 20 West Temple Road, Nantong 226001, Jiangsu Province, China. hjzhang@ntu.edu.cn

Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver cancer with a dismal prognosis, especially when diagnosed at advanced stages. Annexin A2 (ANXA2), is found to promote cancer progression and therapeutic resistance. However, the underlining mechanisms of ANXA2 in immune escape of HCC remain poorly understood up to now. Herein, we summarized the molecular function of ANXA2 in HCC and its relationship with prognosis. Furthermore, we tentatively elucidated the underlying mechanism of ANXA2 immune escape of HCC by upregulating the proportion of regulatory T cells and the expression of several inhibitory molecules, and by downregulating the proportion of natural killer cells and dendritic cells and the expression of several inhibitory molecules or effector molecules. We expect a lot of in-depth studies to further reveal the underlying mechanism of ANXA2 in immune escape of HCC in the future.

Key words: Annexin A2; Hepatocellular carcinoma; Immune microenvironment; Overall survival; Chemotherapy resistance; Checkpoint

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Core tip: Annexin A2 (ANXA2) has been found to promote cancer progression and therapeutic resistance in patients with hepatocellular carcinoma. However, the mechanism by which annexin A2 facilitates the immune escape of hepatocellular carcinoma remains poorly understood. In this opinion review, we discuss in detail the latest findings on the role of annexin A2 in hepatocellular carcinoma immune escape.

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INTRODUCTION

As an aggressive malignancy, liver cancer is the fifth leading cause of death from cancer globally[1]. Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, has a poor prognosis, especially when diagnosed at the advanced stages[2]. One of the leading risk factors for HCC is infection with the hepatitis B virus, particularly in East Asia[3]. Although surgical treatment for HCC may be effective in the early stages, the 5-year overall survival (OS) rate is only 50%-70%[4,5]. According to a recent study based on proteomic and phosphoproteomic profiling, early-stage HCC can be further stratified into three subtypes with different clinical outcomes[6]. The third subtype, which is characterized by disrupted cholesterol homeostasis, is associated with the lowest postoperative OS rate and the greatest risk of a poor prognosis[7,8]. Despite global advancement of social development and implementation of the annual physical examination program to increase the diagnosis of patients with early-stage HCC, the proportion of patients with advanced HCC at first diagnosis remains high[9]. Although first-line drugs such as sorafenib and lovatinib were used in the treatment of advanced HCC, the landscape of advanced HCC management was not optimistic until the advent of immunotherapy and the knowledge gained about the molecular pathogenesis of the disease[2,10,11].

HCC is considered to be an immunogenic tumor resulting from diseases that lead to chronic inflammation of the liver[12]. Therefore, immunotherapeutic strategies may represent a key treatment direction for improving the clinical outcomes of patients with HCC[13,14]. In recent years, immune checkpoint inhibitors have emerged as potential drugs with promising therapeutic effects against advanced HCC[15-17]; examples include nivolumab[18-20] or pembrolizumab[21,22] for programmed cell death protein 1 (PD-1) blockade, atezolizumab (MPDL3280A) for programmed cell death-ligand 1 blockade[23,24], and ipilimumab for cytotoxic T-lymphocyte-associated protein 4 blockade[25]. Of course, the combination of different immunotherapies or of immunotherapies with conventional therapeutic approaches may provide synergistic effects and facilitate the development of personalized medicine[16,26]. However, the molecular mechanisms underlying HCC immune escape remain poorly understood[27-29].

Annexin A2 (ANXA2, also termed annexin II, p36, calpactin 1 heavy chain, or lipocortin II) was originally extracted from human placenta as an inhibitor of phospholipase A2[30]. The human *ANXA2* gene, which is located on chromosome 15q21, is 40 kb in length and has 13 exons. It can be cleaved by chymotrypsin into a 3 kDa amino-terminal domain and a 33 kDa carboxyl-terminal domain. The ANXA2 protein can exist as a monomer, heterodimer, or heterotetramer *in vivo*. The heterodimeric form consists of one subunit of ANXA2 in complex with a molecule of 3-phosphoglycerate kinase, whereas the heterotetrameric form consists of two ANXA2 subunits combined with an S100A10dimer[31].

The function of ANXA2 is closely related to the form in which it exists. It has been shown that the ANXA2 monomer is localized mainly in the cytoplasm but may transition to the intracellular membrane in response to signals such as changes in the Ca^{2+} concentration, pH, or membrane phospholipid composition. However, the specific biological roles it plays in the subsequent processes are still unclear[32]. The ANXA2 dimer is involved in the formation of intracellular vesicles through combination with multiple endosomes and mediation of membrane fusion[33].

Additionally, the dimer is required for the biogenesis of multivesicular bodies as well as being a constituent of exosomes that are frequently cited in proteomic studies[34]. The ANXA2 heterotetramers are the most well studied of the three forms, and it is now well established that they serve as an assembly site for plasminogen and tissue plasminogen activator on the endothelial cell surface, thereby promoting the generation of plasmin and allowing the clearance of fibrin formed on the blood vessel surface in response to more subtle forms of vascular injury[35,36].

In recent years, increasingly more studies have focused on the relationship between ANXA2 and immune-related diseases, such as lupus nephritis, rheumatoid arthritis, and cancer[37-39]. ANXA2 was found to promote various processes related to cancer progression, such as cancer proliferation, migration, epithelial–mesenchymal transition (EMT), invasion, and stem cell formation, as well as their resistance to radiotherapy, chemotherapy, and immunotherapy[40]. There is growing evidence that ANXA2 plays an important role in tumor immune escape[41].

LITERATURE SEARCH

A scientific literature search was conducted using the PubMed, Web of Science, and Google Scholar databases. The keywords used included "cancer," "hepatocellular carcinoma," "ANXA2," "immune escape," "immunotherapy," "overall survival," and combinations of the aforementioned terms.

MOLECULAR FUNCTIONS OF ANXA2 IN CANCER

Upregulation of ANXA2

A high level of ANXA2 is characteristic of malignant salivary gland tumors[42] and pulmonary invasive mucinous adenocarcinoma[43] and is associated with DNA repair as well as metabolic alteration in pancreatic ductal adenocarcinoma[44]. ANXA2 is highly expressed in gastric cancer tissues and is related to the tumor size, histological differentiation, tumor–node–metastasis stage, and lymph node metastasis[45]. The ANXA2–S100A10 heterotetramers are upregulated by the promyelocytic leukemia–retinoic acid receptor alpha fusion protein and promotes plasminogen-dependent fibrinolysis and matrix invasion in acute promyelocytic leukemia[46]. ANXA2 overexpression contributes to the aggressive phenotype of triple-negative breast cancer in the African American population[47]. The ANXA2 protein content harbored by extracellular vesicles represents a promising prognostic biomarker in endometrial cancer[48]. The ginsenoside compound K inhibits nuclear factor-kappa B by targeting ANXA2[49,50].

In a rat model of cirrhosis, after 30 wk of thioacetamide induction, the level of ANXA2 in the liver increased three times over the level before modeling with the dynamic increasing trend being positively correlated with immune factors, such as interleukin and transforming growth factor-beta, indicating the close relationship between ANXA2 and precancerous lesions of HCC[51]. Our previous study results suggested that the circulating levels of ANXA2 in patients with HCC were significantly higher than those in patients with other liver diseases[52]. ANXA2 was frequently found to be upregulated in HCC tissues compared with its levels in benign liver disease tissues and was significantly correlated with the degree of histological differentiation, intrahepatic metastasis, portal vein thrombosis, and tumor–node–metastasis stage[53]. From our present search of The Cancer Genome Atlas database, we have further confirmed that ANXA2 is upregulated in HCC tissues compared with its level in normal tissues (Figure 1A, $P < 0.001$). In addition, *ANXA2* is a critical differentially expressed gene in nonalcoholic fatty liver disease, where it is associated with the disease severity and modifiable lifestyle factors[54].

Signaling pathways

The interaction of human epididymis protein 4 with ANXA2 promotes the migration of various malignant cells[55]. ANXA2 enhances the progression of colorectal cancer and HCC *via* structural rearrangement of the cytoskeleton[56]. The protein also promotes glioma cell proliferation through the signal transducer and activator of transcription 3-cyclin D1 pathway[57]. ANXA2 has been shown to be a specific target of bleomycin, where its binding with the drug impeded the induction of pulmonary fibrosis mediated by the transcription factor EB-induced autophagic flux[58]. The miR155HG-miR-185-ANXA2 loop contributes to glioblastoma progression[59].The long noncoding (lnc) RNA lung cancer-associated transcript 1 promotes tumorigenesis by inhibiting ANXA2 phosphorylation in HCC[60]. The miR-23b-3p-ANXA2 axis inhibits

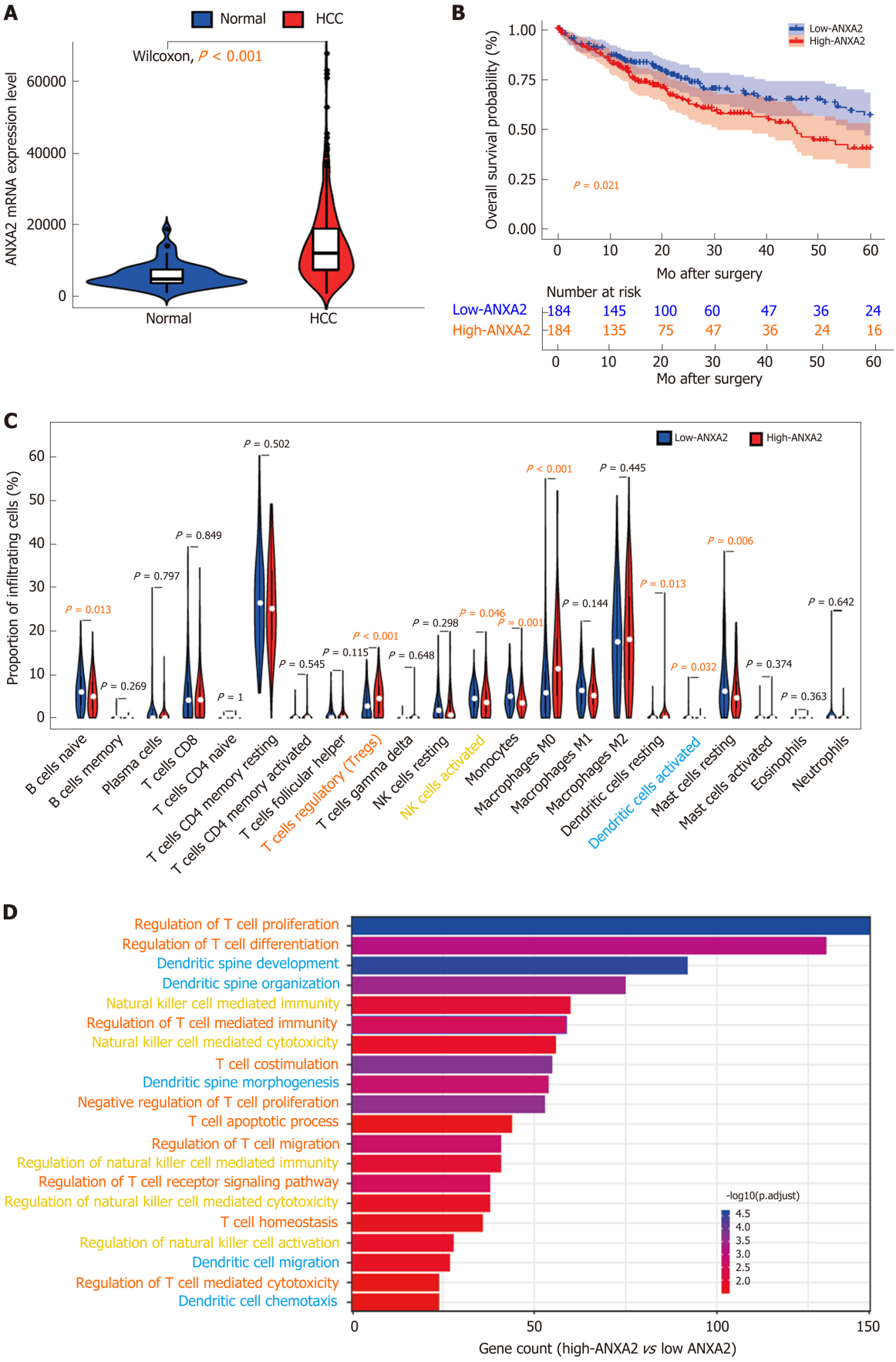

Figure 1 Involvement of Annexin A2 in the change of immune microenvironment. A: The expression of ANXA2 mRNA according to The Cancer Genome Atlas database; B: Increased ANXA2 results in poorer 5-year overall survival; C: The proportion of 22 infiltrating immune cells; D: Gene ontology analysis of differentially expressed genes. ANXA2: Annexin A2; HCC: Hepatocellular carcinoma.

the development and progression of pancreatic ductal adenocarcinoma[61]. ANXA2 was found to promote cancer progression and therapeutic resistance in nasopharyngeal carcinoma[40]. The lncRNA cytoskeleton regulator RNA induces the upregulation of ANXA2 by binding competitively to miR-613, leading to nasopharyngeal carcinoma metastasis[62]. Another lncRNA, colon cancer-associated transcript 1, interacts with ANXA2 to promote beta-catenin translocation to the nucleus where it then activates T-cell factor 4, leading to breast cancer progression[63,64]. The lncRNA small nucleolar RNA host gene 14 potentiates pancreatic cancer progression *via* ANXA2 expression upregulation by acting as a competing endogenous RNA for miR-613[65]. Our previous results have suggested that ANXA2 silencing inhibits the invasion, migration, and tumorigenic potential of hepatoma cells[66].

Epithelial-mesenchymal transition

ANXA2 overexpression is associated with colorectal cancer invasiveness and TGFß-induced EMT through the Src-ANXA2-signal transducer and activator of transcription 3 axis[67]. Mesenchymal stem cells promote hepatocarcinogenesis *via* the interaction of ANXA2 with a novel lncRNA termed mesenchymal stem cell-upregulated factor[68]. ANXA2 inhibition suppresses ovarian cancer progression through the control of beta-catenin and hence EMT[69]. ANXA2 silencing inhibits the proliferation, invasion, and migration of gastric cancer cells[70] as well as non-small cell lung cancer proliferation and EMT through a p53-dependent pathway[71].

Posttranslational modification

The phosphorylation of ANXA2 at its tyrosine residue promotes the invasion and metastasis of drug-resistant breast cancer cells[72]. Highly expressed phosphorylated ANXA2 (Tyr23) also promotes esophageal cancer progression by activating the MYC-hypoxia-inducible factor 1 alpha-vascular endothelial growth factor axis[73]. The tumor suppressor sirtuin 6, which is ubiquitylated and degraded by E3 ubiquitin ligase, contributes to liver tumorigenesis in an ANXA2-dependent manner[74]. Tripartite motif-containing, a novel marker of poor prognosis in ovarian cancer, promotes the malignant progression of the disease by inducing ANXA2 expression[75]. Likewise, tripartite motif-containing 65 supports the aggressiveness of bladder urothelial carcinoma cells by promoting ANXA2 ubiquitination and degradation[76].

ASSOCIATION OF ANXA2 WITH POOR PROGNOSIS

Reduced overall survival

ANXA2 is an independent prognostic biomarker for the malignant progression of laryngeal cancer[77]. The protein may also be a potential prognostic biomarker of liver cancer[78]. The high expression level of ANXA2 in stromal tissue is associated with a reduced OS rate in patients with epithelial ovarian cancer[79], and when highly expressed in cancer cell membranes is associated with poor prognosis in pancreatic cancer[80]. ANXA2 overexpression is predictive of decreased survival in patients with pancreatic cancer[81] and triple-negative breast cancer[82]. According to a quantitative phosphoproteomic analysis, the phosphorylation of ANXA2 Tyr23 is associated with poor prognosis in HCC[83]. Our previous research results confirmed that an increased level of ANXA2 was closely associated with a shortened OS rate in patients with HCC and was therefore identified as an independent prognostic factor of this disease[53]. Herein, working with data from the Cancer Genome Atlas database, we further confirmed that patients with HCC with high ANXA2 expression levels had a shorter OS (Figure 1B, $P < 0.001$).

Drug resistance

A combined *ANXA2*-N-Myc downstream regulated 1-*signal transducer and activator of transcription 1* gene signature predicts the response of patients with cervical cancer to chemoradiotherapy[84]. The interaction of P37 with ANXA2 is required for the mycoplasma-associated multidrug resistance of hepatocarcinoma cells[85]. ANXA2 contributes to cisplatin resistance in cells of non-small cell lung cancer by activating the c-Jun N-terminal kinase-p53 pathway[86] and enhances multidrug resistance in pediatric neuroblastoma by regulating the nuclear factor-kappa B signaling pathway[87]. MiR-101 alleviates the chemoresistance of gastric cancer cells by targeting ANXA2[88]. Chemotherapy combined with bevacizumab can effectively destroy advanced lung adenocarcinoma cells harboring *epidermal growth factor receptor-ANXA2* mutations[89].

ROLE OF ANXA2 IN THE CHAOTIC IMMUNE MICROENVIRONMENT

A study of the antitumor effect of a vaccine prepared from H22 hepatocarcinoma cells induced by cartilage polysaccharides found ANXA2 to be closely related to oncogenesis and cancer development, invasion, and metastasis. A major increase in ANXA2 mRNA was found in the cartilage polysaccharide-induced H22 cells. The data suggested that ANXA2, a specific antigen, may play a key role in the antitumor immune response of HCC and in activating the immune system[90]. ANXA2 was found to be a tumor-associated antigen in patients with lung cancer who had been exposed to asbestos[91]. It has also been implicated in the attachment and entry, genome replication and expression, assembly, and egress of viruses[92].

ANXA2 is essential for the trafficking and capsid disassembly of oncogenic human papillomavirus and protects the virions from lysosomal degradation[93]. The cell-surface translocation of ANXA2 contributes to bleomycin-induced pulmonary fibrosis through its mediation of the inflammatory response in mice[94]. Stromal cell-derived factor-1 alpha triggers the *engulfment and cell motility 1* gene-dependent membrane translocation of ANXA2 for the regulation of HCC chemotaxis and metastasis[95].

Cancer-associated fibroblasts promote EMT and epidermal growth factor receptor–tyrosine kinase inhibitor resistance in non-small cell lung cancers *via* hepatocyte growth factor-insulin-like growth factor-1-ANXA2 signaling[96]. A study on the pathogenesis of immune-mediated liver fibrosis found that after modeling of the disease in rats by injection with pig serum, the ANXA2 concentration increased continually in the rat liver during the process of fibrosis. Similarly, the serum levels of ANXA2 in patients with liver fibrosis were upregulated by 1.4-fold compared with the levels in healthy individuals. When Huh7 cells were exposed to the hepatitis B virus *in vitro*, ANXA2 translocated from the nucleus and cytoplasm to the cytoplasmic membrane, which suggested that it was involved in the immune-mediated liver injury caused by the virus[97]. Dendritic cells (DCs) respond to nasopharyngeal carcinoma cells through an ANXA2-recognizing C-type lectin, named DC-specific intercellular adhesion molecule 3-grabbing nonintegrin[98].

In this present review, our analysis of Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts data[99] revealed that elevated ANXA2 levels resulted in a higher proportion of Treg cells ($P < 0.001$) and lower proportions of activated natural killer (NK) cells ($P = 0.046$) and DCs ($P = 0.032$) than those found in the low-ANXA2 group and in some nonfunctional immune cells (Figure 1C). Furthermore, our Gene Ontology analysis of differentially expressed genes (false discovery rate < 0.05; fold change = 2) suggested that signatures of functional regulation in Treg, NK, and DC cells were enriched (Figure 1D) in patients with HCC.

In addition, ANXA2 plays a key role in nontumorous immunological diseases. Soluble ANXA2 activates human macrophages *via* mitogen-activated protein kinases and may be capable of acting as an inflammatory mediator[100]. ANXA2 expression was downregulated in myeloid cells that had been induced to differentiate through stimulation with all-trans retinoic acid[101]. An immune response mediated by ANXA2 autoantibodies resulted in high circulating levels of interleukin-6 in serum samples from patients with lung cancer[102].

ROLE OF ANXA2 IN IMMUNE ESCAPE

A recent study has shown that T-cell activation, proliferation, and cluster formation are dependent on the proteases tissue plasminogen activator and plasmin[103]. The tissue plasminogen activator treatment of T cells increased the cleavage of ANXA2, which regulates the actin cytoskeleton. Live cell imaging of the activated T cells further indicated a negative role of the ANXA2-regulated actin cytoskeleton in T-cell clustering. This may be one of the mechanisms by which the upregulation of ANXA2 in tumors leads to decreased T-cell activation and an imbalance of the tumor microenvironment[103].The soluble ANXA2 released by tumor cells has novel immunosuppressive properties in patients with renal cell carcinoma[104]. Elevated serum levels of ANXA2 may be important for the suppression of the immune response[105]. A *Listeria*-based ANXA2-targeting immunotherapy in combination with anti-PD-1 antibodies demonstrated high efficacy against pancreatic tumors[39]. ANXA2 in the cancerous cell membrane was identified as the direct antigenic ligand of the Vγ8Vδ3 T-cell receptor of γδ T cells, which make up the first line of defense of stressed cells[41].

At present, there are limited published studies on the role of ANXA2 in immune escape. In this review, we analyzed the correlations of partially labeled genes of Treg

cells (Figure 2A), activated NK cells (Figure 2B), and DCs (Figure 2C). We further confirmed that an elevated ANXA2 level results in the upregulation of several checkpoint molecules, such as PD-1, hepatitis A virus cellular receptor 2, gelectin-9, cytotoxic T-lymphocyte-associated protein 4, CD86, and CD80 (Figure 2D). Moreover, we also found that elevated ANXA2 levels result in the downregulation of several inhibitory molecules (*e.g.*, TGFβ and interleukin-10), and effector molecules (*e.g.*, perforin 1, granzyme B, interferon-gamma, and tumor necrosis factor-alpha; Figure 2E). These results suggest that elevated ANXA2 levels contribute to HCC immune escape.

CONCLUSION

ANXA2 is usually overexpressed in cancerous tissue and results in shorter OS and chemotherapy resistance in patients with HCC[106]. Furthermore, an elevated ANXA2 level results in the upregulation of both the proportion of Treg cells and the expression of several checkpoint molecules as well as the downregulation of both the proportions of activated NK cells and DCs and of several inhibitory molecules. Although there are few research studies to date on the role of ANXA2 in tumor immune escape, we expect a future increase in the number of in-depth studies being carried out to reveal the mechanism through which ANXA2 mediates the immune escape of HCC.

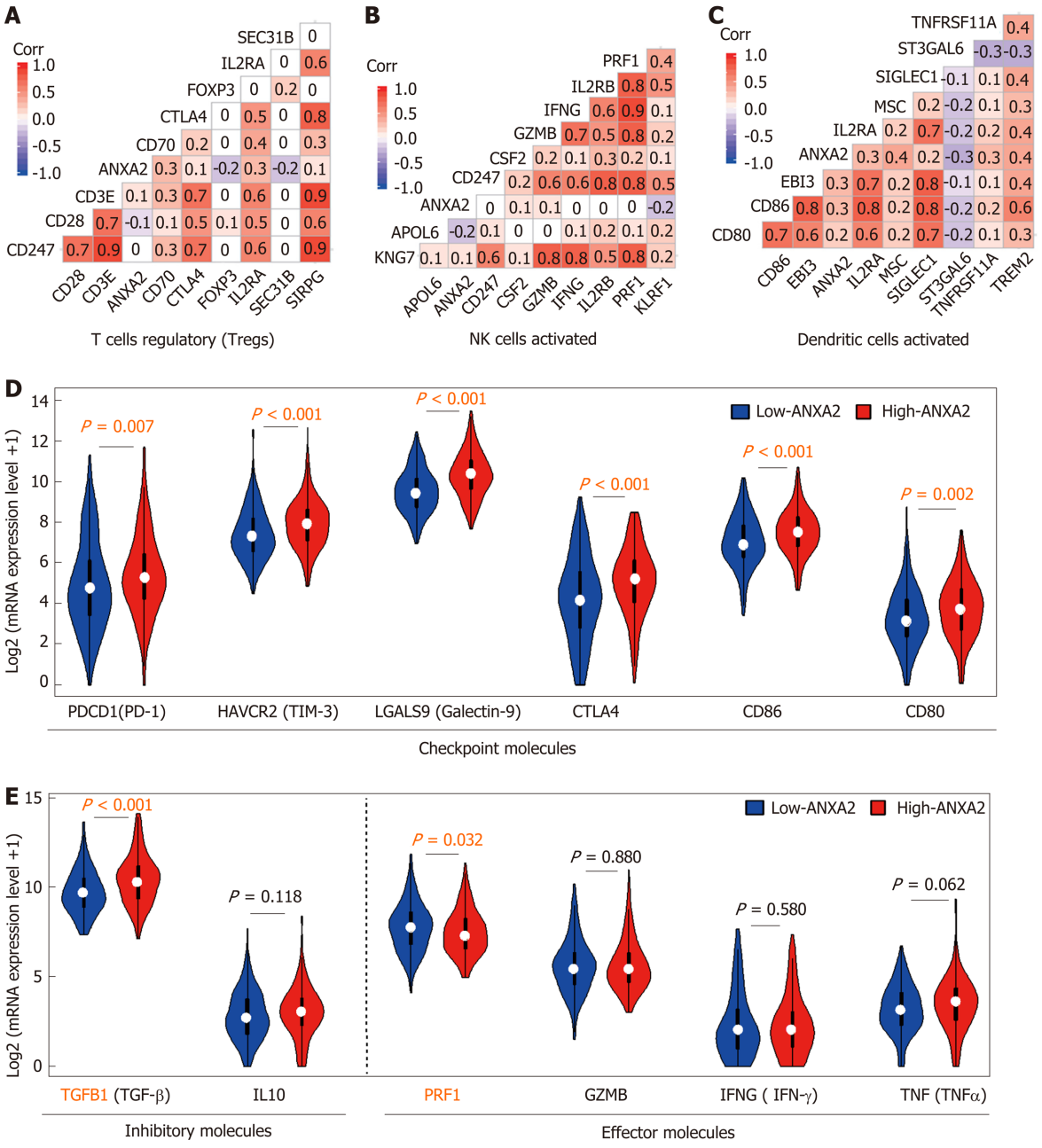

Figure 2 Increased Annexin A2 promotes immune escape in hepatocellular carcinoma patients. A: Correlation of partial labeled genes of regulatory T cells; B: Correlation of partial labeled genes of activated natural killer cells; C: Correlation of partial labeled genes of activated dendritic cells; D: The expression of partial checkpoint molecules; E: The expression of partial inhibitory molecules and effector molecules. ANXA2: Annexin A2; NK: Natural killer.

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REVIEW

Medical management of metabolic and cardiovascular complications after liver transplantation

Chiara Becchetti, Melisa Dirchwolf, Vanessa Banz, Jean-François Dufour

ORCID number: Chiara Becchetti (0000-0002-8262-0304); Melisa Dirchwolf (0000-0002-9083-3561); Vanessa Banz (0000-0002-4887-5776); Jean-François Dufour (0000-0002-8062-1346).

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Chiara Becchetti, Melisa Dirchwolf, Jean-François Dufour, Hepatology, Department of Visceral Surgery and Medicine, Inselspital, University Hospital Bern, Bern CH-3008, Switzerland

Chiara Becchetti, Melisa Dirchwolf, Jean-François Dufour, Department of Biomedical Research, University of Bern, Bern CH-3008, Switzerland

Melisa Dirchwolf, Hepatology, Hepatobiliary Surgery and Liver Transplant Unit, Hospital Privado de Rosario, Rosario S2000GAP, Santa Fe, Argentina

Vanessa Banz, Department of Visceral Surgery and Medicine, Inselspital, University Hospital Bern, Bern CH-3008, Switzerland

Vanessa Banz, Department of Clinical Research, University of Bern, Bern CH-3008, Switzerland

Corresponding author: Chiara Becchetti, MD, Research Associate, Hepatology, Department of Visceral Surgery and Medicine, Inselspital, University Hospital Bern, MEM F807 Murtenstrasse, 35, Bern CH-3008, Switzerland. chiara.becchetti@insel.ch

Abstract

Liver transplantation represents the only curative option for patients with end-stage liver disease, fulminant hepatitis and advanced hepatocellular carcinoma. Even though major advances in transplantation in the last decades have achieved excellent survival rates in the early post-transplantation period, long-term survival is hampered by the lack of improvement in survival in the late post transplantation period (over 5 years after transplantation). The main etiologies for late mortality are malignancies and cardiovascular complications. The latter are increasingly prevalent in liver transplant recipients due to the development or worsening of metabolic syndrome and all its components (arterial hypertension, dyslipidemia, obesity, renal injury, *etc.*). These comorbidities result from a combination of pre-liver transplant features, immunosuppressive agent side-effects, changes in metabolism and hemodynamics after liver transplantation and the adoption of a sedentary lifestyle. In this review we describe the most prevalent metabolic and cardiovascular complications present after liver transplantation, as well as proposing management strategies.

Key words: Solid organ transplantation; Hypertension; New-onset diabetes after transplantation; Obesity; Orthotopic liver transplantation; Post-transplantation metabolic syndrome

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Core tip: Recently there has been an increasing interest in extra hepatic-related complications after liver transplantation because they widely affect late morbidity and mortality. Metabolic and cardiovascular diseases and *de novo* neoplasia are considered to be among the main complications affecting long- and mid-term prognosis after liver transplantation. In this review, we will assess the prevalence of metabolic and cardiovascular complications after liver transplantation, their impact on post-transplant morbidity and mortality, and the optimal medical management currently available.

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INTRODUCTION

Liver transplantation represents the only curative treatment option for end-stage liver disease, chosen cases of acute liver failure and selected patients with hepatocellular carcinoma, providing patients with a complete recovery of their liver function with excellent survival and quality of life[1,2]. Advances in surgical techniques and post-operative medical management have resulted in very good early post-transplant survival rates in the last decades; however, late mortality has remained unchanged[3]. In Europe current reported survival rates are 83%, 71%, 61% and 51% at 1, 5, 10 and 15 years, respectively, with rates increasing up to 86% at 1-year and 74% at 5-year if the period from 2010 to 2014 is considered[4]. Similarly, data from the United States indicates 85%, 68% and 50% 1-year, 5-year and 10-year survival rates after liver transplantation, respectively, with significant differences according to the etiology of the underlying liver disease[5]. These excellent survival rates in the early post-transplantation period underline the importance of understanding the causes and risk factors for late post-transplant mortality, in order to improve overall survival.

Late mortality is traditionally defined as death occurring 5 years or more after liver transplantation[6]. Late mortality is predominantly not related to the liver graft (63%), with high rates of cardiovascular causes and malignancies[7] . Although these findings are in keeping with the main causes of mortality of the general population, patients who underwent liver transplantation show higher risk for developing metabolic, cardiovascular and neoplastic complications[8]. This is partially explained by the need for chronic immunosuppressive drugs, the majority of which are associated with the worsening or development of new-onset hypertension, dyslipidemia and diabetes[9,10]. However, the massive adoption of the Western-world lifestyle and diet have dramatically affected metabolic changes, predisposing and increasing the development of cardiovascular diseases[11]. Therefore, the unmet goal in the management of the post-liver transplantation follow-up is the prevention of these long-term complications. In this review, we aim to review the prevalence of these late-onset complications, their impact on post-transplant morbidity and mortality, and the optimal management currently available.

METABOLIC SYNDROME

Metabolic syndrome is defined as a cluster of interrelated risk factors of metabolic origin, involving insulin resistance and inflammation, which directly promote the development of atherosclerotic cardiovascular disease and type 2 diabetes mellitus[12]. There are different definitions, but most of them consider hypertension, obesity, dyslipidemia, and diabetes mellitus as the main components of metabolic syndrome. Its initial defining criteria, known as the World Health Organization criteria, have not been consistently used because of the need to measure serum insulin and urinary microalbumin to allow for the diagnosis, two expensive analyses[13]. Later on, the Third Report of the National Cholesterol Education Program and the Adult Treatment Panel III[14], proposed a more practical classification (initially described in 2001 and successively revised in 2006[12]), that was widely accepted by the scientific community. According to the National Cholesterol Education Program and the Adult Treatment Panel III modified classification, metabolic syndrome is diagnosed when at least ≥ 3 of

the following criteria are met: (1) Impaired glucose tolerance: Fasting plasma glucose ≥ 100 mg/dL (5.6 mmol/L); (2) Abdominal obesity: Waist circumference > 102 cm (40 in) in men, > 88 cm (35 in) in women; (3) Hypertriglyceridemia: ≥ 150 mg/dL (1.7 mmol/L) or drug treatment for high triglycerides; (4) Low levels of high-density lipoproteins (HDL): < 40 mg/dL (1 mmol/L) in men, < 50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL; and (5) High blood pressure: ≥ 130/85 mmHg or drug treatment for hypertension.

Although originally these considerations on metabolic syndrome were described for the general population, they are currently also adopted in transplanted patients. Another attempt to classify this syndrome was made in 2005 by the International Diabetes Federation criteria, establishing specific national cut-offs, in order to make the classification uniform all over the world[15].

The prevalence of metabolic syndrome is higher in liver transplant recipients when compared to the general population. Retrospective studies assessing the presence of metabolic syndrome post liver transplantation detected this problem in 43%-58% of recipients[16], compared to 30% of non-transplanted patients, with slight variations according to different geographical areas[17]. A recent meta-analysis evaluated eight original publications on metabolic syndrome after liver transplantation, underlining some modifiable and non-modifiable risk factors[16]. Male gender, and components present prior to transplantation such as high BMI[18], type 2 diabetes mellitus[19] and hypertension were all related to the development of *de novo* metabolic syndrome. In particular, patients suffering from diabetes mellitus before transplantation had a six-fold higher risk for developing *de novo* metabolic syndrome[19]. When considering the etiology of the underlying liver disease resulting in the indication for liver transplantation, patients affected by hepatitis C, cryptogenic cirrhosis [group that possibly could include patients with misdiagnosed non-alcoholic steatohepatitis (NASH)] and alcohol related cirrhosis were at higher risk of developing metabolic syndrome after liver transplantation[16,19].

Although the data are not completely conclusive on the effect of immunosuppressive therapy on metabolic syndrome, the metabolic effects of these drugs are well established. Prolonged exposure to these drugs may increase the risk of metabolic complications and/or affect the reversibility of comorbidities present before transplantation. Corticosteroids, usually used in the early post-transplant phase, can act directly on pancreas beta cells increasing insulin resistance, while calcineurin inhibitors can affect the development both of diabetes mellitus (particularly for tacrolimus) and of hypertension (mainly true for cyclosporine). Dyslipidemia is often related to the use of mammalian target of rapamycin (mTOR) inhibitors, whereas the use of anti-metabolites such as mycophenolate have fewer detrimental effects on metabolic syndrome related comorbidities[20]. Considering that all these metabolic side effects are related to immunosuppression, it is reasonable to think that these agents may be the cause of metabolic syndrome. Nevertheless, there is no robust data to support this relationship[21]. Minimizing the effective dose of immunosuppression and supporting a healthy lifestyle are all measures recommended in order to prevent and reduce the development of metabolic syndrome and its related comorbidities.

In the general population, metabolic syndrome is recognized as an independent risk factor for cardiovascular morbidity and mortality. Regardless of the single components of metabolic syndrome, which represent themselves cardiovascular risk factors, metabolic syndrome is a cluster of metabolic dysfunctions that play a multiplicative impact on cardiovascular prognosis[22]. In keeping with these findings, metabolic syndrome has been extensively studied in the setting of liver transplantation. In the aforementioned meta-analysis by Li *et al*[16], liver transplant recipients patients with metabolic syndrome exhibited a higher rate of cardiovascular events, but not poorer survival rates. Patients who are at high risk of developing metabolic syndrome after liver transplantation should undergo regular surveillance in order to achieve an earlier diagnosis and treatment. An early diagnosis of metabolic syndrome will limit possible comorbidities, thereby reducing the risk of cardiovascular events. Additionally, patients who develop metabolic syndrome after liver transplantation are at a higher risk of developing graft steatosis, leading to an increase in the recurrence or in the development of *de novo* non-alcoholic fatty liver disease (NAFLD). NAFLD *de novo* rates range from 20% to 40%[23], but they can increase to 78% when we consider patients transplanted for NASH[24]. This wide variability depends on the methodology used for liver steatosis diagnosis[25]. Nevertheless, in the majority of cases, the recurrence of NAFLD and NASH are harmless, without an evolution towards cirrhosis[26]. Notably, patients with recurrent NAFLD/NASH are more prone to develop cardiovascular comorbidities, type 2 diabetes mellitus and suffer from increased infection-related morbidity and mortality[27]. Interestingly, recipient genetic predisposition might play a role in the recurrence of NAFLD and NASH. The presence of the rs738409-G allele of the patatin-

like phospholipase in liver transplant recipients represents an independent risk factor for post-procedure development of obesity and steatosis[28].

DIABETES MELLITUS TYPE II

New-onset diabetes mellitus type II after-liver transplantation is increasingly recognized as a complication of solid organ transplantation. It is defined by a hemoglobin A1c (HbA1c) level ≥ 6.5% in the transplanted populations[29]. Data on the prevalence of type 2 diabetes in patients after liver transplantation are still controversial. This is due to the heterogeneity of the criteria used for the diagnosis of diabetes mellitus and to the variability in the follow-up time points in the different studies. Nevertheless, the prevalence of type 2 diabetes mellitus ranges from 31% to 38% in post-liver transplantation patients, while the incidence of new onset type 2 diabetes ranges from 13% to 28% in the first three years following transplantation[29,30]. Diabetes mellitus has been demonstrated to have significant consequences in both the early and late post-liver transplantation periods. When present, it was associated with a higher 10-year mortality, compared to non-diabetic liver transplant patients[31].

Patients with diabetes mellitus are more prone to experience complications with an increased risk of cardiovascular events, nephropathy, infections and death[32]. In addition, they experience a higher number of acute rejection episodes compared with non-diabetic patients, with higher rates of graft lost[33]. There are several well-established risk factors associated with the development of diabetes after liver transplantation. Male gender[34], ethnicity, family history[35], hepatitis C[36], citomegalovirus infections[10], and immunosuppressive drugs significantly contribute to the development of new-onset diabetes mellitus or worsening of pre-existing diabetes.

Among the available immunosuppressive drugs, corticosteroids are undisputedly known to increase the risk of new-onset diabetes in a length and dose-dependent manner[37]. This diabetogenic effect represents one of the most worrisome side-effects of glucocorticoids, justifying a strategy of rapid steroid withdrawal. Calcineurin-inhibitors also have a known diabetogenic effect, by directly damaging pancreatic islets cells. Although tacrolimus and cyclosporine share this mechanism of damage, the risk of developing or worsening of diabetes is significantly higher with tacrolimus than with cyclosporine (16.6% *vs* 9.8%, respectively), valid findings for all solid organ recipients[38]. There is convincing evidence from the non-liver transplant population that target glycemic levels significantly reduces morbidity and mortality in patients with type 2 diabetes mellitus[39]. Although this approach has not been specifically proven in the liver transplant population, and little information exists on the use of anti-diabetic drugs in this subset of patients, it is reasonable to assume that euglycemic status is a goal to achieve in post-liver transplantation management. Expert consensus and guideline recommendations suggest screening transplanted patients with basal glycaemia at weekly intervals during the first month following transplantation and subsequently at 3, 6, and 12 mo, with additional annual screening of diabetic complications[35,40]. The oral glucose test remains the best available test to definitely assess new-onset diabetes mellitus[41]. It should be noted that diagnosis of new-onset diabetes is not feasible in the first two months after liver transplantation[41], since in the immediate post-transplant period, insulin requirement is usually increased, being the safest and most effective therapy to treat hyperglycemia. However, once patients have returned to a regular eating pattern and stable immunosuppression, hyperglycemia may either disappear or, in the case of new onset diabetes mellitus, persist. In concordance, use of HbA1c test is recommended 3 mo post-liver transplantation due to possible peri-transplantation transfusions that render the test invalid[40]. The goal for transplanted patients with established type 2 diabetes mellitus should be an HbA1c level of less than 6.5%-7%[35]. An HbA1c level < 6.5% is recommended for patients with a shorter disease duration, younger age and fewer comorbidities. In older patients with multiple comorbidities and a high risk of hypoglycemia, an HbA1c of < 8.0% is considered a safer goal[42]. At present, there is insufficient data to recommend a specific algorithm of anti-diabetic agents in post-transplant diabetes mellitus, as studies addressing this specific population are lacking. However, if current guidelines for the treatment of type 2 diabetes in the general population are extrapolated to liver transplant patients, the choice of the anti-hyperglycemic agent should be tailored to patients' preference and clinical characteristics[43]. Lifestyle changes represent the first line treatment for glycemic control, starting with a balanced diet low in calories and simple carbohydrates accompanied by moderate exercise, although this is often difficult in this patient population with general frailty persisting many years post-liver transplantation.

When these measures are not sufficiently effective, pharmacological therapy with hypoglycemic agents and/or insulin needs to be considered. As the majority of oral diabetic medications are metabolized in the liver, they should be used with caution in patients in whom graft function is reduced[44]. Rosiglitazone, pioglitazone and sulfonylureas have been studied in the post-transplant population showing a possible minimization in insulin requirement[45,46]. Pioglitazone might also be considered in patients at risk of developing *de novo* or recurrent NAFLD after liver transplantation[47]. Metformin has not been extensively studied in the post-liver transplant setting despite its common use as the first-line therapy choice in type 2 diabetes. A single retrospective analysis of 24 renal transplant recipients treated with metformin reported a high rate of drug discontinuation due to gastrointestinal complaints or an increase in serum creatinine. However, no serious adverse events or severe alteration in immunosuppression drug levels were recorded[48]. Interestingly, *in vitro* analysis revealed that metformin optimally reverts diabetogenic genes that are dysregulated in the context of immunosuppression, which is something to take into account when evaluating the choice of therapy[49]. More recently, glucagon-like peptide-1 receptor agonist (GLP-1 RAs, *i.e.*, liraglutide) and inhibitors of dipeptidyl peptidase 4 (DPP-4I or gliptins) were introduced as part of the current antidiabetic therapy. According to recent guidelines, GLP-1 RAs are recommended in the presence of established atherosclerotic cardiovascular disease and might be considered for their additive weight-loss properties. In this scenario, DPP-4I may also be useful because they do not affect body weight[50]. Sitagliptin or vildagliptin use after liver transplantation are not thought to have any significant effect on calcineurin inhibitor or mTOR inhibitor availability. A possible exception includes use of sitagliptin and cyclosporine as well as tacrolimus and vildagliptin, drug combinations that warrants further investigation[51,52]. Finally, there is next to no experience with sodium-glucose cotransporter type 2 (SGLT-2) inhibitors (*i.e.*, canagliflozin, dapagliflozin and empagliflozin) in the setting of liver transplantation. All these drugs work by increasing urinary glucose excretion and are considered highly safe. Nevertheless, we have to consider that SGLT-2 inhibitors are associated with an increased risk of genital and urinary (the latter only for high doses of dapagliflozin) tract infections[53], leading to some controversy in a possible use in liver transplant patients. Moreover, drug elimination mainly occurs through hepatic and biliary excretion, making difficult the use of this medication when liver enzymes alterations are present[50]. The use of SGLT-2 inhibitors may have a positive effect in the setting of heart failure or renal impairment, with a specific dose adjustment needed according to renal function[50]. However, the only direct assessment of the potential interaction with immunosuppressive drugs to date was described in a study including healthy volunteers. Co-administration of cyclosporine resulted in a 23% increase in the mean canagliflozin area under the operating curve (AUC)[54]. The same mechanisms may result in an increased exposure to calcineurin inhibitors and mTOR inhibitors, although further studies are warranted to clarify this possible interaction. In addition, as impaired insulin secretion is a major determinant for liver transplantation, when hepatogenous diabetes[55,56] is suspected, drugs capable to improve β-cell function such as incretins or SGLT-2 inhibitors might be considered.

If therapeutic goals are not met with lifestyle changes and oral anti-diabetic medication, or if the patient becomes metabolically decompensated (symptomatic hyperglycemia with ketosis), insulin must be administered[57]. Ideally, glucagon-like peptide-1 receptor agonists should be used, in combination with basal insulin, in order to reduce the insulin requirement.

In summary, in addition to pharmacological treatment of diabetes mellitus, the adjustment of immunosuppressive regimens can aid in reducing the risk of post-liver transplantation diabetes and improve glycemic control. The possible strategy to adopt consists in minimizing the use of corticosteroids or adopting cyclosporine rather than the more diabetogenic tacrolimus, as well as titrating tacrolimus by adding other immunosuppressive agents (*i.e.*, mycophenolate mofetil or mTOR inhibitors). These possible combinations may help improve glycemic control[57]. Careful attention also needs to be taken into account with regard to other cofactors, such as the occurrence of graft rejection, the concomitant presence of renal failure, *etc*. As mentioned, all immunosuppressive regimen adjustments should be combined with lifestyle modifications, and a carefully selected antidiabetic therapy (Figure 1).

OBESITY

Obesity is defined by the World Health Organization as a body mass index (BMI) > 30 kg/m^2 and morbid obesity as a BMI of > 35 kg/m^2[58]. In the past decade, obesity as a

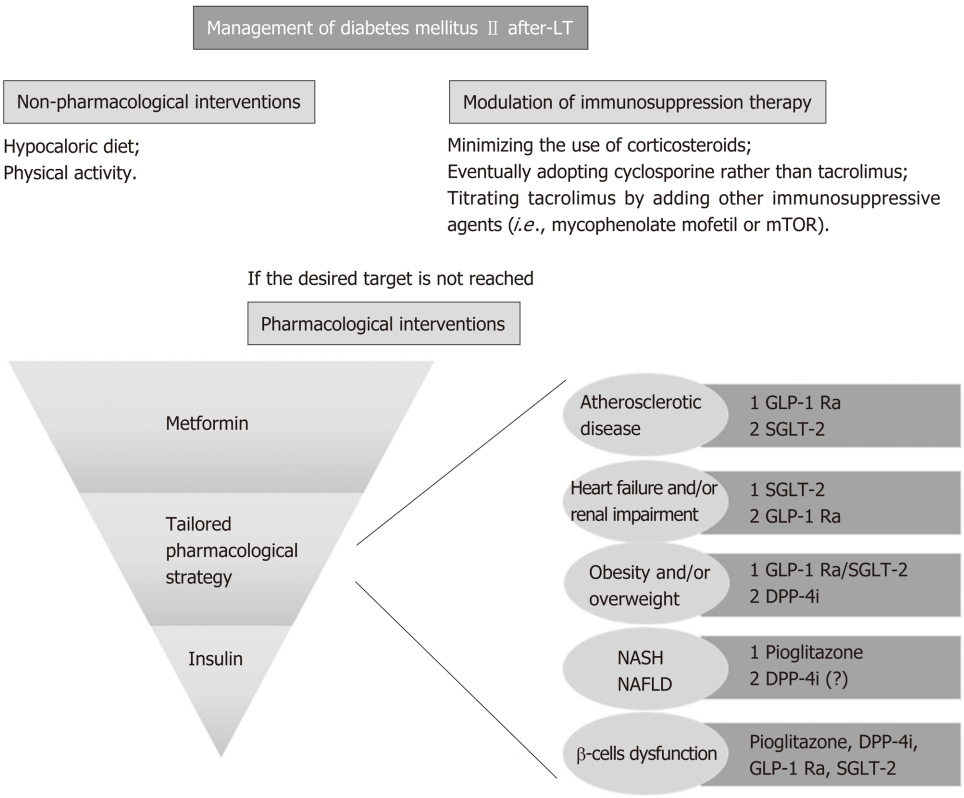

Figure 1 Proposal of a treatment algorithm for diabetes mellitus type II in liver transplant recipients. GLP-1 RA: Glucagon-like peptide-1 receptor agonist; SGLT2 inhibitors: Sodium-glucose cotransporter type 2; DPP-4I: Inhibitors of dipeptidyl peptidase 4; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

whole has progressively become a worldwide epidemic and a relevant comorbidity in the pre-liver transplant setting, with an overt adverse impact on the post-transplant outcome. Although dry weight is not always measurable in patients with decompensated cirrhosis, it is estimated that 15% to 30% of pre-transplant patients meet the criteria for obesity[59]. Following liver transplantation, weight gain tends to increase progressively over time. At one-year post-surgery, 33.7% of patients meet criteria for obesity, and by 5 years, 40.3% of patients are obese[60]. It is frequent that patients with a history of obesity prior to liver transplantation maintain this tendency after the surgical intervention. But additionally, one-third of patients with normal weight prior to transplantation become obese following the procedure[59]. Weight gain in the early post-transplantation period is related to several factors, such as an increased appetite as a result of the resolution of cirrhosis and thus, absence of a catabolic state, and the orexigenic effect of steroids. Risk factors for post-liver transplantation obesity include age greater than 50 years, obesity or type 2 diabetes prior to transplant and NASH as indication to liver transplantation[61]. Patients should be advised to achieve a healthy body weight prior to liver transplantation, as obesity is associated with numerous negative postoperative outcomes such as development of fatty deposits in the graft, development of diabetes, higher cardiovascular and oncogenic risk. Furthermore, patients should be made aware of the tendency to gain weight after transplantation and the problems associated herewith in order to adopt early preventive measures. Modulating immune suppression, such as adopting a regimen with a rapid withdrawal of steroids, is one of the possible strategies in patients at risk. However, it should be noted that steroids are needed not only in the early post-liver transplantation period, but also in the long-term follow-up as treatment of severe cellular rejection, where its use outweighs its associated risks. While corticosteroids are a well-known cause of weight increase, the effect of the exposure to the other available immunosuppressant drugs is not completely defined. Compared with tacrolimus, cyclosporine is associated with more weight gain in the first year following transplantation, whereas this difference is mitigated 2 years post-liver transplantation[60]. Recently, in a randomized trial by Charlton *et al*[62], the authors found that an early introduction of everolimus with reduced-exposure to tacrolimus at 1 month post liver transplantation reduced weight gain assessed at 1 and 2 years post-liver transplantation. When this reduced-exposure to tacrolimus arm was

compared with the absent-exposure to tacrolimus arm, the weight gain was even lower, suggesting a beneficial effect of isolated everolimus therapy. In addition to selecting the best immunosuppression therapy, the fundamental approach to treat and prevent weight gain are lifestyle modifications. Supervised physical activity is considered safe and effective in stable liver transplant recipients[63]. It is proven that increasing aerobic capacity and muscle strength has a favorable impact on glucose homeostasis[64]. There are studies that describe a modified behavior in food intake before and after liver transplantation, with a positive energy balance in the first year after the surgery[65]. Therefore, ongoing physical exercise and the adoption of a healthy low-calorie diet are essential for the management of obesity in the post-transplant setting. Similar to the goals in the pre-transplant setting, the objectives of treating obesity after liver transplantation are to prevent obesity-related comorbidities and mortality, as well as to reduce the incidence of *de novo* NAFLD. When diet and exercise fail to achieve the proposed goal, pharmacologic therapies and/or bariatric surgery should be considered. Orlistat was tested in the post-liver transplant setting and was considered well-tolerated, safe, with no need for a close supervision of immunosuppressant drug levels, and dietary adherence. However, there was no significant evidence regarding its efficacy[66]. Liraglutide was recently approved for the treatment of obesity in non-diabetic patients[67], but to date there is no experience available in liver transplant recipients. Bariatric surgery is considered feasible, when indicated, although some issues remain. Potential problems include the presence of extensive adhesions, rendering surgery technically difficult, as well as the increased risk of wound complications in the setting of steroids or mTOR inhibitors[68]. Whenever possible, steroids should be tapered and stopped and mTOR inhibitors switched to other immunosuppressive agents to reduce the risks of wound healing problems. In the published literature, only case series are available describing the implementation of this therapy in liver transplant recipients (Table 1). Despite weight loss being observed in all reported series (range 21%-75%), high complication rates (30-40% of complications > grade III of the classification of Clavien-Dindo)[69] were documented for all types of procedures, particularly for sleeve gastrectomy[70]. Regarding mortality, there were no reports for sleeve gastrectomy, whereas gastric by-pass showed a mortality rate of 20%[71]. Regarding the pharmacokinetic of immunosuppressant drugs, studies have shown that the kinetics of tacrolimus and mycophenolate mofetil was not impacted by the performance of a sleeve gastrectomy[72]. On the other hand, patients with a gastric by-pass had significantly modified immunosuppressive drugs serum levels[73]. With currently limited data available on the effect of both bariatric surgery and pharmacological treatment on major outcomes such as survival in the post-liver transplant setting, diet and exercise are still considered the cornerstone treatment option for tackling and preventing weight gain.

DYSLIPIDEMIA

High blood lipid levels are unusual in the pre-liver transplant population, due to the impaired hepatic synthetic function in end-stage liver disease. On the other hand, dyslipidemia is a very common finding in the post-liver transplant setting. The definition of hyperlipidemia varies widely among different studies in the post-liver transplant era and only a few consider the standard NCEP-ATP III criteria. In view of this lack of standardization, dyslipidemia is reported as present in 45% to 71% of liver transplant recipients[74]. In most cases, hypertriglyceridemia occurs in the first six months after transplantation, maintaining its prevalence throughout the first year, while it decreases in subsequent years. On the other hand, hypercholesterolemia and low levels of high-density lipoprotein concentration appear later, with an increasing prevalence that affects about 30% of patients at the end of the first post-transplant year[74].

Dyslipidemia has multiple causes after liver transplantation that have been previously discussed in this review: Frequent body weight increase, poor glycemic control, genetic predisposition, donor related factors, early post-liver transplantation renal dysfunction[75] and the effect of immunosuppressant medication[76]. With regard to the latter, long-term corticosteroid use can contribute to hyperlipidemia[77]. Cyclosporine is more frequently associated with hyperlipidemia (14% *vs* 5%) and hypertriglyceridemia (49% *vs* 17%) when compared to tacrolimus, with a dose-dependent relationship[78]. The possible explanation for this cyclosporine effect is related to the inhibition of bile salt synthesis. In the case of tacrolimus, since this drug is known to cause hyperinsulinemia, this effect theoretically may lead to the development of hypertriglyceridemia. mTOR inhibitors are also known to increase

Table 1 Series reporting bariatric surgery after liver transplantation

Ref.	Year	Number of patients	Follow-up, mean (range)	Type of bariatric surgery	Improvement of comorbidities	Complications, *n* (%)	Mortality, *n*
Duchini *et al*[119], United States	2001	2	27 (18-36)	RYGB	Yes	0	0
Tichansky *et al*[120], United States	2005	1	4	RYGB	Yes	0	0
Butte *et al*[121], Chile	2007	1	6	SG	NE	0	0
Gentileschi *et al*[122], Italy	2009	1	9	BPD	NE	0	1 (myocardial infarction)
Elli *et al*[123], United States	2013	1	3	SG	NE	0	0
Lin *et al*[124], United States	2013	9	5 (3-12)	SG	Yes	3 (33.3) (1 incisional hernia, 1 bile leakage, 1 dysphagia)	0
Al-Nowayalati *et al*[71], United States	2013	7	59 (6-103)	RYGB	Yes	4 (57.1) (2 incisional hernia, 2 wound infections)	2 (1 septic shock at 6 mo after, 1 esophageal carcinoma at 9 mo after)
Pajecki *et al*[125], Brazil	2014	1	5	SG	Yes	0	0
Elli *et al*[126], United States	2016	2	2	SG	NE	0	0
Khoraki *et al*[127], United States	2016	5	33.7 (13-79)	SG	Yes	1 (20) (gastrointestinal bleeding)	0
Osseis *et al*[128], France	2017	6	41 (12-94.4)	SG	Yes	2 (33.3) (1 gastric fistula, 1 parietal mesh infection)	1 (multi-organ failure at 19 mo after)
Tsamalaidze *et al*[129], Mexico	2018	12	24	SG	Yes	4 (33.3) (2 dysphagia, 1 late drainage removal, 1 gastrostomy)	0

RYGB: Roux-en-Y gastric bypass; SG: Sleeve gastrectomy; BPD: Biliopancreatic diversion; NE: No effect.

the risk of hyperlipidemia (especially hypertriglyceridemia), through changes in insulin signaling pathways resulting in an excess of triglycerides production and secretion[79]. The combination therapy of mTOR inhibitors and tacrolimus results in lower rates of dyslipidemia[80], something also observed when switching from cyclosporine to tacrolimus[81]. Post-transplant dyslipidemia is generally resistant to dietary interventions, but it responds to traditional lipid-lowering agents. The most recent guidelines of the European Society of Cardiology[82] recommended the use of low-density lipoprotein concentrations (LDL-C) as the primary target for treatment of dyslipidemia, while for patients with elevated triglycerides, HDL-C is recommended as a secondary goal. In primary and secondary prevention for patients catalogued as having very-high risk for cardiovascular events, an LDL-C reduction of ≥ 50% from baseline and an LDL-C goal of < 1.4 mmol/L (< 55 mg/dL) are recommended, whereas in patients at high risk an LDL-C goal of < 1.8 mmol/L (< 70 mg/dL) is sufficient. This last version of the European Society of Cardiology guidelines dedicated a specific session on the management of dyslipidemia in solid organ recipients, although the recommendations are mostly based on studies on kidney recipients. They conclude that the management of dyslipidemia in transplant recipients should be comparable to that recommended for high or very-high risk patients, with an additional caution for possible drug-drug interactions (Figure 2). Statins are unanimously considered as a first line therapy for dyslipidemia in liver transplant patients, preferably pravastatin and fluvastatin because of the lack of interaction with cytochrome P450 and calcineurin inhibitors metabolization[21]. Generally, cyclosporine increases the blood levels of all statins, even more so than tacrolimus. Nevertheless, statins, with particular reference to pravastatin, have been

established to be safe, efficacious and well tolerated in solid organ transplant recipients[83]. The concomitant use of other drugs metabolized by the cytochrome CYP3A4 should be carefully used in patients receiving both calcineurin inhibitors and statins[82], because a perturbation in the cytochrome P450 metabolic pathway can increase immunosuppressive drugs toxicity[84]. Ezetimibe may be considered in recipients who do not tolerate statins, although the experience is scant[85]. Concomitant use of calcineurin inhibitors may result in increased statin levels in the blood. Isolated hypertriglyceridemia can also be present post- liver transplantation and it generally responds well to fish oil. Omega 3 has less drug-drug interactions with immunosuppressive therapy. In addition, omega 3 oil has other pleiotropic effects, such as anti-inflammatory and anti-proliferative properties, which can improve hepatic steatosis[86]. With regard to other lipid-lowering drugs, such as fibric acid derivatives, they are usually well tolerated, although there is scarce data available on their use in liver transplant patients. Importantly, the combination of fibrates with statin therapy increases the risk of myopathies and is thus not recommended. Patients on both these medications should be counseled regarding myalgia as a potential early symptom of rhabdomyolysis[87].

ARTERIAL HYPERTENSION

Arterial hypertension, which is defined as a systolic blood pressure greater than 140 mmHg and/or a diastolic blood pressure greater than 90 mmHg[88], occurs in 30%-50% of patients after liver transplantation, increasing up to 70% when evaluating patients in the long term[89,90]. Features such as high cardiac output, low systemic vascular resistance and low mean arterial pressure characterize end-stage liver disease. After patients are transplanted, this hemodynamic situation changes, leading to an increase in systemic blood pressure. Nevertheless, the etiology of post-liver transplantation hypertension is multifactorial and includes not only this change in hemodynamics, but also the use of immunosuppressive medications. These drugs are a well-known risk factor for hypertension, in particular calcineurin inhibitors. Although there are numerous pathogenetic mechanisms related to the development of hypertension in such patients, vasoconstriction seems to be the main causal factor. Vasoconstriction is caused by the excessive secretion of endothelin-1 and thromboxane and decreased production of prostacyclin, leading to an increase in sympathetic nervous system activity. In addition to these mechanisms, cyclosporine and tacrolimus act on sodium retention, resulting in an increase in effective-volume[90]. Nevertheless, cyclosporine seems to have a more deleterious effect compared to tacrolimus, showing a higher prevalence rate of arterial hypertension (73% *vs* 63%, respectively)[91]. Glucocorticosteroids are also a known cofactor for the development of arterial hypertension. They increase blood pressure through the renin-angiotensin-aldosterone system, causing a reduction in prostacyclin and nitric oxide production, and an increase in the quantity of angiotensin II receptors[92]. However, considering the usually short time of exposure to steroids in these patients, calcineurin inhibitors are the main agent responsible for the long-term development of arterial hypertension. The main concern about arterial hypertension is related to the direct damage on organs and its established association to increased risk for cardiovascular events[93]. Elevated blood pressure may lead to endothelial damage, atherosclerosis, kidney damage and left ventricle remodeling. Hypertensive control is essential to the improvement of long-term survival of both the graft and the recipient, related to the non-negligible risk of developing major cardiovascular events. The withdrawal of steroid therapy, the down-titration of calcineurin inhibitors (when adding mycophenolate mofetil or mTOR inhibitors) are possible strategies to reduce the increase in blood pressure values. Lifestyle modifications (*i.e.*, low-sodium diet, cessation of smoking, avoidance of alcohol, and weight loss) should always be clearly explained to the patient. Nevertheless, when these measures are unsuccessful, medical therapy is mandatory. A blood pressure goal lower than 130/80 mmHg should be targeted to minimize cardiovascular risk[94]. Dihydropyridine calcium channel blockers are the preferred first-line agents in patients who do not exhibit proteinuria, in order to directly counteract the vasoconstriction associated with calcineurin inhibitors[95]. If proteinuria is present, liver transplant recipients benefit from angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers as a first line choice[95]. If a single-agent therapy is ineffective, a combination therapy should be evaluated, taking into account that the addition of beta-blockers is particularly indicated when a cardiac complication is well established[96]. Furthermore, angiotensin-converting enzyme inhibitors and angiotensin-2 blockers may magnify the collateral effects of calcineurin inhibitors such as hyperkalemia and metabolic

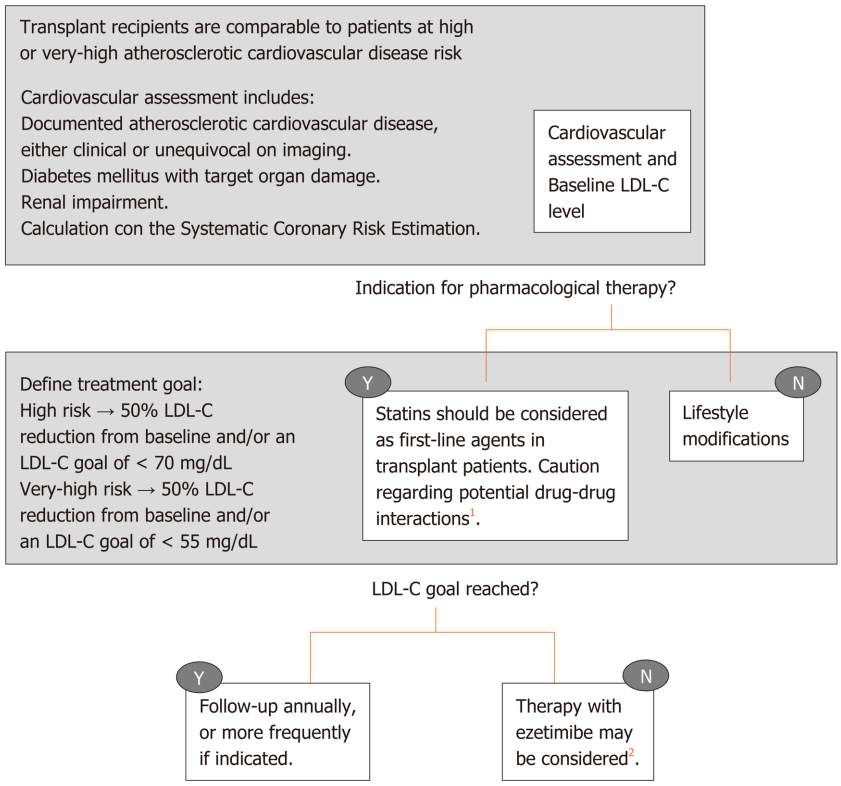

Figure 2 Proposal of a treatment algorithm for low-density lipoprotein cholesterol lowering strategy in liver transplant recipients. [1]Class of recommendation: IIa; level of evidence: B, according to 2019 ESC/EAS Guidelines for the management of dyslipidaemias[118]. [2]Class of recommendation: IIb; level of evidence: C, according to 2019 ESC/EAS Guidelines for the management of dyslipidaemias[118]. LDL-C: Low-density lipoprotein cholesterol.

acidosis, so it is advisable to use them during the long-term follow-up after liver transplantation, when the activation of the renin-angiotensin system becomes more evident. Diuretic therapy is typically avoided, due to the fact that volume contraction in the face of renal vasoconstriction may lead to further impairment in renal function[97].

It is important to keep in mind that drug-drug interactions are frequent in this subgroup of patients. Certain antihypertensive medications, namely beta-blockers, can significantly impact levels of immunosuppressive medication, thus they should be monitored during the introduction of any new drug. Independently from the chosen approach for blood pressure control, the end goal is to find a balance between antihypertensive therapy and immunosuppressive therapy modulation.

RENAL IMPAIRMENT

One of the most frequent medium- and long-term medical complications after liver transplantation is the development of nephrotoxicity, which is estimated to be 8%, 13.9% and 18.1% at 12, 36 and 60 mo post-transplant respectively[98]. Major causes of renal injury include the diagnosis of renal failure and/or hepatorenal syndrome prior to liver transplantation, critical intraoperative variables such as the need for vasopressors[99], donor-related variables such as donation after circulatory death, cold ischemia time, and graft steatosis[100,101]. All of these features are well established predictors of renal insufficiency after liver transplantation, particularly in the early post-surgical phase. However, in the majority of the cases, renal impairment is strongly associated with the direct side effects of immunosuppressive drugs in a dose dependent manner, such as calcineurin inhibitors. Nevertheless, it can also occur with combination regimens that aim to achieve low serum levels of tacrolimus. These findings have suggested the existence of different types of kidney damage based on different, partially reversible, mechanisms. The first type of damage is early and reversible, characterized by vasoconstriction of the kidney afferent arteriole, with a consensual reduction in glomerular filtration rate. A second and often irreversible damage is characterized by hyaline degeneration of renal arterioles, leading to

glomerulosclerosis[102]. In this setting, the withdrawal of cyclosporine or tacrolimus is not effective for the recovery of kidney damage. Thus, early detection of renal impairment after liver transplantation is essential in order to implement different strategies to reduce calcineurin inhibitors levels. The introduction of immunosuppressive combination therapies such as adding mTOR inhibitors to low doses of calcineurin inhibitors represents a possibility to minimize the exposure to nephrotoxic agents, sparing kidney function, without an increase in graft rejection rates[103]. Recently, new findings regarding the relationship between cardiovascular events after liver transplantation and renal impairment have been documented, underlying that even mild renal disease at the time of liver transplantation is a risk factor for post-transplant all-cause and cardiovascular mortality[104]. In a retrospective study on 202 transplant candidates pre-transplant renal impairment was found to be an independent predictor of post-transplant cardiac events (HR = 2.19, 95%CI: 1.25-3.85) and reduced cardiac event-free survival (HR = 2.27, 95%CI: 1.31-3.94)[105]. In addition, the velocity of the decline of glomerular filtrate rate after liver transplantation strongly correlated with the risk of adverse cardiovascular outcomes, highlighting the need to preserve early renal function in order to reduce these complications[104].

CARDIOVASCULAR DISEASES

Cardiovascular diseases affect long-term prognosis after solid organ transplantation. Nevertheless, this risk is substantially different for liver transplant recipients compared to other solid organ recipients. This is partially related to hemodynamic and metabolic changes associated with chronic liver disease[106]. The marked peripheral vasodilatation present in patients with decompensated end-stage cirrhosis makes difficult the detection of a latent myocardial dysfunction with cardiac abnormalities, such as an attenuation in the systolic and diastolic contractile responses leading to the so-called cirrhotic cardiomyopathy. These changes, combined with reduced serum cholesterol, can mask pre-liver transplant cardiovascular risk factors, increasing the challenge to identify those patients at highest risk for cardiovascular diseases[97]. The relevance is notable when analyzing mortality after liver transplantation: It is estimated that 12%-16% of deaths one year after liver transplantation in the USA is due to cardiovascular disease[7]. In Europe, the median estimated 10-year risk of fatal cardiovascular disease is 1% (range: 0%-9%) and 10% of the affected patients have a high risk for these events[107]. A detailed cardiovascular assessment during pre-liver transplant evaluation is thus mandatory to not only assess the perioperative risk but also to allow for an early intervention, if needed, to ensure a good long-term outcome. Despite no guidelines being available in the pre-liver transplant assessment for cardiovascular disease, every transplant center adopts different routines for cardiovascular assessment, in order to stratify the population risk.

Standard evaluation before liver transplantation normally includes a full history and clinical examination, peripheral artery oxygen saturation, 12 lead electrocardiogram and a complete transthoracic ultrasound with assessment of left ventricular, right ventricular and valvular function (with an estimation of systolic pulmonary artery pressure). Further investigations, such as stress echocardiography, cardiac computerized tomographic scan, cardiac magnetic resonance or angiography are solicited based on medical history, cardiology indication and findings from the initial screening tests[108]. It should be noted that second line tests such as dobutamine stress echocardiography have shown a poor predictive value for coronary artery disease screening (sensitivity: 28%), although with high specificity (specificity: 82%), compared with the gold standard coronary angiography[109]. When using a protocol angiography in the pre-transplant cardiac evaluation, 36% of patients showed coronary artery disease in a recently published study, with NASH and hepatitis C being independent risk factors[110]. However, another study showed that the incidence of major cardiovascular events and overall survival after liver transplantation are similar between patients with and without coronary evaluation[111]. In another study where a control group without cardiovascular risk factors was matched with a group with coronary artery disease showed that the severity or extent of coronary artery disease does not affect post liver transplantation survival, if appropriately re-vascularized. However, early postoperative cardiac events could be associated with lower survival rate, irrespectively of underlying coronary artery disease[112]. Hence, it is unclear how many pre-liver transplant asymptomatic cardiovascular abnormalities could influence long-term outcome after transplantation. On the other hand, it seems that cardiac complications are significantly more frequent in patients with a pre-liver transplant known heart disease compared with those without pre-existing

cardiovascular disease[113]. Since liver transplantation is a significantly stressful procedure from a cardiovascular standpoint, cardiovascular mortality is of the utmost importance, particularly when cirrhotic cardiomyopathy is unknown or underestimated[114]. Following liver transplantation, peripheral vascular resistance and blood pressure rapidly increase; these changes may cause an overt cardiac failure leading to pulmonary edema in predisposed patients. Other possible cardiovascular complications include the development of post-operative atrial fibrillation, defined as a new-onset atrial fibrillation during liver transplantation surgery or within 30 d after this procedure in a patient without previous episodes. This phenomenon can drive to hemodynamic and thromboembolic events, significantly affecting the prognosis of the patients in the early post-liver transplantation[115]. Although the impact of these early events on the long-term cardiovascular prognosis has not been explored in detail, a recent retrospective study in over 1000 liver transplant patients found that the development of postoperative atrial fibrillation is an independent risk factor for post-liver transplant mortality (OR = 2.0; 95%CI: 1.3-3.0; $P < 0.01$)[116]. Furthermore, as might be expected, NASH as an indication for liver transplantation had a significantly higher risk of a cardiovascular event 1 and 3 years after liver transplant. Even with a relatively low prevalence, major cardiac events are significantly associated with a lower 5-year survival rate, thus stressing the importance of identifying and stratifying high-risk patients prior to liver transplantation and offering targeted postoperative interventions. A study of Patel *et al*[117] has recently shown that despite that the presence and severity of pre-transplantation coronary artery disease may not affect post transplantation survival, the use of statins in the post-transplantation period might confer a survival benefit (HR = 0.25; 95%CI: 0.12-0.49; $P < 0.001$). This is independent of the use of aspirin, which did not show an effect on mortality. Nonetheless, the study highlighted that statin therapy is still very much underused, with only 46% of patients with proven coronary artery disease benefitting from this therapy. The medication was well tolerated (only 12% of recipients needed to stop the therapy due to side effects)[117], suggesting a promising role of statins in improving the outcomes after liver transplantation.

CONCLUSION

The proper identification of liver transplant recipients at risk of metabolic and cardiovascular morbidity and mortality after liver transplantation is still far from being satisfactory. Literature devoted to this topic is scarce and often of low quality, making it difficult to provide recommendations or to develop appropriate guidelines. Moreover, the efficacy and safety of the current treatment strategies for these metabolic complications needs to be validated in this specific population, as well as finding adequate surrogates which can be considered suitable targets to impact on the prognosis of liver transplant recipients.

However, as survival rate after liver transplantation increases, it seems clear that the management of metabolic complications and cardiovascular disease requires heightened attention. These comorbidities have a major impact on the morbidity, mortality and quality of life of liver transplant recipients in the late postoperative period. Early identification and proper management of these metabolic alterations, initially acting on lifestyle modifications, immunosuppression titration, and tailored medical therapy remain crucial to improve the outcome of liver transplant patients.

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REVIEW

Natural products that target macrophages in treating non-alcoholic steatohepatitis

Chun-Lin Li, Wen-Jun Zhou, Guang Ji, Li Zhang

ORCID number: Chun-Lin Li (0000-0003-3111-4919); Wen-Jun Zhou (0000-0003-4167-0337); Guang Ji (0000-0003-0842-3676); Li Zhang (0000-0002-5338-6096).

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Chun-Lin Li, Wen-Jun Zhou, Guang Ji, Li Zhang, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Corresponding author: Li Zhang, MD, PhD, Senior Scientist, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Shanghai 200032, China. zhangli.hl@163.com

Abstract

Nonalcoholic steatohepatitis (NASH) is the progressive subtype of non-alcoholic fatty liver disease and potentiates risks for both hepatic and metabolic diseases. Although the pathophysiology of NASH is not completely understood, recent studies have revealed that macrophage activation is a major contributing factor for the disease progression. Macrophages integrate the immune response and metabolic process and have become promising targets for NASH therapy. Natural products are potential candidates for NASH treatment and have multifactorial underlying mechanisms. Macrophage involvement in the development of steatosis and inflammation in NASH has been widely investigated. In this review, we assess the evidence for natural products or their active ingredients in the modulation of macrophage activation, recruitment, and polarization, as well as the metabolic status of macrophages. Our work may highlight the possible natural products that target macrophages as potential treatment options for NASH.

Key words: Nonalcoholic steatohepatitis; Macrophages; Natural products; Inflammation; Metabolism

Core tip: Macrophages play a pivotal role in the pathogenesis of nonalcoholic steatohepatitis. Here we discuss the evidence for natural products or their active ingredients in the modulation of macrophage activation, recruitment, and polarization, as well as the metabolic status of macrophages. Our work may highlight the possible natural products that target macrophages as potential treatment options for nonalcoholic steatohepatitis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common liver disease worldwide[1]. Approximately one-quarter of the population suffer from NAFLD[1], and approximately 30% of patients with NAFLD progress to the inflammatory subtype-nonalcoholic steatohepatitis (NASH)[2]. NASH is characterized by steatosis, inflammation, and fibrosis, and serves as a potential risk factor for hepatocellular carcinoma[3]. Lifestyle interventions, such as dieting and exercise, are the general recommendation for NAFLD[4]. Weight control is of great importance, and a weight loss of 7% to 10% can histologically attenuate NASH in patients[5]. Even without weight loss, patients with NAFLD benefit from exercise by improving insulin sensitivity and reducing hepatic lipid content[3,6,7]. However, not all patients are willing or suitable for such interventions, thus making pharmacological agents urgently needed. Although the pathophysiology and treatment of NASH have been extensively investigated, authorized pharmacological agents that are specific for NASH are not yet available.

Macrophages are versatile innate immune cells. As scavengers, they engulf worn-out cells and debris. As secretory cells, they produce a wide array of powerful chemical substances, such as enzymes and complement proteins. In addition, macrophages can present antigens and, along with dendritic cells, initiate adaptive immune response. Tissue macrophages are mainly derived from embryonic progenitors and blood monocytes[8]. Since macrophages obtain phagocytosis and immunoregulating properties, they are involved in tissue development and homeostasis with high plasticity[9]. According to their functions, macrophages are generally divided into two subpopulations, namely, classically activated (M1-type) macrophages and alternatively activated (M2-type) macrophages. The microenvironment determines the phenotype, and the dynamic self-metabolism state inversely regulates its response to the microenvironment. For instance, high levels of lipopolysaccharide (LPS) and interferon γ (IFN-γ) promote M1-type macrophage polarization, whereas interleukin (IL)-4, IL-10, and IL-13 promote M2-type macrophage polarization[9-11]. The predominant phenotype may change at different periods of disease. M1-type macrophages become dominant during inflammation and injury, whereas M2-type macrophages are abundant in the tissue repair and recovery periods.

Macrophages are the main source of inflammatory mediators in the liver, and the activation of macrophages also induces insulin resistance and metabolic dysfunctions. In addition, high titers of immunoglobulin G exist in 40% of adult NAFLD/NASH patients, 60% of pediatric NASH patients, and diet-induced NASH animals, suggesting that adaptive immune responses also take an active part in NASH development[12-14]. The versatile macrophages integrate metabolic and inflammatory responses, as well as adaptive immunity, thus serving as critical targets for the treatment of NASH[15]. Natural products are potential candidates in NASH therapy owing to their safety and multitarget properties, and a series of natural products are reported to modulate macrophages, which may contribute to their effects in preventing or treating NASH.

ROLE OF LIVER MACROPHAGES IN NASH

NASH is characterized by infiltration of inflammatory cells in the liver, and liver macrophages play a central part in the process[16]. Liver macrophages consist of resident Kupffer cells (KCs) and recruited macrophages derived from circulating monocytes. KCs and recruited macrophages have different features in the progression of NASH[17]. KCs are the first line of defense in the liver, and endogenous and exogenous pathogens induce KC activation. The activated KCs clear pathogens depending on their phagocytic activity. Simultaneously, KCs secrete pro-inflammatory cytokines and chemokines, promote the inflammatory response, and recruit peripheral blood monocytes to the liver. With the progression of disease, monocyte-derived macrophages become the dominant macrophages in the liver[17]. Generally, macrophage activation serves as a protector by engulfing pathogens and secreting cytokines or mediators in the early stage of host immunity[18]. However,

continuous stimulation induces cell death, liver injury, and related diseases[19].

NATURAL PRODUCTS THAT TARGET MACROPHAGES IN NASH TREATMENT

Natural products regulating macrophage activation

In NASH, pathogen-associated molecular patterns or damage associated molecular models such as gut-derived endotoxins, adipose tissue-derived adipokines, and debris from injured or dead hepatocytes induce KC activation, activated KCs secrete chemokines to recruit monocytes to the liver, and the expanding liver macrophage pool may further promote liver injury[20,21]. KC depletion has been reported to protect mice from hepatic steatosis and insulin resistance upon high fat diet (HFD) feeding, suggesting that KCs play an important role in NAFLD development[22]. Several natural products are reported to inhibit KC activation. Sparstolonin B is an ingredient of *Sparganium stoloniferum*, and administration of Sparstolonin B to HFD-fed mice decreases the expression of cluster of differentiation 68 (CD68) and chemokine 2 (CCL2) in KCs and reverses NASH features accordingly[23]. Curcuminoids, extracted from the plant *Curcuma domestica Val*., are found to inhibit KC activation in LPS-treated BALB/C mice[24]. In carbon tetrachloride-induced acute liver injury rats, *S. miltiorrhiza* extract administration obviously suppresses p38 and nuclear factor-kappa B (NF-κB) signaling in KCs[25]. A six-week supplementation of methanolic extract of *Graptopetalum paraguayense* was reported to reduce nitric oxide, tumor necrosis factor (TNF)-α, and IL-6 generation and improve liver inflammation and fibrosis in dimethylnitrosamine- or carbon tetrachloride-induced NASH rats[26].

Excess lipids in the liver may cause lipotoxicity, and lipid intermediate metabolites such as palmitate, ceramides, and free cholesterol are crucial contributors to macrophage activation, oxidative stress, and apoptosis[27-29]. Natural products with the function of reducing lipotoxicity are candidates for NASH treatment. The tuber of *Alisma orientalis (Sam.) Juzep*. is a commonly used herbal medicinal material, and administration of its extract prevents endoplasmic reticulum stress and lipogenic gene expression in palmitate-stimulated HepG2 cells as well as in diet-induced NAFLD mice[30,31]. Serine palmitoyltransferase is a key enzyme in ceramide metabolism, and the fungal compound myriocin inactivates serine palmitoyltransferase by forming a C18 aldehyde and prevents sphingolipid biosynthesis in hepatocytes[32].

In addition to endogenous liver stress, liver macrophage activation can also be mediated by extrahepatic stimuli, such as gut-derived endotoxins, translocated bacteria and microbiota metabolites, and adipose-derived cytokines. Blocking or alleviating these stimuli is expected to suppress macrophage activation and improve the NASH phenotype[27,33-36]. Certain natural products may affect the structure of the gut microbiome, and the related metabolites are reported in NASH treatment. Gallic acid is a naturally abundant plant phenolic compound in vegetables and fruits; it partially reshapes gut dysbiosis, reduces the choline metabolites dimethylamine and trimethylamine, and prevents NAFLD development in HFD-fed mice[37]. The natural plant alkaloid berberine can be found in plants, such as *Coptis chinensis Franch*. and *Phellodendron chinense Schneid*. Short-term berberine exposure in mice reshapes gut microbiota by reducing *Clostridium* clusters XIVa and IV, and shows beneficial effects on NASH mice[38]. In *db/db* mice, administration of *dendrobium* extract increases the *Bacteroidetes* to *Firmicutes* ratio and the relative abundance of *Prevotella* and *Akkermansia*, and reduces the relative abundance of *S24-7*, *Rikenella*, and *Escherichia coli*., thus alleviating hepatic steatosis in mice[39]. Certain natural products are found to improve NAFLD/NASH through modulation of adipokines. Dihydromyricetin is the main ingredient of the edible medicinal plant *Ampelopsis grossedentata*. In a double-blind clinical trial, dihydromyricetin treatment reduces resistin levels and improves insulin intolerance in patients with NAFLD[40]. Korean Red Ginseng is found to increase adiponectin and reduce pro-inflammatory TNF-α levels in patients with NAFLD[41]. Total alkaloids of *Rubus alceaefolius Poir* have beneficial effects on NAFLD by reducing serum leptin and resistin and increasing adiponectin levels in HFD-induced rats[42]. Additionally, the edible plants *Opuntia ficus indica* (nopal), umbelliferone, and piperine have been reported to improve insulin resistance and oxidative stress by upregulating serum adiponectin and downregulating leptin levels in obese animals[43-45].

Natural products regulating liver macrophage recruitment

In NASH, classical LY6C^{high} (mice) and CD14^{+} (human) monocytes are recruited to the inflamed area in the liver through chemokines[46]. CCL2 is present at a very low level in the physiological state but is significantly increased in NASH. The interaction of

CCL2 with its receptor C-C motif receptor 2 (CCR2) is required for monocyte migration to the liver, and knockout of CCL2 or CCR2 significantly reduces macrophage accumulation and mitigates NASH severity in mice[21]. Therefore, inhibiting macrophage recruitment to the liver is considered an effective strategy for NASH treatment. Chemokine antagonists have been found in natural products, suggesting that natural products play a positive role in this process[47]. Flavonoids derived from modified apple reduce the transcription of *CCR2*, chemokine ligand 10, and *CCR10* in mice[48]. Dietary broccoli can reverse dextran sulfate sodium-evoked CCR2 upregulation in mice[49]. Berberine reduces CCL2 levels and inhibits macrophage recruitment in HFD-fed rats[50]. In high refined carbohydrate-containing diet-fed BALB/c mice, supplementation with crude extract of *Rudgea viburnoides* (Cham.) *benth*. (Rubiaceae) leaves lowers hepatic CCL2, reduces macrophage recruitment, and improves the inflammatory response in NASH animals[51]. In HFD-induced NASH mice and *ApoE-/-* mice, administration of Long ya Aralia chinensis L-derived total saponins of *Aralia elata* (Miq) Seem for 12 wk decreases CCL2, blocks the inositol-requiring enzyme-1α (IRE1α)-mediated c-Jun N-terminal kinase pathway and significantly improves hepatic steatosis[52].

Natural products regulating macrophage polarization

Polarization of macrophages is determined by the local environment[53]. The inflammatory microenvironment with LPS and IFN-γ induces macrophage polarization to the pro-inflammatory M1-type, characterized by increased pro-inflammatory cytokines, chemokines, and reactive nitrogen and oxygen intermediates[54]. IL-4, IL-10, and IL-13 induce polarization towards the anti-inflammatory M2-type (*e.g.*, M2a, M2b, and M2c) characterized by increased scavenger receptors and enhanced phagocytosis activity[11,55]. In addition, PPAR-γ regulates M2-type polarization, and low levels of IFN-γ or high levels of CSF-1 induce recruited monocytes to differentiate into M2-type macrophages[56,57]. Certain stimuli may switch macrophages from M1-type to M2-type, or *vice versa*[53,58-60]. Failure to appropriately control this switch may cause progression of the disease[61]. In NASH, rapid and abundant pro-inflammatory macrophages are required and of benefit in the early stage; however, the constant existence of pro-inflammatory macrophages results in aggravated inflammation and fibrogenesis[62,63]. A series of natural products have been proven to regulate macrophage polarization and thus alleviate NASH and related complications. Celastrol is found to attenuate lipid accumulation and improve insulin sensitivity in NAFLD mice and regulate macrophage polarization through mitogen-activated protein kinase-NF-κB pathways in mice[64]. Smiglaside A is a phenylpropanoid glycoside isolated from *Smilax riparia*, and it has been found to upregulate M2-type and downregulate M1-type macrophage biomarkers in LPS-stimulated RAW264.7 cells and mouse peritoneal macrophages[65]. Asperlin isolated from marine *Aspergillus versicolor* LZD4403 fungus significantly reduces the expression of pro-inflammatory mediators such as inducible nitric oxide synthase, IL-1β, and TNF-α, and increases expression of IL-4 and IL-10 in LPS-stimulated RAW264.7 cells[66]. The pentacyclic triterpene lupeol regulates macrophage polarization by reducing pro-inflammatory and increasing anti-inflammatory cytokines in intestinal epithelial cells[67]. Baicalin upregulates IL-10, arginase 1, and IFN regulatory factor 4 (IRF4), downregulates TNF-α, IFN regulatory factor 5, IL-6, and IL-23, and enhances the phagocytosis and efferocytosis of macrophages, thus promoting macrophage polarization to the M2-type in mice with inflammatory bowel disease[68,69]. The *Salvia miltiorrhiza* ingredient tanshinone IIA and *Tabebuia avellanedae Lorentz ex Griseb* extract were found to promote M2-type macrophage polarization in colitis mice[70,71]. Emodin can be found in Chinese herbs such as *Rheum palmatum* and *Polygonum multiflorum*; it bidirectionally modulates the polarization of primary mouse macrophages, inhibits pro-inflammatory genes when challenged with LPS/IFN-γ, but increases pro-inflammatory genes under IL-4 stimulation in macrophages[10]. Inactivation of the Notch signaling pathway contributes to M2-type polarization[72]. Natural products such as *Trichosanthes kirilowii* lectin and oridonin are reported to deactivate Notch signaling, induce M2-type macrophage polarization, and inhibit the inflammatory response in rodents[73,74].

NATURAL PRODUCTS THAT MODULATE METABOLIC STATUS OF MACROPHAGES

The liver is an important metabolic organ and provides a favorable environment for macrophages[19,75,76]. As immune cells have high plastic functions, macrophages autonomously change their self-metabolism state to adapt to the micro-

environment[77-79]. Alterations in the metabolic state influence the energy supply as well as the function and phenotype of macrophages[80]. Metabolic pathways in macrophages include amino acid metabolism, glycolysis, mitochondrial oxidative phosphorylation (OXPHOS), pentose phosphate pathway, fatty acid synthesis, and fatty acid oxidation[81]. Activated macrophages are characterized by abnormal amino acid metabolism, upregulated glycolytic metabolism, and damaged OXPHOS[80,82-84]. M1-type macrophages display activated pentose phosphate pathway and a broken tricarboxylic acid (TCA) cycle[85]. M2-type macrophages show enhanced OXPHOS and normal TCA cycle function[86-88]. The damaged TCA cycle promotes the accumulation of succinate and citrate, followed by the generation of IL-1β, and thus contributes to the M1-type macrophage response[89].

Macrophages acquire energy to support their functions; M2-type macrophages obtain energy mainly from OXPHOS, whereas M1-type macrophages obtain energy through glycolysis. Glycolysis is inefficient at ATP generation, so the process is enhanced, and substrate production is accelerated to guarantee the functions of M1 macrophages in the inflammatory state[90]. Accumulated substrates act as stimulants that strengthen the macrophage response and activate other signaling pathways. Pyruvate is one of the end products of glycolysis, and an increase in pyruvate dehydrogenase kinase-2 (PDK2) and pyruvate dehydrogenase phosphorylation decreases pyruvate/acetyl-CoA conversion, reactive oxygen species secretion, and IL-1β production[91-93]. Several natural products are reported to affect the metabolic status of macrophages in NASH treatment. *Ampelopsis brevipedunculata* (Vitaceae) berries are a medicinal plant for treating liver disease, and its ethanol extract decreases pyruvate, superoxide dismutase, and dimethyl sulfoxide levels in ferrous iron-stimulated liver injury rats[94]. *Aim Scutellariae Radix* and *Coptidis Rhizoma* are found to upregulate pyruvate kinase activity in the liver, and thus improve the dysfunctional lipid metabolism in diabetic rats[95]. Hyacinth bean (*Dolichos lablab L*) ameliorates pyruvate-derived amino acid metabolism and prevents obesity in HFD-fed mice[96]. PDK1 is associated with the M1-type response and aerobic glycolysis, and inhibition of PDK1 promotes M2-type polarization[97]. It has been reported that methanol extracts of *Mycetia cauliflora Reinw*. (Rubiaceae) and *Dipterocarpus tuberculatus Roxb*. (Dipterocarpaceae) target PDK1 and suppress the NF-κB signaling pathway in LPS-stimulated RAW264.7 cells[98,99]. Pyruvate kinase M2 inhibits LPS-induced M1-type polarization while evoking M2-type polarization by inhibiting IL-1β and increasing IL-10 generation. Natural products that regulate pyruvate kinase M2 may also benefit NASH therapy, and further studies are needed to explore such agents[100].

SUMMARY AND PERSPECTIVES

Macrophages play a pivotal role in NASH development. Macrophages in the liver integrally regulate immune and metabolic responses and have become attractive targets for NASH treatment. Natural products are important candidates for NASH and are involved in regulating macrophage activation, recruitment, and polarization. Inversely, metabolic status affects the function of macrophages, and enzymes that modulate metabolic processes can also be regulated by natural products (Figure 1, Table 1).

There are plenty of reports about natural products for treating liver-related diseases, and on the basis of the available experimental results, curcumin, berberine, flavonoids, sparstolonin B, baicalin, and emodin are among the most promising agents in NASH treatment. Actually, several natural products are already under clinical investigation. Curcumin is currently in phase II/III clinical trials, expecting to improve liver steatosis, fibrosis, and liver inflammatory mediators in NAFLD patients[101,102]. Administration of berberine plus lifestyle intervention has been proven to reduce body weight, hepatic fat content, and serum lipid profiles, improve insulin sensitivity, and increase brown adipose tissue mass in NAFLD patients[103,104].

Although the effects of natural products on NASH are confirmed, available studies lack consensus standards and specifications, leading to the evaluation system being in an immature state and the potential mechanisms remaining unclear. The variance of patient choice and adherence, dosing methods, as well as test cycle may cause inconclusive results, and large-scale, multicenter random control trials are needed. In addition, many natural products show low bioavailability, thus strategies in promoting drug utilization or improving dosage form (nanoparticle and biological vector) need to develop. Considering the complex pathology of NASH, natural products are quite feasible to solve the problems. However, more work should be done to connect and integrate the two complex systems.

Table 1 Natural products that target macrophage for nonalcoholic steatohepatitis therapy

Section	Drugs	Model	Functions	Ref.
Macrophage activation	Sparstolonin B	HFD-fed mice	↓CD68, MCP-1	[23]
	Curcuminoids	LPS-treated BALB/C mice	↓Phagocytic activity of KCs	[24]
	S. miltiorrhiza extract	CCl4-induced liver injury rats	↓p38, NF-κB signaling	[25]
	Extract of *Graptopetalum paraguayense*	Liver fibrosis rats, primary HSCs and KCs	↓KC activation, nitric oxide, TNF-α, IL-6	[26]
	Alisma orientale extract	PA-stimulated HepG2 cells, NAFLD mice	↓ER stress, lipogenic gene expression	[30,31]
	Myriocin	Co-culture SPT with myriocin	↓SPT activation	[32]
	Gallic acid	HFD-induced NAFLD mice	↓TMA, DMA	[37]
	Berberine	NAFLD mice	↓*Clostridium cluster* XIVa and IV;	[38]
	Dendrobium extract	*db/db* mice	↑The *bacteroidetes* to *firmicutes* ratio, *Prevotella*, *Akkermansia*; ↓*S24-7*, *Rikenella*, *Escherichia coli*.	[39]
	Dihydromyricetin	Adult NAFLD patients	↓Resistin, IR	[40]
	Korean Red Ginseng	NAFLD patients	↑Adiponectin, ↓TNF-α	[41]
	Total alkaloids of *Rubus alceaefolius Poir*	HFD-fed NAFLD rats	↑Adiponectin; ↓Leptin, resistin	[42]
	Opuntia ficus indica	Obese Zucker (*fa/fa*) rats	↑Adiponectin; ↓leptin, IR	[43]
	Umbelliferone	HFD- and STZ-induced type 2 diabetic rats	↑Adiponectin; ↓leptin, IR	[44]
	Piperine	HFD-induced obese rats	↑Adiponectin; ↓leptin, IR	[45]
Macrophage recruitment	Flavonoids	Mice	↓CCR2, CXCL10, CCR10	[48]
	Broccoli	DSS-induced colitis mice	↓CCR2	[49]
	Berberine	HFD-fed rats	↓CCL2	[50]
	Rudgea viburnoides (Cham.) *Benth.* (Rubiaceae) leaves	HC-diet fed BALB/c mice	↓CCL2	[51]
	Total aralosides of *Aralia elata* (Miq) seem	HFD-induced NASH mice, *ApoE-/-* mice	↓CCL2, JNK signaling pathway	[52]
Macrophage polarization	Celastrol	RAW264.7 cells and diet-induced obese mice	↓TNF-α, IL-6, IL-1β, iNOS, MAPK activation, NF-κB nuclear translocation; ↑Nrf2 and HO-1	[64]
	Smiglaside A	LPS-stimulated RAW264.7 cells, mouse peritoneal macrophages	↑AMPK-PPARγ, M2-type macrophages; ↓M1-type macrophages	[65]
	Asperlin	LPS-stimulated RAW264.7 cells	↓TNF-α, IL-1β, iNOS; ↑IL-4, IL-10	[66]
	The pentacyclic triterpene lupeol	DSS-induced colitis mice	↓TNF-α, IL-6, IL-1β, IL-12, p38, MAPK, CD86, IRF5; ↑IL-10, CD206	[67]
	Baicalin	BMDMs, PMs, colitis mice	↓TNF-α, IL-6, IL-23, IRF5; ↑IL-10, Arg-1, IRF4	[68,69]
	Tanshinone IIA	HFD fed *ApoE-/-* mice	↑M2-type macrophage, ↓miR-375	[70]
	Tabebuia avellanedae Lorentz ex Griseb extract	Mesenteric lymph nodes of DSS-induced colitis mice	↑M2-type macrophage	[71]
	Emodin	Primary mouse macrophages	↓NF-κB/IRF5/STAT1 and IRF4/STAT6 signaling, H3K27 acetylation; ↑H3K27 trimethylation	[10]
	Trichosanthes kirilowii lectin	STZ-induced diabetic DN rats	↓Notch signaling	[73]
	Oridonin	LPS-stimulated RAW264.7 cells	↓Notch signaling	[74]
Macrophage metabolism	*Ampelopsis brevipedunculata* (Vitaceae) berries	Ferrous iron-stimulated rat hepatocyte	↓Pyruvate, superoxide dismutase, dimethyl sulfoxide	[94]
	Aim Scutellariae Radix and *Coptidis Rhizoma*	HFD-induced diabetic rats	↑Pyruvate kinase activities	[95]

	Hyacinth bean	HFD-fed mice	↓Pyruvate-derived amino acids metabolism [96]
	Mycetia cauliflora Reinw.	LPS-activated RAW264.7 cells	↓PDK1, NF-κB signaling pathway [98]
	Dipterocarpus tuberculatus Roxb.	LPS-activated RAW264.7 cells	↓PDK1, NF-κB signaling pathway [99]

HFD: High fat diet; LPS: lipopolysaccharide; KCs: Kupffer cells; CCl4: Carbon tetrachloride; HSCs: Hepatic stellate cells; NAFLD: Non-alcoholic fatty liver disease; SPT: Serine palmitoyltransferase; DMA: Dimethylamine; TMA: Trimethylamine; CCR2: C-C motif receptor; CXCL10: Chemokine ligand 10; DSS: Dextran sulfate sodium; HC: High refined carbohydrate; JNK: c-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; iNOS: Inducible nitric oxide synthase; IRF: Interferon regulatory factor; PDK: Pyruvate dehydrogenase kinase; IL: Interleukin; TNF: Tumor necrosis factor; CCR2: C-C motif receptor 2; MCP: Monocyte chemotactic protein; NF-κB: Nuclear factor-kappa B.

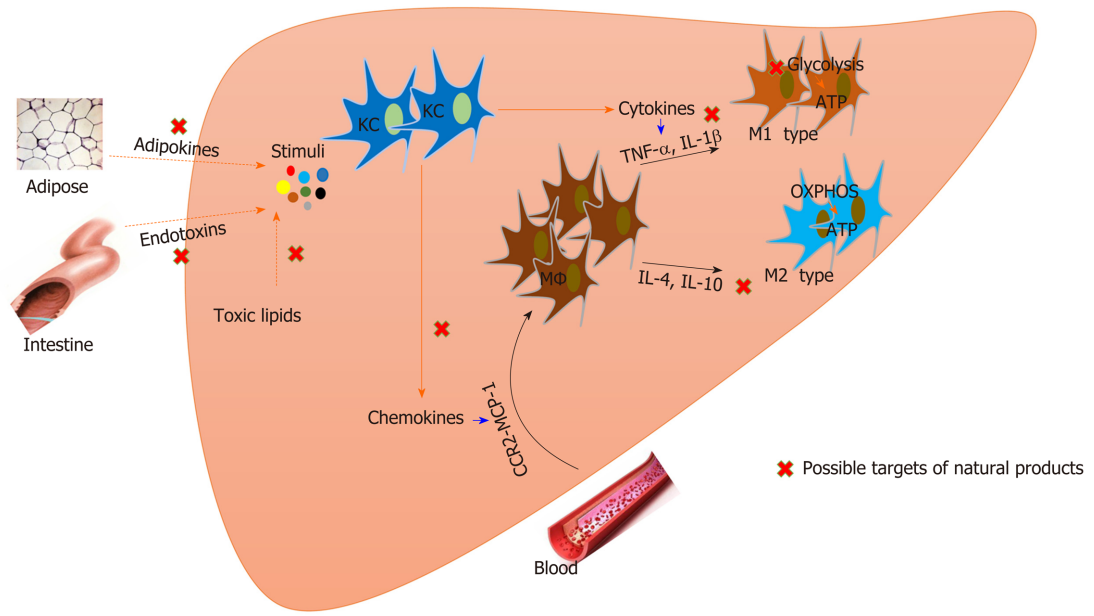

Figure 1 Natural products that target macrophages for nonalcoholic steatohepatitis treatment. Both resident Kupffer cells and recruited macrophages are involved in the pathogenesis of nonalcoholic steatohepatitis. Modulation of macrophage activation, polarization, and recruitment by natural products contributes to nonalcoholic steatohepatitis improvement. Metabolic status affects the function of macrophages, and natural products also regulate macrophage metabolism. KC: Kupffer cell; MΦ: Macrophage; OXPHOS: Mitochondrial oxidative phosphorylation; IL: Interleukin; TNF: Tumor necrosis factor; CCR2: C-C motif receptor 2; MCP: Monocyte chemotactic protein.

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MINIREVIEWS

Hepatitis B virus recurrence after liver transplantation: An old tale or a clear and present danger?

Ilaria Lenci, Martina Milana, Giuseppe Grassi, Tommaso M Manzia, Carlo Gazia, Giuseppe Tisone, Roberta Angelico, Leonardo Baiocchi

ORCID number: Ilaria Lenci (0000-0001-5704-9890); Martina Milana (0000-0003-2027-0481); Giuseppe Grassi (0000-0001-9182-8759); Tommaso M Manzia (0000-0002-4636-3478); Carlo Gazia (0000-0002-3543-4170); Giuseppe Tisone (0000-0001-8860-5909); Roberta Angelico (0000-0002-3439-7750); Leonardo Baiocchi (0000-0003-3672-4505).

Author contributions: Lenci I made the analysis and interpretation, and drafted the manuscript; Lenci I, Milana M, Grassi G, Manzia TM, Gazia C, Tisone G, and Angelico R made the acquisition of data and critical revision; Baiocchi L made the proposal of study, study conception, correction of manuscript and critical revision.

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Ilaria Lenci, Martina Milana, Giuseppe Grassi, Leonardo Baiocchi, Hepatology and Liver Transplant Unit, University of Tor Vergata, Rome 00133, Italy

Tommaso M Manzia, Carlo Gazia, Giuseppe Tisone, Roberta Angelico, Hepato-Pancreato-Biliary and Transplant, Department of Surgery, University of Rome Tor Vergata, Rome 00133, Italy

Corresponding author: Leonardo Baiocchi, MD, PhD, Assistant Professor, Hepatology and Liver Transplant Unit, University of Tor Vergata, Viale Oxford 81, Rome 00133, Italy. baiocchi@uniroma2.it

Abstract

Hepatitis B virus (HBV) recurrence after liver transplantation (LT) has been described more than 50 years ago. Similarly, to other clinical conditions, in which impairment of host immune defense favors viral replication, early reports described in details recurrence and reactivation of HBV in liver transplant recipients. The evidence of a possible, severe, clinical evolution of HBV reappearance in a significant percentage of these patients, allowed to consider, for some years, HBV positivity a contraindication for LT. Moving from the old to the new millennium this picture has changed dramatically. Several studies contributed to establish efficient prophylactic protocols for HBV recurrence and with the advent of more potent anti-viral drugs an increased control of infection was achieved in transplanted patients as well as in the general immune-competent HBV population. Success obtained in the last decade led some authors to the conclusion that HBV is now to consider just as a "mere nuisance". However, with regard to HBV and LT, outstanding issues are still on the table: (1) A standard HBV prophylaxis protocol after transplant has not yet been clearly defined; (2) The evidence of HBV resistant strains to the most potent antiviral agents is claiming for a new generation of drugs; and (3) The possibility of prophylaxis withdrawal in some patients has been demonstrated, but reliable methods for their selection are still lacking. The evolution of LT for HBV is examined in detail in this review together with the description of the strategies adopted to prevent HBV recurrence and their pros and cons.

Key words: Liver transplant; Hepatitis B virus; Viral recurrence; Prophylaxis; Minimization; Antiviral drug

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Core tip: Liver transplantation for hepatitis B virus (HBV) has greatly evolved in the last 50 years. Several studies contributed to establish efficient prophylactic protocols for HBV recurrence and with the advent of more potent anti-viral drugs an increased control of infection was achieved. In this review we examined in detail the results obtained in preventing HBV reappearance in liver transplanted patients and the possible future directions of research in this field.

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INTRODUCTION

The hepatitis B virus (HBV) is a small DNA virus belonging to the *Hepadnaviridae* family[1]. Despite the adoption, in several countries, of an extended vaccination campaign starting in 1992, HBV infection still represents an important health problem with 350-400 million people infected in the world[2]. Without treatment, at least one third of patients are estimated to progress to significant liver disease, including end-stage liver cirrhosis and tumors. In fact, the natural history of HBV liver disease includes a spectrum of clinical conditions ranging from a non-frequent fulminant hepatitis to HBV-related hepatocellular carcinoma and/or end-stage liver disease[3,4]. While vaccination and the new antiviral drugs are effective, respectively, in avoiding HBV infection and preventing the most severe sequelae of HBV disease, liver transplantation (LT) remains the main therapeutic option in patients with more severe forms of HBV liver injury[5]. However, in the early 90's the possibility to offer LT to HBV candidates was an argument of debate. In fact, it was evident that HBV disease recurrence in the graft was severe in a significant proportion of patients[6]. Moreover, an aggressive clinical form of viral reactivation, named Fibrosing Cholestatic Hepatitis, was also described in nearly 25% of HBV transplanted patients, leading to a dramatic and rapidly progressive course[7]. Therapeutic advancement and prophylactic strategies against HBV radically changed this picture in the last three decades, allowing the consideration of HBV recurrence after LT to no longer be of concern. In this review we will describe HBV viral features, its natural history, and current outcome of HBV after LT.

NATURAL HISTORY OF HBV

HBV, a double stranded small DNA virus, replicating by reverse transcription, is able to convert its DNA in a covalently closed circular (ccc) form when reaching the hepatocyte's nucleus. cccDNA represents a mini-chromosome containing information for antigens (HBsAg, HBeAg, and HBcAg), X protein, and polymerase production[8]. The infection route is mainly represented by vertical transmission in endemic areas. The estimated risk of acquiring the infection from an HBeAg+ mother is around 80%[9]. On the other hand, sexual or needle transmission are important paths in non-vaccinated adult patients of western countries[10]. Evolution of infection is dependent on the host, the viral genetics and virus/host interaction[3,11,12]. Vertical transmission at birth is associated (without peri-natal treatment) with a lifetime infection, usually with an immune-tolerant state[11]. This clinical situation is characterized by HBeAg positivity, high levels of HBV-DNA and normal liver function tests. Conversely, in adult normal subjects, immuno-tolerance usually lasts for 2-4 wk, the time span corresponding to the HBV incubation phase. Activation of the immune system against HBV determines: (1) Decreased HBV-DNA levels; (2) Increased liver inflammation; and (3) Elevation of serum levels of liver function tests. These features characterize the immune-active phase. This stage may evolve into: (1) Infection resolution with production of high titers of HBsAb (this target is reached by more than 90% of healthy adult individuals within 6 mo of initial HBV contact); (2) Fulminant hepatitis (rarely, ≤ 0.5%); or (3) HBsAg persistence and evolution to chronic hepatitis[4]. During chronic hepatitis, the seroconversion to the HBeAg negative state (with development of HBeAb titer) represents an important achievement as it corresponds to decreased

levels of HBV-DNA, liver inflammation and injury[13-15]. Moreover, HBeAg seroconversion with the consequent drop in HBV-DNA serum levels has been related to reduced fibrosis progression, histological staging, and onset of cirrhosis and hepatocellular carcinoma[16-18]. A subject with an acquired HBeAg negative state is usually defined as an inactive HBV carrier, referring to a remission state of the liver disease. Unfortunately, seroreversion to an HBeAg positive condition may occur over time (in approximately 20% of patients), also transiently, leading to a "*de novo*" immune-active inflammatory stage. Moreover, HBeAg loss (both spontaneous and drug induced) may determine selection in the host of pre-core mutants of HBV (not producing HBeAg). These strains are not affected, during their replicative phases, by anti-HBe antibodies, thus they determine progression of liver injury in approximately 10 to 30% of patients obtaining HBeAg loss[3,5,19]. The main clinical and virological features, in the different phases of HBV chronic infection, are reported in Table 1. From the above, it is evident that host-immune-system/virus interaction is a major determinant of the presence and severity of liver injury. This result is far more evident in subjects undergoing immune system changes related to biological or immuno-suppressive therapies, including the majority of transplanted patients. In this setting severe reactivation of HBV is an element of concern[20].

HBV DURING IMMUNE SYSTEM SUPPRESSION OR MODIFICATION (DRUG-INDUCED IMPAIRMENT OF THE IMMUNE SYSTEM AS A RISK FACTOR FOR HBV REACTIVATION)

Reactivation of HBV is represented by sudden reappearance or increase of viral DNA in the serum of a patient with a resolved or clinically silent HBV infection[20]. This condition, which may also occur spontaneously, has been reported more frequently in patients undergoing immunosuppressive therapy for malignant or non-malignant disease[21]. In an early study in non-Hodgkin lymphoma patients under chemotherapy, HBV reactivation accounted for 72% of cases in HBsAg positive subjects[22]. More importantly, viral reappearance was also observed in HBsAb/HBcAb or only HBcAb positive subjects, thus suggesting the possibility of HBV reactivation also in conditions in which the infection was considered resolved in the past. Further studies also demonstrated HBV reappearance in non-neoplastic clinical settings such as Crohn's disease[23] or rheumatologic affections[24]. Treatment with biological agents, such as B-cell depleting (*i.e.*, rituximab) or anti-tumor necrosis factor drugs (infliximab), carries a significant risk of HBV reactivation[25]. However, standard steroid treatment may also be responsible for HBV reappearance[26]. Evolution of viral reactivation is generally thought to occur in three separate phases[20,21]. At the beginning, a rise in HBV-DNA (at least ten-fold in comparison with baseline values) is observed during immunosuppressant treatment. In the second phase, when drugs are tapered or discontinued, the inflammatory damage begins, being triggered by the host immune defense that is also, in part, restored. In the last phase, the liver damage is repaired or may progress to end-stage liver failure. The evidence of a possible dramatic evolution of HBV reactivation in liver failure prompted the adoption of strategies to counteract this preventable occurrence. First of all, an adequate screening for HBV virus, including HBcAb, is proposed in individuals undergoing chronic therapy with immunologic modifiers. Secondly, antiviral agents able to prevent or cure this clinical condition are administered according to both viral and patient's features[27-29]. However, the clinical strategies commonly employed to prevent HBV reinfection are still lacking significant scientific evidence[27]. Therefore, the question of the best approach in different clinical scenarios remains open.

Finally, the most important clinical setting in which HBV reappearance is a relevant issue is that of transplant. Transplanted patients usually require long-term high-dose immunosuppression to prevent rejection. In HBsAg positive patients undergoing bone marrow transplantation, HBV reactivation accounts for nearly the totality of cases[30]. Even in HBsAb/HBcAb+ subjects, reappearance of active HBV is not rare, accounting for nearly 20% of cases[31]. Starting from the early eighties, HBV reactivation was reported to be very frequent in the setting of kidney and heart transplantation, and it was characterized by the insurgence of HBV chronic hepatitis[20]. Indeed, HBV reactivation or recurrence also represents an important issue in liver transplanted patients. These subjects, in fact, share the same immuno-suppressive need as other transplanted patients but, at the same time, are suffering the most important sequelae of HBV before surgery. This setting probably represents the most important clinical scenario in which dramatic HBV resurgence was observed

Table 1 Main virological and biochemical features in the different clinical phases of chronic hepatitis B in HBsAg+ patients

Phase 1: Immune tolerant	Phase 2: Immune active	Phase 3: Asymptomatic carrier	Phase 4: HBV reactivation
HBeAg+	Attempt to remove HBeAg	HBeAg-	HBeAg+ or -
HBV-DNA ≥ 10^8 IU/mL	HBV-DNA ↓	HBV-DNA < 2000 IU/mL	HBV-DNA ↑
Normal LFTs→ no damage	Altered LFTs→ damage	Normal LFTs→ no damage	Altered LFTs→ damage

HBV: Hepatitis B virus; LFT: liver function tests

and prophylactic measures were firstly pursued. At the same time, LT was the setting in which the risk of transplant with anti-core-HBV positive liver graft was identified.

HBV RECURRENCE IN THE EARLY TIMES OF LIVER TRANSPLANTATION (THE PURSUIT OF AN EFFECTIVE PROPHYLACTIC STRATEGY)

HBV recurrence/reactivation after LT was already recognized almost 50 years ago[32]. In the early 90's the feasibility of LT in HBV patients remained a crucial question since several reports observed viral recurrence in nearly all transplanted subjects, with an aggressive course in the larger part of them. While graft replacement was able to transiently reduce viral load, viral resurgence in the course of immunosuppression was related to significant liver damage and cirrhosis development[6,33]. So, at that time, LT in HBsAg positive patients was considered a high risk procedure for graft and patient loss, with an unacceptable hazard in particular in HBeAg+ subjects[34]. The disappointing results, and the need to pursue a solution for HBV patients with end-stage liver disease, stimulated the research for a possible prophylactic therapy after LT. In a pioneering study conducted at Paul Brousse Hospital (Villejuif, France) in the eighties, an extended passive immune-prophylaxis was tested in HBsAg positive patients after LT[35]. Despite the monthly HBsAb immunoglobulin (HBIG) administration, 29% of patients experienced HBsAg and HBV-DNA reappearance in serum, however these data demonstrated the possibility to reduce HBV recurrence after LT. In a further European retrospective study on 372 HBV liver transplanted patients (between 1977 and 1990), a reduced rate of HBV reactivation was statistically associated with the absence of HBV-DNA before transplant and again to long-term passive immune prophylaxis with HBIG[36]. The exact mechanisms of the beneficial effects of immunoglobulin in this setting are not completely clear at present. Both reduced deletion of infected hepatocytes and prevention of viral aggression of liver cells have been suggested as possible effects[37]. Starting from the mid 90's, evidence was gathered on the role of lamivudine (Lam) treatment in repressing HBV replication[38,39]. Since, at that time, only HBIG-based prophylaxis was available after LT, and this therapy was a life-long, suboptimal, expensive treatment, the evaluation of the Lam effect in this clinical setting began. In an English study, 17 HBsAg positive patients were enrolled to receive Lam 4 wk before liver transplant and to continue 1 year thereafter[40]. Twelve out of seventeen patients were transplanted. In them, Lam induced a loss of HBsAg and undetectable HBV-DNA serum levels within 4 wk of treatment and after transplant. Moreover, liver histology did not show features suggesting HBV recurrence after LT, and these results were obtained without concomitant HBIG immune prophylaxis. Unfortunately, in the same study, selection of a resistant strain to Lam was observed in one patient after 20 wk of treatment. This occurrence was characterized by reactivation of HBV and evidence of chronic hepatitis on liver tissue after 1 year. Similar to that observed in HIV therapy[41], HBV strains not-responding to Lam were characterized by mutation of polymerase at the highly conserved YMDD motif[42-44]. With regard to liver transplanted HBV patients, extended follow up of Lam resistant patients was lately reported. Resistance to Lam began to occur, typically, six months after its introduction and was sometimes characterized by severe disease recurrence[45,46]. A combination of Lam therapy with HBIG was then attempted in order to further reduce HBV recurrence after LT. In a study, fourteen HBsAg positive LT patients were treated with Lam plus HBIG[47]. In a median follow-up of one year, all patients were HBV-DNA negative in serum, thus demonstrating the superiority of combination therapy in comparison with monotherapy with either Lam or HBIG. These data were also confirmed in a study with an extended (average 31 mo) follow-up[48]. Thus, the past millennium ended with the positive perspective that prevention of HBV recurrence/reactivation in HBsAg

transplanted subjects was feasible. On the basis of these results, the possible exclusion of HBV subjects from transplant lists was largely reexamined.

HBV RECURRENCE/REACTIVATION AFTER LIVER TRANSPLANTATION IN THE THIRD MILLENNIUM (TESTING NEW THERAPEUTIC APPROACHES AND DRUGS)

The efficacy of passive immunization, in association with Lam, was again demonstrated in retrospective studies after the year 2000[49,50]. However, since this strategy was flawed by the relevant cost of HBIG and the need of life-long administration, the possibility to induce active immunization in HBV liver transplanted patients was examined.

HBV vaccination

In a study on 17 HBsAg+, HBeAg and HBV-DNA negative liver transplanted patients (after at least 18 mo of HBIG treatment), the double dose administration of HBV vaccine at baseline, 1 and 6 mo was tested[51]. After vaccination 84% of patients developed an HBsAb titer. During a further follow-up of 14 mo, HBsAg reappearance was not observed. These positive results were not replicated in a following study in which three reinforced and sequential cycles of HBV vaccination determined only a 17.6% HBsAb seroconversion in HBV transplanted patients[52]. In an editorial in the same journal, the limits of this strategy in transplanted patients were discussed, underscoring the scarce vaccine efficacy during immunosuppression and the long time needed to reconstitute the immune system after its depression[53]. In conclusion, it was confirmed that HBIG and antiviral therapy were regarded as the most appropriate measures against HBV recurrence after LT[54].

Adefovir dipivoxil

With regard to antiviral agents, in those years, a new drug implemented the armamentarium for the therapy of HBV. Adefovir dipivoxil (ADV), a nucleotide analog inhibiting viral reverse transcriptase that was abandoned for treatment of HIV because of kidney damage when used at high dose, was licensed for HBV treatment since it was active at lower, non-toxic levels for this virus (10 mg/d). ADV treatment in the majority of immune-competent HBsAg patients (both HBeAg positive or negative) determined a clear reduction of HBV-DNA, improvement of liver histology, and normalization of liver enzymes after a 48 wk course[55,56]. Moreover, emergence of ADV resistant mutants was not observed during these trials. Despite the fact that possible long-term viral resistance to ADV remained to be assessed, the efficacy of this new antiviral drug allowed hope for a new era in which HBV could be regarded as just a "mere nuisance"[57]. Soon, ADV was employed for the treatment of Lam resistant HBV after transplant[58]. Again, a significant improvement of liver function was recorded in nearly 90% of patients, and no resistant HBV strains were selected after 48 wk of therapy. However, ADV viral resistance was then observed with prolonged follow-up[59,60]. This was characterized by a novel *N236T* mutation of HBV polymerase. In spite of this, the clinical evolution in patients was not worrisome since these ADV resistant strains were easily suppressed by Lam concomitant therapy. On the base of these findings, a possible Lam + ADV concomitant treatment for HBV was suggested[61]. Data from a systematic review including 2162 HBV LT patients[62] identified the following as possible risk factors for HBV recurrence: (1) Being HBV-DNA positive at transplant (8.5% *vs* 4%); (2) Administration of low dose HBIG in the first week after LT (6.1% *vs* 3.5%); and (3) Combination therapy with HBIG + Lam *vs* HBIG + ADV (6.1% *vs* 2%). This picture was destined to undergo further changes with the advent of new nucleos(t)ide analogues with high genetic barriers.

New high genetic barrier nucleos(t)ide analogues.

Starting from 2012, entecavir (ETV) and tenofovir dipivoxyl (TDF) were proposed by several guidelines as a first line of treatment for chronic HBV hepatitis[5,63]. In fact, both drugs were demonstrated to be very effective in clinical studies, to have an excellent safety profile, and to be affected by a minimal or absent emergence of resistant HBV strains[64-68]. In a systematic review[69] on nucleos(t)ide analogues for HBV prophylaxis after LT, the comparison between Lam + HBIG *vs* the association of ETV or TDF with HBIG demonstrated the superiority of the latter treatments (HBV recurrence rate 6.1% *vs* 1%, $P < 0.001$). Moreover, in the same analysis, preliminary data evidenced slightly better results with either ETV or TDF monotherapy (after HBIG discontinuation) in comparison with the canonical Lam + HBIG prophylaxis (HBV recurrence rate 3.9% *vs* 6.1%, difference not statistically significant). These findings introduced the concept of

a possible minimization of HBV prophylaxis after LT, stimulating research with this target.

TOWARD HBV PROPHYLAXIS MINIMIZATION AFTER LT

Several strategies have been designed to minimize HBV prophylaxis after LT. The most relevant are described in the following subparagraphs with the corresponding results. Main studies on this issue are also summarized in Table 2.

HBIG dose reduction

Since long-term administration of HBIG was a critical point for its high cost, that would easily reach $100.000/pts/year[70], several attempts were carried out to reduce HBIG administration and acceptable results obtained. In a 2004 study conducted in our Unit (Liver Transplant Center, University of Rome Tor Vergata), we evaluated the possibility to prevent HBV recurrence after LT by administering HBIG on demand (when HBsAb serum levels were ≤ 70 IU/L) instead of the standard monthly administration[71]. Moreover, in the same study, two different HBIG doses (5000 IU or 2000 IU) were employed. In eleven HBV patients, at low risk for reactivation (HBsAg, HBV-DNA negative) and under concomitant Lam therapy, this strategy did not determine any HBV reactivation for 1 year follow up. On the other hand, the treatment based on administration of 2000 IU HBIG on demand reduced the cost of passive immune-prophylaxis by more than 50%. In 2007, the Australasian Liver Transplant Study Group assessed the association of very-low HBIG doses (400-800 IU) + Lam on HBV recurrence after LT[72]. This strategy accounted for a modest HBV recurrence risk of 4% in 5 years, and the results were considered highly satisfactory since the majority of patients (85%) were HBV-DNA positive at transplant.

High-genetic barrier nucleos(t)ide analogues monotherapy

The advent of high-genetic barrier nucleos(t)ide analogues ETV and TDF, allowed speculation on a possible prophylaxis without HBIG. ETV monotherapy, tested on 80 patients undergoing LT for HBV, was able to suppress HBV-DNA (under the lower detection limit) in nearly 99% of cases after 24 mo[73]. Extended follow up of this study (8 years) demonstrated a 92% loss of HBsAg, while HBV-DNA was undetectable in all[74]. On the other hand, discontinuation of HBIG in transplanted patients treated with TDF + HBIG did not change any viral or patient profile in a 72 wk follow-up[75]. Good results with either ETV and TDF were also replicated in other studies[76,77]. In a 5-year follow up in patients discontinuing HBIG and commencing either ETV or TDF after LT, HBsAg+ seroconversion occurred in 8% of cases, while HBV-DNA reappearance was not observed[78]. On the basis of these results, the most authoritative guidelines in the field now contemplate ETV or TDF monotherapy as an efficient prophylactic measure in subjects at low risk of HBV recurrence after LT[78,79].

Complete withdrawal of HBV prophylaxis

In the past years, our group examined a more radical approach to HBV prophylaxis minimization. This was characterized by the complete withdrawal of antiviral drugs in well selected HBV transplanted patients. We started with the assumption that reappearance of HBV after transplantation was dependent on the presence of cccDNA in the graft. Contrary to a North American study, (including several HBeAg/HBV-DNA+ patients at LT) in which total HBV-DNA and cccDNA were detected in liver tissue in 83% and 18% of cases, respectively[80], in a preliminary evaluation of HBsAg patients transplanted in our center, only 1 out of 44 was found to be positive for cccDNA[81]. Among those that were negative for liver ccc-DNA, 30 were selected and underwent sequential withdrawal of HBIG and Lam. The majority of patients (83%, n = 25) did not experience any HBV recurrence in a median follow up longer than 2 years. Five patients came back to an HBsAg positive status. Prompt resumption of HBV prophylaxis allowed infection control, avoiding any significant clinical impairment[82]. From this study, we concluded that complete withdrawal of HBV prophylaxis after LT was feasible in patients with negative serum HBV-DNA and tissue cccDNA at transplant. An editorial, in the same journal, wisely observed that the time had come for an individualized prophylaxis in HBV transplanted patients[83]. In fact, recurrence of HBV was mainly reported in patients who were HBV-DNA positive at transplant (> 100.000 copies/mL) and/or HBeAg+[36,72,84]. On the other hand, those not falling in the above category were considered at low risk for HBV recurrence. In this perspective, the target of HBV-DNA negativity was to be pursued before transplant in order to perhaps minimize prophylaxis after grafting. Conversely, for high-risk patients (HBeAg, HBV-DNA positive), more robust antiviral protocols were to be considered.

Table 2 Main studies examining prophylaxis minimization in hepatitis B virus liver transplanted patients, employing different approaches

Specific aim	Ref.	Number of patients	Method	Main results
HBIG minimization	Di Paolo *et al*[71]	11	HBIG administration on demand (when HBsAb < 70 IU/L) with Lam	No HBV reactivation (1 yr F/U)
	Gane *et al*[72]	147	Very-low HBIG dose (400-800 IU monthly) with Lam	4% of HBV recurrence (5 yr F/U)
High-genetic barrier nucleos(t)ide analogues monotherapy	Fung *et al*[73,74]	80	ETV monotherapy	92% HBsAg-100%HBV-DNA undetectable (8 yr F/U)
	Teperman *et al*[75]	40	TDF monotherapy after HBIG discontinuation	No change (72 wk F/U)
	Manini *et al*[77]	77	ETV or TDF monotherapy after HBIG discontinuation	100%HBV-DNA undetectable 9% HBsAg reappearance (5 yr F/U)
Complete withdrawal of HBV prophylaxis	Lenci *et al*[81,82]	30	Sequential discontinuation of HBIG and Lam in low risk (cccDNA negative) patients	90% successful withdrawal 60% HBsAb > 10 IU (6 yr F/U)

cccDNA: Covalently closed circular DNA; HBV: Hepatitis B virus; ETV: Entecavir; F/U: Follow-up; HBIG: HBsAb immunoglobulin; Lam: Lamivudine; TDF: Tenofovir dipivoxyl.

More recently, data on longer (6-year) follow up of this original cohort were published by our group[85]. Only 3 patients needed prophylaxis resumption (10%). Of the whole cohort, 93% remained HBsAg negative and 100% had undetectable HBV-DNA. More interestingly, 60% of patients spontaneously developed an HBsAb titer > 10 IU/L. This was probably related to the minimization or withdrawal of anti-rejection therapy that is routinely pursued in our center in patients transplanted for several years. Comment on this study appeared in a new editorial[86]. While these data were encouraging, it underscored that limits remained in the identification of low risk patients. cccDNA techniques, in fact, needed to be standardized to be widely and consistently applicable in clinical settings, but on the other hand, extra-hepatic HBV reservoirs were still a possible issue of concern.

CONCLUSION

Several important achievements were obtained in the last fifty years with regard to HBV liver transplanted patients. The original exclusion of these subjects from LT waiting lists, due to poor outcome, was counteracted by the adoption of effective measures to prevent HBV recurrence. At present, high genetic barrier anti-viral drugs are giving an important contribution in transplanted patients, as well as in the HBV immune-competent population. Recently, tenofovir alafenamide, a TDF analog with improved renal safety and increased ability to reduce alanine aminotransferase, was employed in LT patients with good results[87]. However, of concern, HBV mutants with resistance to TDF (the drug with the highest genetic barrier) were recently identified, underscoring the need of a new generation of HBV agents to be employed, at least, as a rescue therapy[88]. The future of LT for HBV is not completely predictable at this stage. It will, however, depend on the global burden of HBV and the possible discovery of HBV eradicating drugs.

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MINIREVIEWS

New role for ceramide in hypoxia and insulin resistance

Qing-Song Xia, Fu-Er Lu, Fan Wu, Zhao-Yi Huang, Hui Dong, Li-Jun Xu, Jing Gong

ORCID number: Qing-Song Xia (0000-0002-8948-2573); Fu-Er Lu (0000-0003-3372-4024); Fan Wu (0000-0003-3938-493X); Zhao-Yi Huang (0000-0003-4234-3872); Hui Dong (0000-0003-4771-0846); Li-Jun Xu (0000-0002-2013-9419); Jing Gong (0000-0003-0757-212).

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Qing-Song Xia, Fu-Er Lu, Fan Wu, Zhao-Yi Huang, Hui Dong, Li-Jun Xu, Jing Gong, Department of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Corresponding author: Jing Gong, MD, Doctor, Department of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095, Jiefang Road, Wuhan 430030, Hubei Province, China. jgongtcm@126.com

Abstract

Ceramides are significant metabolic products of sphingolipids in lipid metabolism and are associated with insulin resistance and hepatic steatosis. In chronic inflammatory pathological conditions, hypoxia occurs, the metabolism of ceramide changes, and insulin resistance arises. Hypoxia-inducible factors (HIFs) are a family of transcription factors activated by hypoxia. In hypoxic adipocytes, HIF-1α upregulates *pla2g16* (a novel HIF-1α target gene) gene expression to activate the NLRP3 inflammasome pathway and stimulate insulin resistance, and adipocyte-specific *Hif1a* knockout can ameliorate homocysteine-induced insulin resistance in mice. The study on the HIF-2α—NEU3—ceramide pathway also reveals the role of ceramide in hypoxia and insulin resistance in obese mice. Under obesity-induced intestinal hypoxia, HIF-2α increases the production of ceramide by promoting the expression of the gene *Neu3* encoding sialidase 3, which is a key enzyme in ceramide synthesis, resulting in insulin resistance in high-fat diet-induced obese mice. Moreover, genetic and pathophysiologic inhibition of the HIF-2α—NEU3—ceramide pathway can alleviate insulin resistance, suggesting that these could be potential drug targets for the treatment of metabolic diseases. Herein, the effects of hypoxia and ceramide, especially in the intestine, on metabolic diseases are summarized.

Key words: Ceramide; Intestinal hypoxia; Insulin resistance; Diabetes mellitus; Hypoxia-inducible factors; Obesity

Core tip: Hypoxia is an essential risk factor that promotes insulin resistance in a variety of tissues, such as adipocytes, intestines, and the liver. In hypoxic adipocytes, hypoxia-inducible factor-1α upregulates *pla2g16* gene expression to activate the NLRP3 inflammasome pathway, leading to insulin resistance. In obese animals or people, increased ceramide further results in insulin resistance under hypoxia. In intestinal epithelial cells, hypoxia-inducible factor-2α is activated and accumulates under hypoxia

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in high-fat diet-fed mice, which upregulates the target gene *Neu3*, accelerating the process of insulin resistance.

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INTRODUCTION

Diabetes mellitus is caused by abnormal secretion or utilization of insulin, resulting in disorders of carbohydrate, protein, and fat metabolism. Hyperglycemia is the primary symptom that can induce visual lesions and impair the kidney, heart, brain, and other organs. Diabetes is characterized by high morbidity and mortality, which brings serious economic and medical burdens to modern society. According to the latest report of the International Diabetes Federation, there were 463 million patients with diabetes in the world in 2019, which is expected to reach 700 million in 2045 at a growth rate of 51%. According to the Global Burden of Disease Study 2013, in 2013, globally, 1.47 million people died because of diabetes and its complications[1]. In 2019, the International Diabetes Federation estimates that 4.2 million people worldwide died from diabetes every year, which was one of the three major causes of noncommunicable diseases worldwide[2].

Insulin resistance is another essential clinical feature of diabetes mellitus. Both weight gain and obesity are important risk factors for metabolic diseases such as type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD)[3]. It is universally known that low-grade inflammation, abnormal glucose and lipid metabolism, endoplasmic reticulum stress, and oxidative stress are involved in insulin resistance[4]. Recently, it was reported that the regulator of hypoxia [hypoxia-inducible factor (HIF)] and the corresponding changes in lipid metabolism, especially ceramide, promote the progression of insulin resistance and NAFLD[5].

HYPOXIA-INDUCIBLE FACTOR IN HYPOXIA

Normoxia refers to physiological oxygen levels (PO_2) in normal tissue in a healthy state, but the oxygen content of different tissues varies in the physiological state, creating a wider range of oxygen levels (range from 13 kPa in the pulmonary vein to 2.7 kPa in the interstitial spaces), such as the intestinal mucosa PO_2 being significantly lower than that of the lung mucosa[6]. Hypoxia refers to the phenomenon of insufficient oxygen in tissues or blood relative to physiological conditions.

HIF is a pivotal intracellular transcriptional regulator in response to hypoxia in metazoan development, physiology, and disease pathogenesis[7]. Most species that breathe oxygen express the highly conserved transcription complex HIF-1[8]. HIF-1, a heterodimer composed of an alpha and a beta subunit, belongs to the Per-Arnt-Sim (PAS) subfamily of the basic helix-loop-helix (bHLH) family of transcription factors. The structure of HIF consists of the following three parts: An N-terminal basic helix-loop-helix domain for deoxyribonucleic acid binding, a central region PAS domain that facilitates heterodimerization, and a C-terminus for recruitment of transcriptional coregulatory proteins[9]. There are six members of the human HIF family: HIF-1α, HIF-1β, HIF-2α, HIF-2β, HIF-3α, and HIF-3β. Many cells express HIF-1α and HIF-2α, especially intestinal epithelial cells[10].

There are two major regulatory mechanisms under normoxia. One way is the degradation of HIFα protein. Hydroxylated by the prolyl hydroxylase domain, HIFα binds to the E3 ubiquitin ligase complex containing the von Hippel-Lindau disease tumor suppressor protein, resulting in expeditious degradation of HIFα. The other way is suppression of transcriptional activity. After hydroxylation by HIFα asparaginyl residue with factor inhibiting HIF1 enzyme, the interaction of HIFα with the transcriptional coactivator cAMP-response element binding protein-binding protein and histone acetyltransferase p300 is incapacitated, thus impeding transcription. However, in hypoxia, HIFα subunits remain stabilized and are not hydroxylated by prolyl hydroxylase domain and factor inhibiting HIF1, which are O_2-dependent oxygenases, resulting in the accumulation of HIFα and the upregulation of

target gene expression[5,11].

HYPOXIA AND INSULIN RESISTANCE

Metabolic syndrome is a clustering of central obesity, insulin resistance, dysglycemia, and a proatherogenic plasma lipid profile, which are associated with the risk of developing cardiovascular disease and T2DM and are presumably caused by chronic inflammation[12,13]. Low-grade inflammation was due to hypoxia, lipids and metabolites, reactive oxygen species, and endoplasmic reticulum stress[4]. At the onset of obesity, resident M2 macrophages contribute to tissue and vascular remodeling to help adipocytes accommodate the new environment of overnutrition to protect adipose tissue from hypoxia and ischemia. Owing to the imbalance between M1 macrophages and M2 macrophages (decrease in protective M2 macrophages and increase in deleterious M1 macrophages), obesity promotes the development of hypoxia in adipose tissue. Moreover, M1 macrophages generate reactive oxygen species and nitrogen monoxide (NO), which influence endothelial cells, compromising the angiogenesis needed to confront hypoxia[14]. In hypoxic tissues, for example, adipose tissue, chronic low-grade inflammation enhances the expression of HIF-1α, which stimulates inflammatory genes to amplify the "meta-inflammatory" reaction, leading to insulin resistance[15,16].

Increased uncoupled respiration by saturated fatty acids binding to adenosine diphosphate/adenosine-triphosphate translocase 2 (ANT2) is the original event in hypoxic adipocytes. A mass of free fatty acids provokes an ANT2-dependent increase in uncoupled mitochondrial respiration and oxygen consumption in obese/high-fat diet (HFD) mice, which stimulates the production of HIF-1α and relative hypoxia in adipocytes. Increased HIF-1α stimulates NO production by inducing iNOS expression. Then, insulin resistance could emerge by NO nitrosylation of the insulin-signaling molecule protein kinase B (Akt/PKB), which suppresses Akt-phosphorylated activation[17]. In addition, abundant HIF-1α increases lactate production in hypoxic adipocytes, giving rise to higher fasting blood glucose levels and accumulation of basal hepatic glucose[18]. Simultaneously, the activation of ANT2 plays a crucial role in furthering adipose inflammation and fibrosis and metabolic dysfunction. Nevertheless, adipocyte-specific ANT2 knockout seems to be effective in preventing inflammation and fibrosis in adipose tissue and improving glucose tolerance and insulin sensitivity in mice[19,20].

At length, two pathways dominate the accumulation of HIF-1α to generate insulin resistance in hypoxic adipocytes: The JAK-signal transducer and activator of transcription 3 (STAT3) signaling pathway and the phospholipase A2 group 16-lysophosphatidylcholine pathway. In the JAK-STAT3 signaling pathway, stabilization and accumulation of HIF-1α enhance the expression of suppressor of cytokine signaling 3 in the nucleus. Suppressor of cytokine signaling 3 protein phosphorylates STAT3, which downregulates the expression of adiponectin (encoded by *ADIPOQ*)[21,22]. In the phospholipase A2 group 16-lysophosphatidylcholine pathway, HIF-1α mediates homocysteine-induced adipose *pla2g16* (a novel HIF-1α target gene) gene expression to elevate lysophosphatidylcholine (lyso-PC), which acts as a second signal activator in homocysteine-induced activation of the NLRP3 inflammasome pathway. Lysophosphatidylcholine (lyso-PC) not only further activates NLRP3 inflammasomes in adipocytes but also stimulates adipose tissue macrophage NLRP3 inflammasomes in a paracrine manner to induce insulin resistance[23] (Figure 1).

CERAMIDE AND INSULIN RESISTANCE

Ceramides, a family of waxy lipid molecules that are composed of sphingosine and fatty acid, are important pathogenic lipids in obesity-related disorders. Starting with saturated fatty acids and palmitate intake, *de novo* synthesis of ceramide undergoes four major steps. This begins with the condensation of palmitate and serine to form 3-keto-dihydrosphingosine. This reaction is catalyzed by the enzyme serine palmitoyl transferase and is the rate-limiting step of the pathway. In turn, 3-keto-dihydrosphingosine is reduced to dihydrosphingosine, followed by acylation through (dihydro) ceramide synthase to produce dihydroceramide. Then, ceramide synthesis is catalyzed by dihydroceramide desaturase[24]. Ceramide is also produced through the sphingomyelinase and salvage pathways. *Via* hydrolysis of sphingomyelin, which is catalyzed by the enzyme sphingomyelinase, ceramide can be generated. In addition, the salvage pathway reutilizes long-chain sphingoid bases to form ceramide through the action of ceramide synthase[25] (Figure 2).

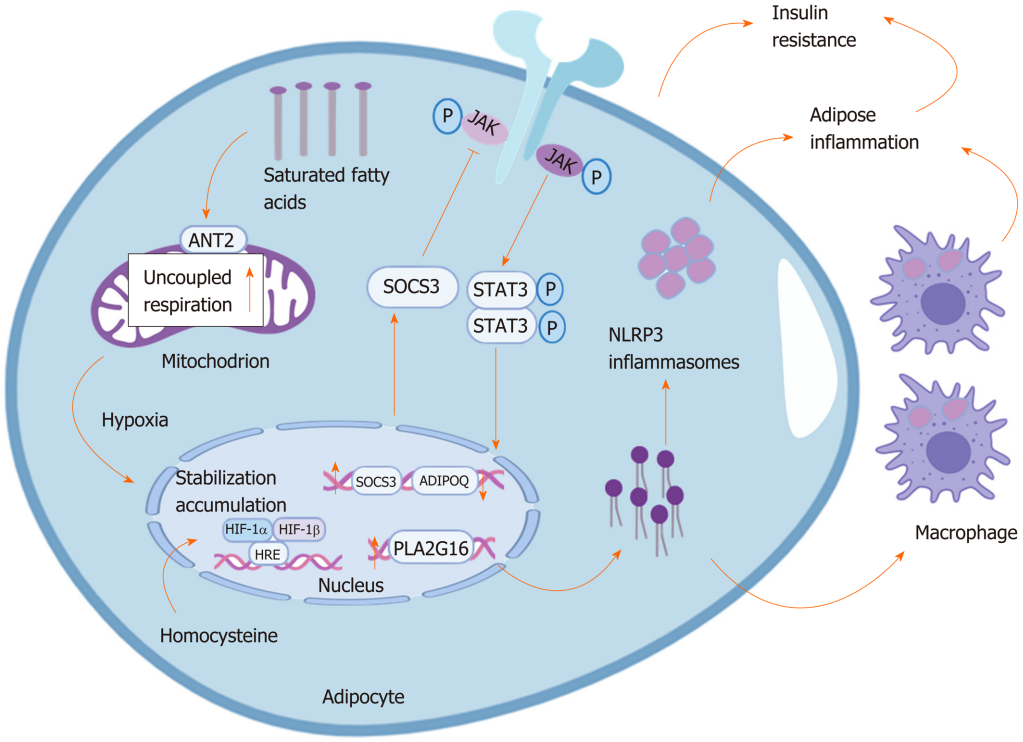

Figure 1 Accumulation of hypoxia-inducible factor 1α induces insulin resistance in hypoxic adipocytes. Owing to excess saturated fatty acids binding to adenosine diphosphate/adenosine-triphosphate translocase 2 in mitochondria, which increases uncoupled respiration leading to hypoxia in adipose tissue, the stabilized and accumulated hypoxia-inducible factor 1α (HIF-1α) further regulates relative target genes. On the one hand, HIF-1α induces expression of suppressor of cytokine signalling 3, which in turn activates signal transducer and activator of transcription 3, and dimerized signal transducer and activator of transcription 3 enters the cell nucleus and inhibits the transcription of ADIPOQ, resulting in insulin resistance. On the other hand, HIF-1α up-regulates the expression of pla2g16 to increase the level of lyso-PC, which in turn activates NLRP3 inflammasomes and stimulates NLRP3 inflammasomes in macrophages of adipose tissue, promoting insulin resistance. ANT2: Adenosine diphosphate/adenosine-triphosphate translocase 2; HIF-1α: Hypoxia-inducible factor 1α; SOCS3: Suppressor of cytokine signalling 3; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; HRE: Hypoxia-inducible factor regulating element; P: Phosphate; lyso-PC: Lyso-phosphatidylcholine.

In obese rodents, the production of ceramide increased compared with that in lean controls[26], especially glucosylceramide[27]. Similarly, studies performed in insulin-resistant human subjects demonstrate aberrant ceramide accumulation[28]. In subjects with T2DM, investigators observed elevations in serum ceramide compared with healthy control subjects[29]. It was reported that exercising training improved insulin sensitivity in obese and T2DM patients, and decreased the level of plasma ceramide especially C16:0 and C14:0. C16:0 was reduced from 2.5 nmol/mL to 1.75 nmol/mL and C14:0 reduced from 0.213 nmol/m to 0.185 nmol/mL[30]. In another clinical trial, it was found that in the group treated with berberine, the weight, body mass index, and ceramide of patients with T2DM significantly decreased compared with the lifestyle intervention group[31]. However, due to the small sample size and limitations of ceramide detection methods, there is no consistent clinical data on the specific ceramide concentration in obese or diabetic patients.

Risk factors that associate with obesity, such as saturated fatty acids and inflammatory cytokines, selectively promote sphingolipid synthesis enzymes. Moreover, lipidomic profiling reveals the relationship between sphingolipid levels and metabolic diseases, and sphingolipid is shown to be involved in insulin resistance, pancreatic beta cell failure, cardiomyopathy, and vascular dysfunction in *in vivo* and *in vitro* studies[32,33]. Adiponectin modulates ceramide by controlling its rate of degradation[34].

Mechanism of ceramide synthesis affecting insulin resistance

Ceramide is produced in response to almost all stress stimuli, including those associated with obesity (*e.g.*, chemotherapy, inflammatory agonists, and saturated fatty acids). Aberrant accumulation of ceramide may lead to the activation of several signals, which may impair normal cellular function, especially insulin[34,35]. How does ceramide synthesis affect insulin resistance in metabolic disease? By blocking translocation of the glucose transporter 4 through the inhibition of Akt/PKB activation, ceramides inhibit insulin-stimulated glucose uptake and glycogen

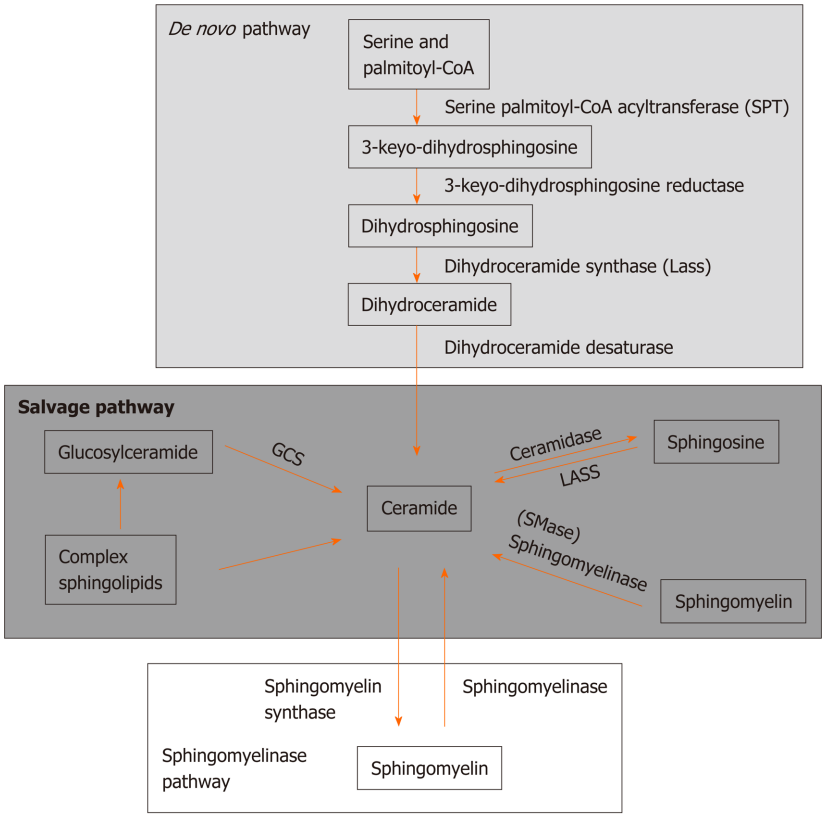

Figure 2 Ceramide synthesis pathways. Ceramides are synthesize through three ways, namely, *de novo* pathway, salvage pathway, and sphingomyelinase pathway. The *de novo* synthesis of ceramide commences with the condensation of serine and palmitate *via* action of serine palmitoyl-coenzyme A acyltransferase, followed by the continuous action of 3-keto-dihydrosphingosine reductase, dihydroceramide synthases, and dihydroceramide desaturase. In the sphingomyelinase pathway, ceramide can be produced from hydrolysis of sphingomyelin through the action of either acid or neutral sphingomyelinase, and ceramide also can synthesize sphingomyelin through the action of sphingomyelin synthase. The salvage pathway is more complex than the other two pathways. Glucosylceramide, complex sphingolipids, sphingosine, and sphingomyelin can generate ceramide from the action of diverse enzymes such as glucosylceramide synthase, LASS, and sphingomyelinase. SPT: Serine palmitoyl-coenzyme A acyltransferase; SMase: Sphingomyelinase; GCS: Glucosylceramide synthase.

synthesis in adipocytes and isolated skeletal muscle[36,37]. Ceramides block the activation of Akt/PKB through two key regulatory mechanisms. First, ceramide activates the atypical protein kinase C isoform protein kinase Cç and stabilizes interactions between Akt/PKB and protein kinase Cç by recruiting the enzymes to detergent-resistant membrane fractions[38]. The enzyme's PH domain of Akt/PKB reduces its affinity for phosphoinositides, resulting in inactivation of Akt/PKB and preventing the translocation of Akt/PKB to the plasma membrane[39,40]. The second mechanism is that activation of protein phosphatase 2A (PPA2, the primary phosphatase responsible for dephosphorylating Akt/PKB) dephosphorylates Akt/PKB. The effects of ceramide on Akt/PKB can be prevented by adding okadaic acid or overexpressing the SV40 small T antigen to inhibit PPA2[41] (Figure 3).

HYPOXIA AND CERAMIDE

HIFα is stabilized and activated under hypoxic conditions[42]. It seems that hypoxia may enhance the level of ceramide in the majority of tissues. Hypoxia leads to ceramide upregulation in NT-2 neuronal precursor cells due to the actions of acid sphingomyelinase and ceramide synthase (LASS-5) to a large extent[43]. In addition, in resistant pulmonary arteries, hypoxia induces increased ceramide and reactive oxygen species[44]. Hypoxia activates neutral sphingomyelinases (nSMases), which are key enzymes in ceramide synthesis, enhancing the production of ceramide and the subsequent ceramide-triggered activation of protein kinase C ζ, which is an early and essential event in the signaling cascade of acute hypoxic pulmonary arteries. Inhibition of nSMase (GW4869) can prevent $p47^{phox}$ phosphorylation induced by

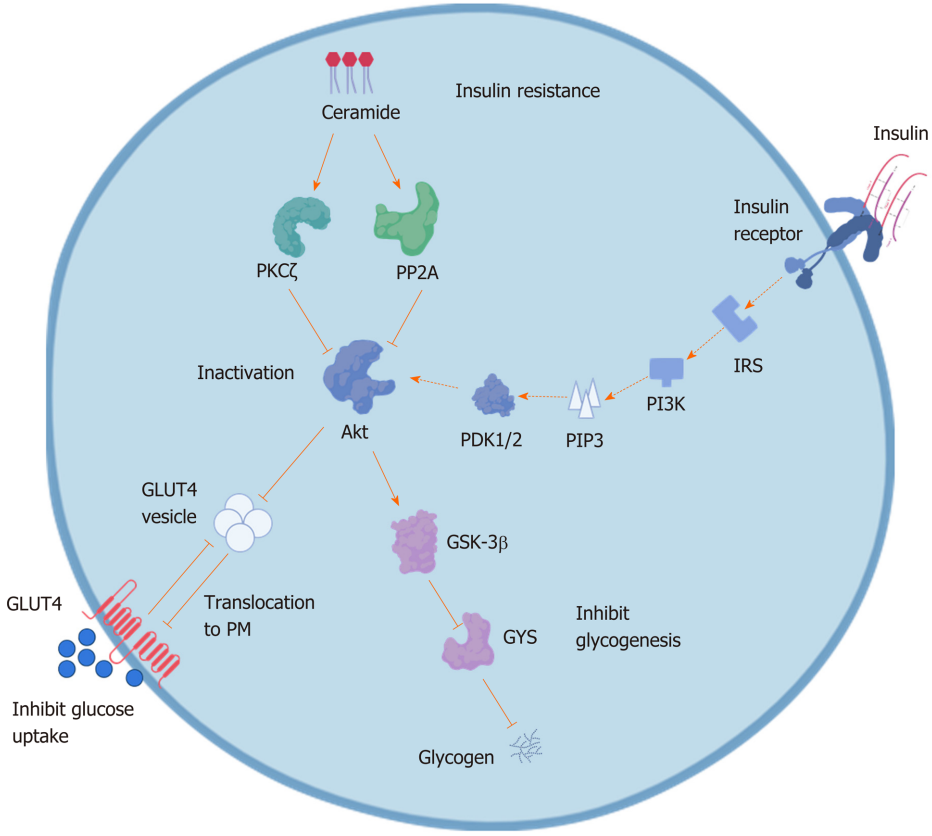

Figure 3 The mechanism of ceramide inducing insulin resistance. Ceramide inactivates protein kinase B (Akt) through stimulating the activity of protein kinase Cç isoform and protein phosphatase 2A which phosphorylates and inhibits the translocation of Akt. The inactivation of Akt prevents from translocation of glucosetransporter4 vesicle to plasma membrane, resulting in inhibiting glucose uptake. Simultaneously, inactivated Akt in turn activates glycogen synthase 3, leading to inactivation of glycogen synthase and thus inhibition of glycogen synthesis and resulting in insulin resistance. PKB: Protein kinase B; PKCç: Protein kinase Cç; PP2A: Protein phosphatase 2A; GLUT4: Glucosetransporter4; PM: Plasma membrane; GSK-3: Glycogen synthase 3; GYS: Glycogen synthase; IRS: Insulin receptor substrate; PI-3K: Phosphoinositide 3-kinase; PIP3: Phosphatidylinositol-3,4,5-trisphosphate; PDK1/2: 3-phosphoinositide-dependent protein kinase 1/2.

hypoxia[45]. Likewise, palmitoyltransferase (SPT) and glucosylceramide synthase (GCS) are the pivotal enzymes of ceramide synthesis, which may regulate the cellular level of ceramide, deciding the fate of the cell exposed to hypoxia. The hypoxia-induced increase in ceramide is partially attributed to the transcriptional upregulation of SPT2. Specific siRNA of SPT2 or GCS can reduce ceramide[46]. Therefore, ceramide synthase inhibitors may be an efficient way to restrain ceramide synthase against hypoxic injury[47].

RELATIONSHIP BETWEEN INTESTINAL HYPOXIA AND CERAMIDE INCREASES WITH INSULUN RESISTANCE

Intestinal mucosal barrier and hypoxia

The intestine is one passageway that communicates between the environment and the external environment of the human body and plays an essential role in the absorption of nutrients and protection from chemical and physical injury. The function of absorption and protection is benefited by the intestinal mucosal barrier, which involves the external physical barrier and internal functional immune barrier[48]. The physical barrier mainly consists of cells and extracellular components, and the cellular components compromise intestinal epithelial cells and the inherent layer. Intestinal epithelial cells consist of absorption (absorbent intestinal cells and M cells) and secretion lines (Pan cells, cup cells, tuft cells, and intestinal endocrine cells)[49], and the inherent layer includes dendritic cells, macrophages, epithelial lymphocytes, regulatory T cells, and B lymphocytes[50]. The functional immune barrier consists of the chemical barrier (antimicrobial peptides, digestive secretions, cytokines, inflammatory mediators, *etc*.), intestinal microbiota barrier, and immune function barrier[51]. The barrier functions of the intestinal mucous membrane are regulated by the availability of oxygen[52].

Intestinal tissue oxygen has several characteristics. First, the intestinal epithelium is located between the underlying mucosa with high oxygen content and the anaerobic lumen of the intestine, forming a cliffy oxygen gradient under physiological conditions[53]. Second, slight changes in blood flow can cause a significant variation in intestinal oxygen, such as an increase in blood flow volume after feeding (5% of total blood flow increased to 30%), which leads to a change in blood flow of the intestinal mucosa being the reason for the distinct change in local oxygen levels[54]. Intestinal epithelial cells have better adaptability and regulation of hypoxia than other tissues, and physiologic hypoxia might be an adaptive regulation mechanism for the steep oxygen gradient[55].

Intestinal hypoxia is divided into physiological hypoxia and pathological hypoxia. Physiological hypoxia refers to a relatively low PO_2 state present in mucosal epithelial cells even at baseline levels because the intestinal mucosa has a wealth of blood vessels, even if a slight reduction in blood flow can lead to a greater reduction in oxygen transport to the intestinal epithelial cells[52,56]. Pathological intestinal hypoxia widely exists in cancer, acute lung injury, inflammatory bowel disease and metabolic diseases[5,54,57]. Intestinal hypoxia is usually associated with the destruction of the intestinal mucosal barrier, such as that occurs in inflammatory intestinal diseases from the reduction in blood supply due to inflammatory immersion, edema, and vasoconstriction, leading to limited oxygen transport of the intestinal epithelium and aggregation of polymorphonuclear cells. At the same time, a large number of neutrophils rapidly deplete local oxygen through respiratory action, leading to hypoxia in the intestines[54]. The HIF-2α—NEU3—ceramide pathway may explain the relationship between intestinal hypoxia and insulin resistance.

The HIF-2α—NEU3—ceramide pathway

In intestinal epithelial cells, HIF-2α is activated and accumulates by hypoxia in HFD mice, which upregulates the target gene *Neu3* encoding sialidase 3. Sialidase 3 hydrolyses gangliosides to form ceramides in the salvage pathway[25]. The HIF-2α—NEU3—ceramide pathway can promote the development of metabolic diseases, such as NAFLD, obesity, and insulin resistance. Ceramides are synthesized through three different pathways: *De novo* pathway, sphingomyelinase (SMase) pathway, and salvage pathway[58]. Increased levels of ceramide cause obesity, insulin resistance, and hepatic steatosis owing to upregulation of fatty acid synthesis. Nevertheless, the target genes of the three ceramide synthesis pathways, including *Degs2, Smpd1, Smpd3, Smpd4, Enpp7, Neu3, Glb1*, and *Gba2*, were substantially downregulated, further resulting in the reduction in ceramide in intestine-specific HIF-2α ablation mice, which significantly ameliorates HFD-induced obesity and hepatic steatosis and improves insulin sensitivity in mice. In addition, treatment with a pharmacological specific inhibitor of HIF-2α (PT2385) or inhibitor of NEU3 (N-acetyl-2,3-didehydro-N-acetyl-neuraminic acid, DANA, or naringin) lessens serum levels of ceramides, reduces obesity and fatty liver, and enhances insulin sensitivity[33,59].

The components of the intestinal barrier are abundant. HIF-1α derived from intestinal epithelial cells is important for intestinal intraepithelial lymphocytes and intestinal flora homeostasis. Whether other mechanisms are involved in insulin resistance under hypoxia requires more research to confirm.

CONCLUSION

The global incidence of T2DM has obviously increased in recent decades with economic development and lifestyle changes, especially in developed countries. Chronic inflammation, hypoxia, and the metabolism of ceramide are closely related to insulin resistance. Many studies have shown that HIFα regulates insulin resistance, for example, in adipocyte-specific *Hif1a* knockout mice, homocysteine-induced insulin resistance is ameliorated, the NLRP3 inflammasome is inhibited, and the production of ceramide is decreased[23]. Meanwhile, intestine-specific *Hif2a* ablation mice show improved HFD-induced insulin resistance[33].

Ceramide is a significant metabolic product of sphingolipids and contributes to insulin resistance and hepatic steatosis[60]. Under hypoxia, HIF-2α can induce ceramide in adipocytes and intestines, resulting in insulin resistance in HFD-induced obesity mice. As a result of a cliffy oxygen gradient in intestinal tissue and inflammatory changes in the intestinal mucosal barrier, hypoxia occurs in the intestine. Intestinal hypoxia may lead to HFD-induced insulin resistance. A study on the HIF-2α—NEU3—ceramide pathway revealed the role of ceramide in hypoxia and insulin resistance in obese mice.

In summary, hypoxia is a key feature of the progression of metabolic disease and

HIF signaling, which can strongly influence metabolic disease by both genetic and pathophysiologic inhibition. Recent discoveries have identified exciting effects of pharmacologic inhibitors of HIF-2α or inhibitors of key enzymes (sialidase 3, NEU3) in ceramide synthesis. This may become a promising approach to the treatment of metabolic diseases, including insulin resistance and NAFLD.

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MINIREVIEWS

Role of gut microbiota on intestinal barrier function in acute pancreatitis

Xue-Yang Li, Cong He, Yin Zhu, Nong-Hua Lu

ORCID number: Xue-Yang Li (0000-0001-8161-4781); Cong He (0000-0002-1185-5456); Yin Zhu (0000-0002-4791-6126); Nong-Hua Lu (0000-0003-4373-551X).

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

Corresponding author: Yin Zhu, PhD, Doctor, Professor, Department of Gastroenterology, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Donghu District, Nanchang 330006, Jiangxi Province, China. zhuyin27@sina.com

Abstract

Acute pancreatitis (AP) is a common gastrointestinal disorder. Approximately 15%-20% of patients develop severe AP. Systemic inflammatory response syndrome and multiple organ dysfunction syndrome may be caused by the massive release of inflammatory cytokines in the early stage of severe AP, followed by intestinal dysfunction and pancreatic necrosis in the later stage. A study showed that 59% of AP patients had associated intestinal barrier injury, with increased intestinal mucosal permeability, leading to intestinal bacterial translocation, pancreatic tissue necrosis and infection, and the occurrence of multiple organ dysfunction syndrome. However, the real effect of the gut microbiota and its metabolites on intestinal barrier function in AP remains unclear. This review summarizes the alterations in the intestinal flora and its metabolites during AP development and progression to unveil the mechanism of gut failure in AP.

Key words: Acute pancreatitis; Gut microbiota; Short-chain fatty acids; Intestinal barrier

Core tip: Acute pancreatitis (AP) is a common clinical acute abdomen disease, and its incidence is increasing year by year. There are several reviews on the pathophysiology, therapeutic options and clinical trials of AP. However, the real effect of the gut microbiota and its metabolites on intestinal barrier function in AP remains unclear. This review summarizes the alterations in the intestinal flora and its metabolites during AP development and progression to unveil the mechanism of gut failure in AP.

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INTRODUCTION

Acute pancreatitis (AP) is a common gastrointestinal disorder. It is a local inflammatory response of the pancreas caused by abnormal activation of pancreatic enzymes by a variety of causes. AP is classified into mild AP (MAP), moderately severe AP (MSAP), and severe AP (SAP) based on the Atlanta Classification of 2012 revision[1]. Approximately 15%-20% of patients develop SAP[2,3], and both systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) may be caused by the massive release of inflammatory cytokines in the early stage of SAP, followed by intestinal dysfunction and pancreatic necrosis in the later stage[4,5]. Most bacteria causing necrotic infection of pancreatic tissue are from the intestinal flora, such as *Escherichia coli* and Enterococci[6]. Therefore, the intestinal flora may play an important role in the development of SAP.

The gastrointestinal tract, the largest organ in the human body, provides a broad colonization surface for the flora. It contains 150 times the total number of human genes[7]. The human intestinal flora has more than 1500 species and more than 50 phyla, with the largest number of *Firmicutes*, followed by Bacteroidetes, and other common phyla are Proteobacteria, Actinomyces, Actinobacteria, Fusobacteria and Verrucomicrobia[7]. In recent years, with the development of metagenomic research, people have become increasingly aware that the intestinal flora plays an important role in human health and diseases, including gastrointestinal diseases, such as inflammatory bowel disease[8], irritable bowel symptoms[9], colon cancer[10], and extragastrointestinal diseases, such as Alzheimer's disease[11], coronary heart disease[12], obesity[13], and diabetes[14]. Some studies have found early dysbiosis of the intestinal flora during the occurrence and development of SAP. In addition to intestinal bacteria, their metabolites, such as short-chain fatty acids (SCFAs), also affect the progression of AP.

This review summarizes the alterations in intestinal flora and its metabolites during the development and progression of AP to unveil the mechanism of gut failure in AP and finally provide a potential therapeutic target for AP.

CHANGES IN THE INTESTINAL FLORA DURING AP

In recent years, an increasing number of studies have found that the intestinal flora changes during the development of AP, which may be related to the severity of the disease. During the AP process, abnormal secretion of trypsin and destruction of pancreatic structure lead to abnormal pancreas secretion, which can cause changes in intestinal homeostasis and the intestinal flora[15,16]. Patients with AP had a greater abundance of the phyla Bacteroidetes and Proteobacteria with lower abundance of Firmicutes and Actinobacteria than healthy controls[17]. Tan *et al*[18] found that the microbial composition shifted significantly between patients with AP and healthy controls (HCs). The abundance of potentially pathogenic bacteria such as *Enterobacteriaceae* and *Enterococcus* was significantly increased, and that of beneficial bacteria such as *Bifidobacterium* was significantly decreased in both the MAP and SAP groups. The abundance of *Enterobacteriaceae* and *Enterococcus* increased by 3.2% and 9.3%, respectively, whereas *Bifidobacterium* abundance decreased by 9.2% in the SAP group compared to that in the MAP group. Our results also showed differences between the AP and HC groups; furthermore, the microbial composition changed further with the worsening of AP, and the abundance of beneficial bacteria such as *Blautia* was decreased in SAP compared with that in MAP and MSAP. It was suggested that the gut microbiota is an important mediator during AP and that its dysbiosis is associated with AP severity[19].

As there were significant changes in the abundance and structure of the intestinal flora in AP patients, researchers continued to study the changes in intestinal flora during AP using animal models. Animal experimental evidence also demonstrated similar intestinal microbiota changes in AP. Chen *et al*[20] applied 16S rRNA high-throughput sequencing analysis to study intestinal microbiota changes in rats in a sham-operated group (SO group) and an acute necrotizing pancreatitis (ANP) group. The SO and ANP groups showed structural segregation, and the microbiota diversity of the ANP group significantly decreased. At the phylum level, the abundance of *Saccharibacteria* and *Tenericutes* decreased significantly. At the genus level, the abundance of *Escherichia-Shigella* and *Phascolarctobacterium* increased significantly, while the abundance of *Candidatus_Saccharimonas, Prevotellaceae_UCG-001, Lachnospiraceae_UCG-001, Ruminiclostridium_5 and Ruminococcaceae_UCG-008* decreased significantly. At the same time, the amount of antimicrobial peptides (AMPs) secreted by panspermia cells decreased significantly and was negatively

correlated with the abundance of *Escherichia coli* and *Shigella*. Deficiencies in Paneth cell AMPs were reported to be associated with intestinal barrier failure, leading to bacterial translocation[21]. Ye *et al*[22] found that obesity could aggravate AP, deteriorate intestinal permeability and aggravate intestinal inflammation. They analysed the faecal microbiota composition and found that obese rats with AP had lower bacterial richness than rats with normal weight. Studies have suggested that faecal bacterial richness is a major marker of gut health[23,24]. Our animal research revealed that antibiotic-treated mice and germ-free mice exhibited alleviated pancreatic injury after AP induction and that subsequent faecal microbiota transplantation in turn exacerbated disease. Moreover, our previous results were supported by animal research, which also found that gut microbiota-depleted AP rats displayed less pancreatic injury and lower levels of interleukin (IL)-17A, tumour necrosis factor-α and IL-1beta in the plasma than AP rats with an intact microbiota[25]. Many recent studies have shown that this may be related to IgA, a key immune protein that is mainly located in the small intestine and protects the intestinal barrier from pathogenic bacteria. The diversity of bacteria can stimulate the body to produce different IgA and combine with the bacteria[26]. Through the combination with bacteria, it can modify the metabolism of bacteria and eliminate the mucosal inflammation response[27], which maintain immune homeostasis. The production of IgA depends on bacterial diversity. Deficiency of IgA in the gut lumen was associated with altered microbiota composition in the small intestine[28], increased susceptibility to induced colitis, and higher bacterial translocation to mesenteric lymph nodes after *Salmonella typhimurium* challenge, which suggested that IgA played a crucial role in the immune regulation between the intestinal flora and the host. Taken together, these studies reveal that the intestinal flora changes during AP and that these changes may be related to the severity of disease.

GUT MICROBIOTA MAY PROMOTE AP PROGRESSION BY AFFECTING INTESTINAL MUCOSAL BARRIER FUNCTION

Normal gut bacteria play a crucial role in maintaining gut mucosal integrity. However, gut mucosal ischaemia and reperfusion during AP can damage gut barrier integrity and lead to gut bacterial translocation to other locations, causing local and systemic infections[29,30]. Studies have revealed that intestinal mucosal barrier injury is one of the major complications of AP. A meta-analysis showed that 59% of AP patients had associated intestinal barrier injury[31], with increased intestinal mucosal permeability, leading to intestinal bacterial translocation, pancreatic tissue necrosis and infection, and the occurrence of MODS. It has been shown that the initial onset of caerulein-driven AP is dependent on the activation of NOD1 in acinar cells by commensal bacteria translocated from the gut, which further induces the expression of inflammatory mediators[32]. The intestinal flora can affect intestinal mucosal barrier function in various ways. First, the biological barrier is composed mainly of the normal intestinal flora and can regulate the intestinal microecological balance. In general, the intestinal flora coexists harmoniously with the human body and does not cause intestinal inflammatory reactions. However, when the intestinal flora is out of balance, the intestinal mucosal barrier can be destroyed by affecting intestinal inflammation and the immune response. Tan *et al*[18] found that serum IL-6 content was positively correlated with the abundance of *Enterobacteriaceae* and *Enterococcus* and negatively correlated with *Bifidobacterium* abundance, whereas plasma endotoxin content was positively correlated with *Enterococcus* abundance. This finding suggests that the inflammatory response may be related to intestinal flora imbalance. Second, the intestinal flora can also influence the mechanical barrier of the intestinal mucosa. Zhu *et al*[33] reported that mice receiving berberine promoted the expression of ZO-1 and Occludin in the intestinal mucosa by increasing the abundance of the beneficial bacteria *Akkermansia* in the intestinal tract, thus thickening the mucous layer of the intestinal mucosa and maintaining the function of the intestinal barrier. Third, *Akkermansia muciniphila* highly produces the pilus-like protein Amuc_1100, which is involved in host immune homeostasis of the intestinal mucosa and improves intestinal barrier function. In summary, the intestinal flora can affect AP progression by influencing the biological, mechanical and immune barriers of the intestinal mucosa[34].

POSSIBLE MECHANISM BY WHICH THE INTESTINAL FLORA AFFECTS THE INTESTINAL MUCOSAL BARRIER

In recent years, with a better understanding of intestinal microecology, studies have shown that not only the intestinal flora itself but also the metabolites of the intestinal flora participate in the regulation of body activities and metabolism. The metabolites of the intestinal flora consist mainly of SCFAs, indole derivatives, polyamines, organic acids, and vitamins. SCFAs are the most common metabolites of the gut microbiota. They include mainly acetate, propionate and butyrate, while formate, valerate, caproate, *etc*., are in the minority[35]. Acetate and propionate are produced mainly by *Firmicutes* and *Bacteroidetes*, which are the most prevalent bacteria, constituting 80% to 90% of the gut microbiota[24]. Acetate and propionate are produced mainly by *Bacteroidetes*, and *Firmicutes* are the primary contributors of butyrate[36]. Our previous study results showed that AP patients had intestinal flora imbalance and decreased SCFA content in the early stage of the disease, and the bacteria producing SCFAs and the SCFA contents in SAP patients were significantly reduced compared to those in MAP patients. With an understanding of SCFAs, it has been found that they can maintain intestinal mucosal barrier function.

SCFAs are the main energy source of intestinal epithelial cells (IECs), and SCFAs can promote the proliferation and differentiation of IECs, reduce cell apoptosis, and play an important role in maintaining the mechanical barrier of the intestinal mucosa[37]. Studies have also shown that SCFAs can promote intestinal epithelial tight junction protein synthesis, increase the protein expression of Zo-1 and Occludin, inhibit intestinal permeability, and enhance the intestinal mucosa mechanical barrier function[38]. Moreover, SCFAs can enhance the intestinal mucosal immune barrier. Antibacterial peptides are small molecular peptides with broad-spectrum antimicrobial activities that are produced by IECs. SCFAs can promote antibacterial peptide production, including lysozyme, defensin and mucin gene expression, and increase the secretion of AMPs to enhance the immunity of the intestinal mucosa[39]. In addition, studies have found that supplementing SCFAs can increase intestinal cross-epithelial resistance, reduce intestinal mucosal permeability, and strengthen the function of the intestinal chemical barrier[40]. SCFAs can also regulate the intestinal biological barrier. SCFAs can reduce the pH of the intestinal tract, which is conducive to the growth of probiotics, while inhibiting the growth and colonization of pathogenic bacteria, such as *Escherichia coli* and *Shigella*[41]. A study revealed that butyrate could ameliorate caerulein-induced AP and intestinal injury[42]. Therefore, SCFAs play an important role in the maintenance of intestinal mucosal barrier function. During AP, gut microbiota dysbiosis with the reduction of SCFAs and intestinal barrier damage further aggravates pancreas damage and promotes the progression of AP (Figure 1).

REGULATION OF THE INTESTINAL FLORA MAY ALLEVIATE DAMAGE TO THE INTESTINAL MUCOSAL BARRIER DURING AP

Changes in the intestinal microbial community lead to alterations of intestinal barrier function, resulting in bacterial overgrowth and impaired immunity[43]. In 2002, a randomized double-blind controlled trial studied the efficacy of probiotic lactobacilli in the treatment of AP. The results showed that the incidence of infectious complications, such as infectious pancreatic necrosis and pancreatic abscess, was significantly lower in the probiotic treatment group than in the control group, suggesting that probiotics can improve the prognosis of AP to some extent[44]. Probiotics can enhance epithelial barrier function by dampening the proinflammatory cytokine and chemokine response, accelerating reconstitution, and altering commensal microbiota in the absence of a functional mucus barrier. However, a few years later, a study obtained the opposite result[45]. Patients who received probiotics had an increased risk of death[46]. Therefore, we need to assess the general situation of patients and then provide appropriate treatment. Lutgendorff *et al*[47] reported that probiotic pre-treatment beginning five days prior to the induction of AP diminished AP-induced intestinal barrier dysfunction and prevented oxidative stress *via* mechanisms involving mainly mucosal glutathione biosynthesis in rats. Faecal microbiota transplantation (FMT) is a method of reconstructing the normal intestinal flora and an important means of treating various diseases caused by intestinal flora disorders. During treatment, the functional flora from a faecal sample from a healthy donor is transplanted into the intestinal tract of patients, and the intestinal flora with

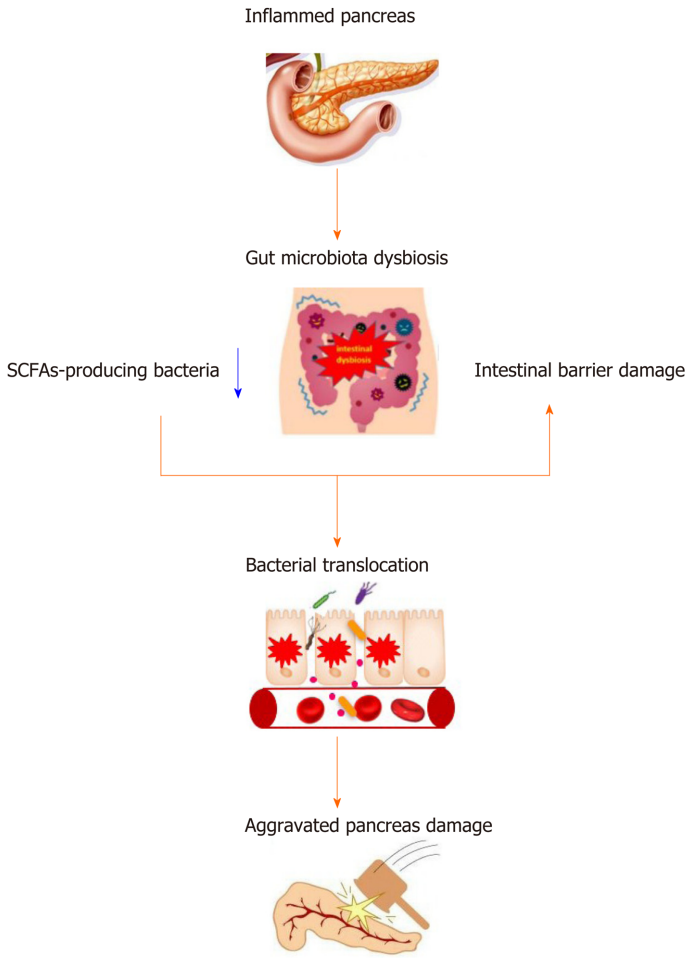

Figure 1 The relationship between acute pancreatitis and gut microbiota. SCFAs: Short-chain fatty acids.

normal functions is reconstructed to treat intestinal and extraintestinal diseases. Li *et al*[48] used ceftriaxone sodium to alleviate intestinal mucosal barrier injury in mice and found that after FMT treatment, intestinal mucosal injury in mice was effectively alleviated, inflammatory cell infiltration was reduced, and the secretory IgA (SIgA, an important component of the intestinal immune barrier) concentration was increased, suggesting that FMT played a certain role in the treatment of intestinal mucosal barrier injury. Our results showed that in gut microbiota-depleted mice treated with normal mouse faeces, AP induction can further damage the intestinal mucosal barrier compared to that in untreated AP mice. In summary, regulation of the intestinal flora may alleviate damage to the intestinal mucosal barrier during AP.

CONCLUSION

In summary, damage to the intestinal mucosal barrier can cause intestinal bacteria to migrate to the blood or other tissues and organs to accelerate the progression of and aggravate AP. Changes in the structure and quantity of the intestinal flora during AP are closely related to damage to the intestinal mucosal barrier, and regulating the intestinal flora to improve intestinal mucosal barrier injury may be an effective method for AP treatment. Although FMT has certain therapeutic effects on some intestinal diseases and parenteral diseases related to intestinal flora imbalance, there is a lack of basic research and clinical trials on AP, and its efficacy and safety need to be identified and confirmed to find an effective way to treat injury to the intestinal mucosal barrier during AP.

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ORIGINAL ARTICLE

Basic Study

Genetic association analysis of *CLEC5A* and *CLEC7A* gene single-nucleotide polymorphisms and Crohn's disease

Nagi Elleisy, Sarah Rohde, Astrid Huth, Nicole Gittel, Änne Glass, Steffen Möller, Georg Lamprecht, Holger Schäffler, Robert Jaster

ORCID number: Nagi Elleisy (0000-0002-0200-2873); Sarah Rohde (0000-0002-6892-1565); Astrid Huth (0000-0002-0858-7948); Nicole Gittel (0000-0002-7134-069X); Änne Glass (0000-0002-7715-9058); Steffen Möller (0000-0002-7187-4683); Georg Lamprecht (0000-0003-0997-3135); Holger Schäffler (0000-0002-8475-3741); Robert Jaster (0000-0002-8220-4570).

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Nagi Elleisy, Sarah Rohde, Astrid Huth, Nicole Gittel, Georg Lamprecht, Holger Schäffler, Robert Jaster, Department of Medicine II, Division of Gastroenterology, Rostock University Medical Center, Rostock 18057, Germany

Änne Glass, Steffen Möller, Institute for Biostatistics and Informatics in Medicine and Ageing Research, Rostock 18057, Germany

Corresponding author: Robert Jaster, MD, Academic Research, Professor, Senior Postdoctoral Fellow, Senior Scientist, Department of Medicine II, Division of Gastroenterology, Rostock University Medical Center, E.-Heydemann-Str. 6, Rostock 18057, Germany.
robert.jaster@med.uni-rostock.de

Abstract

BACKGROUND

Crohn's disease (CD) is characterized by a multifactorial etiology and a significant impact of genetic traits. While *NOD2* mutations represent well established risk factors of CD, the role of other genes is incompletely understood.

AIM

To challenge the hypothesis that single nucleotide polymorphisms (SNPs) in the genes *CLEC5A* and *CLEC7A*, two members of the C-type lectin domain family of pattern recognition receptors, may be associated with CD.

METHODS

SNPs in *CLEC5A*, *CLEC7A* and the known CD risk gene *NOD2* were studied using real time PCR-based SNP assays. Therefore, DNA samples from 175 patients and 157 healthy donors were employed. Genotyping data were correlated with clinical characteristics of the patients and the results of gene expression data analyses.

RESULTS

In accordance with previous studies, rs2066844 and rs2066847 in *NOD2* were found to be significantly associated with CD (allelic *P* values = 0.0368 and 0.0474, respectively). Intriguingly, for genotype AA of rs1285933 in *CLEC5A*, a potential association with CD (recessive *P* = 0.0523; odds ratio = 1.90) was observed. There were no associations between CD and SNPs rs2078178 and rs16910631 in *CLEC7A*. Variants of rs1285933 had no impact on *CLEC5A* gene expression. In contrast, genotype-dependent differences of *CXCL5* expression in peripheral blood mononuclear cells were observed. There is no statistical interaction

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between the tested SNPs of *NOD2* and *CLEC5A*, suggesting of a novel pathway contributing to the disease.

CONCLUSION

Our data encourage enlarged follow-up studies to further address an association of SNP rs1285933 in *CLEC5A* with CD. The C-type lectin domain family member also deserves attention regarding a potential role in the pathophysiology of CD.

Key words: Crohn's disease; Single nucleotide polymorphisms; *NOD2*; *CLEC5A*; Gene expression; *CXCL5*

Core tip: The genetic traits of Crohn's disease (CD) are incompletely understood. Here, we report a potential association of single nucleotide polymorphism (SNP) rs1285933 in *CLEC5A*, a member of the C-type lectin domain family of pattern recognition receptors, with CD. Variants of SNP rs1285933 had no impact on *CLEC5A* gene expression in peripheral blood mononuclear cells but correlated with the expression of *CXCL5*. The SNPs rs2078178 and rs16910631 in *CLEC7A* were not associated with the disease. The role of *CLEC5A* in the pathophysiology of CD deserves further attention.

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INTRODUCTION

Together with ulcerative colitis, Crohn's disease (CD) represents the most common and clinically relevant inflammatory bowel disease (IBD)[1,2]. While it is generally accepted that the pathogenesis of the disease is multifactorial and involves an inappropriate activation of the mucosal immune system, the precise contribution of individual environmental factors and genetic traits remains elusive[1-3]. Mutations in the *NOD2* gene represent the best-characterized genetic association of CD[4-6]. Nucleotide-binding oligomerization domain 2 (NOD2) belongs to the pattern recognition receptor (PRR) family and acts as an intracellular sensor for peptidoglycan[7,8] and its fragment muramyl dipeptide[9,10]. Downstream of NOD2, the transcription factor NF-κB plays a key role in the transduction of receptor-generated signals[11].

C-type lectin domain (CLEC) receptors comprise a large family of carbohydrate-binding proteins[12]. Various CLEC family receptors are considered to exert functions as PRR since they recognize pathogen-associated molecules and may induce intracellular signaling pathways that regulate inflammatory processes. CLEC proteins are crucially involved in the immune response to fungal pathogens, but have also been implicated in anti-bacterial, anti-viral and anti-parasitic defense mechanisms[13,14]. Despite their functional similarities to NOD2, CLEC proteins have not been systematically studied in the context of IBD yet. Interestingly, a single nucleotide polymorphism (SNP) in the *CLEC7A* (*DECTIN-1*) gene, rs2078178, has been reported to be strongly linked to a severe form of ulcerative colitis, and this association was even stronger for the two-marker haplotype rs2078178 to rs16910631[15]. For another *CLEC* gene, *CLEC5A*, we recently observed a CD-associated expression pattern with higher transcript levels in patient-derived peripheral blood mononuclear cells than in corresponding controls. Furthermore, *CLEC5A* showed a *NOD2*-dependent expression profile, supporting the hypothesis that both proteins may act in a regulatory network with a pathophysiological role in CD[16]. Given that defective bacterial clearance may contribute to the pathogenesis of CD[17,18], it is important to note that *CLEC5A* has also been suggested to be essentially involved in innate immunity through neutrophil trap formation and secretion of different proinflammatory cytokines after stimulation with *Listeria monocytogenes*[19]. Interestingly, the SNP rs1285933 in *CLEC5A* is associated with dengue severity[20], and

CLEC5A has been shown to be critical for dengue-virus-induced lethal disease[21].

Here, we have addressed the question if the SNPs rs2078178 and rs16910631 in *CLEC7A* and rs1285933 in *CLEC5A* are associated with CD and have analyzed effects of rs1285933 at the level of gene expression. For comparison and a positive control, the known disease-associated SNPs rs2066844 (SNP8), rs2066845 (SNP12) and rs2066847 (SNP13)[5,6] in *NOD2* were included into the investigations as well.

MATERIALS AND METHODS

Patients

From October 2015 until June 2017, 175 patients (102 females and 73 males; mean age 43.1 ± 14.7 years) with CD from the Department of Gastroenterology of Rostock University Medical Center (Rostock, Germany) were included in the study. This cohort of CD patients represents an extension of a cohort that we have previously characterized regarding relationships between mutations in the *NOD2* gene, the disease phenotype and anti-tumor necrosis factor-α trough levels[22].

The diagnosis of CD was based on clinical, endoscopic, histological and radiological findings of the patients. The following clinical data were collected: Age, sex, age at diagnosis, duration of the disease, disease location, disease behavior, disease activity (assessed by the Crohn's disease activity index[23] and the Harvey–Bradshaw index[24]), disease-specific medications, and previous history of surgery (*i.e.*, colectomy). CD was stratified *via* the Montreal classification[25]. Unrelated and healthy subjects from Germany (n = 157; 101 females and 56 males; mean age 25.3 ± 5.7 years) served as controls. The study was approved by the Local Ethics Board of the University of Rostock (A-2017-0137). We obtained written informed consent from all participants prior to their enrollment.

DNA extraction

EDTA whole-blood samples were subjected to DNA extraction employing the QIAamp DNA blood mini kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany).

Genotyping

Genotyping was performed using TaqMan™ SNP Genotyping Allelic Discrimination Assays with VIC- and FAM-labeled probes (Thermo Fisher Scientific, Karlsruhe, Germany) for rs1285933 (*CLEC5A*, Assay-ID: C__9506735_10), rs2078178 (*CLEC7A*; Assay-ID: C__1932439_10), rs16910631 (*CLEC7A*; Assay-ID: C__33748498_10), rs2066844 (*NOD2*, SNP8, Assay-ID: C__11717468_20), rs2066845 (*NOD2*; SNP12, Assay-ID: C__11717466_20), and rs2066847 (*NOD2*, Assay-ID: SNP13 C__60383785_10). PCR was carried out in 96-well plates, employing a ViiA 7 sequence detection system (Thermo Fisher Scientific). Thermal cycling conditions were: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C/1 min at 60 °C. After PCR, fluorescence was detected and analyzed using TaqManGenotyper software version 1.3. Alternatively, *NOD2* genotypes were determined by Sanger sequencing as described before[22].

In vitro studies with peripheral blood mononuclear cells

In this study, previous data from our laboratory were re-evaluated with respect to the rs1285933 genotype[16]. Briefly, peripheral blood mononuclear cells (PBMC) had been isolated from EDTA venous blood, cultured and treated with lipopolysaccharide (1 μg/mL; Sigma-Aldrich, Deisenhofen, Germany) for 6 h. Afterwards, RNA was isolated, reversely transcribed into cDNA and subjected to real-time PCR employing standard procedures and a ViiA 7 sequence detection system. The following human-specific TaqMan™ gene expression assays with fluorescently labeled MGB probes were used to quantify target cDNA levels: Hs04398399_m1 (*CLEC5A*), Hs01099660_g1 (*CXCL5*), and Hs99999905_m1 (*GAPDH*). PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C/1 min at 60 °C.

Statistical analysis

The data were stored and analyzed employing IBM SPSS Statistics 25.0 (International Business Machines Corporation, Armonk, New York, United States). Differences between patients and controls were assessed for distributions (genotype, allele and sex) using the χ^2 test or Fisher's exact test, and for means using the t-test for independent samples (age, gene expression data), respectively. Pairwise statistical interaction between SNPs in a linear model was studied employing ANOVA. The Hardy-Weinberg equilibrium was assessed using the χ^2 test with 1 degree of freedom. False discovery rates were controlled by using the Benjamini-Hochberg correction.

Values of $P < 0.05$ were considered statistically significant.

RESULTS

SNP genotyping was performed on DNA samples from 175 patients with CD and 157 healthy controls. Both study groups are comparable for distribution of sex ($P = 0.310$), while patients with CD were older than healthy volunteers who served as controls (43.1 ± 14.7 *vs* 25.3 ± 5.7 years; $P < 0.0001$). In the context of this study, this age difference was considered acceptable. For the controls, the distribution of all individual SNP genotypes was in accordance with the Hardy-Weinberg equilibrium.

To study associations of CD with SNP genotypes or allele frequencies, four genetic models (genotype, dominant, recessive, or allelic models) were employed (Table 1). As expected, significant associations with CD were found for SNPs in *NOD2*, specifically rs2066844 (SNP8; genotype $P = 0.0498$, dominant P value = 0.0219, allelic P value = 0.0368) and rs2066847 (SNP13; allelic P value = 0.0474). Intriguingly, the genotype AA of rs1285933 in *CLEC5A* was also potentially associated with the disease (recessive model; $P = 0.0523$). The corresponding odds ratios (ORs) are shown in Table 2. For *NOD2*, the odds of having CD might triple in the presence of the risk allele T (rs2066844: OR = 3.29), and double with allele CC (rs2066847: OR = 2.31). Increased ORs are detectable for *CLEC5A*, too. Genotype AA almost doubles the odds of CD (OR = 1.90). Carrying the risk allele A increases the odds of CD by 39% (OR = 1.39), whereas allele G displays a protective effect (OR = 0.72). We could not detect significant associations between CD and the two SNPs in *CLEC7A* (rs2078178, rs16910631) and also not for rs2066845 (SNP12) in *NOD2* (Table 1). The latter finding might be explained by the rare occurrence of the risk allele C in our cohorts of small size.

We next compared patients with different genotypes of rs1285933 in *CLEC5A* (AA, AG and GG, respectively) regarding their clinical characteristics, employing the following parameters: Age, age at diagnosis, duration of the disease, disease location and behavior according to Montreal classification, Crohn's disease activity index and Harvey–Bradshaw index, history of surgical treatment and treatment with drugs (including antibodies such as tumor necrosis factor-α inhibitors). There were no statistically significant differences between the three genotypes (data not shown).

To study potential functional effects of the rs1285933 polymorphism, we re-evaluated previously published gene expression data from our laboratory. In these studies, PBMC from CD patients and controls had been employed to measure the mRNA expression of a pre-selected set of genes[16]. Using a combined data set from 16 CD patients and 6 healthy controls, we observed no genotype-dependent differences of *CLEC5A* gene expression (Figure 1A). On the other hand, we found that the genotype GG, compared to AG, was associated with significantly lower mRNA levels of the proinflammatory chemokine *CXCL5* (Figure 1B, please note that ΔCt values and expression levels show an inverse and logarithmic relationship that follows the function $2^{-(\Delta Ct)}$).

Located on different chromosomes, the disease-associate SNP of *CLEC5A* is not correlating with disease-associated SNPs of *NOD2* (data not shown). Furthermore, the pairwise contributions to the disease phenotype of the *CLEC5A* SNP and the other SNPs are independent from each other (Table 3).

DISCUSSION

To the best of our knowledge, the results of this study suggest for the first time a potential association of SNP rs1285933 with CD. However, our findings need to be interpreted cautiously since they are based on a relatively small number of patients from a single center.

Given that the SNP is located within the *CLEC5A* gene, our data implicate a PRR beyond *NOD2* into the pathogenesis of the disease. The mechanisms that underlie the effect of the *CLEC5A* polymorphism need to be further elucidated. To this end, reinvestigating data from our past work[16] we can report a *trans* effect of rs1285933 on the expression of the chemokine *CXCL5* in PBMC, but not of *CLEC5A* itself. These data suggest that CLEC5A might be functionally affected, *e.g.*, with respect to its ability of ligand binding or downstream signaling. In accordance with this conclusion, SNP rs1285933 has also been suggested to modulate signaling pathways after interactions between the dengue virus and CLEC5A receptors[20]. Other disease associations of SNP rs1285933 have not been reported yet. In a population of Taiwanese children, neither rs1285933 nor other polymorphisms of *CLEC5A* were

Table 1 Genotype and allele frequencies of single nucleotide polymorphisms in the genes *CLEC5A*, *CLEC7A* and *NOD2* in Crohn's disease patients and controls

Gene	SNP	Genotype	Cases (*n* = 175)	Controls (*n* = 157)	Allele[1]	Cases (alleles)	Controls (alleles)	Genotype *P* value[2]	Dominant *P* value[2,3]	Recessive *P* value[2,3]	Allelic *P* value[2]
CLEC5A	rs1285933	GG	35	36	G, *A*	144, 206	155, 159	0.1093 (0.0285)	0.9727 (0.5921)	0.0523 (0.0091)	0.0900 (0.0352)
		GA	74	83							
		AA	66	38							
CLEC7A	rs2078178	GG	104	100	G, *A*	274, 76	251, 63	1.0000 (0.5713)	0.9033 (0.4320)	0.8344 (0.7618)	0.9107 (0.6335)
		AG	66	51							
		AA	5	6							
CLEC7A	rs16910631	CC	153	139	C, *T*	327, 23	294, 20	0.8078 (0.7024)	0.9056 (0.8662)	0.9269 (0.6045)	1.0000 (1.0000)
		CT	21	16							
		TT	1	2							
NOD2	rs2066844 (SNP8)	CC	146	148	C, *T*	319, 31	305, 9	0.0498 (0.0065)	0.0219 (0.0019)	0.9583 (0.5000)	0.0368 (0.0016)
		CT	27	9							
		TT	2	0							
NOD2	rs2066845 (SNP12)	GG	163	149	G, *C*	338, 12	306, 8	0.8481 (0.6453)	0.8481 (0.6453)	NA	0.7874 (0.6505)
		GC	12	8							
		CC	0	0							
NOD2	rs2066847 (SNP13)	C-C	147	143	C, *CC*	316, 34	300, 14	0.0923 (0.0321)	0.1569 (0.0682)	0.1025 (0.0312)	0.0474 (0.0103)
		C-CC	22	14							
		CC-CC	6	0							

[1]Italic: Minor allele according to database https://www.ncbi.nlm.nih.gov/snp/.
[2]Numbers in brackets refer to the *P* value prior to Benjamini-Hochberg correction (23 tests); significant differences ($P < 0.05$) are indicated in bold.
[3]Refers to the minor allele. SNP: Single nucleotide polymorphism; NA: Not applicable (due to the absence of CC genotype).

associated with susceptibility to Kawasaki disease, coronary artery lesion formation, and intravenous immunoglobulin treatment response[26].

Of note, *CLEC5A* is embedded into an intronic region of another gene, *MGAM*, and the two transcripts are known to correlate[27], so that the effect of SNP rs1285933 is not necessarily exclusively related to the C-type lectin domain family member. Interestingly, decreased maltase activities in the small bowel mucosa are common in children with CD[28], and although this is of course no evidence for a genetic association, the role of *MGAM* in the context of IBD may deserve further attention as well.

We also evaluated possible associations of SNP rs1285933 with different clinical characteristics of our CD patients, including disease location, disease behavior and treatment history, but did not obtain significant results. Given that such effects have been reported for *NOD2* variants[29-32], the studies are nevertheless worth to be continued in larger cohorts of patients. To this end, we conclude that the principal effect of SNP rs1285933 is modulation of CD susceptibility through a different molecular pathway than *NOD2*.

PRRs are key regulators of innate immune responses and inflammatory processes[13,14]. For a prominent member of this family, *NOD2*, a role in the pathogenesis of CD is clearly established[4-6]. Our results suggest an association of a polymorphism in another PRR, rs1285933 in *CLEC5A*, but not of rs2078178 and rs16910631 in *CLEC7A*, with CD. A systematic analysis of PRR functions in the context of CD might reveal novel pathomechanistic insights and help to identify new targets for diagnostic and therapy.

Table 2 Odds ratios of genotypes and alleles of single nucleotide polymorphisms in the genes *CLEC5A* and *NOD2*

Gene	SNP	Genotype/allele	Odds ratio	95%CI[1]	*P* value[1]
CLEC5A	rs1285933	AA	1.90	1.18-3.05	0.009
		GG	0.84	0.50-1.42	0.516
		AG	0.65	0.42-1.01	0.054
		A	1.39	1.03-1.90	0.034
		G	0.72	0.53-0.97	0.034
NOD2	rs2066844 (SNP8)	TT	NA		
		CC	0.31	0.14-0.67	0.003
		CT	3.00	1.36-6.60	0.006
		T	3.29	1.54-7.03	0.002
		C	0.30	0.14-0.65	0.002
NOD2	rs2066847 (SNP13)	CC-CC	NA		
		C-C	0.51	0.26-1.02	0.056
		C-CC	1.47	0.72-2.98	0.287
		C	0.43	0.23-0.82	0.011
		CC	2.31	1.21-4.38	0.011

[1]Unadjusted for multiple testing. SNP: Single nucleotide polymorphism; NA: Not applicable (missing in controls); CI: Confidence interval.

Table 3 Pairwise statistical interaction between single nucleotide polymorphisms in a linear model[1]

SNP	*CLEC5A*	*CLEC7A*		*NOD2*		
	rs1285933	rs2078178	rs16910631	rs2066844	rs2066845	rs2066847
rs1285933	NA	0.6490	0. 7409	0.5266	0.6875	0.2813
rs2078178	0.6490	NA	0.1036	0.8573	0.4040	0.3718
rs16910631	0.7409	0.1036	NA	0.8980	0.6698	0.9270
rs2066844	0.5266	0.8573	0.8980	NA	2.8248e-07	0.9664
rs2066845	0.6875	0.4040	0.6698	2.8248e-07	NA	0.7399
rs2066847	0.2813	0.3718	0.9270	0.9664	0.7399	NA

[1]Disease—single nucleotide polymorphism (SNP) A + SNP B + SNP A: SNP B. The uncorrected *P* values for the last term of an ANOVA are presented in the table for all 30 interactions. The only significance was for two chromosomally neighboring SNPs within *NOD2*. SNP: Single nucleotide polymorphism; NA: Not applicable.

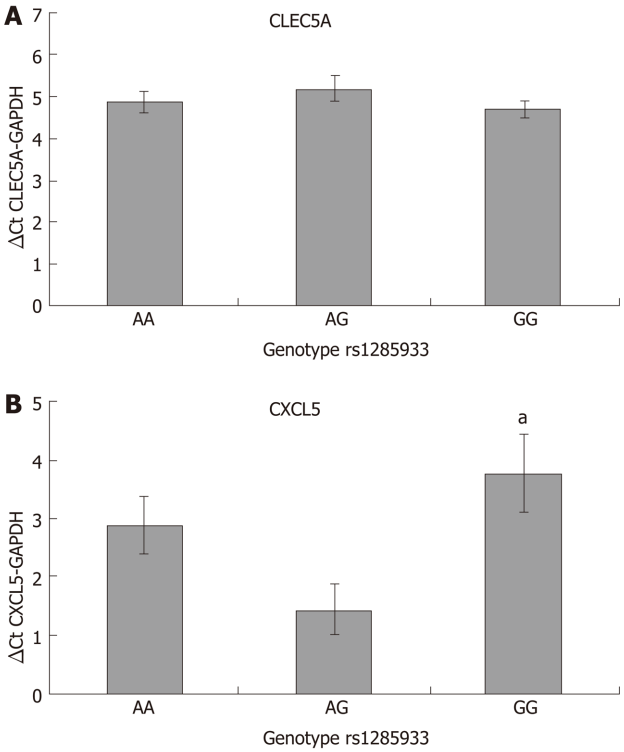

Figure 1 Effects of the rs1285933 genotype on *CLEC5A* and *CXCL5* gene expression. Peripheral blood mononuclear cells were isolated from individuals with genotype AA (n = 8), GG (n = 5), and AG (n = 9), cultured and treated with lipopolysaccharide (1 μg/mL) for 6 h. Subsequently, the mRNA expression of the indicated genes and the house-keeping control *GAPDH* was analyzed by real-time PCR. Data are presented as averaged ΔCt values ± standard error of mean. $^{a}P < 0.05$ *vs* genotype GG.

ARTICLE HIGHLIGHTS

Research background

Crohn's disease (CD) is characterized by a multifactorial etiology and a significant impact of genetic traits. While *NOD2* mutations represent well established risk factors of CD, the role of other genes is incompletely understood.

Research motivation

A better knowledge of the molecular basis of CD is considered as an essential prerequisite for a further improvement of diagnostics and therapy.

Research objectives

Previous studies from our laboratory have pointed to a possible link between CD and the expression of pattern recognition receptors of the C-type lectin domain family (specifically, *CLEC5A*) in peripheral blood mononuclear cells (PBMC). This observation prompted us to ask if single nucleotide polymorphisms in the genes *CLEC5A* and *CLEC7A* might be associated with the disease.

Research methods

DNA samples from patients with CD and healthy donors were subjected to the analysis of single nucleotide polymorphisms in the genes *CLEC5A*, *CLEC7A* and *NOD2*. For studies on gene expression, PBMC from subgroups of both cohorts were employed. Molecular findings were correlated with clinical characteristics of the patients.

Research results

For genotype AA of rs1285933 in *CLEC5A*, a potential association with CD and an increased odds ratio were detected. As expected, risk variants of *NOD2* were associated with an increased occurrence of CD as well. Polymorphisms of rs1285933 correlated with *CXCL5* gene expression but had no effect on *CLEC5A* expression in PBMC.

Research conclusions

SNP rs1285933 in *CLEC5A* may represent a novel genetic association of CD. The finding, however, needs to be reproduced in multicenter studies with larger numbers of CD patients.

Research perspectives

Pattern recognition receptors of the C-type lectin domain family deserve further attention

regarding their potential role in the pathogenesis of CD and their relevance as diagnostic markers and therapeutic targets.

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ORIGINAL ARTICLE

Basic Study

Folic acid attenuates high-fat diet-induced steatohepatitis *via* deacetylase SIRT1-dependent restoration of PPARα

Feng-Zhi Xin, Ze-Hua Zhao, Rui-Nan Zhang, Qin Pan, Zi-Zhen Gong, Chao Sun, Jian-Gao Fan

ORCID number: Feng-Zhi Xin (0000-0001-9674-0481); Ze-Hua Zhao (0000-0002-3708-8881); Rui-Nan Zhang (0000-0001-9049-3010); Qin Pan (0000-0001-5855-4952); Zi-zhen Gong (0000-0001-6979-7888); Chao Sun (0000-0002-0006-1829); Jian-Gao Fan (0000-0001-7443-5056).

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Feng-Zhi Xin, Ze-Hua Zhao, Rui-Nan Zhang, Qin Pan, Chao Sun, Jian-Gao Fan, Center for Fatty Liver, Department of Gastroenterology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Zi-Zhen Gong, Jian-Gao Fan, Shanghai Key Lab of Pediatric Gastroenterology and Nutrition, Shanghai 200092, China

Zi-Zhen Gong, Shanghai Institute for Pediatric Research, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Zi-Zhen Gong, Department of Pediatric Surgery, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Corresponding author: Jian-Gao Fan, MD, PhD, Director, Professor, Center for Fatty Liver, Department of Gastroenterology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai Key Lab of Pediatric Gastroenterology and Nutrition, 1665 Kongjiang Road, Shanghai 200092, China. fanjiangao@xinhuamed.com.cn

Abstract

BACKGROUND

Folic acid has been shown to improve non-alcoholic steatohepatitis (NASH), but its roles in hepatic lipid metabolism, hepatic one-carbon metabolism, and gut microbiota are still unknown.

AIM

To demonstrate the role of folic acid in lipid metabolism and gut microbiota in NASH.

METHODS

Twenty-four Sprague-Dawley rats were assigned into three groups: Chow diet, high-fat diet (HFD), and HFD with folic acid administration. At the end of 16 wk, the liver histology, the expression of hepatic genes related to lipid metabolism, one-carbon metabolism, and gut microbiota structure analysis of fecal samples based on 16S rRNA sequencing were measured to evaluate the effect of folic acid. Palmitic acid-exposed Huh7 cell line was used to evaluate the role of folic acid in hepatic lipid metabolism.

RESULTS

Folic acid treatment attenuated steatosis, lobular inflammation, and hepatocellular ballooning in rats with HFD-induced steatohepatitis. Genes related to lipid *de novo* lipogenesis, β-oxidation, and lipid uptake were improved

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in HFD-fed folic acid-treated rats. Furthermore, peroxisome proliferator-activated receptor alpha (PPARα) and silence information regulation factor 1 (SIRT1) were restored by folic acid in HFD-fed rats and palmitic acid-exposed Huh7 cell line. The restoration of PPARα by folic acid was blocked after transfection with SIRT1 siRNA in the Huh7 cell line. Additionally, folic acid administration ameliorated depleted hepatic one-carbon metabolism and restored the diversity of the gut microbiota in rats with HFD-induced steatohepatitis.

CONCLUSION
Folic acid improves hepatic lipid metabolism by upregulating PPARα levels *via* a SIRT1-dependent mechanism and restores hepatic one-carbon metabolism and diversity of gut microbiota, thereby attenuating HFD-induced NASH in rats.

Key words: Nonalcoholic fatty liver disease; Folic acid; Gut microbiota; PPARα; SIRT1

Core tip: The roles of folic acid in hepatic lipid metabolism, hepatic one-carbon metabolism, and gut microbiota in high-fat diet (HFD)-induced steatohepatitis are still unknown. This study confirmed that folic acid ameliorated HFD-induced steatohepatitis by restoring PPARα levels *via* a SIRT1 dependent mechanism. Moreover, folic acid restored depleted hepatic one-carbon metabolism and the diversity of gut microbiota. All these findings further clarified the improvement effect of folic acid on HFD-induced steatohepatitis and suggested that folic acid may become a therapeutic drug to treat non-alcoholic fatty liver disease in the future.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become one of the main causes of chronic liver disease worldwide[1]. The prevalence of NAFLD in China has increased from 18% to 29% in the past ten years[2,3]. Similar trends have been observed in other parts of world. Non-alcoholic steatohepatitis (NASH), which is a subtype of NAFLD, increases the risk of cirrhosis, hepatocellular carcinoma, and liver-related death[4]. However, there are still no drugs approved for treatment of NASH[5]. Therefore, NAFLD has become a serious global health burden and it is critical to find new drug targets for treatment of NASH.

Folic acid is an important substrate for the synthesis of methyl donors as an essential water-soluble vitamin metabolized by the intestinal flora and the human body[6]. Dietary folic acid could be absorbed and metabolized through the small intestine and liver. Finally, 5-methyltetrahydrofolic acid (5-MTHF) is the active form in blood circulation[7]. Folic acid deficiency could induce hyperhomocysteinemia and NAFLD. Dietary folic acid is essential for whole body folate homeostasis[8]. Additional folic acid supplementation could attenuate liver injury under high-fat diet (HFD)-fed or binge drinking conditions[9,10]. Dietary folic acid has been shown to ameliorate liver lipid accumulation[11-13]. All present data indicates that folic acid may become a potential drug target for treatment of NASH. However, further molecular mechanisms of folic acid on hepatic lipid and one-carbon unit metabolism are still unclear. The effect of folic acid on gut microbiota in NASH is also unknown. Taken together, it is necessary to further access the effect of folic acid on NASH and its possible mechanism.

To address the problems mentioned above, we conducted this research in HFD-induced NASH rats and palmitic acid (PA)-treated Huh7 cell line. Liver histology, hepatic one-carbon metabolism, and gut microbiota were evaluated *in vivo* to investigate the effect of folic acid in NASH. Genes related to lipid metabolism were evaluated both *in vivo* and *vitro* to illustrate the role of folic acid in hepatic lipid

metabolism in NASH.

MATERIALS AND METHODS

Animal experiments

The animal experiments were performed in a way that discomfort for animals was minimized. A total of 24 six-week-old specific-pathogen-free (SPF) male Sprague-Dawley rats (Sippurbec Laboratory Animal Co., Ltd., Shanghai, China) were fed in a controlled environment (24 ± 1 °C, 50% ± 5% humidity, 12-h light-dark cycle, free access to water and standard chow diet). After 1 wk of adaptive feeding, the rats were fed a chow diet or HFD (88% standard diet, 10% lard, and 2% cholesterol) for 8 wk. Then, rats fed an HFD were randomly divided into two groups and fed folic acid (15 mg/kg·d) or saline by gavage once daily for 8 wk. All rats were fasted overnight and then euthanized with pentobarbital sodium at the end of 16 wk.

All animal experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of SHRM (SHRM-IACUC-015).

Gut microbiota analysis

Fecal samples from rats were collected immediately upon defecation and then stored at -80 °C after being snap frozen in liquid nitrogen. Total fecal DNA was extracted using a TIANamp DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The quality and quantity of DNA were verified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States) and agarose gel. Extracted DNA was diluted to a concentration of 1 ng/μl and stored at -20 °C until further processing. The V4-V5 variable regions of 16S rRNA genes were amplified with universal primers 515F and 907R for bacterial diversity analysis. Amplicons were purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using QuantiFluor™-ST (Promega, Wisconsin, United States) according to the manufacturer's instructions. Equal amounts of purified amplicon were pooled for subsequent sequencing. Raw sequencing data were given in FASTQ format. Paired-end reads were preprocessed using Trimmomatic software. Clean reads were subjected to primer sequence removal and clustering to generate operational taxonomic units using Vsearch software with a 97% similarity cutoff. All representative reads were annotated and blasted against the Silva database using the Ribosomal Database Project classifier (confidence threshold was 70%).

Histological analysis

The body weight and liver mass were recorded after the rats were euthanized. Approximately 1.0 cm × 1.0 cm × 1.5 cm liver tissues were fixed in 4% paraformaldehyde for hematoxylin-eosin (HE), Masson, and Sirius red staining. Approximately 1.0 cm × 1.0 cm × 1.0 cm liver tissues were snap frozen in liquid nitrogen and then frozen at -80 °C for oil red O staining. The other liver tissues were stored at -80 °C for further analyses. Steatosis (S), activity (A), and fibrosis (F) (SAF) score was used for analyzing hepatic histological alterations[14]. Approximately 0.5 cm-long sections of the terminal ileum were gently rinsed with phosphate-buffered saline and then fixed in 4% paraformaldehyde for HE and immunohistochemical staining.

Serum and tissue assays

Serum was obtained by centrifugation of whole blood at 3000 r/min at 4 °C. Serum folic acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total bile acid (TBA), and homocysteine (Hcy) were measured with an automated analyzer (Sysmex CHEMIX-180, Japan). The liver TG and cholesterol levels were measured with assay kits (Applygen Technologies Inc., Beijing, China). Samples and the standard curve were measured according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from liver tissue using TRIzol (D9108B, Takara, Dalian, China). The concentration and purity of RNA samples were assessed on a NanoDrop 2000 spectrophotometer (Nanodrop Technologies). Total RNA (1000 ng) was converted to cDNA with RT master mix (RR036A, Takara, Dalian, China). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed with the Applied Biosystems Vii7 with SYBR® Green Master Mix (Low Rox Plus) (11202ES08, YEASEN, Shanghai, China). The primer sequences are shown in Table 1. The specificity of the

primers was determined by dissociation curves using Vii7 system SDS software. RPS18 (B661201-0001, Sangon Biotech) was used as the internal control. The $2^{-\Delta\Delta CT}$ method was used to analyze relative gene expression.

Western blot analysis

Protein levels of methionine adenosyltransferase 1A (MAT1A), silence information regulation factor 1 (SIRT1), peroxisome proliferator-activated receptor alpha (PPARα), carnitine palmitoyltransferase 1A (CPT1α), and fatty acid binding protein 1 (FABP1) in rat liver and SIRT1 and PPARα in the Huh7 cell line were determined by Western blot analysis. Briefly, liver proteins (45 μg) and cell proteins (15 μg) were separated by 8%, 10%, or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane under constant current, cold conditions. The membranes were blocked with Quick-block buffer (P0252, Beyotime, Shanghai, China) for 25 min at room temperature and were then incubated with primary antibody overnight at 4 °C. Primary antibodies include anti-MAT1A polyclonal antibody (AB217005, Abscitech, United States), anti-SIRT1 monoclonal antibody (189494, Abcam, Cambridge, United Kingdom), anti-PPARα polyclonal antibody (A6697, Abclonal, Wuhan, China), anti-CPT1α polyclonal antibody (128568, Abcam), anti-FABP1 polyclonal antibody (A5311, Abclonal), and anti-GAPDH monoclonal antibody (#5147, CST, United States). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Beyotime) were used as secondary antibodies, and the membranes were incubated at room temperature for 1 h. Protein bands were detected using a Western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, United States).

Cell culture and transfection

The Huh7 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, United States) and was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (Gibco, CA, United States). PA powder (Sigma, St. Louis, United States) was dissolved in 1% fatty acid-free BSA (Sigma, St. Louis, United States) Milli-Q water at 70 °C and filtrated with a 0.22 μm filter. The concentration of the stock solution was 5 mmol/L. The concentration of working PA solution was 0.3 mmol/L. The intervention included 0.3 mmol/L PA and 1 or 10 μg/mL 5-MTHF (Sigma-Aldrich, United States). Briefly, after 12 h of serum-free treatment, cells with 5-MTHF or the same amount of phosphate-buffered saline were incubated as pretreatment for 12 h, and then cells were incubated in PA with or without 5-MTHF for another 12 h. The proteins were isolated according to the manufacturer's instructions.

The Huh7 cell line was transfected with 50 nmol/L SIRT1 siRNA (Genomeditech, Shanghai, China) or its negative control (NC; Genomeditech) with Lipofectamine 3000 (Invitrogen, Carlsbad, United States) in Opti-MEM medium (Gibco, CA, United States). After 18 h, the medium was replaced by high-glucose DMEM without fetal bovine serum. Pretreatment and intervention were performed 24 h after transfection.

Statistical analysis

All the data are expressed as the mean ± SE. The results were analyzed using two-tailed Student's *t*-test between two groups and one-way ANOVA followed by Dunnett's test among multiple groups. Nonparametric tests were used for discontinuous data. $P < 0.05$ was considered statistically significant. All the statistical methods mentioned above were reviewed by Guang-Yu Chen from the Clinical Epidemiology Center, Shanghai Jiao Tong University.

RESULTS

Folic acid ameliorates histological alterations in HFD-induced NASH independent of affecting body weight

After a 16-wk experimental period, all rats in the HFD group developed typical NASH characteristics. Body weight (Figure 1A) and liver index (Figure 1B) were significantly elevated in the HFD group compared with the control group. Administration of folic acid had no effect on body weight or epididymis fat of rats (Figure 1A and C). But it ameliorated HFD-induced NASH hepatic lesions in rats. As shown in Figure 1B, liver index showed a certain reduction in the folic acid group compared with the HFD group. Additionally, folic acid improved the liver imaging results to a certain extent and ameliorated hepatic lipid deposition, ballooning degeneration, and inflammatory infiltration (Figure 1D). Moreover, steatosis score (Figure 1E), lobular inflammation score (Figure 1F), and ballooning score (Figure 1G)

Table 1 Primer sequences for real-time quantitative polymerase chain reaction

Gene name	Forward sequence	Reverse sequence
TNF-a	TGCCTCAGCCTCTTCTCATT	GAGCCCATTTGGGAACTTCT
IL-6	AGTTGCCTTCTTGGGACTGA	CCTCCGACTTGTGAAGTGGT
IL-1β	GAAGTCAAGACCAAAGTGG	TGAAGTCAACTATGTCCCG
CCR2	CACCGTATGACTATGATGATG	CAGGAGAGCAGGTCAGAGAT
p47phox	GCCCAAAGATGGCAAGAATA	ATGACCTCAATGGCTTCACC
p67phox	AGCAGAAGAGCAGTTAGCATTGG	TGCTTTCCATGGCCTTGTC
p22phox	GTAGATGCCGCTCGCAATGGCCAG	ATGGGGCAGATCGAGTGGGCCATGT
gp91phox	CTGAGCGAATTGTACGTG	CTTATCACAGCCACAAGC
aSMA	TGTGCTATGTCGCTCTGGAC	CCAATGAAAGATGGCTG GAA
TGFβ1	ATTCCTGGCGTTACCTTGG	AGCCCTGTATTCCGTCTCCT
Col1a1	TGTTCAGCTTTGTGGACCT	CAGCTGACTTCAGGGATGT
Col2a1	ACCTCAGGGTGTTCAAGGTG	CGGATTCCAATAGGACCAGA
Col3a1	GGTGGCTTTCAGTTCAGCTATG	GTCTTGCTCCATTCACCAGTGT
MAT1A	CAATGTGCTCGTGGCTCTGGAG	TCCTCTGTCTCGTCAGTGGCATAG
ALDH1L1	GCACGGCTCCATCATCTACCATC	GTCATCTGGAAGCACCTCACACTC
SREBP1c	CCAGCCTTTGAGGATAACCA	TGCAGGTCAGACACAGGAAG
SCD	AGCTGGTGATGTTCCAGAGG	CAAGAAGGTGCTGACGAACA
ACACA	GAATATCCAGATGGCCGAGA	CCTTCTGCTCTGGCAAGTTC
FASN	GCCTAACACCTCTGTGCAGT	GGCAATACCCGTTCCCTGAA
PPARγ	ACAAGAGCTGACCCAATGGT	GGCTCTTCATGTGGCCTGTT
ACADL	ACTCCGCCTCCGCTTCCATG	TACCACCGTAGATCGGCTGAACTC
FABP1	GTCTGCCTGAGGACCTCATCCAG	TCATGGTCTCCAGTTCGCACTCC
CPT1a	CCACGAAGCCCTCAAACAGA	CACACCCACCACCACGATAA
FATP2	CACGACAGAGTTGGAGACACCTTC	CCGATGCGACCTTCATGACCTG

TNF-α: Tumor necrosis factor alpha; IL: Interleukin; CCR2: Chemokine receptor C-C chemokine receptor type 2; αSMA: α-smooth muscle actin; TGFβ1: Transforming growth factor beta 1; Col1a1: Collagen type I alpha 1; Col2a1: Collagen type II alpha 1; Col3a1: Collagen type III alpha 1; MAT1A: Methionine adenosyltransferase 1A; SREBP1c: Sterol regulatory element binding transcription protein 1c; SCD: Stearoyl-CoA desaturase; ACACA: Acetyl-CoA carboxylase; FASN: Fatty acid synthase; PPARγ: Peroxisome proliferator-activated receptor gamma; ACADL: Long-chain specific acyl-CoA dehydrogenase; FABP1: Fatty acid binding protein 1; CPT1α: Carnitine palmitoyltransferase 1A; FATP2: Fatty acid transport protein 2.

were much lower after folic acid intervention. The rats fed an HFD for 16 wk showed bridging fibrosis through Masson and Sirius red staining (Figure 2A). Treatment with folic acid resulted in less severe fibrosis based on the pathological sections. Furthermore, folic acid downregulated the expression levels of α-smooth muscle actin (Figure 2C), transforming growth factor beta 1 (Figure 2D), collagen type I alpha 1 (Figure 2E), collagen type II alpha 1 (Figure 2F), and collagen type III alpha 1 (Figure 2G). Although folic acid could reduce the fibrosis score, the difference did not reach statistical significance ($P = 0.072$, Figure 2B).

Rats in the HFD group showed significant dyslipidemia. Serum ALT ($P < 0.01$), AST ($P < 0.01$), FBG ($P < 0.01$), TG ($P < 0.01$), TC ($P < 0.01$), and LDL ($P < 0.01$) levels were significantly elevated compared with those in the control group, accompanied by lower HDL ($P < 0.01$) levels (Table 2). The folic acid group showed a significant reduction in FBG ($P < 0.01$), TG ($P < 0.01$), TC ($P < 0.01$), and LDL ($P < 0.01$) levels. However, there was no significant difference in HDL levels between the HFD and folic acid groups. Abnormal bile acid metabolism and Hcy metabolism were detected in the HFD group. HFD rats had higher TBA ($P < 0.01$) and Hcy ($P < 0.01$) levels than the control group. Folic acid significantly reduced serum TBA ($P < 0.05$) and Hcy ($P < 0.01$) levels compared with those in the HFD group (Table 2). The results above suggested that folic acid ameliorates HFD-induced hepatic lipid accumulation, inflammation, and fibrosis.

Folic acid inhibits hepatic lipogenesis and promotes hepatic fatty acid oxidation in rats with HFD-induced NASH

Abnormal hepatic lipid uptake, *de novo* lipogenesis (DNL), and β-oxidation contribute to the progression of NAFLD[15]. To further characterize the effects of folic acid on

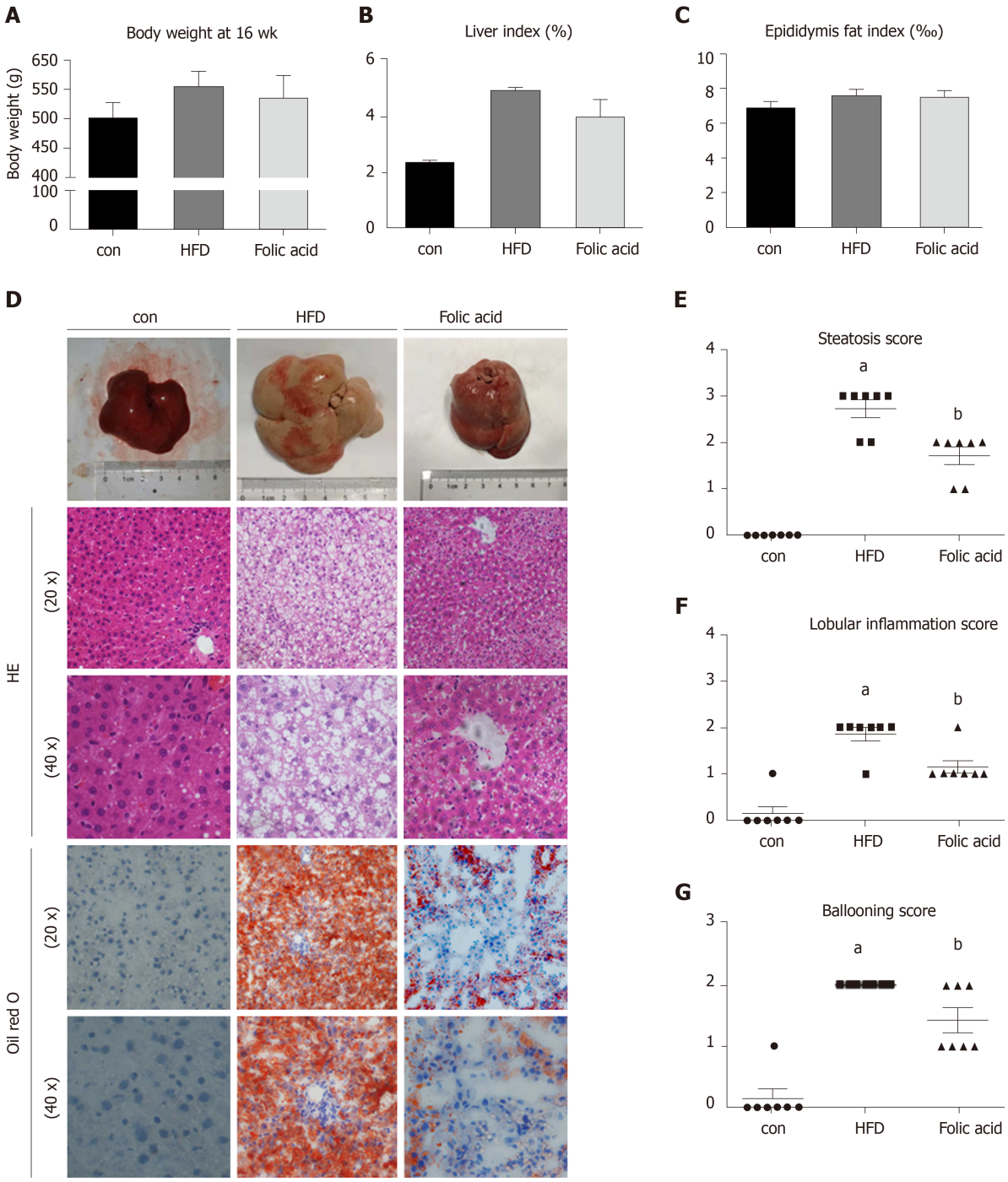


Figure 1 Folic acid ameliorates histological alterations in high-fat diet-induced steatohepatitis independent of affecting body weight. A: Body weight at 16 wk in each group; B: Liver index in each group; C: Epididymis fat index in each group; D: Hematoxylin-eosin and Oil red staining in each group. Scale bars: 50 μm; E-G: Steatosis score, lobular inflammation score, and ballooning score in each group. All the data are expressed as the mean ± SE (n = 4-7). $^{a}P < 0.05$ *vs* con group; $^{b}P < 0.05$ *vs* HFD group. HFD: High-fat diet; HE: Hematoxylin-eosin.

hepatic lipid metabolism in HFD-induced NASH rats, we analyzed the expression levels of genes related to DNL, β-oxidation, and lipid uptake. As shown in Figure 3A-D, folic acid significantly downregulated the expression levels of sterol regulatory element binding transcription protein 1c, stearoyl-CoA desaturase, acetyl-CoA carboxylase, and fatty acid synthase. Moreover, genes related to hepatic lipid β-oxidation and lipid uptake such as PPARγ (Figure 3E), long-chain specific acyl-CoA dehydrogenase (Figure 3F), FABP1 (Figure 3G), CPT1α (Figure 3H), and fatty acid transport protein 2 (Figure 3I) were elevated after folic acid administration. To further confirm the ameliorative effect of folic acid on hepatic lipid β-oxidation. We also detected the expression levels of related genes at the protein level. As shown in Figure 3J-L, CPT1α, and FABP1 levels were strikingly reduced by HFD and significantly restored by folic acid intervention. Furthermore, liver cholesterol (Figure 3M) and

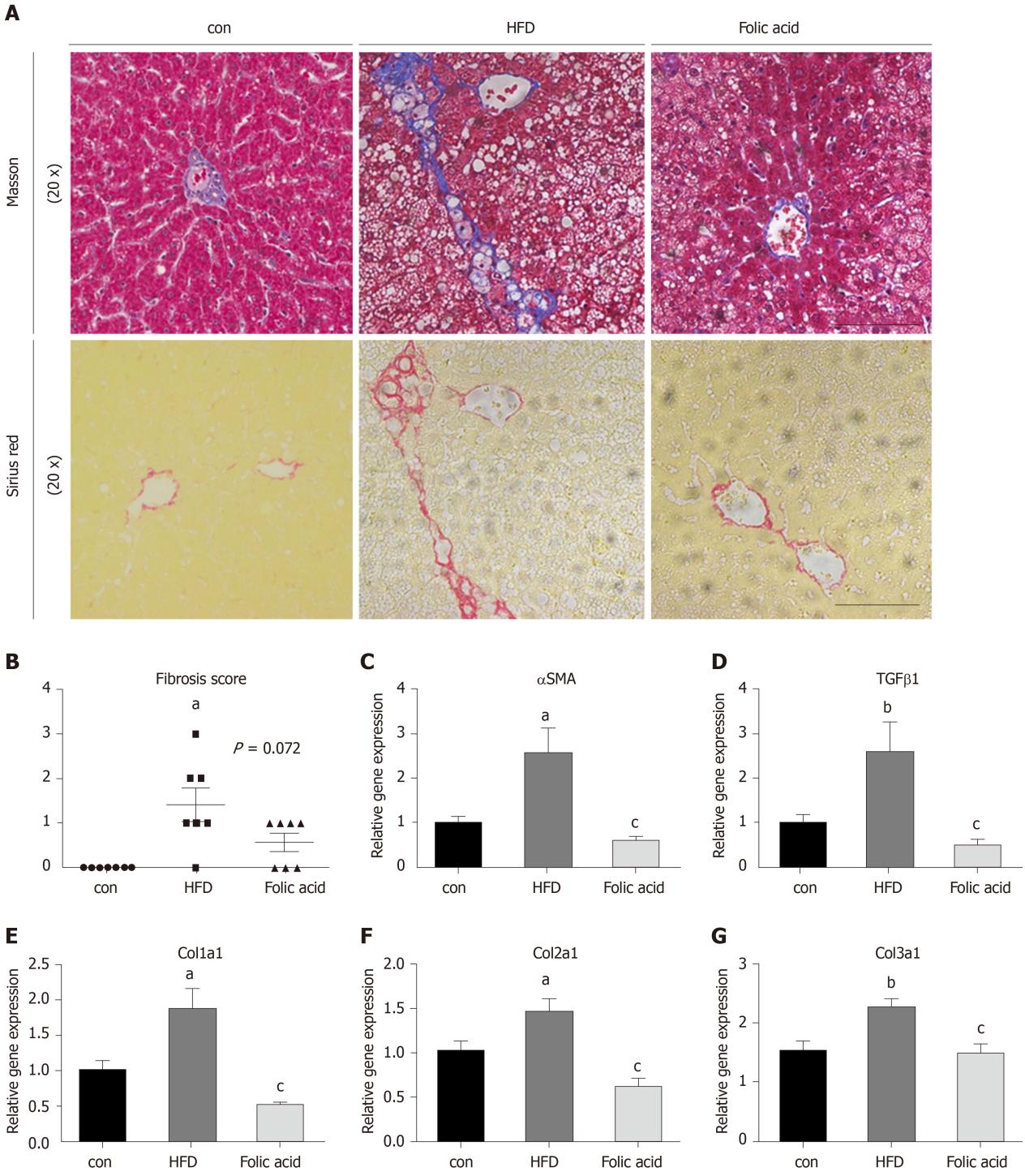


Figure 2 Folic acid ameliorates liver fibrosis in the rat model. A: Masson and Sirius red staining in each group. Scale bars: 100 μm; B: Fibrosis score in each group; C-G: Hepatic αSMA, TGFβ1, Col1a1, Col2a1, and Col3a1 in each group. All the data are expressed as the mean ± SE (n = 4-7). $^{a}P < 0.05$ *vs* con group; $^{b}P < 0.01$ *vs* con group; $^{c}P < 0.01$ *vs* HFD group. HFD: High-fat diet; αSMA: α-smooth muscle actin; TGFβ1: Transforming growth factor beta 1; Col1a1: Collagen type I alpha 1; Col2a1: Collagen type II alpha 1; Col3a1: Collagen type III alpha 1.

triglyceride (Figure 3N) levels were reduced in the folic acid group compared with the HFD group. This part of results suggested that folic acid improves abnormal hepatic lipid metabolism and then reduces hepatic lipid accumulation.

Folic acid restores the expression levels of PPARα via SIRT1 in rats with HFD-induced NASH and Huh7 cell line

Both PPARs and SIRT1 are key regulators in hepatic lipid β-oxidation. To further determine the effect of folic acid on the remission of hepatic β-oxidation in rats with HFD-induced NASH, we first evaluated the expression levels of SIRT1 and PPARα in animal models. As shown in Figure 4A-C, rats in the HFD group displayed lower

Table 2 Serological lipid metabolism indexes in each group

	Control	HFD	Folic acid
ALT (U/L)	38.50 ± 1.58	134.0 ± 8.02[b]	82.13 ± 7.19[d]
AST (U/L)	88.00 ± 4.39	225.4 ± 10.57[b]	176.3 ± 15.3[d]
FBG (mmol/L)	10.45 ± 0.66	13.33 ± 0.40[b]	7.83 ± 0.30[d]
TG (mmol/L)	0.57 ± 0.04	0.76 ± 0.04[b]	0.44 ± 0.02[d]
TC (mmol/L)	1.13 ± 0.04	2.26 ± 0.12[b]	1.48 ± 0.04[d]
HDL (mmol/L)	0.94 ± 0.03	0.75 ± 0.05[b]	0.74 ± 0.04
LDL (mmol/L)	0.24 ± 0.02	1.37 ± 0.10[b]	0.89 ± 0.03[d]
TBA (μmol/L)	37.5 ± 5.57	68 ± 7.49[b]	44.17 ± 3.92[c]
Hcy (μmol/L)	7.35 ± 0.29	13.05 ± 0.52[b]	11.17 ± 0.42[d]

The data are expressed as the mean ± SE (n = 5-8).
[b]P < 0.01 *vs* Control group.
[c]P < 0.05 *vs* HFD group.
[d]P < 0.01 *vs* HFD group. HFD: High-fat diet; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FBG: Fast blood glucose; TG: Triglycerides; TC: Total serum cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; TBA: Total bile acids; Hcy: Homocysteine.

levels of SIRT1 and PPARα than controls. Folic acid could strongly restore the expression levels of SIRT1 and increase the expression of PPARα to a certain extent.

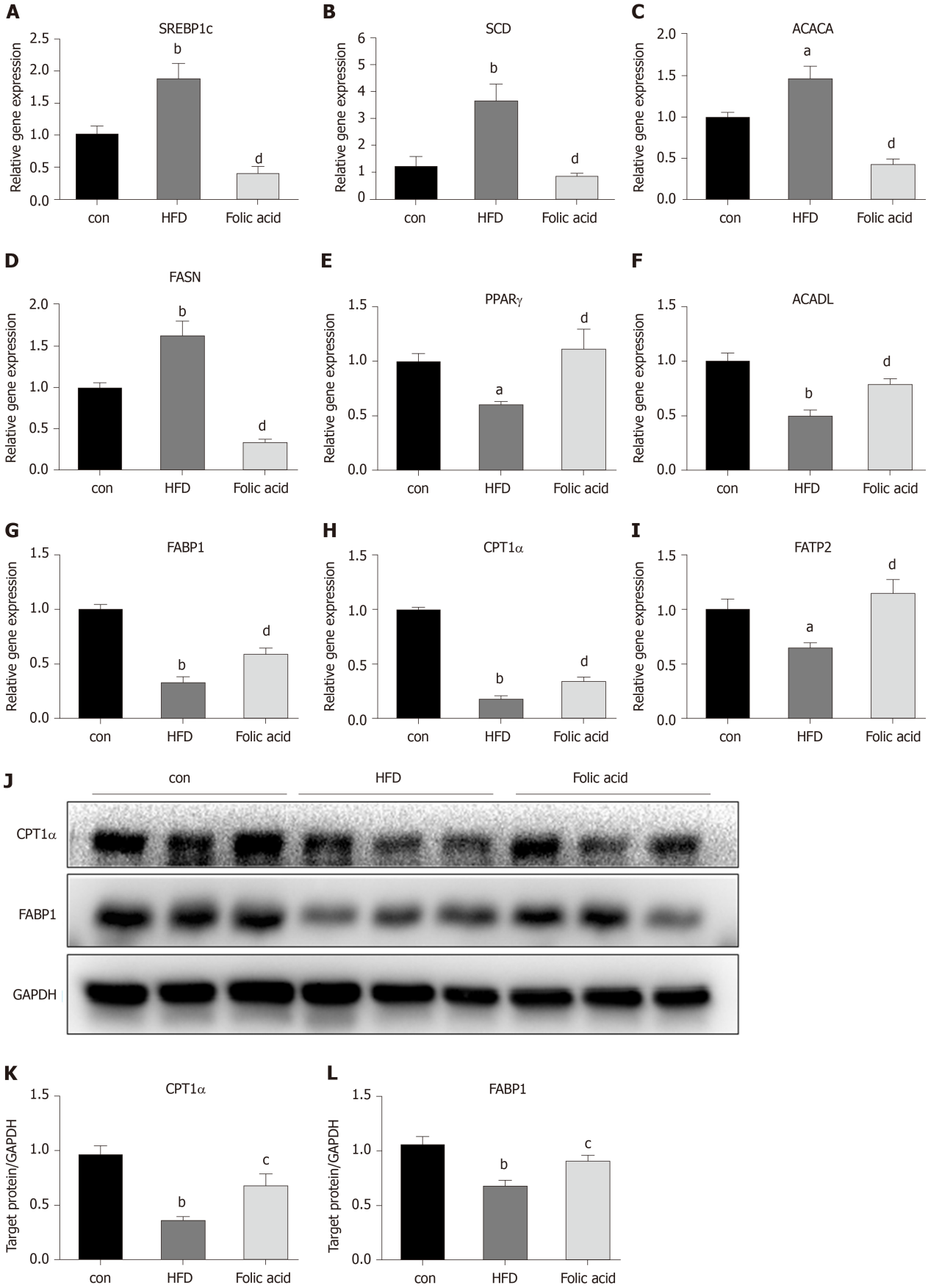
Next, we constructed a PA-induced steatosis cell model using the Huh7 cell line. 5-MTHF, a predominant form of folic acid, was used as an intervention drug. After 12 h of treatment with PA solution, the expression levels of SIRT1 (P < 0.05) and PPARα (P < 0.05) were significantly downregulated. 5-MTHF strongly elevated the expression levels of SIRT1 (1.45-fold in the 1 μg/mL and 1.26-fold in 10 μg/mL 5-MTHF group compared with the levels in the PA treatment group, Figure 4D and F) and PPARα (1.29-fold in the 1 μg/mL and 1.44-fold in 10 μg/mL 5-MTHF group compared with the levels in the PA treatment group, Figure 4D and G). The upregulating effect of PPARα by 5-MTHF was dramatically blocked after knockdown of SIRT1 with a siRNA (Figure 4E, H, and I). Overall, folic acid restores hepatic PPARα levels *via* a SIRT1-dependent mechanism and then improves hepatic lipid metabolism under HFD-feeding conditions.

Folic acid improves hepatic one-carbon metabolism in rats with HFD-induced NASH

We measured the serum folic acid level in each group to further characterize the effect of the folic acid intervention. As shown in Figure 5A, folic acid intragastric administration significantly increased serum folic acid levels, although there was no difference in serum folic acid levels between the control and HFD groups. To further evaluate the effect of folic acid on one-carbon metabolism under HFD conditions, we detected the expression levels of key enzymes involved in one-carbon metabolism. qRT-PCR showed decreased *ALDH1L1* (0.15-fold, P < 0.01, Figure 4C) and *MAT1A* (0.10-fold, P < 0.01, Figure 4D) levels in the HFD group than controls. Western blot confirmed the lower MAT1A level as well (Figure 4B and 4B). Folic acid supplementation could increase the expression of those genes at the transcription (Figure 4C and D) or translation levels (Figure 4B and E). The results above implied that folic acid could partially restore depleted one-carbon metabolism in HFD rats and suggested that folic acid has a direct effect on the liver.

Folic acid restores the diversity of the gut microbiota and the gut barrier and improves endotoxemia and liver inflammation in the NASH rat model

Fecal samples were collected and subjected to 16S rRNA sequencing to detect the effect of folic acid on the gut microbiota. As shown in Figure 6A, folic acid restored the alpha diversity based on PD_whole_tree measurement, which demonstrated that folic acid could restore the HFD-induced depletion of the gut microbiota abundance. Principal coordinates analysis showed that folic acid could alter the composition of the gut microbiota in HFD-fed rats (Figure 6B). The unweighted pair-group method with arithmetic mean analysis showed that folic acid partially restored the alteration in the overall structure of the gut microbiota induced by the HFD (Figure 6C and D). Besides, compared to the control group, lower abundance of Bacteroidetes was detected in the HFD group, and folic acid administration could partially increase levels of Bacteroidetes. Compared with the HFD group, an increase in several genera such as Pseudomonadaceae and Leptotrichiaceae was observed (data not shown).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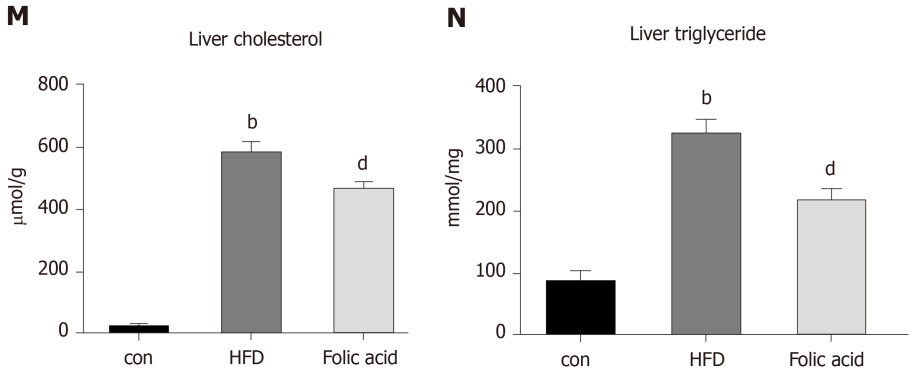


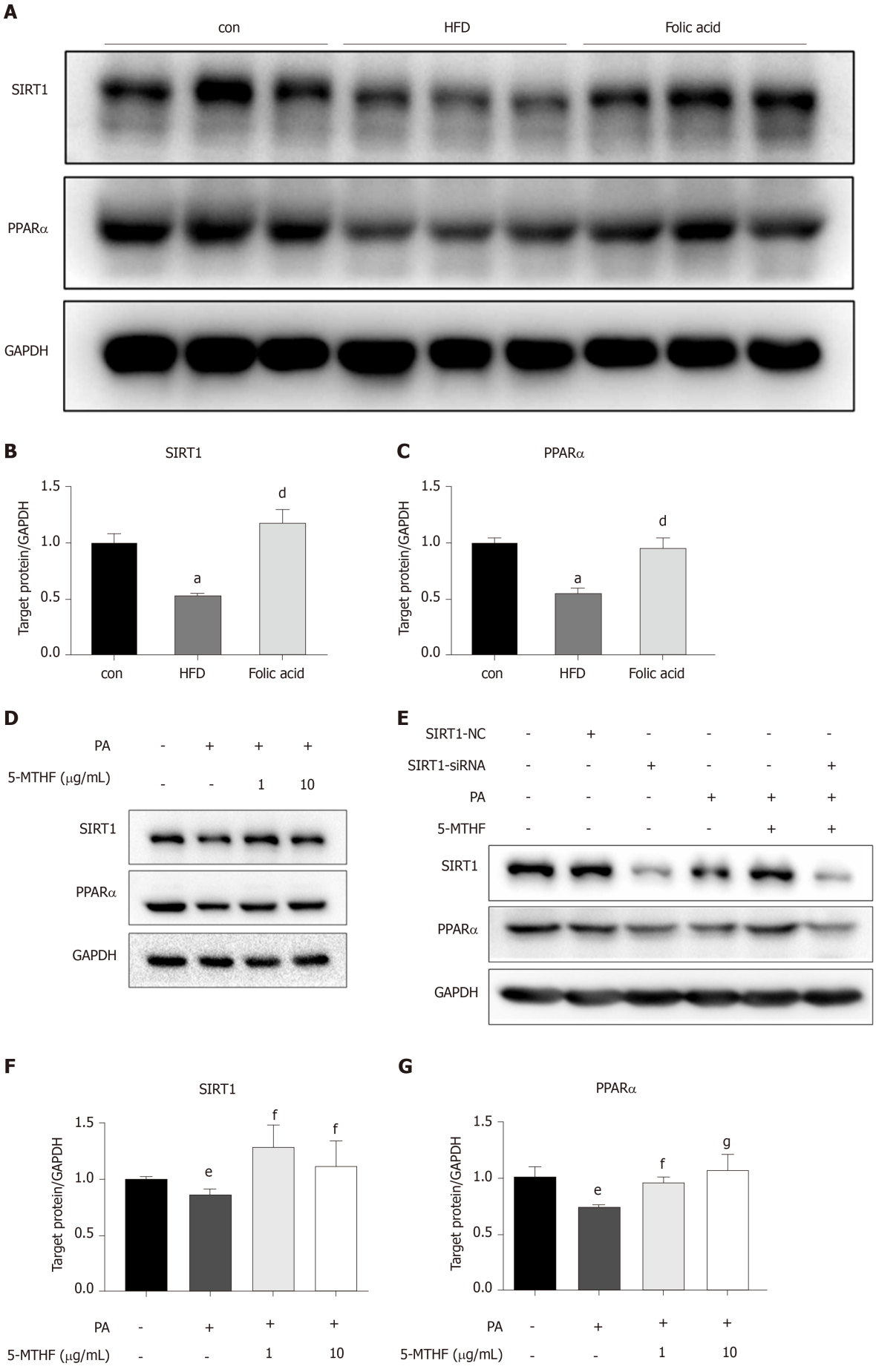
Figure 3 Folic acid inhibits hepatic lipogenesis and promotes hepatic fatty acid oxidation in high-fat diet-induced steatohepatitis rats. A-I: mRNA expression levels of *SREBP1c*, *SCD*, *ACACA*, *FASN*, *PPARγ*, *ACADL*, *FABP1*, *CPT1α*, and *FATP2* in each group; J-L: Protein expression levels of CPT1α and FABP1 in each group; M and N: Liver cholesterol and triglyceride levels. All the data are expressed as the mean ± SE (n = 3-6). $^{a}P < 0.05$ *vs* con group; $^{b}P < 0.01$ *vs* con group; $^{c}P < 0.05$ *vs* HFD group; $^{d}P < 0.01$ *vs* HFD group. HFD: High-fat diet; SREBP1c: Sterol regulatory element binding transcription protein 1c; SCD: Stearoyl-CoA desaturase; ACACA: Acetyl-CoA carboxylase; FASN: Fatty acid synthase; PPARγ: Peroxisome proliferator-activated receptor gamma; ACADL: Long-chain specific acyl-CoA dehydrogenase; FABP1: Fatty acid binding protein 1; CPT1α: Carnitine palmitoyltransferase 1A; FATP2: Fatty acid transport protein 2.

Moreover, HE and Occludin immunohistochemical staining of the ileum showed that folic acid could restore the villus structure and the abundance of the expression of tight junctions (Figure 6E). Moreover, serum endotoxin levels were significantly reduced in the folic acid group (Figure 6F). Then, the expression levels of proinflammatory factors such as tumor necrosis factor alpha (Figure 6G), interleukin-6 (Figure 6H), and interleukin-1 beta (Figure 6I); chemokine receptor C-C chemokine receptor type 2 (Figure 6J); and oxidative stress-related factors such as neutrophil cytosol factor 1 (Figure 6K), neutrophil cytosolic factor 2 (Figure 6L), cytochrome b-245 alpha chain (Figure 6M), and cytochrome b-245 beta chain (Figure 6N) were greatly decreased by folic acid treatment. Overall, folic acid could restore the depleted diversity and the intestinal barrier, ameliorate endotoxemia, and decrease hepatic inflammatory reactions under HFD conditions.

DISCUSSION

We demonstrated that folic acid attenuated hepatic lipid metabolism in rats with HFD-induced steatohepatitis, increased PPARα levels through a SIRT1-dependent mechanism *in vivo* and *vitro*, ameliorated HFD-induced depleted hepatic one-carbon metabolism, and restored the diversity of the gut microbiota, thus contributing to the improvements of HFD-induced NASH in rats.

One of important findings in our present study is that folic acid plays an important role in regulating hepatic lipid metabolism in the HFD-induced NASH model. Lipid metabolism disorder is one of the most important pathophysiological changes in individuals with NAFLD. Either the "two-hit" or "multiple parallel hits" hypothesis confirms that abnormal lipid metabolism is one of the core causes of steatosis[16,17]. Both increased DNL[18] and impaired fatty acid oxidation[19] contribute to the pathogenesis of NAFLD. Previous studies confirmed that folic acid could reduce lipid accumulation in primary chicken hepatocytes[12] and alter lipid metabolism genes in male rat offspring[20]. Studies also indicated that folic acid may alleviate abnormal lipid metabolism and cholesterol deposition in the liver through the LKB1-AMPK pathway[11]. However, the further mechanism for the effect of folic acid in regulating hepatic fatty acid oxidation is still rarely known. PPARs belong to the nuclear hormone receptor superfamily, and of the PPARs, PPARα regulates hepatic lipid metabolism, glucose metabolism, and liver inflammation[21,22]. Numerous rate-limited enzymes associated with fatty acid uptake[23] and mitochondrial β-oxidation[24,25] are regulated by PPARα. Hepatocyte-specific PPARα deletion impaired fatty acid homeostasis and promoted the progression of NAFLD[26]. SIRT1 is an NAD^{+}-dependent deacetylase in mammalian cells that plays a key role in metabolic diseases[27] and regulates the transcription network in free fatty acid oxidation[28]. Microarray analysis confirmed that SIRT1, PPARα, and peroxisome proliferator-activated receptorγcoactivator-1 (PGC1α) played a core role in the regulation of genes responsible for β-oxidation[29]. Hepatic deletion of SIRT1 could impair PPARα signaling, and overexpression of SIRT1 could restore the expression levels of PPARα and its target genes[30]. We confirmed that folic acid restores hepatic PPARα *via* a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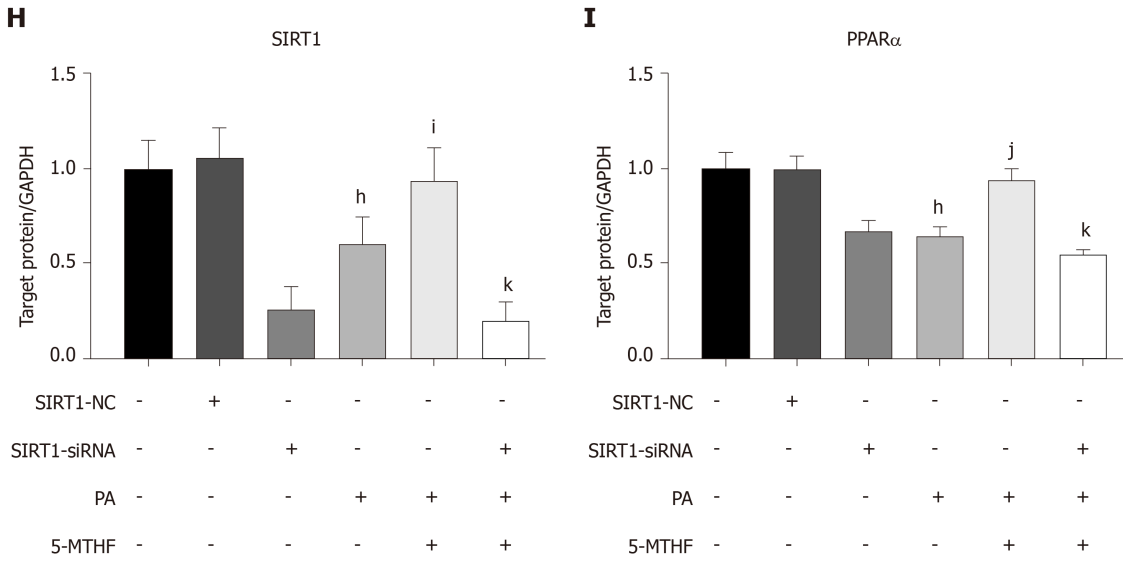


Figure 4 Folic acid restores the expression levels of PPARα *via* SIRT1 in rats with high-fat diet-induced steatohepatitis and Huh7 cell line. A-C: The expression levels of SIRT1 and PPARα in each group of rats; D, F, and G: The expression levels of SIRT1 and PPARα in Huh7 cell line exposed to 0.3 mmol/L PA; E, H, and I: The expression levels of SIRT1 and PPARα in Huh7 cell line transfected with SIRT1 siRNA and then exposed to 0.3 mmol/L PA. All the data are expressed as the mean ± SE (n = 3). [a]P < 0.05 *vs* con group; [b]P< 0.01 *vs* con group; [d]P < 0.01 *vs* HFD group; [e]P < 0.05 *vs* control; [f]P < 0.05 *vs* 0.3 PA group; [g]P < 0.01 *vs* 0.3 PA group; [h]P < 0.01 vs SIRT1-NC group; [i]P < 0.05 *vs* 0.3 PA group; [j]P < 0.01 *vs* 0.3PA group; [k]P <0.01 *vs* 5-MTHF and 0.3 PA group. HFD: High-fat diet; PPARα: Peroxisome proliferator-activated receptor alpha; SIRT1: Silence information regulation factor 1; PA: Palmitic acid; 5-MTHF: 5-methyltetrahydrofolic acid.

SIRT1 dependent pathway, which further reveals the effect of folic acid on hepatic lipid metabolism.

Significantly lower FBG levels in the folic acid group indicated that folic acid may play a role in glucose metabolism in metabolic diseases including NAFLD. Studies showed that chronic folic acid deficiency induced glucose metabolism disorder[31]. Folic acid treatment decreased serum glucose levels in a diabetic rat model[32]. Administration of folic acid improved insulin resistance by altering the DNA methylation profile in HFD-fed mice[33]. This indicated that folic acid could improve glucose metabolism in NASH conditions, but specific mechanisms need further research.

Another finding in our present study is that folic acid could restore one-carbon metabolism in rats with HFD-induced NASH. Several studies have reported that folic acid and other methyl donors have an alleviating effect on chronic liver diseases, such as liver fibrosis[34], cholestasis[34], drug-induced liver injury[6,35], alcoholic liver disease[13], obesity[36], and NAFLD[37,38]. However, serum folic acid level in NAFLD patients is still controversial in recent studies. Some researchers[39] believed that there was no significant difference in serum folic acid and vitamin B12 levels between NAFLD and healthy control groups and that neither folic acid nor vitamin B12 levels were associated with pathological severity. Other studies have found varying degrees of positive results. Hirsch *et al*[40] found a lower serum folic acid concentration in female obese patients with NAFLD than in healthy controls; Mahamid *et al*[41] posited that lower folate or vitamin B12 levels were associated with the histological severity of NASH. An association between serum folic acid levels and the severity of liver steatosis was also found by research on Chinese adult NAFLD patients[42]. The serum folic acid levels in the NAFLD patients from the abovementioned literature varied from 9.3 to 12.6 ng/mL on average, all of which were normal levels. Therefore, we believed that HFD had little effect on folate absorption or serum folate levels. This result was consistent with the lack of a significant difference in serum folic acid levels between the control and HFD groups in our study. However, as a co-enzymatic substrate, folic acid serves a core role in on-carbon transfer reactions. Folic acid-dependent one-carbon metabolism is important for methylation reactions in mammal cells[43]. It has been well demonstrated that differential DNA methylation occurs in individuals with NAFLD[44-46]. Genes involved in one-carbon metabolism showed abnormal DNA methylation, and of these genes, MAT1A and ALDH1L1 showed hypermethylated levels and downregulation[46]. MAT1A participates in the synthesis of S-adenosylmethionine[47] and ALDH1L1 is involved in metabolism in the carbon pathway[48]. Both of them are required for lipid homeostasis. We found strong downregulation of MAT1A and ALDH1L1 in HFD-fed rats in our present study, and additional folic acid supplementation was effective in restoring their expression

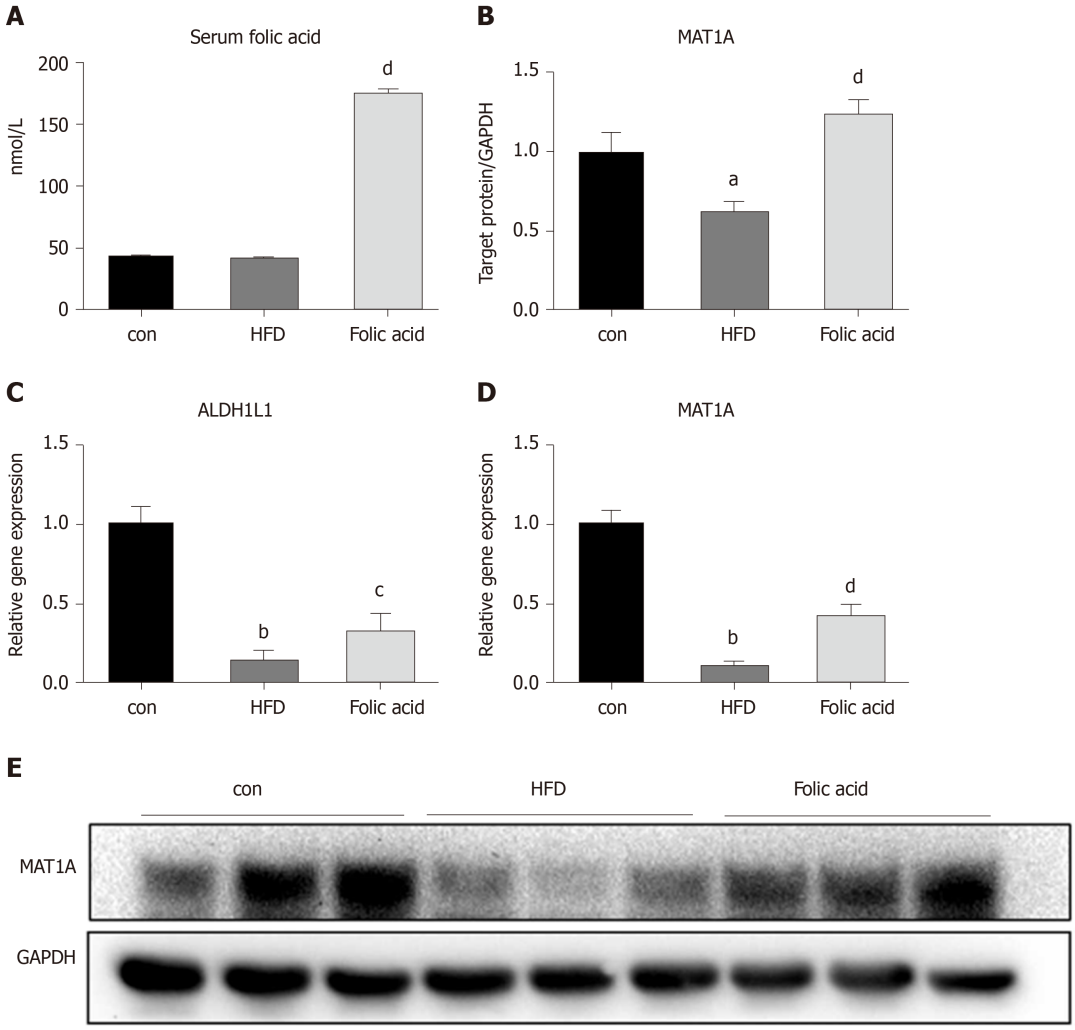


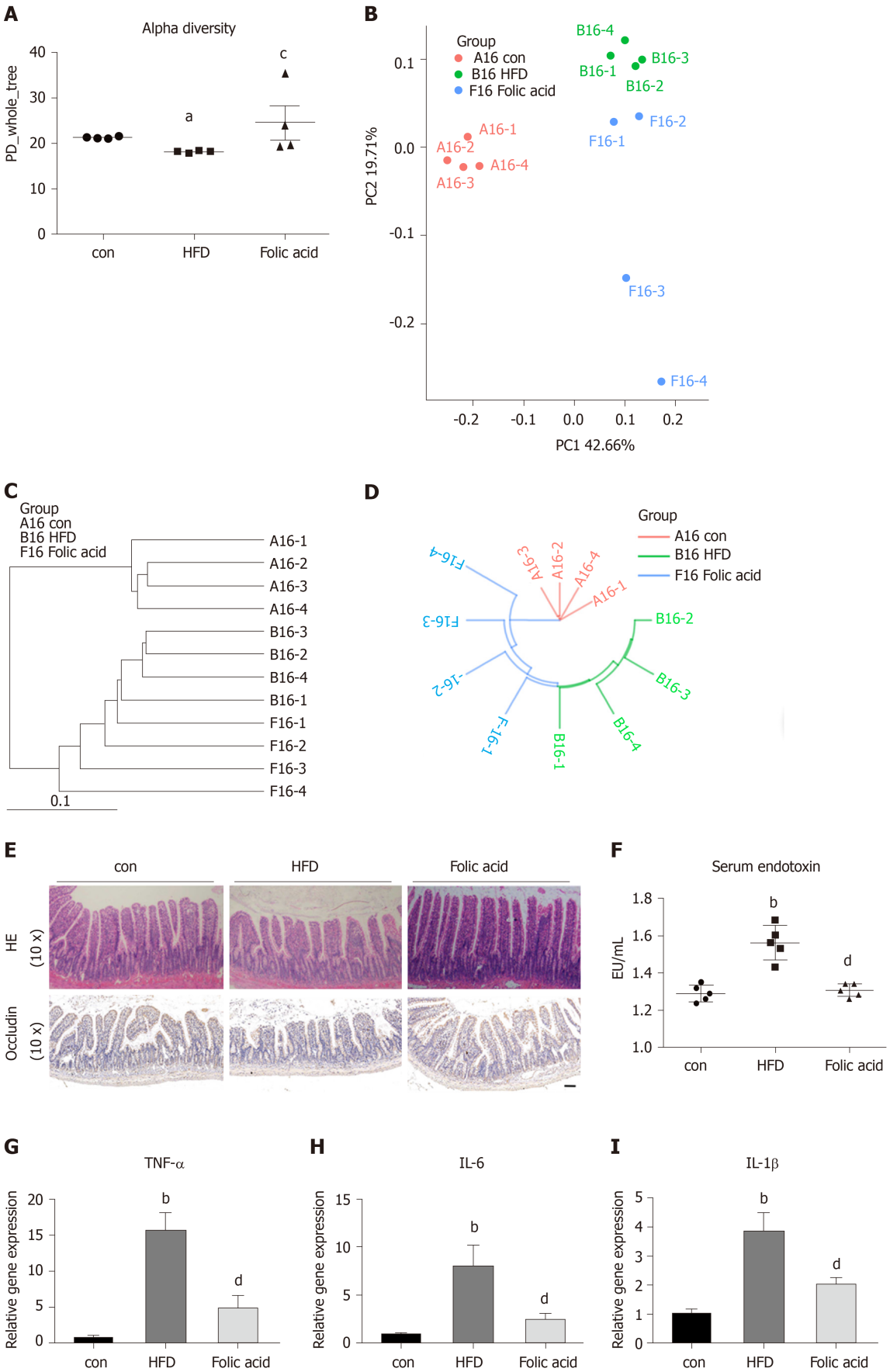
Figure 5 Folic acid improves hepatic one-carbon metabolism in rats with high-fat diet-induced steatohepatitis. A: Serum folic acid levels in each group; C and D: mRNA levels of MAT1A and ALDH1L1 in each group; B and E: Protein levels of MAT1A in each group. All the data are expressed as the mean ± SE (n = 3-8). [a]P < 0.05 *vs* con group [b]P < 0.01 *vs* con group; [c]P < 0.05 *vs* HFD group; [d]P < 0.01 *vs* HFD group. HFD: High-fat diet; MAT1A: Methionine adenosyltransferase 1A.

levels. These findings indicated that folic acid supplementation is required for NASH individuals to improve hepatic lipid metabolism through restoring one-carbon metabolism.

An increasing number of studies have demonstrated that NAFLD has a disease-specific gut microbiome signature[49], of which the depleted diversity of the microbiota and microbial gene richness and differential bacterial clusters were most commonly reported[50,51]. Additionally, HFD consumption disturbed gut permeability by reducing tight-junction proteins such as Occludin and ZO-1, which leads to endotoxemia and chronic systemic inflammation[52] and promotes the progression of NASH. We notably found that folic acid could stabilize the intestinal barrier and the diversity of the gut microbiota, which partially explained the calming effect on the whole body and hepatic inflammation.

There are still some limitations that deserve further study. First, we have not demonstrated the effect of folic acid on the SIRT1-PPARα pathway *in vivo*. SIRT1 conditional knock-out mice should be used in future study to further evaluate the molecular mechanism of folic acid in the improvement of NAFLD. Second, studies have confirmed that several genes related to lipid metabolism, such as *PGC1a*[53], *ZNF274*, and *SREBP2*[44], had enriched DNA methylation in individuals with NAFLD. Therefore, whether folic acid could influence the balance of acetylation and methylation in genes related to free fatty acid oxidation, especially *PPARa* and *PGC1a*, remains an interesting question. Finally, a drug-dose gradient *in vivo* could be considered to evaluate the optimal intervention dose for clinical guidance.

In conclusion, we have confirmed the improvement effect of folic acid on HFD-induced NASH in rats. We demonstrated that folic acid improves hepatic lipid metabolism by upregulating PPARα *via* a SIRT1-dependent mechanism. Meanwhile, folic acid administration restores depleted hepatic one-carbon metabolism and the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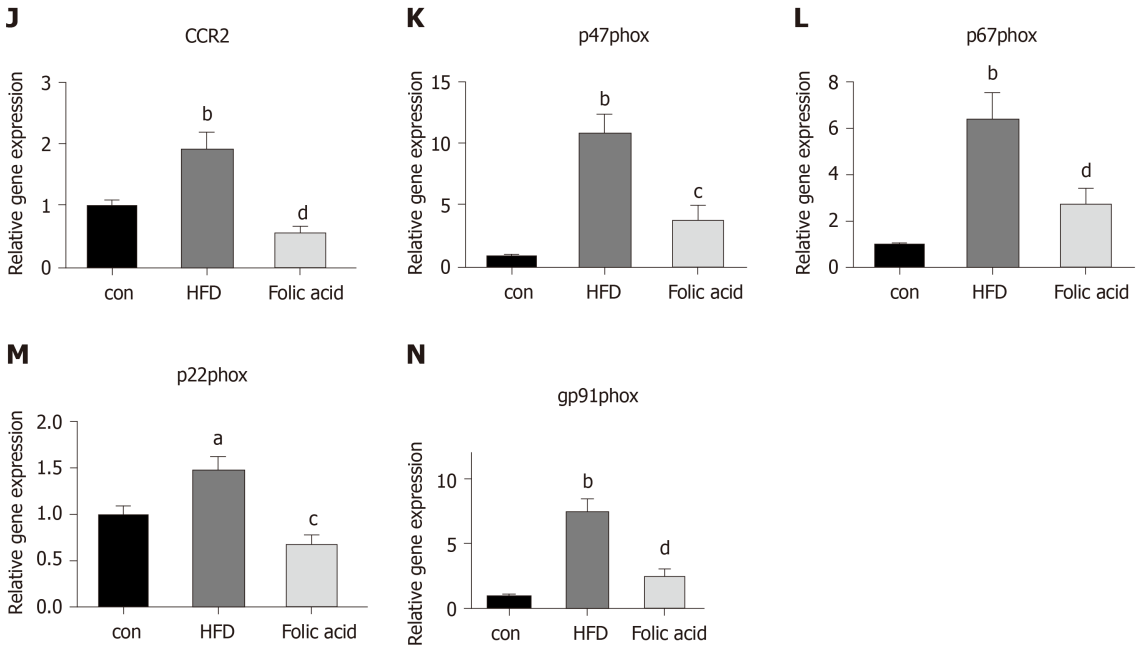

Figure 6 Folic acid restores the diversity of the gut microbiota and the gut barrier and improves endotoxemia and liver inflammation in a non-alcoholic steatohepatitis rat model. A: Alpha diversity of the gut microbiota; B: Principal coordinates analysis; C and D: Unweighted pair-group method with arithmetic mean analysis; E: Hematoxylin-eosin and Occludin immunochemical staining of the ileum. Scale bars: 100 μm; F: Serum endotoxin levels in each group; G-N: Hepatic TNF-α, Il-6, IL-2β, CCR2, p47phox, p67phox, p22phox, and gp91phox levels in each group. All the data are expressed as the mean ± SE (n = 4-6). $^{a}P < 0.05$ *vs* con group $^{b}P < 0.01$ *vs* con group; $^{c}P < 0.05$ *vs* HFD group; $^{d}P < 0.01$ *vs* HFD group. HFD: High-fat diet; HE: Hematoxylin-eosin; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; CCR2: Chemokine receptor C-C chemokine receptor type 2.

diversity of gut microbiota in HFD-fed rats. These results further clarify the therapeutic role of folic acid in NAFLD and its possible mechanism, suggesting that folic acid may become a therapeutic drug to treat NAFLD in the future.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease has become a global burden, but there is still a lack of convinced drug therapy strategies for non-alcoholic steatohepatitis (NASH). As one of essential water-soluble vitamins for the human body, folic acid may become one of the drug targets for treatment of NASH, but the specific mechanism is not fully understood.

Research motivation

As one of essential vitamins absorbed by the intestine mainly, food-sourced folic acid improved high-fat diet (HFD)-induced steatohepatitis in previous studies, but further mechanism of folic acid on host hepatic lipid metabolism and the effect of folic acid on lipid one-carbon metabolism and gut microbiota remains rarely understood.

Research objectives

We aimed to evaluated the effect of folic acid on HFD-fed rat models and further clarify the mechanism of folic acid on hepatic lipid metabolism and gut microbiota.

Research methods

An HFD-induced rat model of NASH was used in the present study. Treatment of folic acid by oral administration lasted for 8 wk. Hepatic lipid metabolism was evaluated by real-time quantitative polymerase chain reaction (qRT-PCR). Expression levels of silence information regulation factor 1 (SIRT1) and peroxisome proliferator-activated receptor alpha (PPARα) were measured by Western blot analysis in HFD-induced rat models and palmitic acid-induced Huh7 cells. SIRT1 siRNA was transfected in Huh7 cells to examine whether folic acid restored PPARα levels through a SIRT1-dependent mechanism. Genes and proteins related to hepatic one-carbon metabolism were detected by qRT-PCR and Western blot. 16S rDNA sequencing was used to evaluate the effect of folic acid on gut microbiota profile.

Research results

Administration of folic acid ameliorated HFD-induced steatohepatitis. Folic acid repaired impaired hepatic lipid β-oxidation and hepatic one-carbon metabolism. SIRT1 and PPARα levels were restored by folic acid treatment. The restoration effect of PPARα by folic acid was blocked after SIRT1 knockdown *in vitro*. Furthermore, folic acid restored the diversity and altered the

overall structure of gut microbiota profile.

Research conclusions

Folic acid restores PPARα levels *via* a SIRT1-dependent mechanism, ameliorates HFD-induced impaired hepatic lipid metabolism and hepatic one-carbon metabolism, and improves the diversity of gut microbiota, thus acting a protective role in HFD-induced NASH in rats.

Research perspectives

Folic acid may become one of drug targets for treatment of NASH. Research about folic acid in epigenetic regulation may further clarify the mechanism of folic acid on NASH.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic significance of hepatic encephalopathy in patients with cirrhosis treated with current standards of care

Anuj Bohra, Thomas Worland, Samuel Hui, Ryma Terbah, Ann Farrell, Marcus Robertson

ORCID number: Anuj Bohra (0000-0003-2913-8225); Thomas Worland (0000-0001-5122-5750); Samuel Hui (0000-0002-2839-6209); Ryma Terbah (0000-0003-3654-2437); Ann Farrell (0000-0002-4123-5661); Marcus Robertson (0000-0002-8848-1771).

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Anuj Bohra, Thomas Worland, Samuel Hui, Ann Farrell, Marcus Robertson, Department of Gastroenterology, Monash Health, Clayton 3168, Victoria, Australia

Thomas Worland, Ryma Terbah, Marcus Robertson, Department of Gastroenterology, Austin Health, Heidelberg 3084, Victoria, Australia

Marcus Robertson, Department of Medicine, School of Clinical Sciences, Monash University, Clayton 3168, Victoria, Australia

Corresponding author: Anuj Bohra, BMed, Doctor, Department of Gastroenterology, Monash Health, 246 Clayton Road, Clayton 3168, Victoria, Australia. anujbohra@hotmail.com

Abstract

BACKGROUND

Hepatic encephalopathy (HE) is a reversible neuropsychiatric complication of liver cirrhosis and occurs in up to 50% of cirrhotic patients. Studies examining the prognostic significance of HE are limited despite the high prevalence in cirrhosis.

AIM

To define the clinical outcomes of patients after an episode of HE treated with current standards-of-care.

METHODS

All patients hospitalised with HE requiring Rifaximin to 3 tertiary centres over 46-mo (2012–2016) were identified *via* pharmacy dispensing records. Patients with hepatocellular carcinoma and those prescribed Rifaximin prior to admission were excluded. Medical records were reviewed to determine baseline characteristics and survival. The Kaplan-Meier method was used to calculate survival probability. Univariate survival analysis was performed with variables reaching statistical significance included in a multivariate analysis. The primary outcome was 12-mo mortality following commencement of Rifaximin.

RESULTS

188 patients were included. Median age was 57 years (IQR 50-65), 71% were male and median model for end stage liver disease and Child Pugh scores were 25 (IQR 18-31) and 11 (IQR 9-12) respectively. The most common causes of cirrhosis were alcohol (62%), hepatitis C (31%) and non-alcoholic fatty liver disease (20%). A precipitating cause for HE was found in 92% patients with infection (43%), GI bleeding (16%), medication non-compliance (15%) and electrolyte imbalance (14%) the most common. During a mean follow up period of 12 ± 13 mo 107 (57%) patients died and 32 (17%) received orthotopic liver transplantation. The

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most common causes of death were decompensated chronic liver disease (57%) and sepsis (19%). The probability of survival was 44% and 35% at 12- and 24-mo respectively. At multivariate analysis a model for end stage liver disease > 15 and international normalised ratio reached statistical significance in predicting mortality.

CONCLUSION
Despite advances made in the management of HE patients continue to have poor survival. Thus, in all patients presenting with HE the appropriateness of orthotopic liver transplantation should be considered.

Key words: Hepatic encephalopathy; Cirrhosis; Portal hypertension; Prognosis; Rifaximin; Lactulose

Core tip: The development of hepatic encephalopathy in cirrhotic patients continues to be associated with an extremely poor prognosis despite current standards-of-care and newer therapeutic options such as Rifaximin. In this study, the probability of survival at 12-mo was 44% after an episode of acute hepatic encephalopathy requiring hospital admission. Thus, in all patients with hepatic encephalopathy the appropriateness of urgent liver transplantation assessment should be considered.

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INTRODUCTION

Prognosis in patients with liver cirrhosis varies significantly depending on whether a patient has compensated or decompensated cirrhosis[1,2]. In patients with compensated cirrhosis, median survival is greater than 12 years. By contrast, in patients experiencing a decompensation, commonly defined by ascites, hepatic encephalopathy (HE), variceal haemorrhage and jaundice, survival is far shorter at two years or less[3-5].

HE is defined as a reversible neuropsychiatric complication of liver cirrhosis. It represents the second most common decompensating event after ascites and will occur in 30%-45% of cirrhotic patients during their lifetime[6,7]. HE manifests as a wide spectrum of neuropsychiatric abnormalities and motor disturbance, ranging from mild alterations in cognitive function to coma and death[8,9]. The clinical features of HE define the grade of encephalopathy, with the West Haven criteria most commonly employed to stage the severity of disease[10]. Plasma ammonia levels are typically elevated in patients with HE, however this is not a reliable sign and is not used in the diagnosis of HE[11]. The current treatment priorities in HE are to identify and reverse the underlying precipitants, which include sepsis, gastrointestinal bleeding, medications such as benzodiazepines, opiates and anti-histamines, acid-base disturbances, renal impairment and constipation[2]. Traditionally, pharmacological therapies have aimed to decrease plasma ammonia levels. Lactulose, which decreases colonic pH and plasma ammonia levels has been the mainstay of treatment for many years. More recently, Rifaximin, a broad-spectrum semisynthetic derivative of rifamycin with minimal systemic absorption, has been added to the therapeutic armamentarium in addition to the use of lactulose[12-14]. Multiple other therapeutic options require further trials to clearly define their role in the management of HE[15-18].

The natural history and prognosis of patients with ascites and variceal bleeding has been extensively studied, however, despite its prevalence there is a paucity of literature related to the prognostic significance of HE. Two sentinel studies published prior to the development of rifaximin demonstrated that development of HE was associated with an extremely poor survival in patients with cirrhosis who did not receive liver transplantation[3,19]; Bustamante *et al*[19] demonstrated a survival

probability of 42% and 23% at one and three years respectively in cirrhotic patients after development of a first episode of acute HE. In the post-Rifaximin era, there is a paucity of literature investigating the prognosis of cirrhotic patients following an episode of HE[20]. To our knowledge, survival of patients with a presentation of HE in the era of rifaximin has yet to be studied in an Australian real-world cohort.

In this study, we evaluated the clinical outcomes and probability of survival amongst cirrhotic patients who presented with acute HE requiring hospital admission and were commenced on rifaximin. In addition, we aimed to identify factors associated with mortality.

MATERIALS AND METHODS

Patient selection and data collection

All patients admitted with HE to three Australian tertiary centres, including one transplant centre, over a 42-mo period from May 2012 to March 2016 who were prescribed rifaximin were identified retrospectively *via* pharmacy dispensing records. Inclusion criteria were that rifaximin must have been commenced during an inpatient admission for HE and continued upon discharge from hospital. Patients with hepatocellular carcinoma (HCC) diagnosed prior to or during the index admission with HE were excluded from the study. Diagnosis of HCC was established by standard imaging techniques (CT Quad Phase Liver or MRI Liver) and/or serum alpha foetoprotein and/or histological examination. The Human Research Ethics Committee at Monash Health approved the study as audit activity and the committee provided a waiver for informed consent.

For each patient, medical records were manually reviewed to collect baseline demographic data, medical comorbidities, aetiology of liver disease, medication use, laboratory results, evidence of decompensated liver disease, precipitating causes of HE and previous and current treatments of HE. Patient outcome data up to 48-mo following the index admission was collected. Death was determined through hospital medical records and confirmed with a patient's Local Medical Officer if required. Each medical record was independently reviewed by two reviewers and any discrepancies in data were referred to a third reviewer. All patients were risk stratified using the model for end stage liver disease (MELD) and Child-Pugh (CP) scores; when calculating the CP score, the serum albumin level prior to intravenous albumin administration was used. The diagnosis and grade of HE was determined using established West Haven criteria[9].

All patients were followed-up from the date of rifaximin commencement until the date of death, liver transplantation or last known survival up to 48-mo following index admission. The primary outcome was 12-mo survival following the commencement of rifaximin. The secondary outcome was to identify patient-specific prognostic factors at the time of commencement of rifaximin that would be useful in determining suitability for a liver transplant.

Treatment protocols for hepatic encephalopathy

Patients with cirrhosis and HE are admitted under a specialist Gastroenterology or Liver Transplant Unit. In all patients treatment of HE consists of identification and correction of possible precipitating factors. Intravenous albumin (1.5 g/kg per day) is typically administered consistent with evidence that it shortens the duration of acute HE[21]. In our centres, regular Lactulose (administered orally or rectally in the setting of reduced mental state) is given as first-line therapy and rifaximin is typically reserved for patients with recurrent or refractory HE.

Statistical analysis

Survival probability curves were calculated using the Kaplan-Meier method. Univariate survival analysis was performed using the Cox proportional hazards model to analyse each considered variable, which included demographic data, maximal grade of HE, precipitating factors of HE, MELD and CP scores and clinical and laboratory data at the time of HE. Variables which reached statistical significance ($P \leq 0.05$) in the univariate analysis were subsequently included in a multivariate analysis to identify variables independently associated with survival. We used the stepwise Cox regression procedure (variables entered if $P \leq 0.10$, variables removed if $P \geq 0.15$). Statistical analysis was carried out using *R* for windows (version 1.1.419) through the survival package as well as through MedCalc (version 19.0.7).

RESULTS

Total 365 patients with acute HE necessitating hospital admission were prescribed rifaximin during the study period. Total 177 (48%) patients were excluded from the study, leaving a total of 188 patients for analysis (Figure 1). Reasons for exclusion included: pre-existing use of rifaximin prior to admission in 134 (37%) patients, the presence of HCC in 41 (11%) patients and no identifiable start date for rifaximin in 2 (0.5%) patients.

Characteristics of patients

There were 133 males and 55 females with a median age of 57 years (IQR 50–65). All patients had established cirrhosis based on hospital records compiled from previous histological and radiology data. The most common aetiologies of cirrhosis were: Alcohol (70 patients), non-alcoholic fatty liver disease (24 patients) and hepatitis C (20 patients) (Table 1). Four patients had previously received a transjugular intrahepatic portosystemic shunt procedure. The median CP score was 11 (IQR 9-12) and 3, 39 and 120 patients had Child A, B and C cirrhosis respectively on admission; 26 patients had insufficient documentation to accurately calculate a CP score. The median MELD score was 25 (IQR 18-31) with 77% patients having a MELD score ≥ 15. Baseline patient characteristics and laboratory data are shown in Tables 1 and 2.

A likely precipitant of decompensated cirrhosis with acute HE was identified in 173 (92%) patients (Table 3); in many patients this was felt to be multi-factorial with more than one precipitant identified. Alone or in combination, the most commonly identified causes for HE were: Infection (including spontaneous bacterial peritonitis) in 81 (43%) patients, gastrointestinal bleeding in 31 (16%), constipation in 35 (19%) and non-compliance with prescribed medications in 29 (15%). In relation to the severity of HE, the West Haven HE grades were available in 162 (86%) patients (Table 1), with 22 (14%), 93 (57%), 38 (23%) and 9 (5%) patients recording a maximal HE grade of 1, 2, 3 and 4 respectively. Thirty-three (18%) patients required admission to an intensive care ward. In addition to rifaximin, 166 (88%) patients received either oral or rectal lactulose with a mean dose of 177 mL, 13 (7%) patients received macrogol (polyethylene glycol "3350") and 19 patients received other forms of aperients.

Documentation of resolution of encephalopathy was identified in 133 patients prior to discharge with a median duration to resolution of symptoms of 7 d (IQR 2–9 d). Of the remaining 55 patients, 20 died prior to resolution of HE and in the other 35 documentation was insufficient to determine whether HE has resolved at the time of discharge.

Mortality and prognostic factors

Within a mean follow-up period of 12 ± 13 mo, 107 (57%) patients died and 32 (17%) received liver transplantation. 42 patients died during or within 30-d of the index admission in which rifaximin was commenced. Causes of death included liver failure in 61 (57%) patients, sepsis in 19 (18%), unknown cause in 12 (11%), non-HCC malignancy in 4 (4%), cerebrovascular accidents in 4 (4%), gastrointestinal bleeding in 4 (4%), ischemic gut in 1 (1%) and cardiopulmonary arrest in 2 (1%) patients. The probably of survival in the entire cohort was 44% at 12-mo, 35% at 24-mo and 29% at 36-mo (Figure 2).

Twenty-seven variables were included in the univariate analysis, of which 10 were significantly associated with a poor prognosis: Hepatitis C infection, infection as the precipitant for HE, serum bilirubin, urea, creatinine, international normalised ratio (INR), white cell count, CP score, MELD and a MELD score ≥ 15 (*vs* ≤ 15). These variables were subsequently introduced into the multivariate analysis. The multivariate analysis (performed in the 159 patients in whom all variables were available) identified two variables as statistically significant, independent prognostic factors: A MELD score ≥ 15 and INR (Table 4).

DISCUSSION

Hepatic encephalopathy remains a common complication in patients with liver cirrhosis. Our study demonstrates that development of HE necessitating hospital admission in cirrhotic patients is associated with a short life expectancy in the absence of liver transplantation despite current standards-of-care. The cumulative survival at 12-, 24- and 36-mo were 44%, 35% and 29% respectively with the majority of patients dying from complications of decompensated liver disease or liver failure. At multivariate analysis the variables significantly associated with mortality were a MELD score ≥ 15 and INR.

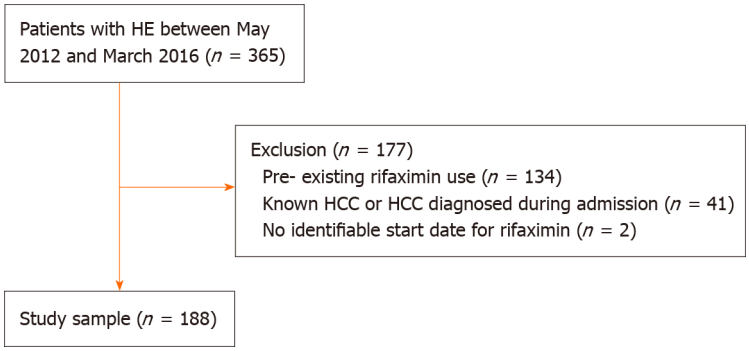

Figure 1 Recruitment flowchart. HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma.

Our study represents one of the largest real-world studies to investigate the prognostic significance of HE in the era of rifaximin. Study inclusion criteria were broad and simple, including all cirrhotic patients admitted with acute HE and commenced on rifaximin to three metropolitan tertiary centres in Australia, including one transplant centre, with a total catchment area of approximately two million people. The study population consisted of patients with decompensated cirrhosis managed on a specialist Gastroenterology ward who received treatment consistent with recent practice guidelines. Study results thus represent real-world data and should be widely applicable to other treating centres.

The results of this study correlate with sentinel studies from the pre-rifaximin era. Bustamante *et al*[19] demonstrated a similar 12-mo survival probability of 42% amongst patients experiencing their first presentation with HE where lactulose was the primary pharmacological management option. In addition, Stewart *et al*[3] demonstrated that higher grades of HE corresponded to increased mortality rates with an overall survival of less than 20% at 36-mo in patients presenting with Grade 3 HE[3,19].

Following the introduction of rifaximin various studies have sought to assess whether the survival probability has improved in cirrhotic patients following an episode of HE. Sharma *et al*[8] demonstrated in a randomised control trial that the 10-d survival following the commencement of rifaximin plus lactulose for the management of HE was superior to patients receiving lactulose alone. A larger retrospective cohort study by Kang *et al*[22], of 421 patients with HE of whom 145 received rifaximin found rifaximin use to be independently associated with a decreased risk of death. Despite a similar median CP score (10 *vs* 11), this study demonstrated a survival probability at 12-, 24-, 36- and 48-mo of 70%, 68%, 64% and 63% respectively[22], significantly higher than the cumulative survival found in our cohort. The likely reason for this discrepancy in survival is that in the Kang *et al*[22] study, patients were enrolled after discharge from the index HE admission and patients who died within 2-d of recovery were excluded. Consistent with the study by Bustamante *et al*[19], we elected to enrol patients during the index admission in which rifaximin was commenced and patients in our cohort had a 22% 30-d mortality. Furthermore, in Australia, prescribing guidelines necessitate that rifaximin be used only in recurrent or refractory episodes of HE and thus it is typically employed as a second-line agent after Lactulose. Consistent with this, 40% of our patient cohort had experienced an episode of HE prior to the index admission.

Within our cohort, multiple clinical and standard laboratory variables were significantly associated with a poor prognosis at univariate analysis. Five laboratory variables were independently associated with a poor prognosis: Increased serum bilirubin, urea, creatinine, INR and decreased white cell count. Of these variables, bilirubin, renal function and INR are commonly utilised in prognostic risk stratification algorithms and have clear relationships with poor prognosis in patients with liver cirrhosis[23,24]. In addition, Childs Pugh C class cirrhosis and a MELD score ≥ 15 were both associated with a poor prognosis which is in keeping with their known value in prognosticating survival in advanced liver disease[23,24]. The prognostic significance of leukopaenia in HE requires further investigation. Other studies have not found a low white cell count to be a significant prognostic factor[19], however Qamar *et al*[25] demonstrated that leukopenia in patients with compensated cirrhosis predicted and increased mortality. Following multivariate analysis, a MELD score ≥ 15 and INR were found to be independently associated with a poor prognosis. A MELD score ≥ 15 was selected as the cut-off given data that patients with a MELD > 15 have higher mortality and shortened survival compared to those who proceed to

Table 1 Baseline characteristics of study patients

Parameter	*n* (%)
Age (yr)	57 (IQR 50–65)
Male	133 (71)
Current smoker	70 (37)
Co morbidities	
IHD	16 (9)
DM	64 (34)
CCF	16 (9)
Previous CVA	15 (8)
COPD	14 (7)
Non-HCC malignancy	20 (11)
CKD	39 (21)
Ascites	138 (73)
Aetiology of cirrhosis	
Alcohol	117 (62)
HBV	6 (3)
HCV	59 (31)
NASH	37 (20)
PSC	6 (3)
AIH	4 (2)
PBC	1 (1)
Other	11 (6)
Child Pugh score	11 (IQR 9-12)
CPA	3 (2)
CPB	39 (21)
CPC	120 (64)
Unknown	26 (14)
MELD	25 (IQR 18-31)
Hepatic encephalopathy	
Grade 1	22 (5)
Grade 2	93 (23)
Grade 3	38 (57)
Grade 4	9 (14)
Unknown grade	26
Previous episode of encephalopathy	75
Median duration of encephalopathy (d)	7 (IQR 2-9)

Continuous variables are presented as median (interquartile range). IHD: Ischemic heart disease; DM: Diabetes mellitus; CCF: Congestive cardiac failure; CVA: Cerebrovascular event; COPD: Chronic obstructive pulmonary disease; HCC: Hepatocellular cancer; CKD: Chronic kidney disease; HCV: Hepatitis C; HBV: Hepatitis B; NASH: Non-alcoholic steatohepatitis; PSC: Primary sclerosing cholangitis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; CPA: Child's Pugh A; CPB: Childs Pugh B; CPC: Childs Pugh C; MELD: Model for end-stage liver disease.

liver transplantation assessment[26]. All measured components of the MELD were found to be prognostically significant within the univariate analysis but only an elevated INR was independently significant at multivariate analysis.

Interestingly, in our study the grade of HE did not reach statistical significance in predicting mortality in either univariate of multivariate analysis. This finding is discordant with some previous studies including the paper by Bustamante *et al*[19], in which higher grades of HE were found to be significant at univariate analysis but not on multivariate analysis. In addition, Bajaj *et al*[13] performed a large retrospective analysis of patients with alcohol-related cirrhosis and HE, finding that higher grades of HE were associated with a higher 30-d mortality. By comparison, Stewart *et al*[3] found on multivariate analysis that in hospitalised patients with HE, the presence of HE was a strong predictor of mortality however there was no difference detected between Grades 2 and 3 HE. One possible reason for our findings may be a type-2

Table 2 Baseline laboratory parameters

Parameters	mean ± SD
Haemoglobin (g/L)	90 ± 20
Platelet (× 10^9/L)	86.1 ± 64
White cell count (× 10^9/L)	8 ± 6
Bilirubin (μmol/L)	151 ± 168
Albumin (g/L)	25 ± 7
ALT (U/L)	135 ± 468
ALP (U/L)	166 ± 106
GGT (U/L)	154 ± 262
INR	2.1 ± 1.0
Urea (mmol/L)	11 ± 7
Creatinine (micromol/L)	161 ± 138
Sodium (mmol/L)	133 ± 7
Potassium (mmol/L)	4.5 ± 1.0

ALT: Alanine transaminase; ALP: Alkaline Phosphatase; GGT: Gamma glutamyl transferase; INR: international normalised ratio.

error with insufficient patient numbers to demonstrate a statistically significant difference between Grades of encephalopathy. In our cohort, 80% patients had a maximum encephalopathy grade of 2 or 3 with few patients diagnosed with Grades 1 or 4.

In our cohort, the vast majority of patients had an identifiable precipitant for the development of HE. Overwhelmingly, HE occurred in patients with advanced, decompensated cirrhosis and portal hypertension and was most commonly associated with other co-existing complications of decompensated cirrhosis such as ascites with spontaneous bacterial peritonitis and gastrointestinal bleeding. This is consistent with previous observations that HE occurs relatively late in the natural history of cirrhosis and previous studies have also demonstrated an association between MELD score and the development of HE[14]. Indeed, it has been postulated that for HE to occur, decreased hepatic function and portosystemic shunting are necessary to allow putative toxic molecules to reach the cerebral circulation[3].

Our study has certain limitations including its retrospective design, meaning all data collection was ascertained through existing clinical records which were generated by multiple health practitioners in a non-standardised fashion. Inherent with retrospective studies, not all data points were available in all patients which potentially affects the statistical analysis. Errors were minimised by using a small number of data collectors who entered information into a standardised database and each medical record was independently reviewed by two researchers. Secondly, our study population was recruited from tertiary centres and consisted entirely of patients with decompensated liver cirrhosis with portal hypertension. All patients required hospital admission for acute HE and 73% had concurrent ascites. The median Child Pugh score of 11 and MELD score of 25 reflects that our population had advanced liver disease and were unwell at the time of hospital admission. Patients with advanced liver disease have a poor prognosis irrespective of the development of encephalopathy. The 30-d mortality in this study was 22% which is higher than that recorded by patients with acute variceal bleeding in recent studies[27,28] and again reflects that acute HE is associated with a very guarded prognosis.

Finally, due to the retrospective nature of the study it was not possible to accurately assess nutritional therapy during the acute course of encephalopathy and this is obviously an important factor in any patient with decompensated cirrhosis.

In conclusion, the development of HE in patients with cirrhosis still confers an extremely poor prognosis with low probability of transplant-free survival despite current standards-of-care. In all cirrhotic patients, development of HE should prompt consideration of the appropriateness of urgent liver transplantation assessment. Further prospective studies are required to investigate whether there is a survival benefit of rifaximin in patients with advanced cirrhosis and encephalopathy.

Table 3 Precipitating factors for hepatic encephalopathy (alone or in combination with other factors)

Precipitating factors	*n* (%)
Infection including SBP	81 (43)
Gastrointestinal bleeding	31 (16)
Constipation (opiate-induced)	10 (5)
Constipation (not opiate induced)	25 (13)
Benzodiazepine use	10 (5)
Noncompliance to regular medications	29 (15)
Electrolyte imbalance	27 (14)
Other	24 (13)
Unknown	15 (8)

SBP: Spontaneous bacterial peritonitis.

Table 4 Hazard ratio for the different variables investigated by univariate analysis and multivariate analysis as possible prognostic factors in 188 cirrhotic patients presenting with hepatic encephalopathy and commenced on rifaximin

Variable	Univariate hazard ratio using cox regression (95%CI)[1]	Multivariate hazard ratio (95%CI)[1]
Age	1.014 (0.99, 1.03)	
Sex (male *vs* female)	1.087 (0.75, 1.58)	
Aetiology of cirrhosis[2]		
HBV infection	0.76 (0.28, 2.05)	
HCV infection	0.62 (0.42, 0.91)[a]	
Alcohol	0.92 (0.65, 1.30)	
Precipitating factors[2]		
Gastrointestinal bleed	0.75 (0.46, 1.22)	
Infection	1.49 (1.03, 2.15)[a]	
Diuretic therapy	1.47 (0.94, 2.32)	
Constipation	1.19 (0.73, 1.95)	
Ascites at the time of HE[2]	1.11 (0.77, 1.59)	
Maximal grade of HE (grade 3 and 4 *vs* grade 1 and 2)	0.80 (0.53, 1.21)	
Serum values[3]		
Bilirubin	1.001 (1, 1.002)[a]	
ALT	1 (0.99, 1.003)	
GGT	1 (1, 1)	
Albumin	0.97 (0.95, 1.002)	
Urea	1.05 (1.02, 1.09)[a]	
Creatinine	1.001 (1, 1.002)[a]	
Sodium	0.99 (0 .97, 1.03)	
Potassium	1.16 (0.96, 1.40)	
INR	1.5 (1.21, 1.85)[a]	1.27 (1.04, 1.54)[a]
Hb	0.98 (0.97, 1.01)	
WCC	1.06 (1.02, 1.10)[a]	
Plt	1.00 (0.99, 1.01)	
Child Pugh Score (C *vs* A/B)	1.57 (1.02, 2.41)[a]	
MELD	1.03 (1.01, 1.06)[a]	
MELD (≥ 15 *vs* ≤ 15)	2.41 (1.20. 4.85)[a]	2.17 (1.07, 4.43)[a]

[1]In brackets: 95% confidence interval.
[2]Presence *vs* absence.
[3]Hazard ratio per unit increase.
[a]$P \leq 0.05$. HE: Hepatic encephalopathy; HCV: Hepatitis C; HBV: Hepatitis B; ALT: Alanine transaminase; GGT: Gamma glutamyl transferase; INR: international normalised ratio; Hb: Haemoglobin; WCC: White cell count; Plt: Platelet count; MELD: Model for end-stage liver disease.

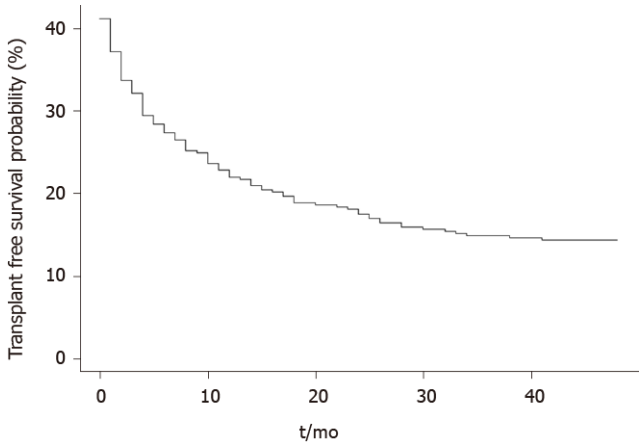

Figure 2 Transplant-free survival probability following commencement of rifaximin.

ARTICLE HIGHLIGHTS

Research background

Hepatic encephalopathy (HE) is a common neuropsychiatric complication in patients with liver cirrhosis and represents the second most common decompensating event after ascites. The current treatment approach for HE includes the reversal of identifiable underlying precipitants and the use of ammonia-lowering agents such as lactulose and rifaximin.

Research motivation

Previous sentinel studies have demonstrated that development of HE is associated with extremely poor transplantation-free survival. There remains a paucity of literature examining the natural history and prognosis of HE in the post-rifaxamin era.

Research objectives

We aimed to evaluate the clinical outcomes and survival probability of cirrhotic patients who developed acute HE requiring admission to hospital and were treated with rifaxamin in addition to current standards-of-care. In addition, we aimed to identify factors at the time of HE that could predict mortality and highlight the need to consider liver transplantation.

Research methods

We performed a retrospective, multi-centre analysis of 188 patients admitted with HE and commenced on rifaxmin with a mean follow-up period of 12 ± 13 mo. Survival probability curves were calculated using the Kaplan-Meier method. Univariate survival analysis was performed using the Cox proportional hazards model. Variables which reached statistical significance ($P \leq 0.05$) were subsequently included in a multivariate analysis to identify factors independently associated with survival using the stepwise Cox regression procedure.

Research results

In patients with acute HE requiring hospital admission and treated with current standards-of-care, the probability of survival remains poor with a 1- and 3-year survival probability of 44% and 29% respectively. The majority of patients have an identifiable precipitant for HE and the most common cause of death was liver failure or complications of decompensated cirrhosis. Baseline international normalised ratio and a model for end stage liver disease score ≥ 15 reached statistical significance on multivariate analysis to predict mortality.

Research conclusions

Despite advances in treatment, the development of acute HE in cirrhotic patients continues to confer an extremely poor prognosis and a low probability of survival in the absence of liver transplantation. Both international normalised ratio, a marker of synthetic liver dysfunction, and model for end stage liver disease score, which is well-validated to prognosticate survival in advanced liver disease, were able to independently predict survival probability at the time of admission.

Research perspectives

The development of HE in a cirrhotic patient is an extremely serious complication that typically occurs late in the disease process and confers an extremely poor prognosis. Inpatient management of HE with current standards-of-care can successfully resolve the episode of HE in the majority of cases but has limited ability to affect the natural sequalae of the advanced disease state. In all cirrhotic patients, the development of HE should prompt consideration of the appropriateness of liver transplantation. Further prospective studies would be useful to investigate the survival benefits of rifaxamin in patients with advanced cirrhosis and HE.

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ORIGINAL ARTICLE

Retrospective Study

Optimal proximal resection margin distance for gastrectomy in advanced gastric cancer

Amy Kim, Beom Su Kim, Jeong Hwan Yook, Byung Sik Kim

ORCID number: Amy Kim (0000-0002-7021-9214); Beom Su Kim (0000-0002-3656-2086); Jeong-Hwan Yook (0000-0002-7987-5808); Byung Sik Kim (0000-0001-9579-9211).

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Informed consent statement: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent from patients for this retrospective study was waived by the institutional review board.

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Amy Kim, Beom Su Kim, Jeong Hwan Yook, Byung Sik Kim, Division of Stomach Surgery, Department of Surgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Amy Kim, Department of Surgery, Korea University Medical Center Ansan Hospital, Ansan, Gyeonggi-do 15355, South Korea

Corresponding author: Beom Su Kim, MD, PhD, Surgeon, Associate professor, Division of Stomach Surgery, Department of Surgery, University of Ulsan College of Medicine, Asan Medical Center, 88, Olympic-Ro 43-Gil, Songpa-Gu, Seoul 05505, South Korea. bskim0251@naver.com

Abstract

BACKGROUND

The conventional guidelines to obtain a safe proximal resection margin (PRM) of 5-6 cm during advanced gastric cancer (AGC) surgery are still applied by many surgeons across the world. Several recent studies have raised questions regarding the need for such extensive resection, but without reaching consensus. This study was designed to prove that the PRM distance does not affect the prognosis of patients who undergo gastrectomy for AGC.

AIM

To investigate the influence of the PRM distance on the prognosis of patients who underwent gastrectomy for AGC.

METHODS

Electronic medical records of 1518 patients who underwent curative gastrectomy for AGC between June 2004 and December 2007 at Asan Medical Center, a tertiary care center in Korea, were reviewed retrospectively for the study. The demographics and clinicopathologic outcomes were compared between patients who underwent surgery with different PRM distances using one-way ANOVA and Fisher's exact test for continuous and categorical variables, respectively. The influence of PRM on recurrence-free survival and overall survival were analyzed using Kaplan-Meier survival analysis and Cox proportional hazard analysis.

RESULTS

The median PRM distance was 4.8 cm and 3.5 cm in the distal gastrectomy (DG) and total gastrectomy (TG) groups, respectively. Patient cohorts in the DG and TG groups were subdivided into different groups according to the PRM distance; ≤ 1.0 cm, 1.1-3.0 cm, 3.1-5.0 cm and > 5.0 cm. The DG and TG groups showed no

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statistical difference in recurrence rate (23.5% *vs* 30.6% *vs* 24.0% *vs* 24.7%, P = 0.765) or local recurrence rate (5.9% *vs* 6.5% *vs* 8.4% *vs* 6.2%, P = 0.727) according to the distance of PRM. In both groups, Kalpan-Meier analysis showed no statistical difference in recurrence-free survival (P = 0.467 in DG group; P = 0.155 in TG group) or overall survival (P = 0.503 in DG group; P = 0.155 in TG group) according to the PRM distance. Multivariate analysis using Cox proportional hazard model revealed that in both groups, there was no significant difference in recurrence-free survival according to the PRM distance.

CONCLUSION
The distance of PRM is not a prognostic factor for patients who undergo curative gastrectomy for AGC.

Key words: Stomach neoplasms; Gastrectomy; Margins of excision; Prognosis; Recurrence

Core tip: The conventional guidelines suggest the surgeons to obtain an extensive resection margin during surgery for gastric cancer. The objective of this study was to investigate the influence of the proximal resection margin (PRM) distance on the oncologic outcomes of advanced gastric cancer patients, thus to prove the safety of the PRM distance shorter than the conventional literatures suggest. The length of the PRM did not affect the prognosis of patients who underwent a curative gastrectomy for advanced gastric cancer.

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INTRODUCTION

Although the worldwide gastric cancer incidence has been declining over the past few decades, gastric cancer remains the third leading cause of cancer mortality[1-3] and surgery is still the mainstay curative treatment for gastric cancer patients[4]. While radical surgery with adequate resection of the stomach and lymph nodes is the prime focus of treatment, quality of life after surgery has been receiving increased attention due to improvements in the postoperative survival of gastric cancer patients. Several studies have revealed that subtotal gastrectomy leads to better nutrition and quality of life after surgery than total gastrectomy (TG)[5,6], and a recent report showed the relationship between the remnant volume of the stomach and nutritional status after surgery[7]. Thus, surgeons should consider these factors when determining the optimal extent of resection.

Bozzetti *et al*[8] reported that a proximal resection margin (PRM) of at least 6 cm should be obtained for tumors invading the serosa to ensure an infiltration-free margin. However, this was published back in 1982 and may not accurately reflect the current state of gastric cancer treatment where values such as function preservation, nutrition, and quality of life are emphasized. The 2014 Japanese gastric cancer treatment guidelines (Version 4) suggest that a gross margin of at least 3 cm should be obtained for T2 or deeper tumors with Bormann type 1 or 2, and 5 cm should be obtained for Bormann type 3 or 4 tumors[9]. The National Comprehensive Cancer Network recommends a PRM of > 4 cm for a safe microscopic margin[10]. These guidelines do not specify the clinical studies, making it difficult to assess the reliability of the suggested PRMs.

In 2014, it was reported that as long as negative margins were obtained by intraoperative frozen-section examination, PRM is not related to patient survival or local recurrence[11]. However, a 2017 study revealed that PRM is an independent prognostic factor for the overall survival (OS) of gastric cancer patients and a PRM of at least 2.1 cm should be obtained[12]. Several other studies have examined the relationship between the PRM and the prognosis of patients with gastric cancer, but

the results were inconsistent[13-18], particularly for patients with advanced gastric cancer (AGC).

This study is based on extensive retrospectively collected data and aims to investigate the relationship between PRM and the recurrence-free survival (RFS) or OS after surgery and thus determine the optimal PRM for patients with AGC.

MATERIALS AND METHODS

Patients

Between June 2004 and December 2007, 1518 patients in total underwent total or distal gastrectomy (DG) with curative intent for AGC at the Division of Stomach Surgery in Asan Medical Center. Patients with stage IV AGC or evident gross residual tumor were observed intraoperatively and those who underwent palliative gastrectomy were not included in the study. We excluded gastroesophageal junction cancer (Siewert I or II) patients, patients with a history of previous stomach surgery, patients who underwent neoadjuvant chemotherapy and patients whose pathologic report confirmed fewer than 15 lymph nodes retrieved. Cases in which grossly positive resection margins were observed, and those where the final biopsy reports confirmed a positive resection margin were excluded. We also excluded cases without data for PRM.

To evaluate patient characteristics, we collected data on the sex, age, preoperative body mass index (BMI), history of previous operations on the stomach, medical history of hypertension (HTN), diabetes mellitus (DM), American Society of Anesthesiologists (ASA) score, history of smoking, preoperative value of CEA, CA 19-9 and CA 72-4, tumor location, type of surgery (TG or DG), and type of reconstruction. Clinicopathologic outcomes included the Borrmann classification of the tumor, the number of synchronous tumors in the stomach, tumor size, depth of invasion, number of lymph nodes collected (CLN), number of positive lymph nodes (PLN), histology according to differentiation, status of lymphovascular invasion (LVi) and Perineural invasion (PNi), distance of the tumor from the PRM and distal resection margin (DRM), TNM stage based on the American Joint Committee on Cancer (AJCC) staging Manual 7th edition, recurrence status, and survival.

The extent of resection was determined according to the surgeon's preference, primarily based on the Japanese gastric cancer treatment guidelines. The tumor location was defined according to equally divided sections for the upper-third, middle-third, and lower-third of the stomach. For multiple cancers, the location was defined based on the most proximal tumor. The distances of the PRM and DRM were defined as the shortest distance from the most proximal or distal end to each resection line, measured on formalin-fixed surgical specimens by pathologists. Recurrence was classified as locoregional (anastomosis site, remnant stomach, gastric bed, regional lymph nodes, adjacent organ, or paraaortic lymph node), hematogenous (distant organs), peritoneal (peritoneal seeding or Krukenberg's tumor), distant lymph nodes (extra-abdominal lymph nodes), and mixed. The main patterns of recurrence were determined based on the site at the time of diagnosis.

This study was approved by the Institutional Review Board of Asan Medical Center and the University of Ulsan College of Medicine (No. 2019-1036).

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, United States). To analyze the demographics and clinicopathologic features depending on different PRM categories, one-way ANOVA and Fisher's exact test were used for continuous and categorical variables, respectively. Kaplan-Meier survival analysis and Cox proportional hazard analysis were performed to assess the impact of PRM on RFS and OS. Any P value < 0.05 was considered statistically significant. The study was reviewed by a biomedical statistician from Department of medical statistics, University of Ulsan College of Medicine.

RESULTS

Table 1 summarizes the patients' baseline demographics and clinicopathologic characteristics. There were 859 patients who underwent DG and 659 patients who underwent TG. The median age at the time of operation was 60 and 57 in the two groups, respectively. In the DG group, there were 626 patients (72.9%) with tumors located in the lower third of the stomach. In the TG group, 586 (88.9%) had cancer in the upper or middle third of the stomach. After DG, anastomosis was performed

using the Billroth I reconstruction method for 71.0% of patients, Billroth II for 15.9% and Roux-en-Y gastrojejunostomy (RYGJ) for 13.0%.

The median PRM distance was 4.8 cm and 3.5 cm in the DG and TG groups, respectively. There were 220 (25.6%) and 251 (38.1%) cases of recurrence during the follow-up period of 59 (0-127) and 58 (0-129) months in each group.

Patient cohorts in the DG and TG groups were subdivided into different groups according to the length of the PRM: ≤ 1.0 cm, 1.1-3.0 cm, 3.1-5.0 cm and > 5.0 cm. Tables 2 and 3 present the clinicopathologic factors in the different PRM subgroups. In both the DG and TG groups, there were no significant differences in age, sex, T stage, or N stage according to the PRM distance. Among patients who underwent DG, the tumor location ($P < 0.001$), reconstruction type ($P = 0.004$) and tumor size ($P = 0.004$) differed between the PRM subgroups. Additionally, there were more undifferentiated tumors ($P = 0.023$) and perineural invasion ($P = 0.010$) in the PRM ≤ 1 cm subgroup. In the TG group, there were statistical differences in the tumor location ($P < 0.001$), tumor size ($P < 0.001$), proportion of linitis plastica ($P < 0.001$), and perineural invasion ($P = 0.002$) between the PRM subgroups. There were no significant differences in the recurrence rate or local recurrence rate according to the PRM distance in either the DG or TG group.

Kaplan-Meier analysis was performed to assess the impact of PRM distance on RFS and OS. In the DG group, the mean RFS was 83.8, 90.9, 96.0, and 94.9 mo with a five-year RFS of 35.3%, 41.8%, 47.0%, and 41.0% in the PRM ≤ 1 cm, 1.1-3.0 cm, 3.1-5.0 cm, and > 5 cm subgroups, respectively. In the TG group, the mean RFS was 73.8, 78.5, 88.3, and 83.7 mo with a five-year RFS of 42.2%, 33.0%, 45.9%, and 39.3%, respectively. Neither the DG nor TG group showed statistical differences in either RFS or OS according to the PRM distances (Figures 1 and 2).

Univariate and multivariate analyses were performed to investigate the impact of the PRM distance and other factors on OS (Tables 4 and 5) and RFS (Tables 6 and 7) using the Cox proportional hazard model. Variable selection for multivariate analysis was done using the backward elimination method with a likelihood ratio test. This revealed that among patients who underwent DG, a higher T stage (T3; $P = 0.003$, T4; $P < 0.001$) and N stage (N2, N3; $P < 0.001$) were associated with worse RFS. Other risk factors included older age ($P = 0.012$) and reconstruction type; Billroth II ($P = 0.016$) and RYGJ ($P = 0.003$) reconstructions resulted in worse RFS than Billroth I reconstruction (Table 6). In the TG group, higher T stage (T4; $P = 0.014$) and N stage (N2; $P = 0.001$, N3; $P < 0.001$) were risk factors associated with RFS. Older age ($P = 0.032$), linitis plastica ($P < 0.001$) and the presence of lymphovascular invasion ($P = 0.013$) were also associated with worse RFS (Table 7). However, neither group showed a significant difference in either RFS or OS according to the distance of the PRM.

DISCUSSION

It is widely accepted that sufficient resection margins should be achieved for curative resection of gastric cancer. The optimal length for the proximal margin is often suggested to be at least 4-6 cm[8-10]. Over the years, surgical skills and technologies have developed and fields of minimal, less invasive approaches are quickly growing. Guidelines suggest laparoscopic gastrectomy should be performed for early gastric cancer (EGC) in the distal third of the stomach[9] and laparoscopic TG was recently demonstrated to be safe and feasible for EGC. Moreover, there are ongoing trials and studies for laparoscopic approaches in advanced cancer, particularly in eastern countries. However, surgeons still abide by conventional rules and try to achieve the recommended margin length, even in difficult conditions.

Several studies are rooted in this discrepancy in the appropriate PRM distance. In 2006, Ha *et al*[19] reported that PRM had no significant influence on the prognosis of EGC patients; however, a PRM length of > 3 cm improved the survival rates in AGC patients. Squires *et al*[15] reported their findings from a 2015 study based on 465 patients who underwent curative-intent gastrectomy for distal gastric cancer. Their results indicated that a proximal margin distance > 3 cm is associated with better OS and RFS in stage I disease, whereas the proximal margin distance did not significantly improve prognosis in either stage II or III disease. The authors concluded that a proximal margin of > 3 cm is optimal for distal gastric cancer. Wang *et al*[12] reported that a proximal margin of 2.1-4.0 cm and 4.1-6.0 cm should be obtained for patients with solitary- and infiltrative-type tumors, respectively, for better prognoses. In 2017, based on 974 patients with gastric and esophago-gastric junction cancer, Bissolati *et al*[17] reported that a resection margin, either proximal or distal, that is < 2 cm for T1 cancer and < 3 cm for T2-4 cancer is associated with resection margin involvement, which was demonstrated in previous literature to have a negative prognostic

Table 1 The basic demographics and clinicopathologic characteristics of patients who underwent distal and total gastrectomy with curative intent for gastric adenocarcinoma, *n* (%)

Variables	Distal gastrectomy (*n* = 859)	Total gastrectomy (*n* = 659)
Age (yr; median) at operation	60 (23-87)	57 (22-86)
Sex		
Male	603 (70.2)	441 (66.9)
Female	256 (29.8)	218 (33.1)
BMI (kg/m^2, median)	23.2 (16.0-36.2)	23.4 (13.4-36.0)
ASA		
1	246 (28.6)	213 (32.3)
2	571 (66.5)	427 (64.8)
3	39 (4.5)	17 (2.6)
4	3 (0.3)	2 (0.3)
Tumor location		
Upper 1/3	8 (0.9)	266 (40.4)
Middle 1/3	225 (26.2)	320 (48.6)
Lower 1/3	626 (72.9)	73 (11.1)
Reconstruction		
Billroth I	610 (71.0)	
Billroth II	137 (15.9)	
RYGJ	112 (13.0)	
RY		659 (100.0)
Bormann classification		
I	14 (1.6)	14 (2.1)
II	162 (18.9)	66 (10.0)
III	660 (76.8)	499 (75.7)
IV	23 (2.7)	80 (12.1)
Tumor size (cm, median)	5.0 (0.8-18)	6.0 (0.7-24)
CLN	27 (15-75)	30 (15-106)
PLN	2 (0-49)	3 (0-101)
T stage		
T2	288 (33.5)	110 (16.7)
T3	370 (43.1)	310 (47.0)
T4a	195 (22.7)	226 (34.3)
T4b	6 (0.7)	13 (2.0)
N stage		
N0	308 (35.9)	203 (30.8)
N1	181 (21.1)	110 (16.7)
N2	173 (20.1)	128 (19.4)
N3a	159 (18.5)	133 (20.2)
N3b	38 (4.4)	85 (12.9)
AJCC stage		
Stage I	155 (18.0)	66 (10.0)
Stage II	336 (39.1)	235 (35.7)
Stage III	368 (42.8)	358 (54.3)
PRM (cm; median)	4.8 (0.3-17)	3.5 (0.1-18.5)
DRM (cm; median)	3.2 (0.2-19)	9.4 (0.3-27)
Histology		
Differentiated	351 (40.9)	192 (29.1)
Undifferentiated	508 (59.1)	467 (70.9)
Lymphovascular invasion	413 (48.1)	360 (54.6)
Perineural invasion	368 (42.8)	344 (52.2)
Recurrence	220 (25.6)	251 (38.1)
Locoregional recurrence	60	41
Hematogenous metastasis	74	83

Extra-abdominal LN metastasis	2	1
Peritoneal metastasis	74	1
Mixed	10	17

RYGJ: Roux-en-Y gastrojejunostomy; RY: Roux-en-Y esophagojejunostomy; CLN: Total number of collected lymph nodes; PLN: Total number of positive lymph nodes; PRM: Proximal resection margin; DRM: Distal resection margin; AJCC: American Joint Committee on Cancer.

impact[20-24]. However, Kim *et al*[13] reported in 2014 that the length of the proximal margin did not affect the OS or local recurrence and several subsequent studies have arrived at similar conclusions[11,14,18].

The conclusions regarding the safe length of PRM, particularly for AGC patients, are not consistent even among recent papers. Thus, we designed a large-scale study to determine the optimal length of the PRM for patients with AGC. Cross-tabulation analysis with our data showed that the incidence of recurrence or local recurrence according to the distance of the PRM did not differ ($P > 0.05$) in patients who underwent DG or TG for AGC. We performed Kaplan-Meier survival analysis to assess the effect of the PRM distance on RFS and our results showed no statistical difference in RFS between the PRM subgroups. Multivariate analysis using the Cox proportional hazard model revealed consistent results. Although previous reports do not agree on the safety of short resection margins, particularly in AGC, our results demonstrate that the distance of the PRM did not affect the prognosis of AGC patients who underwent curative gastrectomy.

Our multivariate analysis of influential factors in RFS and OS for patients who underwent DG showed significant differences between different reconstruction methods; this is inconsistent with previous literature. Billroth I was the most preferred reconstruction method after gastrectomy for gastric cancer patients at our institution. When a tumor involved pylorus or the stomach stump was too short for gastroduodenostomy, Billroth II or RYGJ was applied. Therefore, there is a chance that cases with B-II and RYGJ anastomosis were associated with larger and more progressed tumors. Another possible reason is that because Billroth I is the most preferred method in our institution, surgeons were more comfortable with the procedure, resulting in better outcomes. Although there is no consensus, a number of studies reveal more gastric stump cancer in patients who underwent Billroth II reconstruction rather than Billroth I after gastrectomy either due to carcinoma or benign lesions[25-27]. There is also an RCT from Japan that shows more hematogenous recurrence in B-II compared to B-I[28]. This is an important result that warrants further investigation with a careful design, taking many factors such as recurrence patterns, recurred time after surgery, histology of the initial tumor, and many other factors into consideration.

There is a limitation in the retrospective design of this study. Another limitation is that the length of the resection margin used in the study may not accurately portray the gross distance we observe intraoperatively. We used the PRM as described on the pathologic report, which was measured under formalin fixation. We chose to use the pathologic report because measurements from the operation room are expected to be less consistent depending on the measured time after resection or in cases with indistinctive tumor margins such as linitis plastica. Additionally, for TG, we used circular staplers that produce doughnut specimens that are not added to the length of PRM, so the actual PRM may be few millimeters longer than measured.

In conclusion, the distance of PRM is not a prognostic factor for AGC patients; it does not affect the incidence of recurrence or local recurrence. A greater PRM distance was not associated with better survival outcomes and a distance of < 1 cm did not correlate with worse OS or RFS.

Table 2 Clinicopathologic factors depending on the distance from the proximal resection margin in patients who underwent curative distal gastrectomy, *n* (%)

Variables	PRM (cm)				*P* value
	≤ 1.0 (*n* = 17)	1.1-3.0 (*n* = 170)	3.1-5.0 (*n* = 287)	> 5.0 (*n* = 385)	
Age (yr)[1] at operation	59.7 ± 3.4	57.2 ± 1.0	58.2 ± 0.7	59.0 ± 0.6	0.416
Sex					0.279
Male	9 (52.9)	116 (68.2)	199 (69.3)	279 (72.5)	
Female	8 (47.1)	54 (31.8)	88 (30.7)	106 (27.5)	
Tumor location					< 0.001
Upper 1/3	1 (5.9)	3 (1.8)	3 (1.0)	1 (0.3)	
Middle 1/3	9 (52.9)	74 (43.5)	86 (30.0)	56 (14.5)	
Lower 1/3	7 (41.2)	93 (54.7)	198 (69.0)	328 (85.2)	
Reconstruction					0.004
Billroth I	12 (70.6)	101 (59.4)	218 (76.0)	279 (72.5)	
Billroth II	3 (17.6)	32 (18.8)	38 (13.2)	64 (16.6)	
RYGJ	2 (11.8)	37 (21.8)	31 (10.8)	42 (10.9)	
Borrmann type IV	1 (5.9)	6 (3.5)	7 (2.4)	9 (2.3)	0.461
Tumor size (cm)[1]	6.5 ± 0.8	5.8 ± 0.2	5.3 ± 0.1	5.2 ± 0.1	0.004
T stage					0.768
T2	5 (29.4)	56 (32.9)	89 (31.0)	138 (35.8)	
T3	7 (41.2)	75 (44.1)	123 (42.9)	165 (42.9)	
T4	5 (29.4)	39 (22.9)	75 (26.1)	82 (21.3)	
CLN[1]	26.6 ± 2.0	29.3 ± 0.8	29.4 ± 0.6	28.8 ± 0.5	0.612
N stage					0.971
N0	5 (29.4)	61 (35.9)	101 (35.2)	141 (36.6)	
N1	2 (11.8)	36 (21.2)	63 (22.0)	80 (20.8)	
N2	4 (23.5)	36 (21.2)	55 (19.2)	78 (20.3)	
N3	6 (35.3)	37 (21.8)	68 (23.7)	86 (22.3)	
AJCC stage					0.551
Stage I	4 (23.5)	31 (18.2)	52 (18.1)	68 (17.7)	
Stage II	3 (17.6)	65 (38.2)	108 (37.6)	160 (41.6)	
Stage III	10 (58.8)	74 (43.5)	127 (44.3)	157 (40.8)	
Differentiation					0.023
Differentiated	4 (23.5)	64 (37.6)	105 (36.6)	178 (46.2)	
Undifferentiated	13 (76.5)	106 (62.4)	182 (63.4)	207 (53.8)	
LVi	8 (47.1)	75 (44.1)	142 (49.5)	188 (48.8)	0.706
PNi	9 (52.9)	80 (47.1)	138 (48.1)	141 (36.6)	0.010
Recurrence	4 (23.5)	52 (30.6)	69 (24.0)	95 (24.7)	0.765
Local recurrence	1 (5.9)	11 (6.5)	24 (8.4)	24 (6.2)	0.727

[1]mean ± standard error. PRM: Proximal resection margin; RYGJ: Roux-en-Y gastrojejunostomy; CLN: Total number of collected lymph nodes; AJCC: American Joint Committee on Cancer; LVi: Lymphovascular invasion; PNi: Perineural invasion.

Table 3 Clinicopathologic factors depending on the distance from the proximal resection margin in patients who underwent curative total gastrectomy, *n* (%)

Variables	PRM (cm)				*P* value
	≤ 1.0 (*n* = 90)	1.1-3.0 (*n* = 209)	3.1-5.0 (*n* = 146)	> 5.0 (*n* = 214)	
Age (yr)[1] at operation	57.1 ± 1.2	55.0 ± 0.9	54.6 ± 0.9	56.3 ± 0.9	0.330
Sex					0.364
Male	64 (71.1)	135 (64.6)	92 (63.0)	150 (70.1)	
Female	26 (38.9)	74 (35.4)	54 (37.0)	64 (29.9)	
Tumor location					< 0.001
Upper 1/3	81 (90.0)	127 (60.8)	32 (21.9)	26 (12.1)	
Middle 1/3	8 (8.9)	75 (35.9)	103 (70.5)	134 (62.6)	
Lower 1/3	1 (1.1)	7 (3.3)	11 (7.5)	54 (25.2)	
Borrmann type IV	17 (18.9)	37 (17.7)	14 (9.6)	12 (5.6)	< 0.001
Tumor size (cm)[1]	8.1 ± 0.5	7.6 ± 0.3	7.0 ± 0.3	5.9 ± 0.2	< 0.001
T stage					0.873
T2	14 (15.6)	30 (14.4)	24 (16.4)	42 (19.6)	
T3	44 (48.9)	100 (47.8)	67 (45.9)	99 (46.3)	
T4	32 (35.6)	79 (37.8)	55 (37.7)	73 (34.1)	
CLN[1]	30.7 ± 1.1	33.1 ± 1.0	32.2 ± 1.0	31.0 ± 0.7	0.216
N stage					0.495
N0	23 (25.6)	74 (35.4)	47 (32.2)	59 (27.6)	
N1	14 (15.6)	35 (16.7)	21 (14.4)	40 (18.7)	
N2	15 (16.7)	41 (19.6)	30 (20.5)	42 (19.6)	
N3	38 (42.2)	59 (28.2)	48 (32.9)	73 (34.1)	
AJCC stage					0.587
Stage I	8 (8.9)	19 (9.1)	14 (9.6)	25 (11.7)	
Stage II	29 (32.2)	85 (40.7)	53 (35.3)	68 (31.8)	
Stage III	53 (58.9)	105 (50.2)	79 (54.1)	121 (56.5)	
Differentiation					0.082
Differentiated	29 (32.2)	55 (26.3)	34 (23.3)	74 (34.6)	
Undifferentiated	61 (67.8)	154 (73.7)	112 (76.7)	140 (65.4)	
LVi	57 (63.3)	108 (51.7)	75 (51.4)	120 (56.1)	0.231
PNi	54 (60.0)	101 (48.3)	92 (63.0)	97 (45.3)	0.002
Recurrence	44 (48.9)	80 (38.3)	48 (32.9)	79 (36.9)	0.648
Local recurrence	8 (8.9)	10 (4.6)	11 (6.9)	14 (6.2)	0.637

[1]mean ± standard error. PRM: Proximal resection margin; RYGJ: Roux-en-Y gastrojejunostomy; CLN: Total number of collected lymph nodes; AJCC: American Joint Committee on Cancer; LVi: Lymphovascular invasion; PNi: Perineural invasion.

Table 4 Analysis of the risk factors associated with overall survival in patients who underwent distal gastrectomy using the Cox proportional hazard model

	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.04 (1.03-1.05)	< 0.001	1.04 (1.03-1.05)	< 0.001
Female sex	0.89 (0.69-1.15)	0.367		
BMI	0.94 (0.90-0.98)	0.002		
Tumor location				
Upper 1/3	Ref.			
Mid 1/3	3.11 (0.43-22.4)	0.260		
Lower 1/3	3.54 (0.50-25.2)	0.208		
Reconstruction				
Billroth I	Ref.		Ref.	
Billroth II	1.66 (1.26-2.20)	< 0.001	1.40 (1.04-1.87)	0.025
RYGJ	1.34 (0.96-1.86)	0.083	1.45 (1.04-2.03)	0.030
Tumor size	1.11 (1.06-1.16)	< 0.001		
Borrmann type IV	0.96 (0.48-1.94)	0.914		
CLN	0.99 (0.98-1.00)	0.045	0.99 (0.98-1.00)	0.034
T stage				
T2	Ref.		Ref.	
T3	1.36 (1.01-1.83)	0.044	1.08 (0.79-1.48)	0.612
T4	3.02 (2.24-4.06)	< 0.001	1.90 (1.38-2.62)	< 0.001
N stage				
N0	Ref.		Ref.	
N1	1.07 (0.73-1.56)	0.739	0.92 (0.63-1.36)	0.686
N2	2.33 (1.67-3.25)	< 0.001	2.06 (1.46-2.90)	< 0.001
N3	3.74 (2.77-5.05)	< 0.001	3.10 (2.25-4.28)	< 0.001
Diffuse type histology	1.00 (0.79-1.25)	0.967		
LVi	1.82 (1.44-2.29)	< 0.001		
PNi	1.32 (1.06-1.66)	0.015		
PRM (cm)				
0-1.0	Ref.			
1.1-3.0	0.57 (0.27-1.19)	0.134		
3.1-5.0	0.59 (0.29-1.23)	0.162		
> 5.0	0.61 (0.30-1.25)	0.175		

PRM: Proximal resection margin; RYGJ: Roux-en-Y gastrojejunostomy; CLN: Total number of collected lymph nodes; LVi: Lymphovascular invasion; PNi: Perineural invasion.

Table 5 Analysis of the risk factors associated with overall survival in patients who underwent total gastrectomy using the Cox proportional hazard model

	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.02 (1.01-1.03)	< 0.001	1.03 (1.02-1.04)	< 0.001
Female sex	1.03 (0.81-1.30)	0.834		
BMI	0.95 (0.92-0.99)	0.009		
Tumor location				
Upper 1/3	Ref.			
Mid 1/3	0.76 (0.60-0.95)	0.018		
Lower 1/3	0.94 (0.65-1.35)	0.723		
Tumor size	1.09 (1.07-1.12)	< 0.001		
Borrmann type IV	2.21 (1.66-2.94)	< 0.001	1.93 (1.43-2.60)	< 0.001
CLN	1.00 (0.99-1.01)	0.548	0.99 (0.98-1.00)	0.035
T stage				
T2	Ref.		Ref.	
T3	1.67 (1.14-2.45)	0.008	1.21 (0.81-1.81)	0.352
T4	3.24 (2.22-4.72)	< 0.001	1.85 (1.22-2.79)	0.004
N stage				
N0	Ref.		Ref.	
N1	1.11 (0.74-1.68)	0.617	1.03 (0.67-1.57)	0.900
N2	1.73 (1.21-2.46)	0.003	1.48 (1.01-2.18)	0.045
N3	3.87 (2.88-5.19)	< 0.001	2.81 (1.98-3.98)	< 0.001
Diffuse type histology	1.23 (0.96-1.58)	0.103		
LVi	2.09 (1.65-2.64)	< 0.001	1.43 (1.10-1.86)	0.008
PNi	1.60 (1.27-2.00)	< 0.001		
PRM (cm)				
0-1.0	Ref.			
1.1-3.0	0.80 (0.57-1.10)	0.164		
3.1-5.0	0.65 (0.45-0.93)	0.019		
> 5.0	0.81 (0.58-1.12)	0.202		

PRM: Proximal resection margin; RYGJ: Roux-en-Y gastrojejunostomy; CLN: Total number of collected lymph nodes; LVi: Lymphovascular invasion; PNi: Perineural invasion.

Table 6 Analysis of the risk factors associated with recurrence-free survival in patients who underwent distal gastrectomy using the Cox proportional hazard model

	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.02 (1.00-1.03)	0.011	1.02 (1.00-1.03)	0.012
Female sex	0.93 (0.69-1.25)	0.636		
BMI	0.96 (0.92-1.00)	0.068		
Tumor location				
Upper 1/3	Ref.			
Mid 1/3	2.32 (0.32-16.77)	0.403		
Lower 1/3	2.35 (0.33-16.76)	0.395		
Reconstruction				
Billroth I	Ref.		Ref.	
Billroth II	1.90 (1.37-2.64)	< 0.001	1.50 (1.08-2.10)	0.016
RYGJ	1.87 (1.31-2.67)	0.001	1.72 (1.20-2.47)	0.003
Tumor size	1.16 (1.10-1.21)	< 0.001		
Borrmann type IV	1.04 (0.46-2.33)	0.931		
CLN	1.00 (0.99-1.01)	0.91		
T stage				
T2	Ref.		Ref.	
T3	2.61 (1.72-3.96)	< 0.001	1.92 (1.25-2.95)	0.003
T4	6.17 (4.08-9.34)	< 0.001	3.42 (2.21-5.31)	< 0.001
N stage				
N0	Ref.		Ref.	
N1	1.23 (0.75-2.03)	0.415	1.03 (0.62-1.70)	0.92
N2	3.42 (2.26-5.19)	< 0.001	2.55 (1.67-3.89)	< 0.001
N3	5.75 (3.92-8.42)	< 0.001	3.88 (2.59-5.80)	< 0.001
Diffuse type histology	1.19 (0.91-1.57)	0.206	1.05 (0.79-1.39)	0.758
LVi	2.29 (1.73-3.02)	< 0.001		
PNi	1.63 (1.25-2.12)	< 0.001		
PRM (cm)				
0-1.0	Ref.			
1.1-3.0	1.03 (0.37-2.86)	0.949		
3.1-5.0	0.78 (0.29-2.15)	0.633		
> 5.0	0.84 (0.31-2.29)	0.734		

PRM: Proximal resection margin; RYGJ: Roux-en-Y gastrojejunostomy; CLN: Total number of collected lymph nodes; LVi: Lymphovascular invasion; PNi: Perineural invasion.

Table 7 Analysis of the risk factors associated with recurrence-free survival in patients who underwent total gastrectomy using the Cox proportional hazard model

	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.01 (1.00-1.02)	0.071	1.01 (1.00-1.02)	0.032
Female sex	1.23 (0.95-1.56)	0.118		
BMI	0.96 (0.92-1.00)	0.067		
Tumor location				
Upper 1/3	Ref.			
Mid 1/3	0.67 (0.51-0.87)	0.002		
Lower 1/3	0.86 (0.57-1.30)	0.859		
Tumor size	1.11 (1.08-1.14)	< 0.001	1.03 (1.00-1.07)	0.075
Borrmann type IV	2.84 (2.10-3.83)	< 0.001	1.91 (1.32-2.76)	0.001
CLN	1.00 (0.99-1.01)	0.612		
T stage				
T2	Ref.		Ref.	
T3	2.20 (1.36-3.54)	0.001	1.31 (0.90-2.16)	0.289
T4	4.18 (2.60-6.72)	< 0.001	1.90 (1.14-3.18)	0.014
N stage				
N0	Ref.		Ref.	
N1	1.43 (0.89-2.31)	0.139	1.27 (0.77-2.07)	0.348
N2	2.35 (1.55-3.56)	< 0.001	1.68 (1.06-2.65)	0.026
N3	4.90 (3.43-6.99)	< 0.001	2.84 (1.87-4.32)	< 0.001
Diffuse type histology	1.14 (0.86-1.50)	0.357		
LVi	2.41 (1.84-3.15)	< 0.001	1.44 (1.08-1.91)	0.013
PNi	1.71 (1.33-2.21)	< 0.001		
PRM (cm)				
0-1.0	Ref.			
1.1-3.0	0.80 (0.55-1.15)	0.225		
3.1-5.0	0.63 (0.42-0.95)	0.028		
> 5.0	0.74 (0.51-1.07)	0.104		

PRM: Proximal resection margin; RYGJ: Roux-en-Y gastrojejunostomy; CLN: total number of collected lymph nodes; LVi: Lymphovascular invasion; PNi: Perineural invasion.

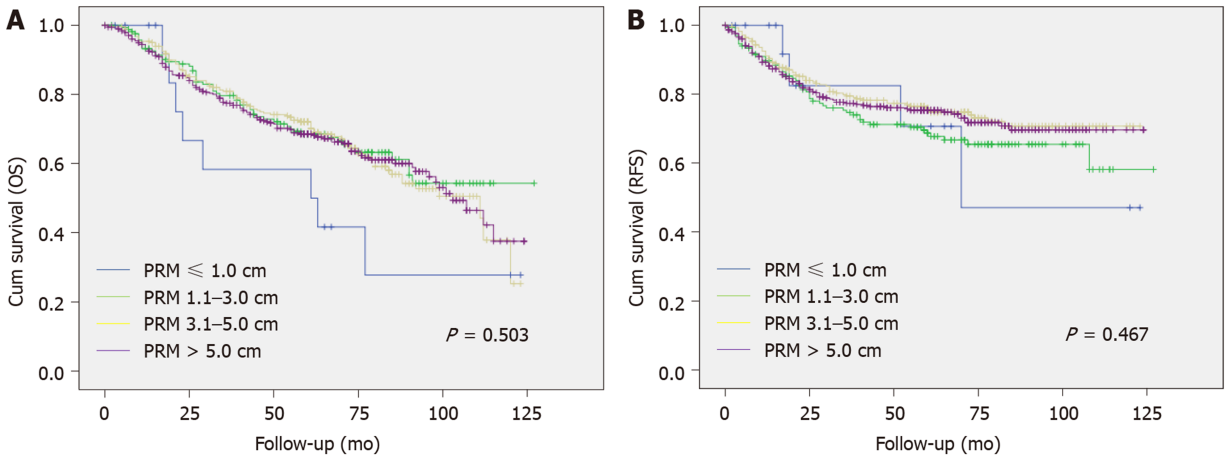

Figure 1 Correlation of overall survival (A) and recurrence-free survival (B) with the distance of proximal resection margin in patients who underwent distal gastrectomy. Kaplan-Meier method was used to analyze OS and RFS according to the distance of PRM. There were no significant differences between the PRM subgroups. OS: Overall survival; RFS: Recurrence-free survival; PRM: Proximal resection margin.

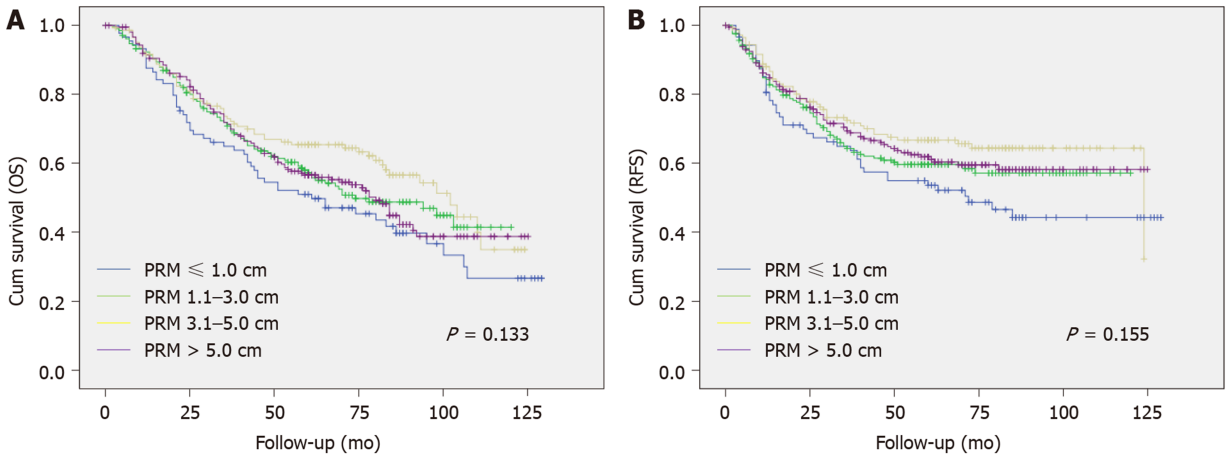

Figure 2 Correlation of overall survival (A) and recurrence-free survival (B) with the distance of proximal resection margin in patients who underwent total gastrectomy. The Kaplan-Meier method was used to analyze OS and RFS according to the distance of PRM. There were no significant differences between the PRM subgroups. OS: Overall survival; RFS: Recurrence-free survival; PRM: Proximal resection margin.

ARTICLE HIGHLIGHTS

Research background

The conventional guidelines suggest the surgeons to obtain an extensive resection margin during surgery for gastric cancer. Several recent studies have raised questions regarding the need for such extensive resection and necessity of total gastrectomy for tumors located on middle-third of stomach, while the consensus has not been reached. There are some studies those demonstrate the unnecessity of longer proximal resection margin (PRM) distance in early gastric cancer. However, there are very few regarding the PRM distance for advanced gastric cancer (AGC).

Research motivation

We would like to discover the optimal PRM distance for patients who undergo gastrectomy for AGC.

Research objectives

The objective of this study was to investigate the influence of the PRM distance on the oncologic outcomes of patients who underwent gastrectomy for AGC, thus to prove the safety of the PRM distance shorter than the conventional literatures suggest.

Research methods

We retrospectively collected data from 1518 patients who underwent total gastrectomy (TG) or distal gastrectomy (DG) for AGC between June 2004 and December 2007. The distances of the PRM and DRM were defined as the shortest distance from the most proximal or distal end to each resection line, measured on formalin-fixed surgical specimens by pathologists. The demographics and clinicopathologic outcomes were compared according to the different PRM categories and an analysis on the influence of PRM on recurrence-free survival and overall survival was performed.

Research results

The DG and TG groups showed no statistical difference in RFS or OS according to the distance of PRM. Multivariate analysis also revealed that in both groups, there was no significant difference in RFS or OS according to the PRM distance.

Research conclusions

The distance of PRM did not affect the incidence of recurrence or local recurrence. A greater PRM distance was not associated with better survival outcomes and a distance as short as < 1 cm did not correlate with worse OS or RFS. Therefore, the PRM distance shorter than conventional literatures suggest may be accepted.

Research perspectives

Further research would be essential to set a guideline for the optimal PRM distance for AGC. A long-term prospective study with detailed data on PRM including measurements done during operation by the surgeons and after fixation by the pathologists should give better answers.

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META-ANALYSIS

Computed tomography *vs* liver stiffness measurement and magnetic resonance imaging in evaluating esophageal varices in cirrhotic patients: A systematic review and meta-analysis

Yue Li, Lei Li, Hong-Lei Weng, Roman Liebe, Hui-Guo Ding

ORCID number: Yue Li (0000-0001-8459-0420); Lei Li (0000-0002-0295-9480); Hong-Lei Weng (0000-0003-1414-303X); Roman Liebe (0000-0002-3199-5595); Hui-Guo Ding (0000-0002-8716-4926).

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Yue Li, Lei Li, Hui-Guo Ding, Department of Gastroenterology and Hepatology, Beijing You'an Hospital Affiliated with Capital Medical University, Beijing 100069, China

Hong-Lei Weng, Department of Medicine II, Section Molecular Hepatology, Medical Faculty Mannheim, Heidelberg University, Mannheim 68167, Germany

Roman Liebe, Department of Medicine II, Saarland University Medical Center, Homburg 66424, Germany

Corresponding author: Hui-Guo Ding, MD, PhD, Chief Doctor, Chief Physician, Professor, Department of Gastroenterology and Hepatology, Beijing You'an Hospital affiliated with Capital Medical University, 8 Xitoutiao, Youanmenwai, Fengtai District, Beijing 100069, China. dinghuiguo@ccmu.edu.cn

Abstract

BACKGROUND

Computed tomography (CT), liver stiffness measurement (LSM), and magnetic resonance imaging (MRI) are non-invasive diagnostic methods for esophageal varices (EV) and for the prediction of high-bleeding-risk EV (HREV) in cirrhotic patients. However, the clinical use of these methods is controversial.

AIM

To evaluate the accuracy of LSM, CT, and MRI in diagnosing EV and predicting HREV in cirrhotic patients.

METHODS

We performed literature searches in multiple databases, including PubMed, Embase, Cochrane, CNKI, and Wanfang databases, for articles that evaluated the accuracy of LSM, CT, and MRI as candidates for the diagnosis of EV and prediction of HREV in cirrhotic patients. Summary sensitivity and specificity, positive likelihood ratio and negative likelihood ratio, diagnostic odds ratio, and the areas under the summary receiver operating characteristic curves were analyzed. The quality of the articles was assessed using the quality assessment of diagnostic accuracy studies-2 tool. Heterogeneity was examined by Q-statistic test and I^2 index, and sources of heterogeneity were explored using meta-regression and subgroup analysis. Publication bias was evaluated using Deek's funnel plot. All statistical analyses were conducted using Stata12.0, MetaDisc1.4, and RevMan5.3.

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RESULTS

Overall, 18, 17, and 7 relevant articles on the accuracy of LSM, CT, and MRI in evaluating EV and HREV were retrieved. A significant heterogeneity was observed in all analyses ($P < 0.05$). The areas under the summary receiver operating characteristic curves of LSM, CT, and MRI in diagnosing EV and predicting HREV were 0.86 (95% confidence interval [CI]: 0.83-0.89), 0.91 (95%CI: 0.88-0.93), and 0.86 (95%CI: 0.83-0.89), and 0.85 (95%CI: 0.81-0.88), 0.94 (95%CI: 0.91-0.96), and 0.83 (95%CI: 0.79-0.86), respectively, with sensitivities of 0.84 (95%CI: 0.78-0.89), 0.91 (95%CI: 0.87-0.94), and 0.81 (95%CI: 0.76-0.86), and 0.81 (95%CI: 0.75-0.86), 0.88 (95%CI: 0.82-0.92), and 0.80 (95%CI: 0.72-0.86), and specificities of 0.71 (95%CI: 0.60-0.80), 0.75 (95%CI: 0.68-0.82), and 0.82 (95%CI: 0.70-0.89), and 0.73 (95%CI: 0.66-0.80), 0.87 (95%CI: 0.81-0.92), and 0.72 (95%CI: 0.62-0.80), respectively. The corresponding positive likelihood ratios were 2.91, 3.67, and 4.44, and 3.04, 6.90, and2.83; the negative likelihood ratios were 0.22, 0.12, and 0.23, and 0.26, 0.14, and 0.28; the diagnostic odds ratios were 13.01, 30.98, and 19.58, and 11.93, 49.99, and 10.00. CT scanner is the source of heterogeneity. There was no significant difference in diagnostic threshold effects ($P > 0.05$) or publication bias ($P > 0.05$).

CONCLUSION

Based on the meta-analysis of observational studies, it is suggested that CT imaging, a non-invasive diagnostic method, is the best choice for the diagnosis of EV and prediction of HREV in cirrhotic patients compared with LSM and MRI.

Key words: Multidetector computed tomography imaging; Magnetic resonance imaging; Liver stiffness measurement; Liver cirrhosis; Esophageal varices; Meta-analysis

Core tip: To date, endoscopy is regarded as the "gold standard" for diagnosis of esophageal varices (EV) and prediction of high-bleeding-risk EV (HREV) in cirrhotic patients. This study came into the conclusion that computed tomography has higher accuracy in diagnosing EV and predicting HREV than liver stiffness measurement and magnetic resonance imaging in cirrhotic patients. It is suggested that computed tomography, a non-invasive diagnostic method, is the best choice for diagnosing EV and predicting HREV in cirrhotic patients compared with liver stiffness measurement and magnetic resonance imaging. However, in future, the head-to-head comparisons of these imaging tools in the same series of patients are required to confirm the predictive value, especially by using artificial intelligence technique.

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INTRODUCTION

Globally, liver cirrhosis is the most common liver disease and the 11th leading cause of death. Approximately two million people die from liver disease every year and 50% of them die from complications of cirrhosis[1]. Portal hypertension (PH) with esophageal varices (EV) and the following lethal variceal hemorrhage is the most serious and common complication of cirrhosis. The incidence of EV in cirrhotic patients is 7% per year and the five-year cumulative incidence rate reaches 21%[2]. Although the treatment of variceal hemorrhage has been improved over the past two decades, the 6-wk mortality is 10%-20%[3]. The confirmation of varices and the most suitable treatment in the early phase is crucial in order to reduce the mortality. To date, endoscopy is regarded as the "gold standard" for diagnosing the presence of varices and predicting bleeding risk. Baveno VI recommends that compensated

cirrhotic patients without varices whose etiological factor has been removed should receive endoscopy every 3 years[4]. Endoscopy, however, is invasive and uncomfortable. In addition to endoscopy, hepatic vein pressure gradient (HVPG) is considered as a "gold standard" in estimating PH and for risk stratification of liver cirrhosis. HVPG is superior to liver biopsy in predicting the occurrence of complications in cirrhotic patients, including EV and variceal hemorrhage[5]. It is promising that with the aid of HVPG-guided precise treatment, physicians can diagnose and treat PH similarly to "high blood pressure"[6]. However, HVPG measurement is also invasive and expensive. Therefore, non-invasive and easy-to-perform diagnostic techniques to predict complications in cirrhotic patients with PH are required in clinical practice.

So far, several models and parameters based on serum markers[7,8] have been proposed. However, poor reliability has prevented their use in clinical practice. Recently, multiple studies evaluated the accuracy of liver stiffness measurement (LSM), computed tomography (CT), and magnetic resonance imaging (MRI) in the diagnosis of EV and prediction of high-bleeding-risk EV (HREV) in cirrhotic patients. There have, however, been controversies regarding the use of LSM, CT, and MRI as non-invasive diagnostic methods for EV and prediction of HREV in cirrhotic patients. Therefore, the aim of this meta-analysis was to evaluate the value of the imaging methods for the diagnosis of EV and prediction of HREV in clinical practice.

MATERIALS AND METHODS

This systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines[9], and the protocol is registered at PROSPERO (CRD42019126278).

Literature search

A systematic literature research based on PubMed, Embase, Cochrane, CNKI, and Wanfang databases using various combinations of Medical Subject Headings and non-Medical Subject Headings terms was performed independently by two reviewers. The search was limited to original full text articles published in English and Chinese.

The articles reporting the diagnostic value of LSM were searched using key words "LS," "liver stiffness," "FibroScan," "esophageal varices", and "cirrhosis", and those reporting the diagnostic value of CT and MRI were searched based on key words "CT," "computed tomography," "esophageal varices", and "cirrhosis" and "MR," "magnetic resonance," "esophageal varices", and "cirrhosis", respectively.

The last search was performed on April 26, 2019.

Eligibility criteria: The inclusion criteria were: (1) Patients were diagnosed with liver cirrhosis; (2) Endoscopy was performed to confirm the presence and/or grade of EV; (3) Relevant examinations, such as LSM, CT, or MRI, were performed; and (4) The diagnostic accuracy was compared between reference and LSM, CT, or MRI. The exclusion criteria were: (1) Duplicate articles; (2) Reviews; (3) Case reports; (4) Noncirrhotic patients; (5) Patients in whom the presence of varices evaluated was not evaluated by endoscopy; and (6) Lack of accuracy assessment.

Data extraction: The primary data were extracted by two reviewers independently. The study characteristics contained country, study design, age, gender, and etiology of liver cirrhosis. The data included patient number, cut-off value, and the sensitivity and specificity in the diagnosis of EV or HREV. The criteria for HREV based on endoscopy were any of the following[10-12]: (1) Varices diameter ≥ 5 mm and snakelike varices with red color signs; and (2) Large varices (diameter ≥ 10 mm) and nodular and tumor-shaped varices with or without red color signs.

Quality assessment

Two reviewers independently assessed the study quality using the Quality Assessment of Diagnostic Accuracy Studies-2 in RevMan5.3. They calculated the risk of bias as high, low, or unclear with regard to the following aspects: Patient selection, index test, reference standard, and flow and timing. Discrepancies were resolved by discussion between the two reviewers. Each question was judged as "yes", "no", or "unclear".

Statistical analysis

First, true positive (TP) value, false positive (FP) value, false negative (FN) value, and true negative (TN) value were extracted from the original articles. Data analyses were conducted using Stata12.0, MetaDisc1.4, and RevMan5.3.

Second, the heterogeneity of all tested parameters was examined by *Q*-statistic test and I^2 index. Heterogeneity was considered significant if $P < 0.05$ (*Q*-statistic test) or $I^2 \geq 50\%$[13]. When heterogeneity was tested, we further evaluated the threshold effects by calculating the Spearman's correlation coefficient. Threshold effects were considered significant if $P < 0.05$. If no threshold effects existed, sources of heterogeneity were analyzed by meta-regression according to study characteristics. Besides, we performed subgroup analysis according to the results of meta-regression.

The analysis was performed using the fixed-effects model or random-effects model if heterogeneity was considered significant. The diagnostic accuracy was evaluated by the area under the summary receiver operating characteristic curve (AUSROC) with 95% confidence interval (CI), summary sensitivity and specificity with 95%CI, summary positive likelihood ratio (PLR) and negative likelihood ratio (NLR) with 95%CI, and summary diagnostic odds ratio (DOR).

Finally, publication bias was evaluated using Deek's funnel plot, with $P < 0.05$ as having significant publication bias[14].

RESULTS

Literature identification

All analyzed cirrhotic patients were diagnosed by histopathology and/or typical clinical symptoms and laboratory and imaging findings. The etiologies of liver cirrhosis included hepatitis B virus, hepatitis C virus, alcohol, autoimmune hepatitis, nonalcoholic steatohepatitis, and miscellaneous.

LSM: According to the aforementioned search strategy, 898 articles relevant to LSM and cirrhosis were identified. Eighteen best-matched articles were chosen for final meta-analysis[15-32]. The selection process is presented in Figure 1A. Fifteen out of eighteen selected publications[15-17,19-25,27-29,31,32] studied the diagnostic value for EV in 1836 patients. These studies were performed in Asia ($n = 6$), Europe ($n = 7$), and Africa ($n = 2$). In addition, 13[15-18,20-23,26,27,30-32] articles reported the predictive value of HREV in 2388 patients. These studies were performed in Asia ($n = 5$), Europe ($n = 6$), and Africa ($n = 2$), respectively.

CT: According to the search strategy, 17 out of 2192 articles relevant to CT imaging and cirrhosis were chosen for meta-analysis[33-49] (Figure 1B). Sixteen articles[33-38,40-49] enrolled 3327 patients (31 groups) and examined the diagnostic value of CT for EV. These studies were performed in Asia ($n = 9$), North America ($n = 3$), and Africa ($n = 4$) (Table 1). Besides, 10[34-36,39-43,45,47] articles reported the predictive value of HREV in 2686 patients (23 groups). These studies were performed in Asia ($n = 5$), North America ($n = 3$), and Africa ($n = 2$) (Table 2).

MRI: According to the search strategy, 7 out of 601 articles that evaluated MRI in liver cirrhosis were included in the meta-analysis[50-56] (Figure 1C). Four manuscripts reported the diagnostic value of MRI for EV, which included 750 patients (7 groups)[50-52,54]. These studies were performed in Asia ($n = 3$) and Africa ($n = 1$). Besides, 4 articles comprising 9 groups and 1053 patients studied the predictive value of HREV[53-56], which were performed in Asia ($n = 3$) and Europe ($n = 1$).

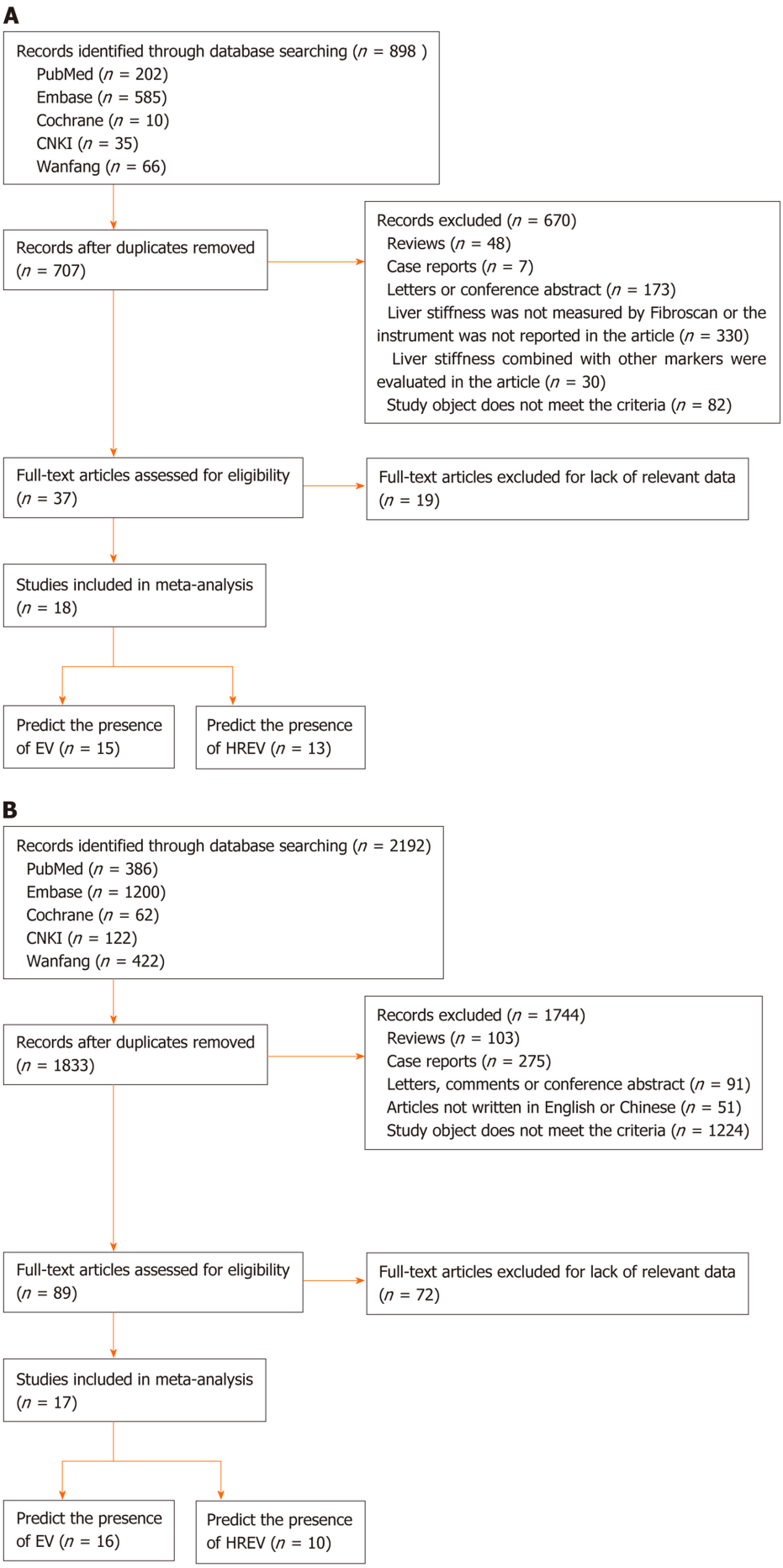
The quality of the eligible articles is shown in Figure 2.

Meta-analysis

The results of meta-analysis are shown in Table 3. Significant heterogeneity was observed in all analyses ($P < 0.05$), except summary sensitivity in diagnosing EV and summary NLR in evaluating both EV and HREV using MRI ($P > 0.05$). Therefore, the random-effects model was used to combine effect quantity. Threshold effects were not found in all analyses ($P > 0.05$). CT had the highest AUSROC for the evaluation of EV and HREV (Figure 3A and B).

LSM: Using LSM to diagnose EV, the AUSROC was 0.86 (95%CI: 0.83-0.89, I^2 = 97.43%, Figure 3C), with a summary sensitivity of 0.84 (95%CI: 0.78-0.89, I^2 =82.63%; Figure 4A) and summary specificity was 0.71 (95%CI: 0.60-0.80, I^2 = 86.56%; Figure 4B). The summary PLR, NLR, and DOR were 2.91 (95%CI: 2.08-4.06, I^2 = 82.66%), 0.22 (95%CI: 0.16-0.30, I^2 = 79.49%), and 13.01 (95%CI: 7.83-21.64; Table 3), respectively.

As for the predictive value of LSM for HREV, the AUSROC was 0.85 (95%CI: 0.81-0.88, I^2 = 97.13%; Figure 3D), with a summary sensitivity of 0.81 (95%CI: 0.75-0.86, I^2 = 70.93%; Figure 4C) and summary specificity of 0.73 (95%CI: 0.66-0.80, I^2 = 91.65%; Figure 4D). The summary PLR, NLR, and DOR were 3.04 (95%CI: 2.38-3.89, I^2 = 85.63%), 0.26 (95%CI: 0.19-0.34, I^2 = 68.30%), and 11.93 (95%CI: 7.89-18.03; Table 3),


A
Records identified through database searching (n = 898)
PubMed (n = 202)
Embase (n = 585)
Cochrane (n = 10)
CNKI (n = 35)
Wanfang (n = 66)
Records after duplicates removed (n = 707)
Records excluded (n = 670)
Reviews (n = 48)
Case reports (n = 7)
Letters or conference abstract (n = 173)
Liver stiffness was not measured by Fibroscan or the instrument was not reported in the article (n = 330)
Liver stiffness combined with other markers were evaluated in the article (n = 30)
Study object does not meet the criteria (n = 82)
Full-text articles assessed for eligibility (n = 37)
Full-text articles excluded for lack of relevant data (n = 19)
Studies included in meta-analysis (n = 18)
Predict the presence of EV (n = 15)
Predict the presence of HREV (n = 13)
B
Records identified through database searching (n = 2192)
PubMed (n = 386)
Embase (n = 1200)
Cochrane (n = 62)
CNKI (n = 122)
Wanfang (n = 422)
Records after duplicates removed (n = 1833)
Records excluded (n = 1744)
Reviews (n = 103)
Case reports (n = 275)
Letters, comments or conference abstract (n = 91)
Articles not written in English or Chinese (n = 51)
Study object does not meet the criteria (n = 1224)
Full-text articles assessed for eligibility (n = 89)
Full-text articles excluded for lack of relevant data (n = 72)
Studies included in meta-analysis (n = 17)
Predict the presence of EV (n = 16)
Predict the presence of HREV (n = 10)

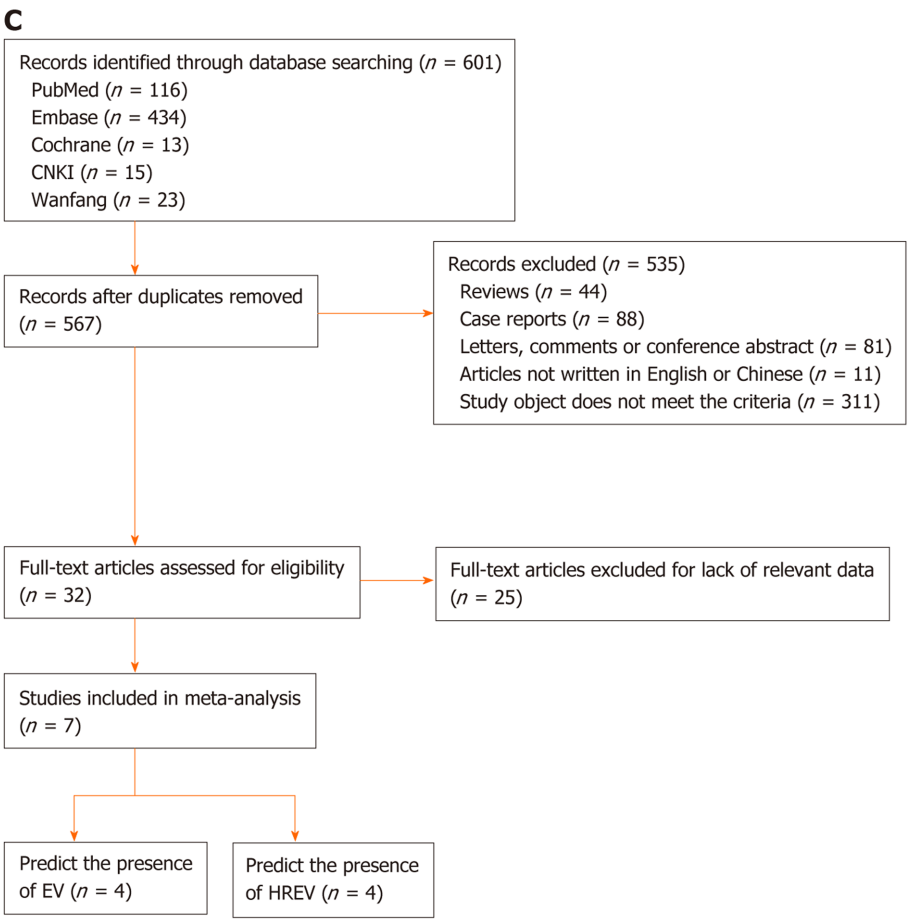


Figure 1 Flow chart of the search and selection of articles. A: Flow chart of the search and selection of articles about liver stiffness measurement; B: Flow chart of the search and selection of articles about computed tomography; C: Flow chart of the search and selection of articles about magnetic resonance imaging.

respectively.

CT: The AUSROC of CT in the diagnosis of EV was 0.91 (95%CI: 0.88-0.93, I^2 = 97.17%; Figure 3E), with a summary sensitivity of 0.91 (95%CI: 0.87-0.94, I^2 = 88.46%) and specificity of 0.75 (95%CI: 0.68-0.82, I^2 = 80.58%; Figure 5A and B). The summary PLR, NLR, and DOR were 3.67 (95%CI: 2.73-4.94, I^2 = 83.81%), 0.12 (95%CI: 0.08-0.18, I^2 = 88.94%), and 30.98 (95%CI: 16.02-59.91; Table 3), respectively.

The AUSROC of CT in the prediction of HREV was 0.94 (95%CI: 0.91-0.96, I^2 = 98.30%; Figure 3F), with a summary sensitivity of 0.88 (95%CI: 0.82-0.92, I^2 = 87.06%) and specificity of 0.87 (95%CI: 0.81-0.92, I^2 = 93.26%; Figure 5C and D). The summary PLR, NLR, and DOR were 6.90 (95%CI: 4.54-10.49, I^2 = 91.04%), 0.14 (95%CI: 0.09-0.21, I^2 = 91.10%), and 49.99 (95%CI: 25.38-98.43; Table 3), respectively.

MRI: The AUSROC of MRI in the diagnosis of EV was 0.86 (95%CI: 0.83-0.89, I^2 = 86.41%; Figure 3G), with a summary sensitivity of 0.81 (95%CI: 0.76-0.86, I^2 = 33.57%) and specificity of 0.82 (95%CI: 0.70-0.89, I^2 = 74.53%; Figure 6A and B). The summary PLR, NLR, and DOR were 4.44 (95%CI: 2.74-7.21, I^2 = 31.66%), 0.23 (95%CI: 0.18-0.28, I^2 < 0.01%), and 19.58 (95%CI: 11.36-33.66; Table 3), respectively.

As for the prediction of HREV by MRI, the AUSROC was 0.83 (95%CI: 0.79-0.86, I^2 = 91.64%; Figure 3H), with a summary sensitivity of 0.80 (95%CI: 0.72-0.86, I^2 = 67.03%) and specificity of 0.72 (95%CI: 0.62-0.80, I^2 = 83.17%; Figure 6C and D). The summary PLR, NLR, and DOR were 2.83 (95%CI: 2.11-3.80, I^2 = 51.94%), 0.28 (95%CI: 0.21-0.38, I^2 = 43.01%), and 10.00 (95%CI: 6.63-15.09; Table 3), respectively.

Based on this meta-analysis, CT had higher accuracy in evaluating the presence of both EV and HREV with an AUSROC of 0.91 and 0.94, respectively.

Meta-regression

Based on the above results, we further focused on CT for diagnosis of EV and prediction of HREV. We performed meta-regression for CT to examine the source of heterogeneity and found that the accuracy of CT in the diagnosis EV was affected by CT scanner (P < 0.05).

Table 1 Characteristics of articles using computed tomography imaging to diagnose esophageal varices

Ref.	Study design	Total patients	Mean age (yr)	Male (%)	Etiology (%)	Child-Pugh class (%)	CT scanner	Interval between CT and endoscopy	Presence of EV			
									TP	FP	FN	TN
Cansu *et al*[33], 2014	Prospective	50	56.8	54	HBV (40.0); HCV (30.0); Biliary (6.0); HBV + HCV (4.0); Alcohol (4.0%); Others (16.0)	A (52.0); B (36.0); C (12.0)	16-slice	Within 4 wk	33	2	0	15
	Prospective	42	56.2	69	HBV (45.2); HCV (23.8); Alcohol (4.8); HBV + HCV (2.4); Biliary (2.4); Others (21.4)	A (38.1); B (31.0); C (31.0)	16-slice	Within 4 wk	25	3	8	6
Deng *et al*[34], 2016	Retrospective	52	55.4	63.5	Alcohol (30.8); HBV (25.0); HBV + Alcohol (9.6); HCV (3.8); HBV + HCV (1.9); Others (28.9)	A (49.0); B (39.2); C (11.8)	NR	NR	43	2	2	5
Dessouky *et al*[35], 2013	Prospective	137	58.7	53.3	HCV (67.9); HBV (19.7); HBV + HCV (10.2); Steatohepatitis (2.2)	A (55); B (31); C (14)	16-slice	Within 24 h	89	1	1	46
Elalfy *et al*[36], 2016	Retrospective	124	56.5	52	HCV (100)	A (62.9); B (37.1)	16-slice	NR	70	4	4	46
Elkammash *et al*[37], 2015	NR	112	51.4	68.8	HBV (46); HCV (44); Schistosomiasis (10)	NR	64-slice	Within 2 wk	97 99	0 0	2 0	13 13
Jiang *et al*[38], 2015	NR	89	57	64	HBV (71.9); Alcohol (11.2); HCV (7.9); Biliary (5.6); Drug (1.1); Unknown (2.3)	NR	64-slice	NR	58	3	8	20

Kim *et al*[40], 2020	Retrospective	104	59	74	HBV (72.1); HCV (12.5); Alcohol (6.7); Others (8.7)	A (41.3); B (30.8); C (27.9)	16 or 64-slice	Within 4 wk	180 169 172	9 9 15	8 19 16	11 11 5
Kim *et al*[41], 2007	Prospective	90	54.8	72.2	HBV (73.3); HCV (21.1); Alcohol (2.2); Others (3.3)	A (81.1); B (18.9)	16-slice	Within 4 h	50 47 46 46	15 12 13 8	3 6 7 7	22 25 24 29
Kim *et al*[42], 2007	Retrospective	67	56.2	58.2	HCV (35.8); HBV (22.4); Alcohol (22.4); HBV + HCV (9.0); Others (10.4)	A (23.9); B (37.3); C (38.8)	NR	Within 4 wk	29 27	6 3	13 15	19 22
Lipp *et al*[43], 2011	Retrospective	299	55.2	64.9	NR	NR	4 or 16 or 64-slice	Within 12 wk	54 41	24 17	7 30	52 77
Moftah *et al*[44], 2014	NR	54	56.8	74.1	NR	NR	8-slice	NR	48	0	2	4
Perri *et al*[45], 2008	Retrospective	101	NR	63.4	Viral (21.8); Alcohol (18.8); Biliary (17.8); NASH (14.9); Others (26.7)	A (44.6); B (39.6); C (15.8)	4-slice or higher	2 d[1]	73 68	10 12	6 11	12 10
Wu *et al*[46], 2009	Prospective	50	57.7	60	HBV (76); Autoimmune (2); Others (22)	A (26.0); B (62.0); C (12.0)	16-slice	Within 4 wk	39 40	3 5	2 1	6 4
Yu *et al*[47], 2011	Retrospective	109	55.9	55	HCV (46.8); Alcohol (17.4); HBV (6.4); Others (29.4)	NR	16 or 64-slice	NR	50 50 49 47	10 12 24 17	12 12 13 15	37 35 23 30
Zhao *et al*[48], 2016	Retrospective	143	52.39	67.1	HBV (70.6); Alcohol (11.2); Autoimmune (4.9); HCV (3.5); Biliary (3.5); Others (6.3)	A (37.8); B (33.6); C (28.7)	64-slice	3.4 d[2]	11 2	3	1	27
Zhu *et al*[49], 2009	Retrospective	127	45.2	75.6	HBV (74.8); Alcohol (10.2); HCV (4.7); Others (10.2)	A (37.8); B (37.0); C (25.2)	4-slice	Within 4 wk	72 67	15 11	14 19	26 30

[1]Median.

[2]Mean. NR: Not reported; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Nonalcoholic steatohepatitis; CT: Computed tomography; EV: Esophageal varices; TP: True positive; FP: False positive; FN: False negative; TN: True negative.

CT subgroup analysis

64-slice scanner *vs* 16-slice scanner in diagnosis of EV: CT performed with a 64-slice scanner showed better accuracy in EV compared with imaging performed with a 16-slice scanner [AUSROC: 0.98 (95%CI: 0.97-0.99, I^2 < 0.01%) *vs* 0.94 (95%CI: 0.92-0.96, I^2 < 0.01%); summary sensitivity: 0.98 (95%CI: 0.91-1.00, I^2 = 92.01%) *vs* 0.94 (95%CI: 0.88-0.97, I^2 = 73.98%); summary specificity: 0.94 (95%CI: 0.82-0.98, I^2 = 64.69%) *vs* 0.78 (95%CI: 0.65-0.87, I^2 = 76.48%); and summary DOR: 904.11 (95%CI: 74.85-11000) *vs* 50.75 (95%CI: 16.21-158.911)]. I^2-values decreased and indicated that there was no significant heterogeneity.

16-slice scanner in prediction of HREV: Based on the diameter of EV, the AUSROC for prediction of HREV using 16-slice CT scanner was 0.96 (95%CI: 0.93-0.97, I^2 = 40.73%). The summary sensitivity, specificity, and DOR were 0.93 (95%CI: 0.89-0.96, I^2 = 17.26%), 0.94 (95%CI: 0.87-0.97, I^2 = 78.08%), and 192.47 (95%CI: 71.03-521.49), respectively. There was no statistically significant heterogeneity.

Publication bias

According to Deeks' funnel plot, there was no evidence of significant publication bias (P > 0.05).

DISCUSSION

Esophageal variceal hemorrhage is a catastrophic and fatal complication of PH with cirrhosis. The current "gold standard" for the diagnosis of EV and HREV is endoscopy in clinical practice. However, periodic endoscopy is expensive and uncomfortable, and therefore not easily accepted by most patients. The advantages of non-invasive diagnostic tools for evaluating EV and HREV are repeatability and better patient acceptance. We therefore performed a meta-analysis to compare the accuracy of evaluating EV and HREV by three non-invasive diagnostic methods: CT, MRI, and LSM.

In this meta-analysis, we identified 18, 17, and 7 articles evaluating the accuracy of LSM, CT, and MRI for diagnosing EV and predicting HREV, respectively. The analysis showed that CT had the highest accuracy for both EV and HREV. The AUSROC was 0.91 and 0.94, and DOR was 30.98 and 49.99 for evaluating the presence of EV and HREV. Baveno VI consensus recommends that patients with a liver stiffness < 20 kPa on transient elastography and with a platelet count > 150 × 10^9/L have a very low risk of having varices requiring treatment, and can avoid screening endoscopy. In studies that validate the criteria, up to 100% of patients who met the criteria had an ultimately negative endoscopy, but it showed a relatively low specificity of 61.5%[57]. Rosman *et al*[58] investigated the utility of incorporating the CT or MR findings of portosystemic collateral vessels to predict HREV in patients who did not meet Baveno VI criteria. The presence of portosystemic collateral vessels to predict HREV yielded a sensitivity of 0.95 and specificity of 0.36 in these patients. Therefore, the use of additional portosystemic collateral vessels from CT or MRI can further help identify patients with compensatory cirrhosis who do not require endoscopy. The weakness of LSM using transient elastography is decreased applicability in obese patients and patients with ascites. Lipp *et al*[43] evaluated the ability of CT and MRI to detect EV and found that CT is a superior imaging modality to MRI. According to a meta-analysis performed by Deng *et al*[7], Lok score had the highest AUSROC of 0.79, followed by FIB-4, Forns, aspartate aminotransferase-to-alanine aminotransferase ratio, and aspartate aminotransferase-to-platelet ratio, for the diagnosis of EV. Aspartate aminotransferase-to-alanine aminotransferase ratio had the highest AUSROC of 0.74, followed by aspartate aminotransferase-to-platelet ratio, Lok, FIB-4, and Forns scores for the prediction of HREV. A significant heterogeneity (I^2 ranged from 86.41% to 98.30%) was found in their meta-analysis. The CT scanner was significantly associated with heterogeneity in diagnosing EV. Subgroup analysis suggested that the accuracy of CT scanner with more slices was critical for diagnosing EV.

Compared with endoscopy, contrast-enhanced CT or MRI can clearly show the portal vein system and collateral circulation[59,60]. In addition to EV, they can be used for the diagnosis of other complications including hepatocellular carcinoma[61,62]. There is no doubt that endoscopy is irreplaceable. It can diagnose esophageal and gastric

Table 2 Characteristics of articles using computed tomography imaging to predict HREV

Ref.	Study design	Total patients	Mean age (yr)	Male (%)	Etiology (%)	Child-Pugh class (%)	CT scanner	Cut-off value (mm)	Interval between CT and endoscopy	Presence of HREV			
										TP	FP	FN	TN
Deng *et al*[34], 2016	Retrospective	52	55.4	64	Alcohol (30.8); HBV (25.0); HBV + Alcohol (9.6); HCV (3.8); HBV + HCV (1.9); Others (28.9)	A (49.0); B (39.2); C (11.8)	NR	EVD 3.9	NR	35	4	4	9
Dessouky *et al*[35], 2013	Prospective	137	58.7	53	HCV (67.9); HBV (19.7); HBV + HCV (10.2); Steatohepatitis (2.2)	A (55); B (31); C (14)	16-slice	EVD 3.0	Within 24 h	38	0	0	99
Elalfy *et al*[36], 2016	Retrospective	124	56.5	52	HCV (100)	A (62.9); B (37.1)	16-slice	PVD 12.5	NR	33	49	13	29
Kim *et al*[39], 2008	Retrospective	110	61	74	HBV (60.9); HCV (29.1) Alcohol (6.4); HBV + HCV (1.8); Others (1.8)	A (63.6); B (26.4); C (10.0)	16-slice	EVD 2.0	8.2 d[1]	123 113 115	15 2 15	9 19 17	61 59 61
Kim *et al*[40], 2020	Retrospective	104	59	74	HBV (72.1); HCV (12.5); Alcohol (6.7); Others (8.7)	A (41.3); B (30.8); C (27.9)	16 or 64-slice	EVD 2.0	Within 4 wk	34 36 32	3 10 4	3 1 5	70 63 69
Kim *et al*[41], 2007	Prospective	90	54.8	72	HBV (73.3); HCV (21.1); Alcohol (2.2); Others (3.3)	A (81.1); B (18.9)	16-slice	Grade 2 and Grade 3[3]	Within 4 h	28 28 27 27	11 5 9 2	2 2 3 3	49 55 51 58
Kim *et al*[42], 2007	Retrospective	67	56.2	58	HCV (35.8); HBV (22.4); Alcohol (22.4); HBV + HCV (9.0); Others (10.4)	A (23.9); B (37.3); C (38.8)	NR	EVD 3.0	Within 4 wk	11 11	9 9	1 1	46 46

Lipp *et al*[43], 2011	Retrospective	299	55.2	65	NR	NR	4 or 16 or 64-slice	EVD 4.0	Within 12 wk	18 12	11 4	12 22	96 127
Perri *et al*[45], 2008	Prospective	101	NR	63	Viral (21.8); Alcohol (18.8); Biliary (17.8); NASH (14.9); Others (26.7)	A (44.6); B (39.6); C (15.8)	4-slice or higher	EVD 5.0	2 d[2]	23 27	5 8	18 14	55 52
Yu *et al*[47], 2011	Retrospective	109	55.9	55	HCV (46.8); Alcohol (17.4); HBV (6.4); Others (29.4)	NR	16 or 64-slice	EVD 2.0	NR	25 25 23 23	24 18 41 17	1 1 3 3	59 65 42 66

[1]Mean.
[2]Median.
[3]Grade 2: Varices show beaded appearance; Grade 3: Varices run in oblique course and are tortuous with tumorlike appearance. EVD: Esophageal varices diameter; NR: Not reported; PVD: Portal vein diameter; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Nonalcoholic steatohepatitis; CT: Computed tomography; HREV: High-bleeding-risk esophageal varices; TP: True positive; FP: False positive; FN: False negative; TN: True negative.

varices as well as other lesions that cause upper gastrointestinal bleeding, such as peptic ulcer. Combined with the ultrasound probe, it was applied to probe the blood vessels around the wall of the esophagus. Zheng *et al*[63] evaluated endoscopic ultrasound probe examinations for the prediction of recurrence of EV after endoscopic therapies by detecting peri-esophageal collateral veins, perforating veins, and para-esophageal collateral veins. The result showed that peri-esophageal collateral veins can predict 1-year variceal recurrence with a sensitivity of 45% and specificity of 86% when using a diameter of 3.5 mm as cut-off value.

There are several limitations of our analysis that should be taken into consideration. First, we searched the databases for articles only written in English and Chinese, which may miss some articles written in other languages. Second, though the Deek's funnel plot asymmetry test showed no evidence of significant publication bias, there are probably studies of negative outcomes which have not been published. These research results may be missed. Third, the included articles had different definitions or cut-off values of HREV. Thus, no standard diagnostic thresholds for CT, MRI, and LSM were defined. Finally, we regarded endoscopy currently as the "gold standard" for diagnosing EV and HREV, nevertheless, there was no head-to-head controlled study of the above-mentioned non-invasive diagnostic methods in the same series of patients. This indirect comparison brought to a statistical bias, thus might attribute to study heterogeneity. Despite the limitations, new analysis techniques of radiomics are likely to improve diagnostic and predictive accuracy of many diseases. Choi *et al*[64] developed a deep learning system for accurate staging of liver fibrosis using CT. These promising results should initiate further studies on CT using artificial intelligence and machine learning technology to reduce the need for endoscopy.

In conclusion, based on this meta-analysis, CT has higher accuracy for evaluating both EV and HREV in cirrhotic patients. However, further head-to-head comparisons of these noninvasive diagnostic tools are required to confirm the predictive value in EV and HREV, particularly in view of the future use of artificial intelligence technology.

Table 3 Overview of results of meta-analysis

Group	LSM for presence of EV	LSM for presence of HREV	CT for presence of EV	CT for presence of HREV	MRI for presence of EV	MRI for presence of HREV
Diagnostic threshold						
Spearman correlation coefficient	0.36	0.21	-0.2	0.12	0.27	0.57
P value	0.19	0.5	0.27	0.58	0.56	0.11
SROC						
AUSROC (95%CI)	0.86 (0.83-0.89)	0.85 (0.81-0.88)	0.91 (0.88-0.93)	0.94 (0.91-0.96)	0.86 (0.83-0.89)	0.83 (0.79-0.86)
I^2	97.43%	97.13%	97.17%	98.30%	86.41%	91.64%
P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Sensitivity						
Summary sensitivity (95%CI)	0.84 (0.78-0.89)	0.81 (0.75-0.86)	0.91 (0.87-0.94)	0.88 (0.82-0.92)	0.81 (0.76-0.86)	0.80 (0.72-0.86)
I^2	82.63%	70.93%	88.46%	87.06%	33.57%	67.03%
P value	< 0.01	< 0.01	< 0.01	< 0.01	0.17	< 0.01
Specificity						
Summary specificity (95%CI)	0.71 (0.60-0.80)	0.73 (0.66-0.80)	0.75 (0.68-0.82)	0.87 (0.81-0.92)	0.82 (0.70-0.89)	0.72 (0.62-0.80)
I^2	86.56%	91.65%	80.58%	93.26%	74.53%	83.17%
P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
PLR						
Summary PLR (95%CI)	2.91 (2.08-4.06)	3.04 (2.38-3.89)	3.67 (2.73-4.94)	6.90 (4.54-10.49)	4.44 (2.74-7.21)	2.83 (2.11-3.80)
I^2	82.66%	85.63%	83.81%	91.04%	31.66%	51.94%
P value	< 0.01	< 0.01	< 0.01	< 0.01	0.01	< 0.01
NLR						
Summary NLR (95%CI)	0.22 (0.16-0.30)	0.26 (0.19-0.34)	0.12 (0.08-0.18)	0.14 (0.09-0.21)	0.23 (0.18-0.28)	0.28 (0.21-0.38)
I^2	79.49%	68.30%	88.94%	91.10%	<0.01%	43.01%
P value	< 0.01	< 0.01	< 0.01	< 0.01	0.53	0.08
Summary DOR (95%CI)	13.01 (7.83-21.64)	11.93 (7.89-18.03)	30.98 (16.02-59.91)	49.99 (25.38-98.43)	19.58 (11.39-33.66)	10.00 (6.63-15.09)

A significant heterogeneity was found in all analyses. There were no threshold effects in all analyses ($P > 0.05$). CT had the largest area under the summary receiver operating characteristic curves in both of the diagnosis of EV and prediction of HREV. SROC: Summary receiver operating characteristic curves; CT: Computed tomography; EV: Esophageal varices; HREV: High bleeding risk esophageal varices; MRI: Magnetic resonance imaging; LSM: Liver stiffness measurement; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; DOR: Diagnostic odds ratio; AUSROC: Area under the summary receiver operating characteristic curves.

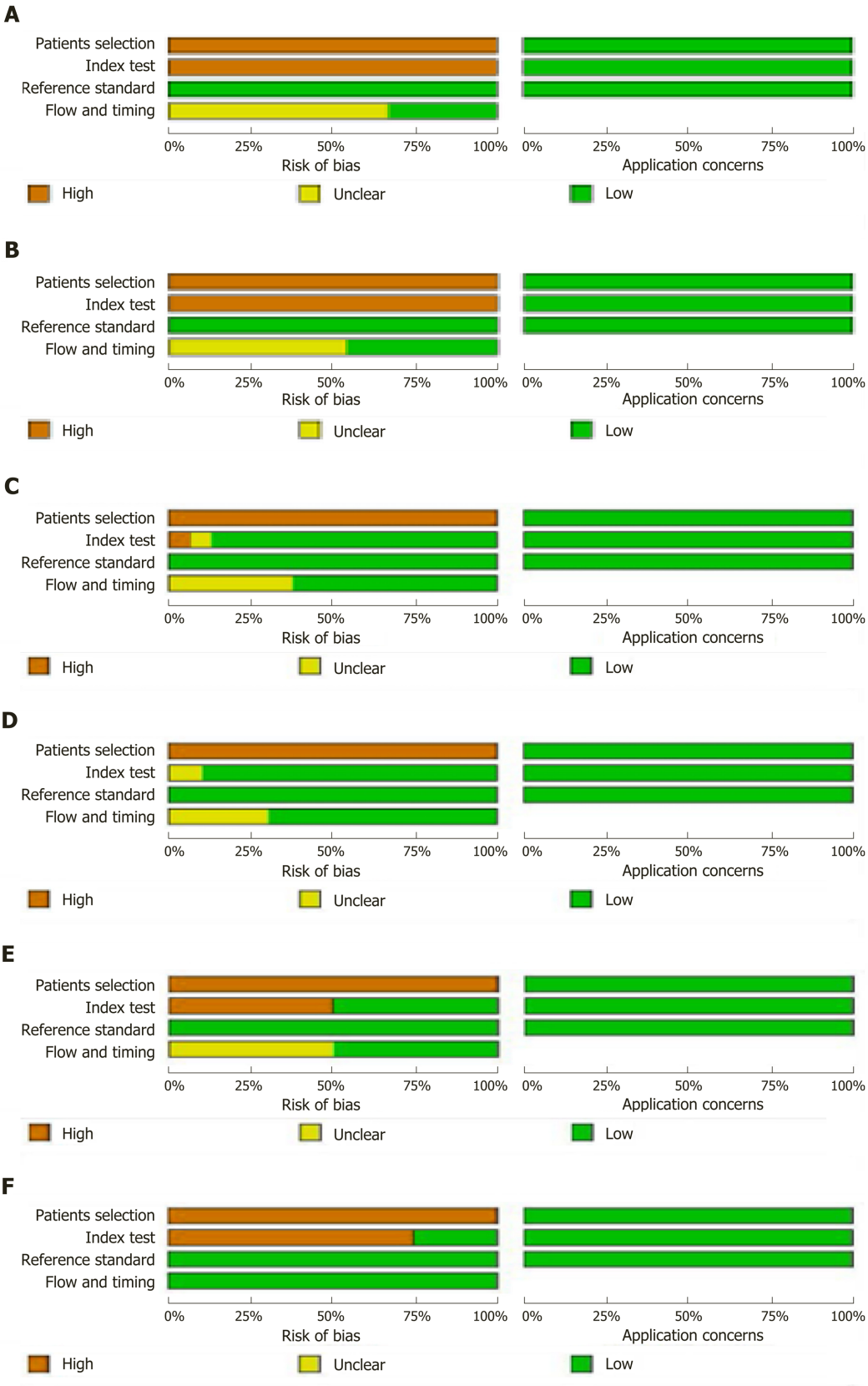


Figure 2 Methodological evaluation according to Quality Assessment of Diagnostic Accuracy Studies-2 of the included articles. A, C, and E: Diagnosis of esophageal varices using liver stiffness measurement, computed tomography, and magnetic resonance imaging, respectively; B, D, and F: Prediction of high-bleeding-risk esophageal varices using liver stiffness measurement, computed tomography, and magnetic resonance imaging, respectively. Articles were identified as having a potential bias risk for patient selection and index text.

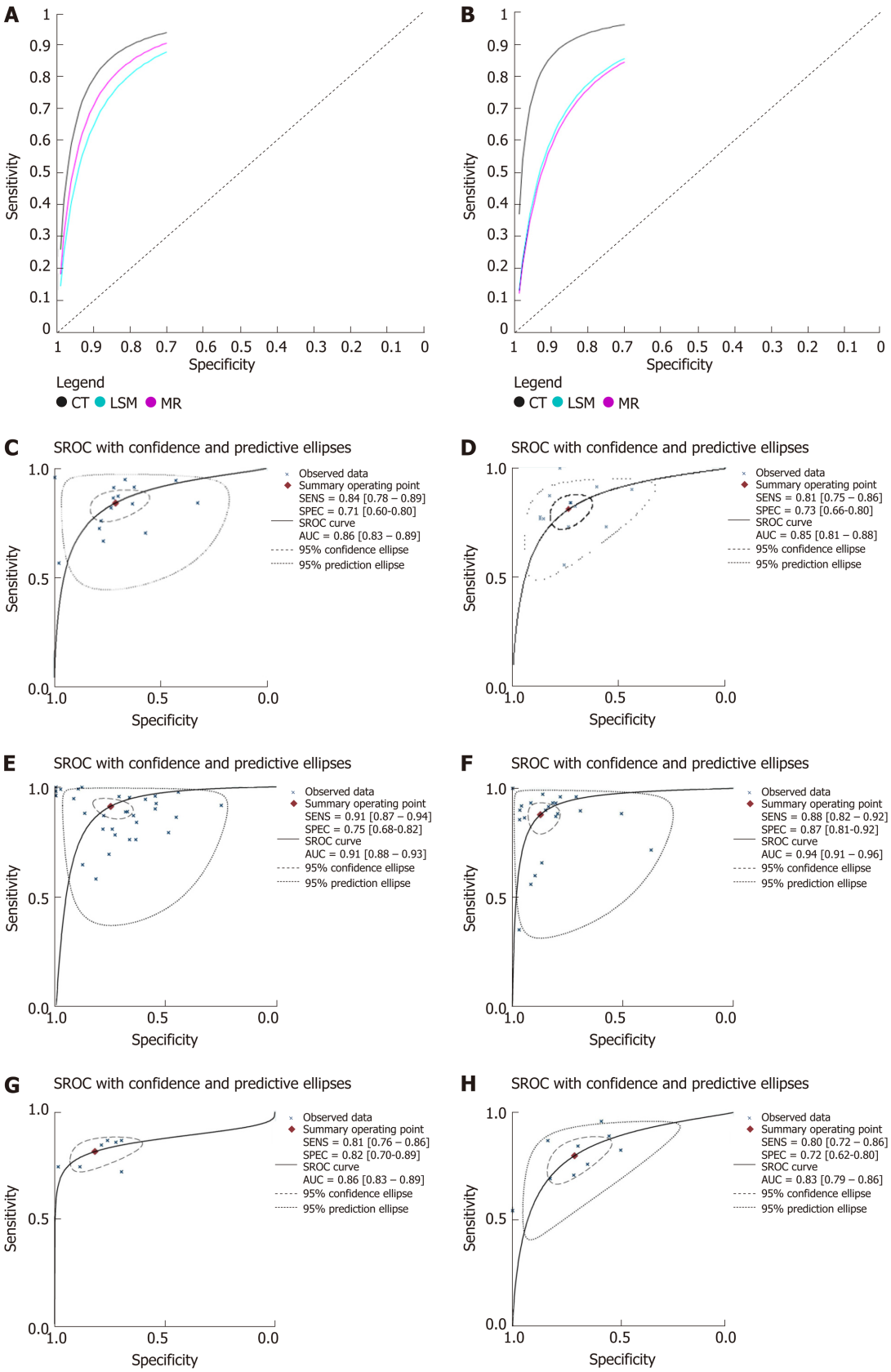


Figure 3 Summary receiver operating characteristic curves. A and B: Summary receiver operating characteristic (SROC) curves of liver stiffness measurement, computed tomography, and magnetic resonance imaging for the diagnosis of esophageal varices (EV) and prediction of high-bleeding-risk EV (HREV); C and D: SROC curves of liver stiffness measurement for the diagnosis of EV and prediction of HREV; E and F: SROC curves of computed tomography for the diagnosis of EV and prediction of HREV; G and H: SROC curves of magnetic resonance imaging for the diagnosis of EV and prediction of HREV.

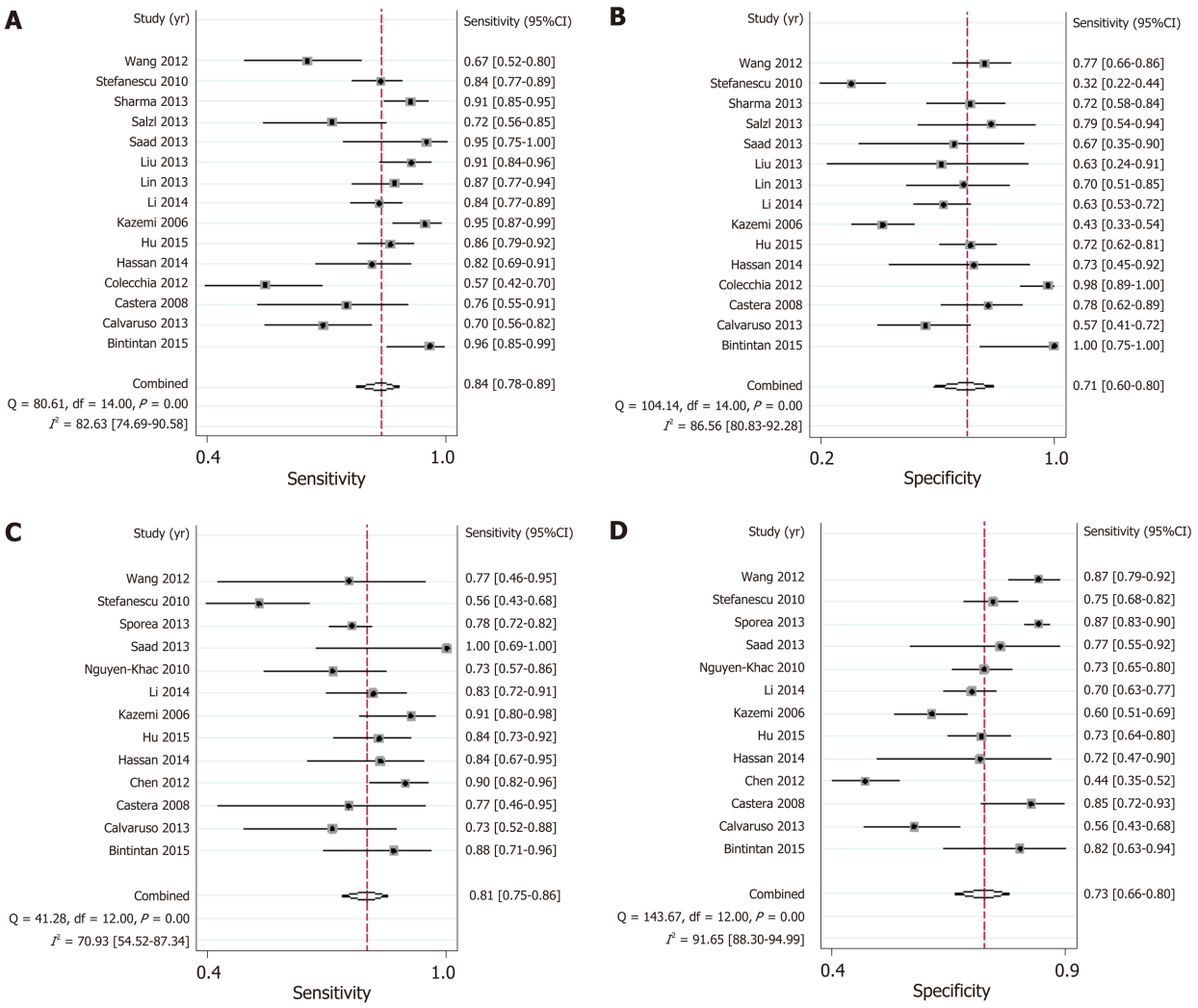


Figure 4 Summary sensitivity and specificity of liver stiffness measurement. A and B: Summary sensitivity and specificity of liver stiffness measurement for the diagnosis of esophageal varices; C and D: Summary sensitivity and specificity of liver stiffness measurement for the prediction of high-bleeding-risk esophageal varices.

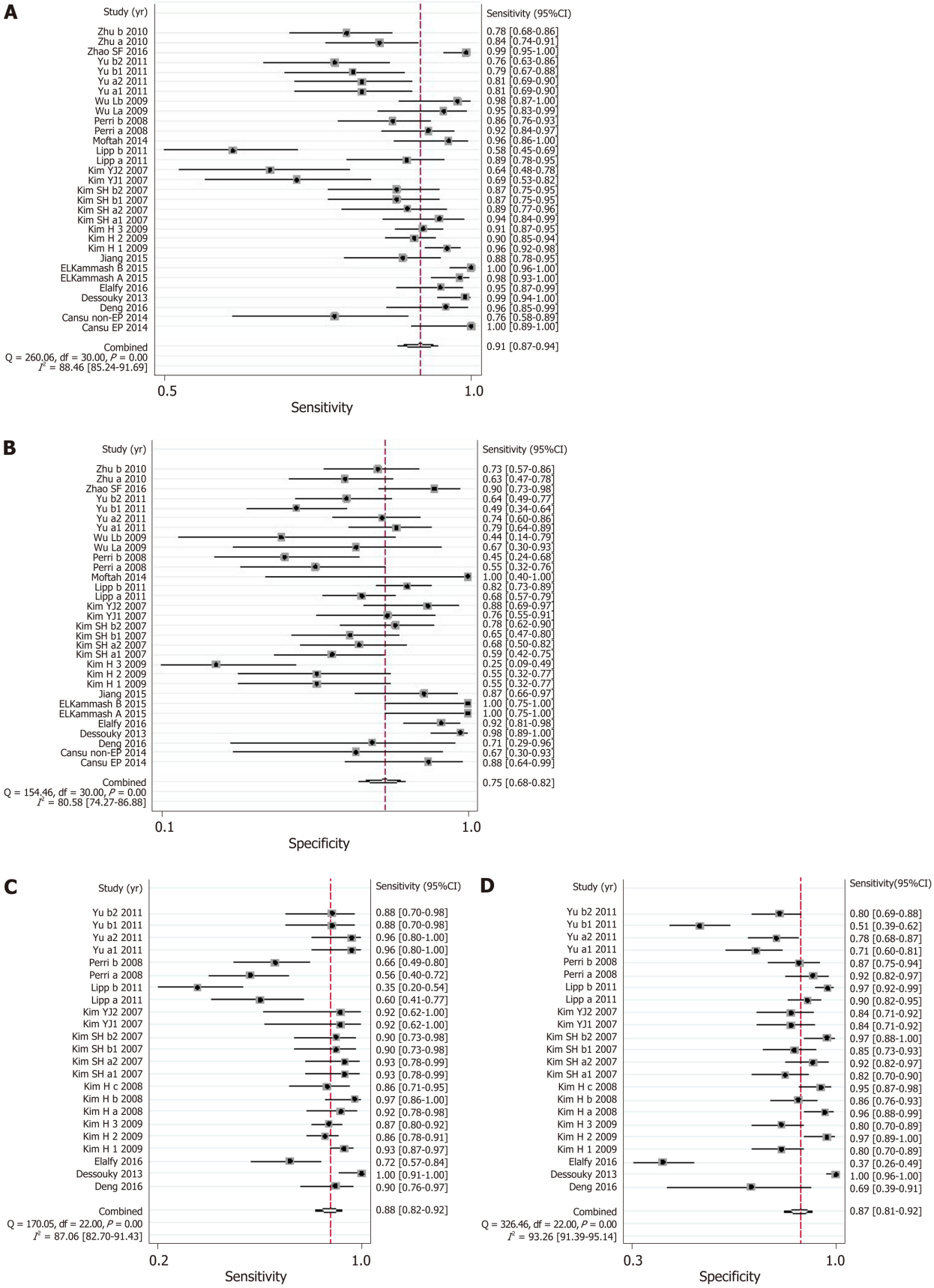


Figure 5 Summary sensitivity and specificity of computed tomography imaging. A and B: Summary sensitivity and specificity of computed tomography for the diagnosis of esophageal varices; C and D: Summary sensitivity and specificity of computed tomography imaging for the prediction of high-bleeding-risk esophageal varices.

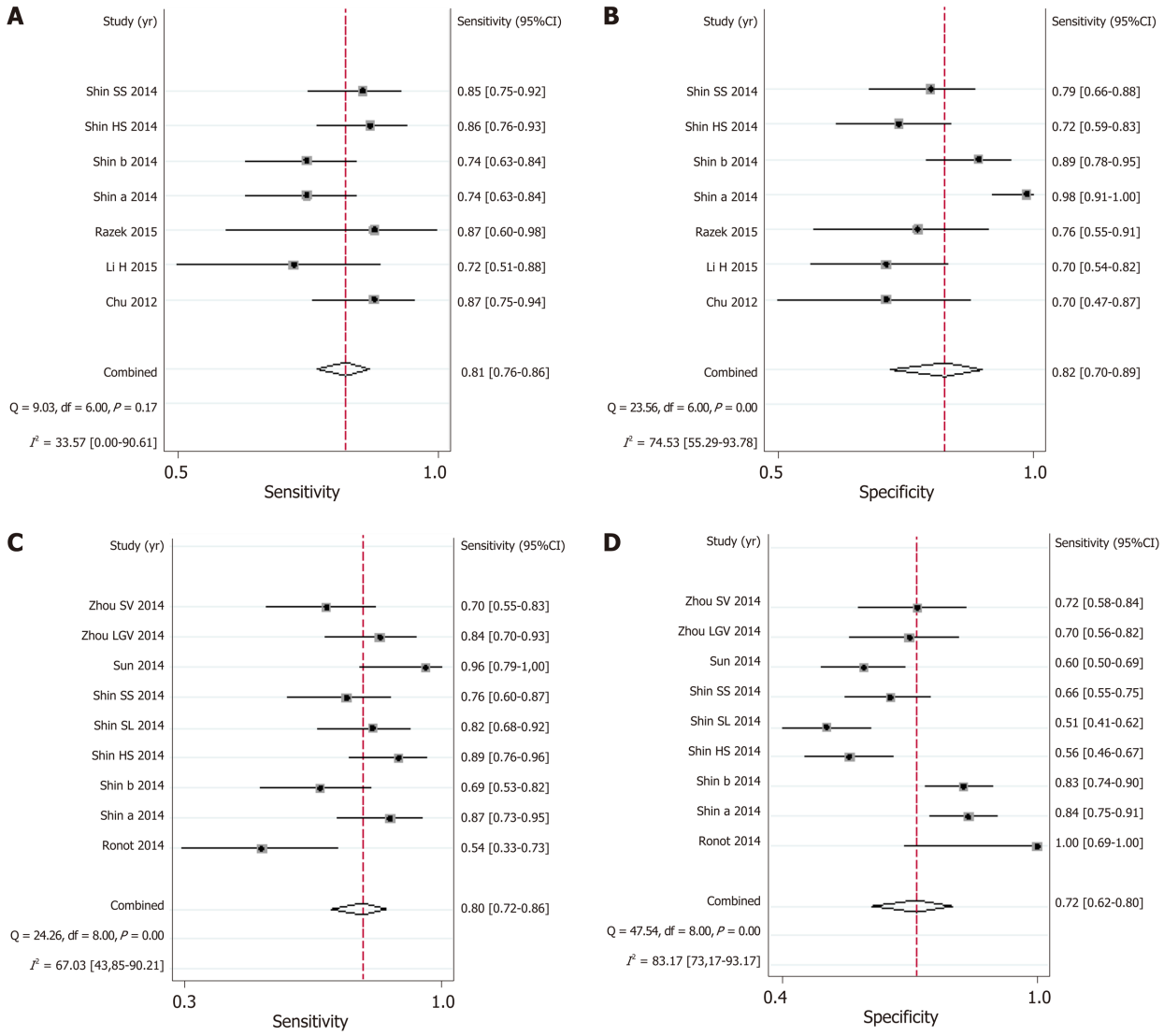


Figure 6 Summary sensitivity and specificity of magnetic resonance imaging. A and B: Summary sensitivity and specificity of magnetic resonance imaging for the diagnosis of esophageal varices; C and D: Summary sensitivity and specificity of magnetic resonance imaging for the prediction of high-bleeding-risk esophageal varices.

ARTICLE HIGHLIGHTS

Research background

The non-invasive and easy-to-perform diagnostic techniques to predict complications in cirrhotic patients are required in clinical practice. Up to now, the clinical use of liver stiffness measurement (LSM), computed tomography (CT), and magnetic resonance imaging (MRI), as non-invasive diagnostic methods to diagnose esophageal varices (EV) and to predict high-bleeding-risk EV (HREV) in cirrhotic patients, is controversial.

Research motivation

The LSM, CT, and MRI for the diagnosis of EV and prediction of HREV, promising non-invasive diagnostic methods to predict complications in cirrhotic patients, are required in clinical practice. However, the accuracy, sensitivity, and specificity varied in different studies. The overall accuracy, sensitivity, and specificity of LSM, CT, and MRI in the diagnosis of EV and prediction of HREV in cirrhotic patients have not stated.

Research objectives

This is a very important and interesting systematic review and meta-analysis aimed to determine the overall accuracy and sensitivity of three non-invasive methods to diagnose EV and predict the risk of bleeding in patients with liver cirrhosis.

Research methods

We performed literature searches by using selected keywords in PubMed, Embase, Cochrane, CNKI, and Wanfang databases for full-text articles published in English and Chinese. All

statistical analyses were conducted using Stata12.0, MetaDisc1.4, and RevMan5.3. Summary sensitivity and specificity, positive likelihood ratio and negative likelihood ratio, diagnostic odds ratio, and the area under the summary receiver operating characteristic curves that evaluated the accuracy of LSM, CT, and MRI as candidates for diagnosing EV and predicting HREV in cirrhotic patients were analyzed. The random-effects model was used to combine effect quantity. The quality of the articles was assessed using the quality assessment of diagnostic accuracy studies-2 tool. Heterogeneity was examined by Q-statistic test and I^2 index, and sources of heterogeneity were explored using meta-regression and subgroup analysis. Publication bias was evaluated using Deek's funnel plot.

Research results

Overall, 18, 17, and 7 relevant articles on the accuracy of LSM, CT, and MRI in diagnosing EV and predicting HREV were retrieved. CT had higher accuracy than LSM and MRI in diagnosing EV and predicting HREV with areas under the summary receiver operating characteristic curves of 0.91 (95%CI: 0.88-0.93) and 0.94 (95%CI: 0.91-0.96), respectively. The sensitivities of LSM, CT, and MRI in diagnosing EV and predicting HREV were 0.84 (95%CI: 0.78-0.89), 0.91 (95%CI: 0.87-0.94), and 0.81 (95%CI: 0.76-0.86), and 0.81 (95%CI: 0.75-0.86), 0.88 (95%CI: 0.82-0.92), and 0.80 (95%CI: 0.72-0.86), respectively. The specificities were 0.71 (95%CI: 0.60-0.80), 0.75 (95%CI: 0.68-0.82), and 0.82 (95%CI: 0.70-0.89), and 0.73 (95%CI: 0.66-0.80), 0.87 (95%CI: 0.81-0.92), and 0.72 (95%CI: 0.62-0.80) , respectively. The positive likelihood ratios were 2.91, 3.67, and 4.44, and 3.04, 6.90, and 2.83, respectively. The negative likelihood ratios were 0.22, 0.12, and 0.23, and 0.26, 0.14, and 0.28, respectively. The diagnostic odds ratios were 13.01, 30.98, and 19.58, and 11.93, 49.99, and 10.00, respectively. A significant heterogeneity was observed in all analyses ($P < 0.05$). CT scanner was identified to be the source of heterogeneity. There was no significant difference in diagnostic threshold effects ($P > 0.05$) or publication bias ($P > 0.05$). To determine the risk for bleeding of EV using a non-invasive method might have important clinical applications in daily practice. The study gives an overall view of the problem, and for sure does give clinical details which could be useful in making decisions in everyday practice.

Research conclusions

Based on the meta-analysis of observational studies, CT has higher accuracy in evaluating EV and HREV than LSM and MRI in cirrhotic patients. It is suggested that CT, a non-invasive diagnostic method, is the best choice for the diagnosis of EV and prediction of HREV in cirrhotic patients compared with LSM and MRI.

Research perspectives

The results are very important with significant applications for clinicians in making decisions in daily practice for treatment of cirrhotic patients with portal hypertension. In future, the head-to-head or direct comparisons of these non-invasive methods in the same series of patients are required to confirm the predictive value, especially by using artificial intelligence technique.

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CASE REPORT

Ectopic hepatocellular carcinoma mimicking a retroperitoneal tumor: A case report

Yuki Adachi, Hiromitsu Hayashi, Toshihiko Yusa, Toru Takematsu, Kazuki Matsumura, Takaaki Higashi, Kensuke Yamamura, Takanobu Yamao, Katsunori Imai, Yo−ichi Yamashita, Hideo Baba

ORCID number: Yuki Adachi (0000-0003-1915-0407); Hiromitsu Hayashi (0000-0002-1832-4287); Toshihiko Yusa (0000-0002-0970-8262); Toru Takematsu (0000-0003-4167-061X); Kazuki Matsumura (0000-0002-6204-9147); Takaaki Higashi (0000-0002-1034-8671); Kensuke Yamamura (0000-0003-0506-6394); Takanobu Yamao (0000-0003-3125-9165); Katsunori Imai (0000-0003-0549-1825); Yo-Ichi Yamashita (0000-0001-8439-6660); Hideo Baba (0000-0002-3474-2550).

Author contributions: Adachi Y and Hayashi H made substantial contributions to conception and design of the study, acquisition of data, or analysis and interpretation of data; Yusa T, Yamao T, Higashi T, Matsumura K, Yamamura K, and Imai K drafted the article and/or made critical revisions related to important intellectual content of the manuscript; all authors approved the final version of the manuscript for submission.

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Yuki Adachi, Hiromitsu Hayashi, Toshihiko Yusa, Toru Takematsu, Kazuki Matsumura, Takaaki Higashi, Kensuke Yamamura, Takanobu Yamao, Katsunori Imai, Yo−ichi Yamashita, Hideo Baba, Department of Gastroenterological Surgery, Graduate School of Life Sciences, Kumamoto University, Kumamoto 8608556, Japan

Corresponding author: Hiromitsu Hayashi, FACS, MD, PhD, Surgeon, Department of Gastroenterological Surgery, Graduate School of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 8608556, Japan. hhayasi@kumamoto-u.ac.jp

Abstract

BACKGROUND

An ectopic hepatocellular carcinoma (EHCC) arises from the ectopic liver which is defined as a hepatic organ or tissue not connected to surrounding tissues. EHCC is a rare disease and it is difficult to diagnose preoperatively. Furthermore, the clinical features are not fully elucidated.

CASE SUMMARY

A retroperitoneal tumor (6 cm) was located at the dorsal side of the pancreas head on abdominal ultrasonography in an 81-year old woman positive for hepatitis C virus antibody. Contrast enhanced-computed tomography and gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging showed viable HCC patterns with early enhancement and delayed washout. The tumor markers — serum alpha-fetoprotein and alpha-fetoprotein-L3% — were increased to 30.1 ng/mL and 83.1%, respectively. Protein induced by vitamin K absence or antagonist-II was within normal levels (17 mAU/mL). Positron emission tomography-computed tomography showed strong accumulation into the tumor (Standardized Uptake Value max: 13.8), and the tumor cytology following endoscopic ultrasound-guided fine needle aspiration showed poorly differentiated carcinoma. Tumor extirpation was performed, and operative findings showed that the retroperitoneal tumor was disconnected from the pancreas and the liver. Swollen lymph nodes near the tumor were histologically normal. On histological examination, the tumor was finally diagnosed as EHCC with Arginase-1 positive expression.

CONCLUSION

We report our experience of a rare EHCC which was difficult to diagnose, and we present a review of the literature.

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Core tip: Ectopic liver tissue is often found on the gallbladder wall. The current case is the first ever reported case of ectopic hepatocellular carcinoma (EHCC) on the dorsal side of the pancreatic head. It is usually difficult to confirm the diagnosis of EHCC preoperatively because of the location of the mass and the rarity of this condition. In this case, we also could not make a definitive diagnosis preoperatively, but the macroscopic findings of the tumor, the immunohistological examination, and the decrease in tumor marker levels after surgery were very useful signs for the definitive diagnosis of EHCC.

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INTRODUCTION

An ectopic hepatocellular carcinoma (EHCC) is defined as an HCC arising from hepatic parenchyma located in an extrahepatic organ or tissue[1]. It can occur in various sites near the liver; for example, gallbladder, hepatic ligaments, omentum, retroperitoneum, and thorax[2].

The incidence of ectopic liver has been reported to be between 0.24% and 0.47% at laparoscopy or autopsy[3-5]. Thus, EHCC is a very rare disease and it is difficult to diagnosis preoperatively, which leads to a clinical issue. The clinical features of EHCC are still not fully elucidated. Here, we report a case of EHCC mimicking a retroperitoneal tumor, and a review the literature concerning EHCC.

CASE PRESENTATION

Chief complaints

The case was an 81-year-old woman positive for a hepatitis C virus (HCV) antibody (HCV-RNA was not detectable), and she had no remarkable chief complaints.

History of present illness

She was followed up regularly by a nearby outpatient clinic as she was an asymptomatic hepatitis C virus carrier, and the abdominal ultrasonography at that clinic revealed the tumor. She was referred to our department for further examination and treatment.

History of past illness

She was positive for HCV antibodies, but HCV-RNA was not detectable. She had previously undergone laparoscopic cho-lecystectomy for cholelithiasis and thyroidectomy at another hospital (details unknown).

Physical examinations

The abdomen was soft and flat. She has a past history of laparoscopic cholecystectomy. Any other digestive symptoms such as abdominal pain or weight loss were not observed (Eastern Cooperative Oncology Group: 0).

Imaging examinations and laboratory examinations

The abdominal ultrasonography revealed the retroperitoneal tumor (6 cm in size) located at the dorsal side of the pancreas head. On the laboratory tests, serum alpha-fetoprotein (AFP) and AFP-L3% were 30.1 ng/mL and 83.1%, respectively. The protein induced by vitamin K absence or antagonist-II (PIVKA-II) level was 17 mAU/mL, carcinoembryonic antigen level was 3.5 ng/mL, and CA19-9 level was 9.6 U/mL. The contrast enhanced-computed tomography scan showed that the

retroperitoneal tumor had enhancement in the arterial phase and was washed out in the venous phase (Figure 1A). Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging displayed enhancement in the arterial phase and a defect in the hepatobiliary phase (Figure 1B). The positron emission tomography-computed tomography revealed strong accumulation into the tumor (Standardized Uptake Value max: 13.8) (Figure 1C).

TREATMENT

Cytology by endoscopic ultrasound-guided fine needle aspiration showed a poorly differentiated carcinoma with unknown origin (Figure 2 and Table 1). We performed tumor extirpation and sampled the surrounding lymph nodes. Intraoperative findings showed that the tumor was disconnected to the liver and the head of pancreas (Figure 3). The resected gross specimen was 7.5 cm × 6.5 cm × 3.5 cm in size and encapsulated in membrane, and the cut surface was reddish-yellow with intratumoral hemorrhage (Figure 4). On microscopic examination, the tumor was composed of polygonal cells and had hyperchromatic nuclei with prominent nucleoli and granular eosinophilic cytoplasm. Very little pancreatic tissue was seen on the surface, so it was assumed that the tumor had not invaded the pancreas. The morphological histologic diagnosis was poorly differentiated carcinoma (Figure 5). On immunohistochemical staining of the tumor, Hep Per-1 and Glypican 3 were negative, but Arginase-1 (Arg-1) was focally positive. Moreover, AE1/AE3 was partly positive, CAM 5.2 was positive, synaptophysin was negative, S100 was negative, p53 was negative, and β-catenin was positive (Figure 6 and Table 2). On post-operative laboratory tests, the levels of AFP and AFP-L3% had decreased to 1.7 ng/mL and 27.3%, respectively.

FINAL DIAGNOSIS

Finally, we diagnosed the tumor as EHCC.

OUTCOME AND FOLLOW-UP

Currently, eight months have passed, but she is still alive without recurrence.

DISCUSSION

We searched "ectopic hepatocellular carcinoma" in PubMed, and 24 case reports were available in full text (Supplementary Table 1)[1,2,4-25]. Preoperative examination revealed no tumor in the mother liver in any patients[1,2,4-25]. There were two cirrhotic cases (one case was viral hepatitis and one case was unknown)[11,25]. Of the 24 cases, 18 underwent surgery[1,2,4,6,8,9,11,15-25] and two received adjuvant therapy[1,19]. There were six cases of recurrence (four cases in the mother liver and two cases in the abdominal cavity)[1,9,16,22,23,25]. Preoperative serum AFPs were often relatively elevated. In addition, AFP L3% was measured in only three cases, but a significant increase was observed in all three cases[11,17,22]. Seven cases had a hepatitis B virus infection[1,6,7,9,13,16,23] and only one case had a hepatitis C virus infection[5]. Immunohistochemical staining showed 17 cases of Hep Per-1 staining, one of which was negative[2,4-8,10-12,14-18,21-23]. There were 14 cases of AFP staining, three of which were negative[1,6,8,10,11,13,15-19,21,22,25]. EHCC is associated with a relatively long-term survival after resection, so surgical treatment should be considered initially if the tumor can be resected. Recurrence often occurs in the mother liver, and it is necessary to follow-up regularly and perform imaging testing after surgery, similar to the follow-up after surgery for HCC.

EHCC is one of the rare carcinomas defined as an HCC arising from ectopic liver tissue, and it is usually discovered incidentally at autopsy or during laparoscopy[3]. Ectopic liver tissue was recognized within the gallbladder, spleen, pancreas, adrenal gland, portal vein hepatic ligament, diaphragm, thorax, retroperitoneum, and omentum. The reported incidence of an ectopic or accessory liver is approximately 0.56% and the gallbladder is the most common location[6,7].

Liver development starts in the middle of the third week of embryonic life. The hepatic diverticulum (liver bud) is formed from the foregut and becomes a hepatocellular cord. Subsequently, a bile duct, a gallbladder, and a gallbladder duct develop from a connection part between the hepatic diverticulum and the foregut. The pancreas is composed of two types of buds: A ventral pancreatic bud that

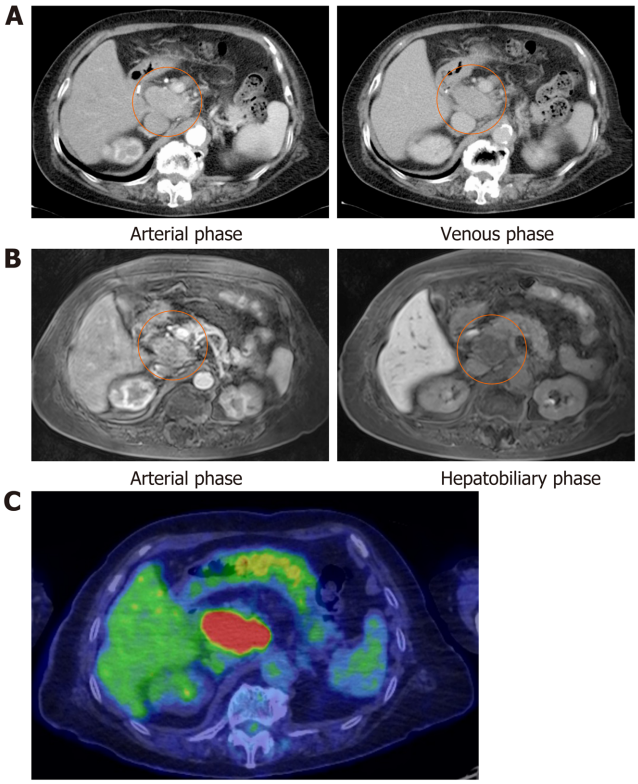

Figure 1 Imaging findings. A: Contrast-enhanced axial computed tomography (CT) scan in arterial phase and venous phase; B: Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging scan in arterial phase and hepatobiliary phase showing a round and smoothly mass which located from the hepatic portal region of the liver to the dorsal of the pancreatic head (orange circle); C: Positron emission tomography-computed tomography revealed that the mass uptakes strongly (Standardized Uptake Value max: 13.8).

develops from the bile duct and a dorsal pancreatic bud that arises from the foregut. The liver parenchyma differentiates from the hepatocellular cord. The expression of the ability of the foregut to differentiate into liver tissue is blocked by the ectoderm and mesoderm of the heart. However, the function of these inhibitory factors is blocked in the area where the liver would sprout in the future by fibroblast growth factor 2 secreted from the mesoderm of the heart and adjacent angiogenic endothelial cells. Ectopic liver tissue on the gallbladder wall and around the pancreas comprises liver parenchyma due to the influence of fibroblast growth factor 2 when the bile duct and dorsal pancreatic bud are formed from the foregut and when the ventral pancreatic bud is formed from the bile duct.

In previous reports, four out of 24 cases were found in the pancreas, but only one of them was present in the head of the pancreas, and this case was found on the ventral side of the pancreas head[6,10,18,21]. The current report represents the first case that was found on the dorsal side of the pancreatic head. Because it was located on the dorsal side of the pancreas and close to the caudate lobe, it was difficult to diagnose ectopic HCC before surgery. The initial differential diagnosis was malignant lymphoma or pancreatic head mass. It was believed to be an HCC that developed in the caudate lobe. In our literature review, the median overall survival of 24 cases is 18 mo[1,2,4-25]. There are 6 cases with recurrence after tumor resection, whose median overall survival and recurrence-free survival were 18.5 (range 6-48) and 7.5 (range 2-30) mo, respectively[1,9,16,22,23,25]. Among 18 patients who underwent surgery, one cases received postoperative adjuvant therapy[19]. In the case, the adjuvant chemotherapy using cisplatin + etoposide + bleomycin was performed for the EHCC over the left subphrenic space, and the case is without recurrence during 8 mo after surgery[19]. In six cases who did not undergo surgery, three cases received chemotherapy (sorafenib, cisplatin+etoposide+bleomycin, or unknown regimen), two cases underwent just a biopsy and one case inserted a biliary stent as a palliative care[5,7,10,12-14]. The survival outcomes in the two cases with multiple EHCCs in the spleen treated by cisplatin + etoposide + bleomycin and with multiple EHCCs in the thoracic and abdominal cavities treated by sorafenib, are 34 and 13 mo, respectively[7,14]. Although tumor resection had been performed in the majority of EHCC (18 of 24 cases, 75%), the clinical benefit of tumor resection for EHCC is still unclear from our literature review.

Table 1 Details of immunohistochemical staining of the tumor biopsy by endoscopic ultrasound-guided fine needle aspiration

Variables	Results
Hep Per-1	Negative
Arginase-1	Negative
Glypican 3	Negative
AE1/AE3	Focal positive
CK5/6	Negative/negative
CK7/20	Negative/negative
Vimentin	Negative
Synaptophysin	Negative
S100	Negative

Further accumulation of EHCC cases need to elucidate the epidemiologic aspects of EHCC.

Some reports have noted that EHCC is observed in about 7%–30% of cases of ectopic liver[2,9]. Carcinogenesis is a multistep process that appears to be accelerated within these tissues. It is theorized that due to the lack of a normal vascular and ductal system, the foci of ectopic liver tissue may be metabolically handicapped, leading to longer exposure to various carcinogenic factors[6,7,10]. The underlying microenvironment would cause persistent cellular stress, which may result in cell death and compensatory cell proliferation. An increased cell turnover may lead to genetic mutations and subsequent development of carcinoma[11]. The reason is that both morphological features are similar, and if the carcinoma cell is poorly differentiated or undifferentiated, it will be extremely difficult to distinguish HCC from adenocarcinoma cells morphologically. The effectiveness of Arg-1 against poorly differentiated HCC. In particular, Arg-1 is the most sensitive and specific marker (greater than 90%) of hepatocellular differentiation and should be the first-line marker of HCC *vs* other tumors[12,13].

CONCLUSION

The preoperative diagnosis of EHCC is often very difficult. Specific tumor markers can be useful to diagnose EHCC preoperatively if there is any possibility of another tumor from radiological findings. Early surgical treatment for EHCC would provide favorable long-term outcomes.

Table 2 Details of immunohistochemical staining of the resected tumor specimen

Variables	Results
Hep Per-1	Negative
Arginase-1	Focal positive
Glypican 3	Negative
AFP	Negative
AE1/AE3	Partly positive
CAM 5.2	Positive
Synaptophysin	Negative
S100	Negative
p53	Negative
β-catenin	Positive

AFP: Alpha-fetoprotein.

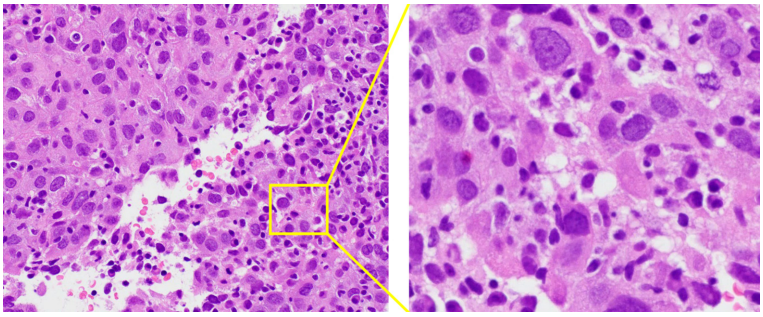

Figure 2 The microscopic examination of the tumor by endoscopic ultrasound-guided fine needle aspiration. Hematoxylin and eosin stain, magnification × 200 (left) and × 400 (right). Microscopic examination of the tumor confirmed poorly differentiated carcinoma.

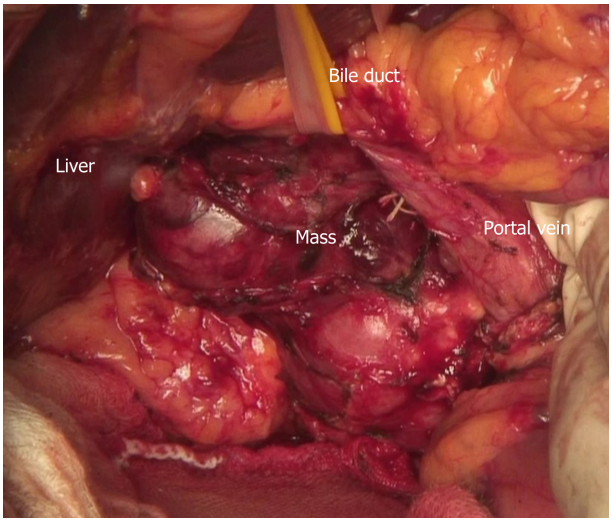

Figure 3 The tumor is located at between the hepatic portal region and the dorsal of the pancreatic head and has no connection to the surrounding organ.

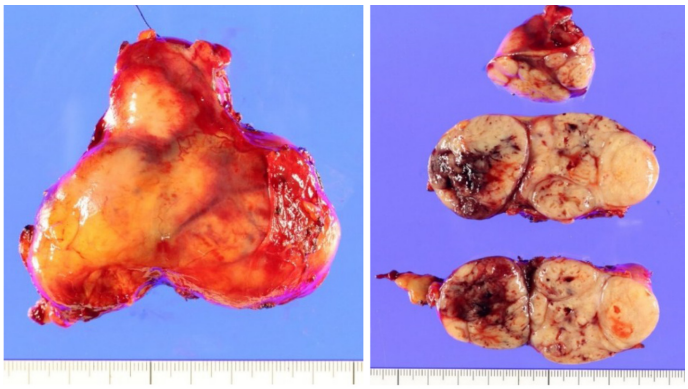

Figure 4 Macroscopic features of the excised hepatocellular carcinoma demonstrating a solid multinodular tumor with a fibrous capsule and intratumoral hemorrhage.

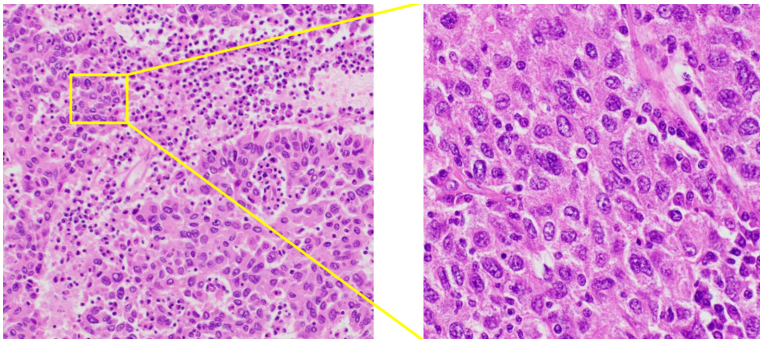

Figure 5 The microscopic examination of the tumor. Microscopic examination of the tumor confirmed poorly differentiated carcinoma morphologically similar to the tumor biopsy by endoscopic ultrasound-guided fine needle aspiration Hematoxylin and eosin stain, magnification × 100 (left) and × 400 (right).

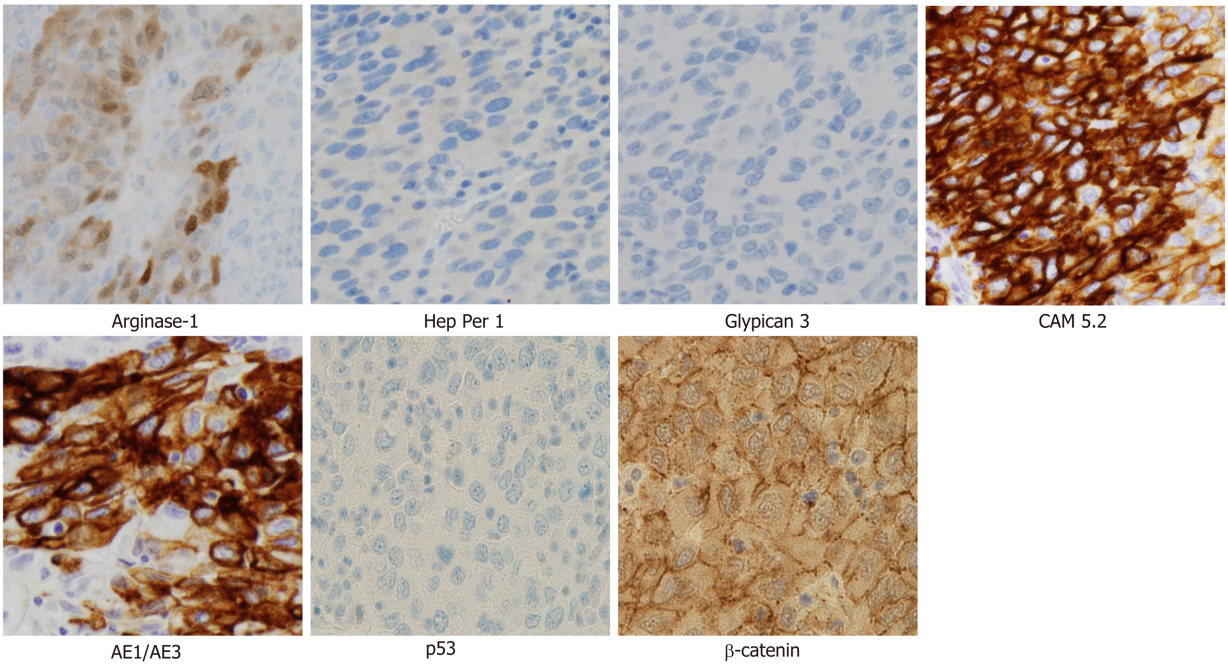

Figure 6 Immunohistochemical findings. Tumor cells are negative for Hep Per-1, Glypican 3 and p53, but focal positive for Arginase-1. Moreover, CAM 5.2, AE1/AE3, and β-catenin are positive (Magnification × 400).

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